9th International Barley Genetics Symposium

Proceedings

Edited by
Ing. Jaroslav Spunar, CSc.
Jarmila Janikova

Technical and computer processing
Ing. Antonin Pospisil
Ing. Antonin Soucek
Agricultural Research Institute Kromeriz, Ltd.

Brno 2004

Published in 500 copies

Agricultural Research Institute Kromeriz, Ltd.

http://www.vukrom.cz

Oral Presentations

Proceedings - Part I

Oral presentations are ordered according to the sessions and the program.

Authors are fully responsible for the content of their contributions.
No language corrections were made.

INTRODUCTORY SESSION

Brno – City of Gregor Mendel[1]

O. Chloupek

Mendel University of Agriculture and Forestry,
613 00 Brno, Czech Republic

Most of the world´s inhabitants hear the name of our town only in lectures from biology in connection with Gregor Mendel – the founder of genetics.

1. Mendel's Life and His Motives for Genetics

Johann Mendel was born 1822 in a farmer´s family in the small village of Hynčice[1] about 120 km to the north of Brno[2]. Priest in his birth village introduced him into gardening[3]. Johann studied grammar school, "*Gymnasium",* in Opava; this school was headed by a member of the Augustinian community in Brno.

Mendel described his life in Opava in his curriculum vitae (Mendel 1850): *"Owing to several successive disasters, his parents were completely unable to meet the expenses necessary to continue his studies, and it therefore happened that the respectfully undersigned, then only sixteen years old, was in the sad position of having to provide for himself entirely. For this reason he attended the course for School Candidates and Private Teachers…. When he graduated from the Gymnasium in the year 1840, his first care was to secure for himself the necessary means for the continuation of his studies. Because of this, he made repeated attempts in Olomouc to offer his services as a private tutor, but all his efforts remained unsuccessful because of a lack of friends and references. ...Having finished his philosophical studies, he felt himself compelled to enter a station in life that would free him from the bitter struggle of existence. His circumstances decided his vocational choice. He requested and received in the year 1843 admission to the Augustinian monastery of St Thomas in Brno"*, where he took the name Gregor.

The monastery was founded in 1350 by the brother of the Emperor Charles IV who in 1348 founded the Prague University, the first in Central Europe. The monastery received by the Emperor the famous painting *Black Madonna*[4]. About two hundred years ago the monks were forced to move to the dissolved Cistercian nunnery[5] in *Old* Brno with large gardens.

As a novice Mendel was entrusted to the care of a member of the Agricultural Society in Brno. This kind of education was a custom since the order owned a lot of agricultural grounds. Mendel, as a student of theology, studied theology, philosophy and agricultural sciences. In 1847 Mendel was ordained a priest[6]. Soon Mendel began his University study in Vienna. His professor of physics published a textbook *Combinatorial analysis*, in which he stressed the importance of mathematical generalization of experimental results. Professor of physics Doppler explained the general principle of science: complicated phenomena should be explained by means of their elements.

---

[1] Picture: Village Vražné
[2] Picture: Birth-house of Gregor Mendel, today his museum
[3] Picture: The church in Vražné
[4] Picture: The famous painting of the Black Madonna
[5] Picture: The Augustinian monastery, prelate quarters
[6] Picture: The cistercian church of Virgin Mary, "basilica minor"

Modern type of secondary education, *Realschule*, was opened in 1851. Mendel became a teacher of physics and natural history there. A new building was opened in Jánská - street. The staff of the Technical Institute and the teachers of the *Realschule* founded the *Natural Science Society* in Brno in 1862, where Mendel presented his experiments with pea hybrids[7] three years later.

2. Discovery and Rediscovery of Mendel's Laws of Genetics

On February and March 1865 Gregor Mendel lectured in the *Natural Science Society* Brno on his *Experiments with plant hybrids* (*Versuche über Pflanzen-Hybriden)*[8]. The motivation for the experiments was breeding, in particular breeding of flowers[9].

Mendel selected the pea since the species had clear distinguished characters, was easy to castrate, pollinate and to protect against cross-pollination, no problems with fertility occurred in a progeny, was easy to grow and had a short vegetation period[10]. He grew in each of the 10 experimental years about 4-5 thousand plants of peas in the monastery garden. Constancy of parental varieties was proved in the first two years and their hybrids were evaluated in the eight years later.

Mendel first summarized[11] in his lectures the experience of those who bred new plant varieties to obtain new colours[12]. In his second sentence he recalled "the striking regularity with which the same hybrid forms always reappeared whenever fertilisation between the same species took place"[13]. He also pointed to the significance of research for the developmental history of organic forms, including the enigma of generation, heredity, and fertilisation[14].

To study the change of two different characters in the progeny and to find out the law for the occurrence of the characters in the progeny was the aim of the experiment[15]. But some characters were not suitable since it was difficult to distinguish them; it was only possible to evaluate them „more or less"[16].

Only one of the seven alternative hybrid characters occurred in progeny of the crosses[17]. The characters which whole or nearly without change pass over into hybrids were named

---

[7] Picture: The building of the former Realschule

[8] *Künstliche Befruchtungen, welche an Zierpflanzen vorgenommen wurden, um neue Farbenvarianten zu erzielen, waren die Veranlassung zu den Versuchen, die hier besprochen werden sollen.*

[9] Picture: The experimental garden with the museum Mendelianum situated in the monastery

[10] *Eine besondere Aufmerksamkeit wurde gleich Anfangs den Leguminosen wegen ihres eigenthümlichen Blütenbaues zugewendet. Versuche, welche mit mehreren Gliedern dieser Familie angestellt wurden, führten zu dem Resultate, dass das Genus Pisum den gestellten Anforderungen hinreichend entspreche. Einige ganz selbständige Formen aus diesem Geschlechte besitzen constante, leicht und sicher zu unterscheidende Merkmale, und geben bei gegenseitiger Kreuzung in ihren Hybriden vollkommen fruchtbare Nachkommen.*

[11] Picture: The first page of the famous manuscript

[12] *Künstliche Befruchtungen, welche an Zierpflanzen deshalb vorgenommen wurden, um neue Farbvarianten zu erzielen, waren die veranlassung zu den Versuchen, die hier besprochen werden sollen.*

[13] *Die auffalende Regelmässigkeit, mit welcher dieselben Hybridformen immer wiederkehrten, so oft die Befruchtung zwischen gleichen Arten geschah, gab die Anregung zu weiteren Experimenten, deren Aufgabe es war, die Entwicklung der Hybriden in ihren Nachkommen zu verfolgen.*

[14] *Es gehört allerdings einiger Muth dazu, sich einer so weit reichenden Arbeit zu unterziehen; indessen scheint es der einzig richtige Weg zu sein, auf dem endlich die Lösung einer Frage erreicht werden kann, welche für die Entwicklungsgeschichte der organischen Formen von nicht zu unterschätzender Bedeutung ist.*

[15] *Diese Veränderungen für je zwei differirende Merkmale zu beobachten und das Gesetz zu ermitteln, nach welchem dieselben in den aufeinander folgenden Generationen eintreten, war die Aufgabe des Versuches.*

[16] *Ein Theil der … Merkmale lässt jedoch eine sichere und scharfe Trennung nicht zu, indem der Unterschied auf einem oft schwierig zu bestimenden „mehr oder weniger" beruht.*

[17] *Jedes von den 7 Hybridenmerkmalen gleicht dem einen der beiden Stamm-Merkmale …. Dieser Umstand ist von grosser Wichtigkeit …*

„dominant“ and the characters which were latent „recessive“[18]. It was found that it is no matter if the dominant character belonged to the seed or to the pollen plant[19].

Even if Mendel formulated his findings clearly, his expressions were modest. He stated for example that his experiments need more evidence, since only one observer can omit some differences that could be later important[20]. On contrary he stated clearly that uniformity of developmental plans of organic life is undisputed[21]. He described also back-cross for development of constant forms, even if it depends on the number of experimental plants and on the number of characters which were unified through pollination[22].

Gregor Mendel was elected 1868 for the Abbot of the monastery[23]. He supported then not only his home village, but also three sons of his sister, and was active in the public. However, only in 1883 he became ill and died on January 6, 1884[24]. The funeral requiem for him was conducted by Leoš Janáček, who was educated in the monastery, later famous composer. Mendel is buried in the central cemetery of Brno[25]. Mendel´s statue was established in 1910 in the former monastic square and later transferred to his experimental garden[26]. According to Czech law from 1994 was the Agricultural University in Brno, founded 1919, renamed to Mendel University of Agriculture and Forestry[27].

The impact of the research was not understood by contemporary science. A “layman” from a province could not expect an understanding of scientific world. Only when chromosomes were microscopically observed, before the end of the 19th century, it was found that the behaviour of characters in progeny of crossing, statistically described by Mendel, was relevant to the behaviour of chromosomes in dividing cells. The impact of Mendel´s work was appreciated at the same time by three scientists who published in 1900 their own results supporting them. It was Hugo de Vries, Carl Correns and Erich Tschermak von Seysenegg.

---

[18] *In der weiteren Besprechung werden jede Merkmale, welche ganz oder fast unverändert in die Hybride-Verbindung übergehen … als dominierende und jene, welche in der Verbindung latent werden, als recessive bezeichnet.*

[19] *Es wurde ferner durch sämtliche Versuche erwiesen, dass es völlig gleichgültig ist, ob das dominirende Merkmal der Samen- oder Pollenpflanze angehört.*

[20] *Die Geltung der für Pisum aufgestellten Sätze bedarf allerdings selbst noch der Bestätigung, und es wäre deshalb eine Wiederholung … wünschenswerth … . Dem einzelnen Beobachter kann leicht ein Differentiale entgehen, welches, wenn es auch anfangs unbedeutend scheint, doch so anwachsen kann, dass es für das Gesammt-Resultat nicht vernachlässigt werden darf.*

[21] *… die Einheit im Entwicklungsplane des organischen Lebens ausser Frage steht.*

[22] *Demnach ist für alle derartigen Versuche die Möglichkeit vorhanden, dass schon aus der zweiten Befruchtung eine constante Form gewonnen wird, welche der Pollenpflanze gleich kommt. Ob dieselbe aber wirklich erhalten wird, hängt in jedem einzelnen Falle von der Zahl der Versuchspflanzen ab, sowie von der Anzahl der differirenden Merkmale, welche durch die Befruchtung vereinigt wurden.*

[23] Picture: Gregor Mendel, Prelate and Abbot, before 1880

[24] Record from the meeting of the *Naturforschender Verein* on January 9, 1884 (Verhandlungen des naturforschendes Vereines in Brünn, 1884, Vol. 23, p. 19):

Der Secretär Herr Prof. G. v. Neissl theilt die betrübende Nachricht von dem Tode des hochverdienten Vereinsmitgliedes P. Gregor Mendel, inful. Abt des Augustinerstiftes in Brünn mit. Der Verstorbene gehörte seit der Gründung des Vereines zu seinen eifrigsten und wohlwollendsten Förderern, indem er nicht alleindenselben materiell ausgiebig unterstützte, sondern auch lebhaften Antheil an den wissenschaftlichen Arbeiten desselben nahm. Er benützte die Musse, welche ihm seine glückliche Stellung gewährte, fast ausschliesslich zu sehr eingehenden naturwissenschaftlichen Studien, die durchaus von selbstständiger eigenthümlicher Auffasungsweise zeigten. Hieher gehören insbesondere die Beobachtungen über die von ihm in grossen Mengen cultivirten Pflanzenbastarde. …

[25] Picture: Augustinian vault in the Brno central cemetery, including the grave of Mendel

[26] Picture: Mendel´s statue in the former vegetable monastic garden by Theodor Charlemont, Vienna

[27] Picture: Mendel University of Agriculture and Forestry, main building

Prof. Tschermak founded 1912 Mendeleum in Lednice[28], research institute for genetics and plant breeding of Vienna Agricultural University, which belongs now to the Mendel University.

Plant breeding practice seems to be a product of science, intuition and of experience. However, knowledge of the Mendel's principles of inheritance enabled development of the intuition and critical judgement of the experience. In such a way contribute to a rational appreciation of different breeding approaches.

The principles of genetics by plant breeder G. Mendel used later also human and animal geneticists. Plant geneticists learn in this time principles of gene transfer from them, since genetically modified organisms are widely used for production of important human medicaments.

References

BATESON W. (1909): Mendel´s principles of heredity: Cambridge University Press, Cambridge.

BLIXT S. (1975): Why didn´t Gregor Mendel find linkage? Nature, **256**: 206.

ILTIS H. (1924): Gregor Johann Mendel. Leben, Werk und Wirkung. Springer, Berlin.

KŘÍŽENECKÝ J. (1965): Gregor Johann Mendel, 1822-1884. Barth Verlag, Leipzig. 198 pp.

MATALOVÁ A. (1981): Published primary sources to Gregor Mendel´s biography. Folia Mendeliana, **16:** 239-251.

MENDEL G. (1866): Versuche über Pflanzenhybriden. Verhandlungen des Naturforschenden Vereins Brünn, **4**: 3-47.

OREL V. (1996): Gregor Mendel: The first geneticist. Oxford University Press, Oxford, 363 pp.

VOLLMAN J., Ruckenbauer P. (1997): Von Gregor Mendel zur Molekulargenetik in der Pflanzenzüchtung – ein Überblick. Die Bodenkultur, **48:** 53-65.

[28] Picture: Mendeleum in Lednice

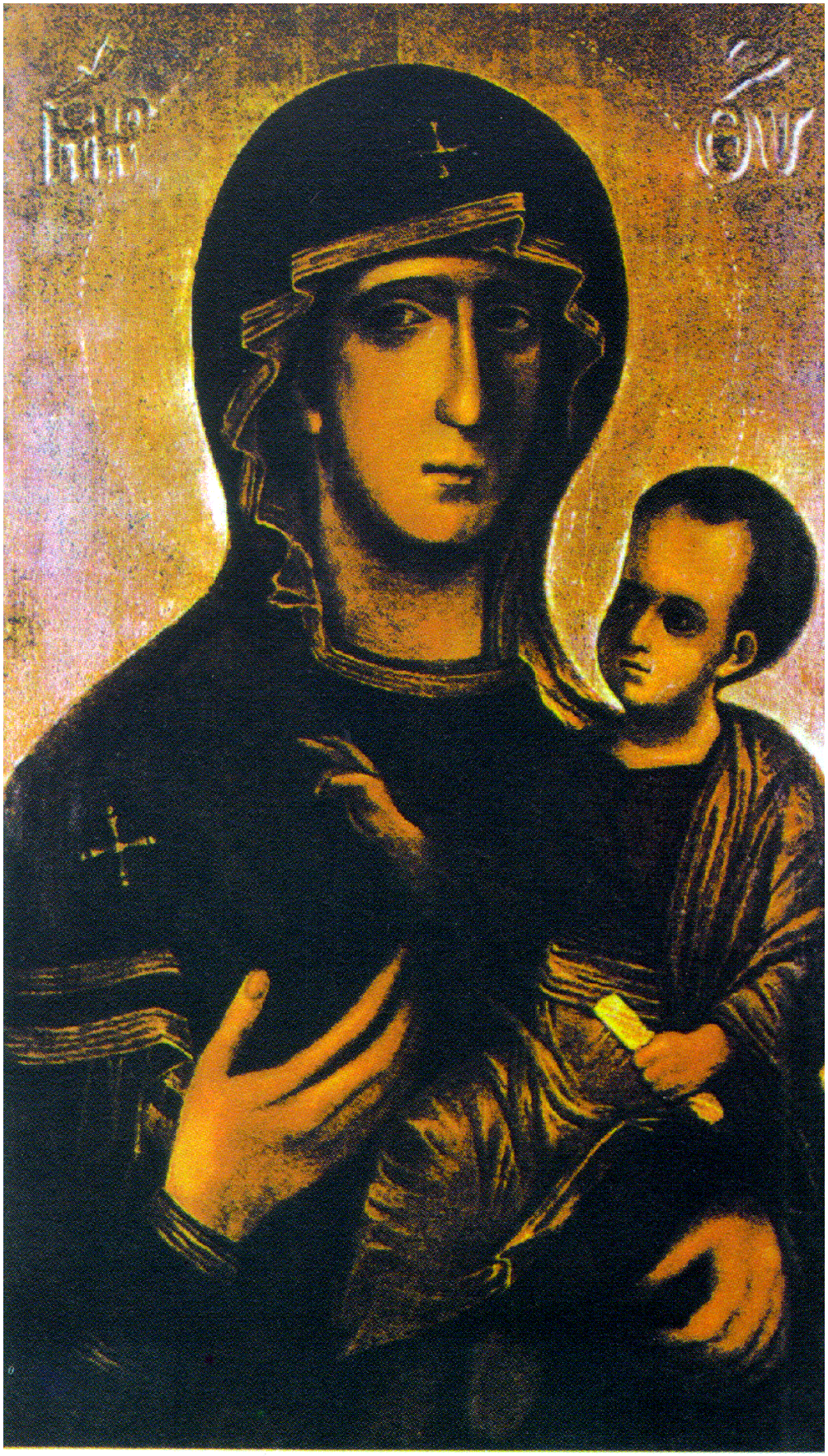
Picture 1: Gregor Mendel, about 1864/1865

Picture 2: Village Hynčice

Picture 3: Birth-house of Gregor Mendel

Picture 4: Church in Vražné

Picture 5: The famous painting of the *Black Madonna*



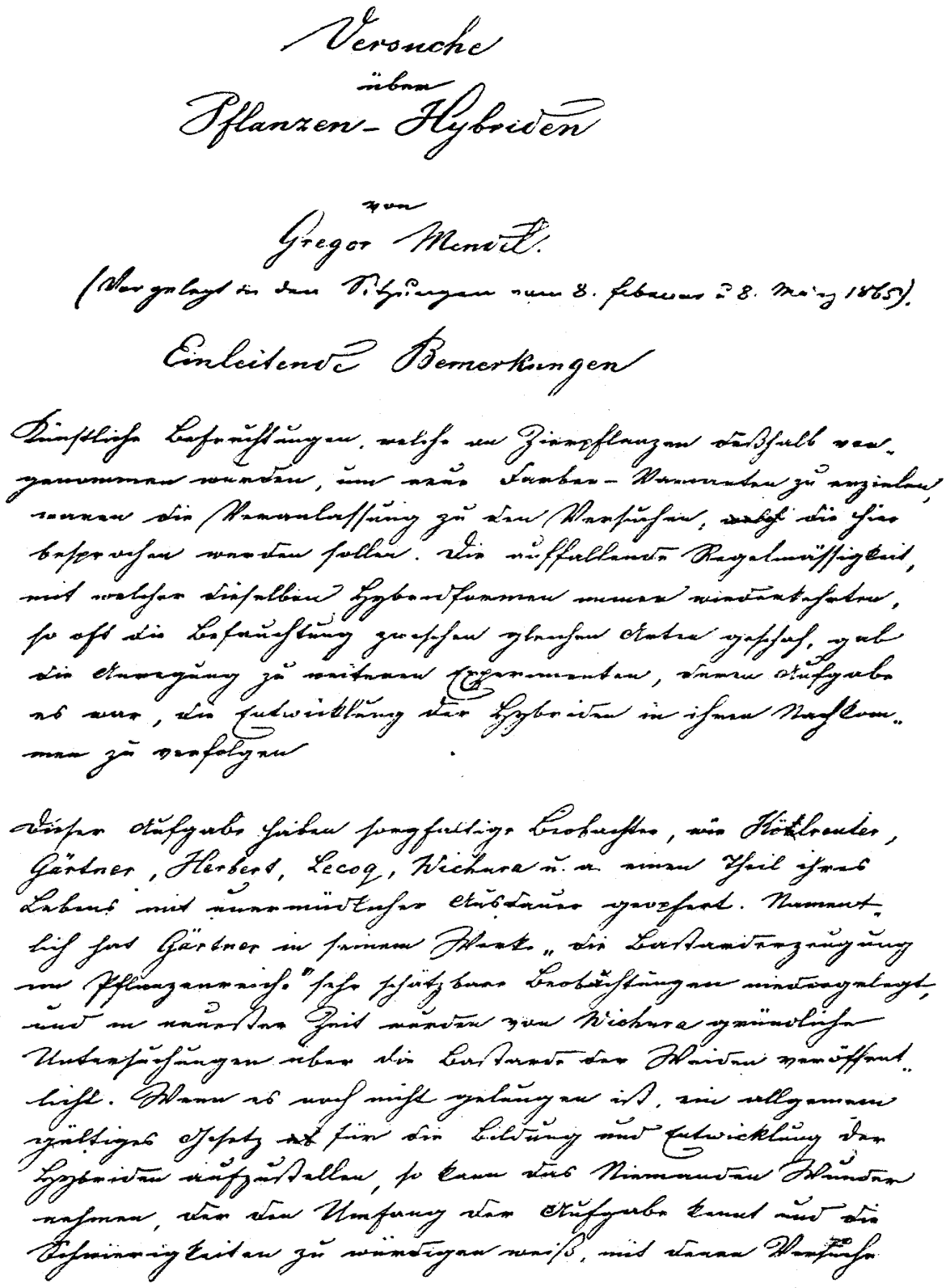
Picture 6: The Augustinian monastery, prelate quarters

Picture 7: The Cistercian Church of Virgin Mary (basilica minor)

Picture 8: The building of the former Realschule

Picture 10: The experimental garden with the Museum Mendelianum situated in the monastery

Picture 13: The first page of the famous manuscript



Versuche
über
Pflanzen-Hybriden

von
Gregor Mendel.

(Vorgelegt in der Sitzung vom 8. Februar u. 8. März 1865).

Einleitende Bemerkungen

Künstliche Befruchtungen, welche an Ziergewächsen deshalb vorgenommen wurden, um neue Farben-Varietäten zu erzielen, waren die Veranlassung zu den Versuchen, welche hier besprochen werden sollen. Die auffallende Regelmässigkeit, mit welcher dieselben Hybridformen immer wiederkehrten, so oft die Befruchtung gewissen Arten gelang, gab die Anregung zu weiteren Experimenten, deren Aufgabe es war, die Entwicklung der Hybriden in ihren Nachkommen zu verfolgen.

Diese Aufgabe stellen sorgfältige Beobachter, wie Kölreuter, Gärtner, Herbert, Lecoq, Wichura u. a. einen Theil ihres Lebens mit unermüdlicher Ausdauer gewidmet. Kölreuter, fast Gärtner in seinem Werke „die Bastarderzeugung im Pflanzenreiche" sehr schätzbare Beobachtungen niedergelegt, und in neuester Zeit wurden von Wichura gründliche Untersuchungen über die Bastarde der Weiden veröffentlicht. Wenn es auch nicht gelungen ist, eine allgemeine gültige Lösung der für die Bildung und Entwicklung der Hybriden massgebenden Verhältnisse zu geben, so kann doch Niemand diese Arbeiten, der den Umfang der Aufgabe kennt und die Schwierigkeiten zu überwinden weiss, mit einem Urtheile

Picture 26: Gregor Mendel, prelate and Abbot, before 1880

Picture 30: Augustinian vault in the Brno central cemetery, including the grave of Mendel

Picture 31: Mendel´s statue in the monastery by Theodor Charlemont, Vienna

Picture 32: Mendel University of Agriculture and Forestry, main building

Picture 33: Mendeleum in Lednice

International Barley Genetics Symposium
Their Mission and Position in Barley Genetics History 1963-2003

J. Špunar[1] and R. Nilan[2]

[1]Agricultural Research Institute Kroměříž, Ltd., Kroměříž, Czech Republic;
[2]Washington State University, Pulman, USA

Nearly 41 years ago in 1963 the First International Barley Genetics Symposium (IBGS) was held in the Netherlands. Thus, at the beginning of the Ninth IBGS is should be worthwhile to present the Mission of the Symposium in barley genetics history, 1963-2003. Especially it is very important that more recent barley geneticists and breeders have some knowledge of how barley genetics and related areas developed over the past 40 years.

The need for an international barley genetics network and Symposium evolved and the planning started in the fall of 1959 in Arne Hagberg´s Cytogenetics Laboratory of the Swedish Seed Association, Svalov, Sweden. Ewald Favret, Castelar, Argentina, and Bob Nilan, Pullman, USA were working there on chromosome aberrations and induced mutations. The three decided to convene a meeting of the worlds barley geneticists prior to the XI International Genetics Congress in the Netherlands. This, with the major assistance of several scientists of the Netherlands, the First International Barley Genetics Symposium was held in Wageningen in August 1963. The beginnings of the International Barley Genetics Symposium is described in detail by Hagberg and Nilan in Barley Genetics VI (1962) pages XIII a XV.

In this brief review, special emphasis is placed on the major new information on barley genetics and related areas presented at each of the eight previous Symposia and recognize those who planned and developed each Symposium. For the sake of accuracy, in the summary of each Symposium we have not listed the scientists connected with each finding or advance. We did not want to inadvertently overlook any scientific participant.

We cannot review the last 40 years of barley genetics without mentioning some of the pioneers who were instrumental in initiating and developing important areas reported in the early Symposia. These include Wiebe (developing and maintaining the world collection of breeding stocks and developing hiproly barley), Ramage (cytogenetics-translocations), Robertson (tracking genes, mapping and developing linkage stocks), Truchiya (cytogenetics-trisomics), Bakhteyev (origin and phylogeny), Hagberg (cytogenetics-use of translocations and haploids), Kasha (cytogenetics-microspore culture and haploids), Shepperd addition lines), Lundquist (mapping, maintaining and cataloguing genetic stocks), and Von Weastein (induced mutations and molecular biology of the chloroplast).

The First Symposium was organized by F. Nijdam (President), H. Lamberst (ecretary), and the organizing Committee of the Netherlands. There were 106 in attendance and 36 presentations. Highlights of this symposium included: origin and phylogeny: use of translocations and trisomics in genetic analyses and breeding, radiation and chemical mutagenesis, interspecific and intergeneric hybrids, host-pathogen interactions, and chromosome mapping. A major development was the selection of co-coordinators for genetic and chromosome stocks and linkage maps.

Pullman, Washington, USA was the site for the Second Symposium in July 1969. It was planned by G. A. Wiebe (President), R. A. Nilan (Secretary) and D. W. Robertson with 157 participants and 69 presentations.

New information included, hybrid barley, high-lysine, high-protein barley, fate and role of exogenous bacterial DNA, molecular biology of the chloroplast, gene pools of naked strains and eceriferum mutants for understanding barley waxy surfaces. The Barley Genetics Newsletter was created at this symposium and was first published in 1970.

The Third Symposium was held in Garching, Germany in July 1975. It was organized and developed by R. Nilan (President), H. Gaul (Secretary), E. Favret, G. Fischbeck and W. Friedt with 180 in attendance and 93 presentations. New scientific developments included, utilization of haploids in genetic analysis and breeding, genetics and physiology of resistance to stress, addition of individual chromosomes to wheat, Giemsa C. banding of chromosomes and breeding and improvement through induced mutations.

Edinburgh, Scotland was the site of the Fourth Symposium in July 1981. It was organized and executed by H. Gaul (President), R. Whitehouse (Secretary) and the local executive committee with 259 participants and 137 presentations. Important new scientific findings included, the development and role of major collections and composite crosses for breeding, beta glucans and proanthocyanidin free strains for improving malting quality and feed quality, phylogeny and taxonomy the genus Hordeum, analyses of rye X barley hybrids, and isolation of genes that could serve as traps for transposable elements.

In October 1986 the Fifth Symposium was held in Okayama, Japan. It was organized and produced by A. Hagberg (President), Y. Yasuda (Secretary), T. Konishi and the local organizing committee with 240 scientists in attendance and 142 presentations. New information on barley genetics and related areas included: Rigorous productive duplications, ideotype breeding, genetics of resistance to barley yellow-dwarf mosaic, four kinds of powdery mildew resistance and major advances in molecular genetics.

The Sixth Symposium was held in Helsingborg, Sweden in July 1991. B. Harvey (President), R. Von Bothmer (Secretary) and the Nordic Oganizing Committee had the major responsibilities of planning and organizing. There were 342 in attendance, 222 presentations and again several new important scientific findings. These included, structure of storage protein genes, breeding for resistance to environmental stresses – frost, drought and salt tolerance, application of microspore cultures in breeding, mutation, transformation and genetic studies, advances in genetic transformation, quantitative imaging techniques for quality malting analyses and advances in the genetics of plant development. Several Nordic collaborators presented advances in genetic, biochemical and physical analyses of starch synthesis. The North American Genome Mapping Project involving 19 collaborators presented a Restriction Fragment Length Polymorphism (RFLP) map and its applications to breeding.

Saskatoon, Canada was the site of the Seventh Symposium in July 1996. Co-chairs B. Harvey and B. Rossnagel, President R. von Bothmer, and the local organizing committee were responsible for the planning and execution. It was held jointly with the Fifth International Oat Conference and there were 425 participants and 327 presentations for both functions.

New advances in barley genetics and related areas included: transformation related to breeding, importance of stem reserves in grain filling, genotype-phenotype associations at major malting quality and quantitative trait loci (QTL) and implications for molecular marker assisted selection (MMAS), molecular mapping of genes conferring resistance to major pathogens, variation in agronomic characters derived from anther culture, improvements in malting quality through engineering improvements in 1-3, 1-4 beta glucanase, genetic analysis

of traits for improvements in feed for beef cattle, and high resolution mapping and rice synteny and new roles for barley in human foods.

The Eight Symposium was held in Adelaide, Australia in October of 2000. Planning and organization was directed by B. Harvey (President). G. Fincher (Secretary) and the local organizing committee including A. Barr, P. Johnson and P. Langridge. There were 325 participants and 255 presentations.

New information in barley genetics and related areas included: durable resistance to spot blotch and stem rust, new areas of genetic diversity, quality malt from hulless strains, protein engineering in breeding and improvement, an easy method for establishing linkage groups, and new breeding methodologies. Many papers dealt with advances in genetics and improvement through transgenic transposon tagging, transposable elements, molecular mapping and quantitative trait loci.

It is fully expected that the Ninth Symposium will reveal many new scientific advances that will add immeasurably to our knowledge and application in barley genetics and related areas.

From One Gene to Thousands of Genes: Perspectives from Another Man's Life with Barley

P.M. Hayes

Dept. of Crop and Soil Science, Oregon State University, Corvallis, Oregon, 97331 USA

It is fitting that the 9th International Barley Genetics Symposium (IBGS) is held in the cradle of malting barley, the home of outstanding beer, and a few hundred meters from the fields of Mendel. This historical perspective is humbling: our unparalleled insights into the structure and function of the barley genome will appear as glimmerings by the next IBGS and primitive by the next anniversary of the publication of Mendel's work. Pursuing this theme of historical perspective, I've used Mendel's first paper and Jack Harlan's book - "One Man's Life With Barley" - as springboards for this talk. I also engaged in a bit of historical review and dusted off the copies of IBGS Proceedings II and V – VIII that grace my bookshelf. I presented a poster at IBGS VI and gave invited talks at IBGS VII and VIII. I am therefore honored to have been invited to give one of the opening session talks at IBGS IX, but I certainly hope that this is not the end point of the regression line.

Returning to the IBGS proceedings of years past, several things caught my eye in these classic volumes. I have a treasured copy of IBGS II (1969), for which Dr. R. Nilan wrote the Introduction. Thirty five years ago, Bob identified four reasons why barley genetics knowledge is useful: it can "aid in the creation of improved varieties for food and malt production, (2) increase the usefulness of barley as an experimental plant in a variety of biological studies, (3) be utilized in understanding the genetics and improvement of other plants, and (4) generally help to advance the science of genetics" (NILAN 1970). Need I say more? It appears that in the old days there were no opening ceremonies, or the opening session speakers did not have to supply text for the symposium proceedings. At any rate, Bob's Introduction is immediately followed by a paper with Dr. R. Allard, and the remainder of the volume is sprinkled with papers by the early pillars of genetics and barley.

Picking up the thread of the IBGS events that I've been privileged to attend, at IBGS VI there were opening session addresses. Dr. R. von Bothmer led off with a lyrical description of the "beautiful billowing landscapes with the often colorful flowering barley fields" of Scandinavia and highlighted the incredibly broad adaptation and multiple end uses of barley. At IBGS VII, the Canadians returned to the North American tradition of no papers presented at the opening ceremonies. The Australians, however, did allow for two "perspective" papers under the heading "International Outlook" for IBGS VIII. Dr. S. Smith of Novartis Seeds Limited concluded his paper with the following prescient observation; "The future and outlook therefore are bright and equally as bright as they ever have been. The only thing necessary to be part of that future is an ability to identify change and adapt to it, and anything else isn't worth a XXX". Well put, I'd say, particularly considering the volatility of the agricultural biotechnology industry. Dr. A. Kleinhofs' paper "The Future of Barley Genetics" follows in the Proceedings of IBGS VII. It is a comprehensive and well-referenced paper in the course of which Andy states that "Predicting the future is always a risky business, however it is fun to try". Unfortunately, Andy wound up in the hospital rather than at the symposium. A consequence, I trust, of the long flight to Australia, not protracted fun. At any rate, when Dr. J. Spunar kindly invited me to give a talk with the same title that Andy had used, I declined.

Instead, I came up with "From One Gene to Thousands of Genes: Perspectives From Another Man's Life With Barley". The one gene part is an obvious choice, given our venue. Mendel, of course, sparked a revolution that still has us reeling. My first exposure to genetics - under the tutelage of Dr. R. Ramage, one of the stalwarts of North American barley genetics - was essentially Mendelian. Later, I was enthused by quantitative genetics, as it promised tools for moving beyond two locus models and into the sorts of traits that plant breeders are concerned with. Such enthusiasm was dampened a bit by one of the heroes of barley, Dr. R. Allard, who observed "Most quantitative genetic models require numerous assumptions, many of which may be invalid, thus causing estimates of genetic parameters to be imprecise and even to exceed their theoretical limits. Whatever the cause or causes, the laborious biometrical experiments I conducted provided little information about numbers of alleles per locus, numbers of loci, types of gene action (additive, dominance, epistatic), the impact of various single-locus or multilocus genotypes on fitness, and other genetic factors about which we must learn more if we are to understand the evolution of adaptedness" (ALLARD 1988).

Fortunately, my despair at Allard's bad news about quantitative genetics was allayed by the advent of molecular tools that allowed for relatively cost-effective genotyping of relatively large populations. One of the fruits of such endeavors was Quantitative Trait Locus (QTL) analysis. The QTL tool promised, and to some extent has delivered, information about the "numbers of alleles per locus, numbers of loci, and types of gene action" that Allard sought. The application of QTL analysis tools to barley genetics breeding has been reviewed elsewhere (HAYES *et al.* 2003) and will be a subject of discussion (and perhaps closure) at this symposium. The advent of even higher-throughput and relatively cost-effective genomics tools, promises (and to some extent may deliver) many more tools for gene discovery and use. A key (buzz) word in the preceding sentence is "high-throughput". Of course we need to remember that today's "high" is tomorrow's "low". Furthermore, "high-throughput" admittedly makes me a bit nervous and queasy: it is daunting for one who came of age in the computer age to deal with thousands of genes simultaneously. After all, when one takes notes in a breeding nursery – the ultimate macroarray – one takes notes on one plot at a time. Nonetheless, the fact remains that if one chooses to do so the technology is available (albeit at a steep cost) to monitor the expression of ~23,000 genes in each plot, and at a plethora of growth stages and tissues. Even the hungriest of data vultures, however, would surely soon succumb to overload when faced with such a barrage of information.

Cheer, history and rare books are always a refuge from the overloads of modern life, and barley researchers are especially blessed to have at hand the exquisite fruits of their labors (in the forms of malt-based brewed and distilled products) and "One Man's Life With Barley" by Dr. H. Harlan. The book was published, posthumously, in 1957, and it recounts thirty-five years of "looking at barley…the mental compost from which our ideas spring like mushrooms". All in all, "One Man's Life With Barley" is an excellent read. I've extracted seven principal themes from Harry's treatise: germplasm is of paramount importance; barley is an excellent pretext to travel to the ends of the earth; genetics is a promising field, but a sideline to plant breeding; plant breeding is a numbers game; bureaucrats are a distasteful and parasitic lot; barley research provides opportunities to meet and enjoy some great colleagues; sometimes one's son will follow in one's barley breeding footsteps.

I'd better set things straight on the last theme first – Dr. J. Harlan (son of Dr. H. Harlan) went on to do great things in plant biology. My son spent one season on the barley summer field crew and discovered that awns and barley itch are not sufficient to entice him into a life of barley science. Harry and I see eye to eye on most of the remaining themes, and certainly when it comes to the taxonomy of bureaucrats (a lower life form related to the amoeba but with a much larger genome comprised entirely of nested retro-elements) and the value of barley

colleagues (inestimable). Of the seven themes, germplasm is the one that may bear some elaboration at this juncture and the one that will allow me to interject a bit of science into an otherwise anecdotal ramble. Looking back on the North American Barley Genome Project's accomplishments, I'd say it is germplasm that has allowed us to make good, and innovative, use of technology.

Steptoe, Morex, Dicktoo, Calicuchima, Shyri, Gobernadora, Galena, Bowman, Wolfe Barley Dominant and Recessive, 88Ab536 – these are all great genetic stocks that are testimony to the skill, persistence, and good fortune of their developers. Other stocks of equal importance have of course been developed by barley researchers around the world. However, as I am most familiar with the North American Barley Genome Project stocks, I'll focus the remainder of the discussion on this germplasm base. These genetic stocks have served as parents of linkage and QTL mapping populations and one or more serve as the basis for an arsenal of genomics tools, including large insert libraries (e.g. the Morex BAC library) and microarrays (e.g. the Morez Affymetrix chip). There is always a delicate balance between choosing (and staying with) a genetic model and applying genetics to the constantly shifting germplasm arrays present in a breeding program. In our breeding program at Oregon State University, I've been fortunate to have the best of both worlds - our "foundation" germplasm for malting quality and winter hardiness happens to have Morex – the "Chinese Spring" of barley genetics – as a backcross parent.

This combination of agronomic relevance and genetic utility has made integrated breeding and genetics a process of fascinating discovery and, optimistically, utility. 88Ab536 was developed by Dr. D. Wesenberg (USDA/ARS, retired). 88Ab536 is derived from the cross of a Nebraska winter feed barley selection backcrossed to Morex; Darrell selected backcross progeny after a particularly severe winter at Aberdeen, Idaho (USA). The selection 88Ab536 went on to become the first U.S. winter barley to pass the American Malting Barley Association (AMBA) pilot program. Unfortunately 88Ab536 - like many an otherwise noble and promising selection - was dropped during plant scale testing due to concerns regarding flavor. In any event, the malting quality profile – particularly the high diastatic power sought by U.S. brewers – of 88Ab536 is quite good, and this selection produces a reasonable quality profile even when sorely afflicted by biotic and/or abiotic stresses. Agronomically speaking, 88Ab536 has an acceptable level of cold tolerance; it is very susceptible to stripe rust, scald, net blotch, and BYDV; and it has low yield potential. Physiologically, 88Ab536 is a curiosity since it has an acceptable level of cold tolerance but it is not photoperiod sensitive and it does not have a vernalization requirement. At the VIII IBGS we proposed the idea of a "winter malting quality footprint" based on 88Ab536, since one could surmise that all favorable quality genes would be contributed by Morex and all winter hardiness genes would be contributed by the Nebraska feed parent.

In order to define the "first footprint", we used the Simple Sequence Repeat (SSR) markers developed by the Scottish Crop Research Institute (SCRI), and the bin map assignments for QTL reported by HAYES *et al.* (2003). Our first footprint was intuitively appealing – Morex alleles were detected in ~ 75% of the genome (on a linkage map basis) at all malting quality QTL regions. 88Ab536 is notable for its high diastatic power, and based on a molecular marker in the *Bmy1* gene, we inferred that the allele for this key determinant of diastatic power traced to Morex. The Nebraska winter feed parent alleles were found on chromosome 5H in the region where low temperature tolerance, vernalization response, and photoperiod sensitivity QTL have been reported in various germplasm combinations in barley and wheat (FRANCIA *et al.* 2003; YAN *et al.* 2003). We could not, however, explain why 88Ab536 is cold tolerant but does not have a vernalization requirement and is not photoperiod sensitive. We

hypothesized that there had been a crossover between the tightly linked vernalization, photoperiod, and cold tolerance loci on 5H.

Recent progress in gene isolation and characterization of allelic diversity has provided new and intriguing insights into the genomic architecture of 88Ab536 that may explain why it is such a unique germplasm accession. In cooperation with Dr. C. Henson we recently proposed a solution to the conundrum that feed barley varieties (and *Hordeum vulgare* subsp. *spontaneum)* contribute favorable QTL alleles for diastatic power at a position coincident with the *Bmy1* locus on the long arm of chromosome 4H (CLARK *et al.* 2003). These unheralded donors of favorable malting quality alleles share single nucleotide polymorphisms (SNPs) at three positions in the coding region of the gene that lead to a more thermostable and active form of the enzyme. In order to understand the allele architecture of the variety Strider, which has extraordinarily low diastatic power, and is the partner of 88Ab536 in the parentage of the STAB mapping population, we sequenced the *Bmy1* alleles in Strider and 88Ab536 and found, to our surprise, that 88Ab536 does not have a Morex allele at *Bmy1*. Instead, it has the more thermostable and higher activity allele, which must have originated from the Nebraska feed barley parent! This led to the question, "why had we inferred from the molecular marker data that the *Bmy1* allele in 88Ab536 originated form Morex?" The answer came when we aligned the 88Ab536, Strider, and Morex sequences for the *Bmy1* locus and found that the molecular marker allele amplicon allele sizes for 88Ab536 and Morex differ by a few bp whereas the Strider allele is much smaller. In our first footprint analysis, we were premature in equating a 2 bp difference with identity by descent. Thanks to the sequence alignment, we now know that although the amplicon allele sizes for 88Ab536 and Morex are quite similar, they are due to very different insertion/deletion events.

Coincidentally, we have been cooperating with Dr. J. Dubcovsky in comparative structural analyses of the vernalization (*Vrn*) loci in wheat and barley. The first result of this work was the discovery that 88Ab536 has a functional *VrnH1* allele on 5H. In the course of our C-repeat binding factor (CBF) gene cloning and sequencing work, we determined that a large region on the long arm of chromosome 5H in 88Ab536 traces to the Nebraska winter feed barley parent. The finding that there were no crossovers in the vicinity of *VrnH1* ruled out the loss-of-vernalization/retention-of-cold tolerance-due-to-crossover hypothesis. Per the *Vrn* epistatic interaction model proposed by YAN *et al.* (2003), the *VrnH2* locus on 4H encodes a repressor of the *VrnH1* locus on 5H, and the effects of this repressor are ameliorated by vernalization. Our next hypothesis, accordingly, was that 88Ab536 has a Morex (non-functional) allele at the *VrnH2* locus. This would, of course, require a crossover between *Bmy1* and *VrnH2*. We proceeded to test this hypothesis by confirmation of SSR alleles in this region, which do indeed trace to Morex. We found further corroboration by comparative allele sequencing at the *Snf2* locus, which is physically linked to the *Vrn2* locus in *Triticum monococcum* (YAN *et al.* 2004). We are currently exploring in greater detail the structural and functional properties of the *VrnH2* locus in 88Ab536 and other important germplasm accessions. As of this writing, we have found that we can amplify the *VrnH2* candidate gene from Strider and Kompolti Korai (varieties with a vernalization requirement) but not from 88Ab536 and Dicktoo (cold tolerant varieties that do not have a vernalization requirement).

Genetically speaking, we have preliminary answers to several puzzling questions – and the answers are non-intuitive. In 88Ab536, the high diastatic power contributed by *Bmy1* traces to the winter parent and the growth habit and winter hardiness are attributable to contrasting "winter" and "spring" alleles at the *VrnH1* and *VrnH2* loci, respectively. This analysis of the genomic architecture of 88Ab536 also leads us to hypothesize that hyper-susceptibility of

88Ab536 to stripe rust may be due to its Morex allele at a major stripe rust resistance QTL which is ~ 15 cM proximal to *VrnH2* (CASTRO *et al.* 2003). Morex is super susceptible to stripe rust and replacement of this region of the genome with QTL allele tracing to our quantitatively resistant spring barley lines has led to acceptable levels of quantitative resistance in a malting quality six-row background (MARQUEZ-CEDILLO *et al.* 2003).

This genetics information has given us a better understanding of 88Ab536, a foundation of germplasm in our breeding program. A next, and intriguing, step to take will be an expression footprint of 88Ab536 using the Morex-based Affymetrix chip. Genetics has, in this case, been at the service of plant breeding: our current strategy is to select in cross progeny for the 88Ab536 alleles in the 5H winterhardiness cluster region and the long arm of 4H from *VrnH2* to *Bmy1*. Proximal to *VrnH2*, we will introgress a stripe rust resistance QTL allele. As the "malting quality footprint" comes into better focus, we anticipate continued progress in fixing favorable alleles in our breeding program and continually introgressing novel alleles from other germplasm. These plans for the future and applications of technology are possible thanks to the germplasm.

A unique attribute of the barley research, production, processing and consuming communities is their simultaneous appreciation for pure science and its applications. As Harlan observed, "At the moment, the field of theoretical genetics may be as far removed from breeding as astronomy, but workers in that field have the possibility of uncovering the mother lode". There are gold mines out there to be discovered and exploited. However, the disturbing trend towards absolute and uncompromising intellectual property protection leads to constrained germplasm, technology, and data exchange. Let us not fall into the trap of the "Treasure of the Sierra Madre" such that our greed for gold finally leads us to dust. To quote Harlan a final time "Tucked away in the hills of China or Nepal may be a barley that will one day save the crop of Montana (USA) from a disease we have never seen" (HARLAN 1957). Substitute your country/state/program and breeding challenge – the imperative is to collect, conserve, characterize, use, and share. Germplasm is the base of barley and barley is the base of beer. Na zdravi!

References

ALLARD R.W. (1988). Genetic changes associated with the evolution of adaptedness in cultivated plants and their wild progenitors. J. Hered., **79**: 225-238.

CASTRO A., CAPETTINI F., COREY A., FILICHKINA T., HAYES P.M., KLEINHOFS A., KUDRNA D., RICHARDSON K., SANDOVAL-ISLAS S., ROSSI C., VIVAR H. (2003). Mapping and pyramiding of qualitative and quantitative resistance to stripe rust in barley. Theor. Appl. Genet., **107**: 922-930.

CLARK S.E., HAYES P.M., HENSON C. (2003). Effects of single nucleotide polymorphisms in Beta-amylase 1 alleles from barley on functional properties of the enzymes. Plant Phys. and Biochem., **41**: 798-804.

HARLAN H.V. (1957). One man's life with barley. Exposition Press, New York.

MARQUEZ-CEDILLO L., CAPETTINI F., COREY A., HAYES P.M., SANDOVAL-ISLAS S. (2003): Validation of barley stripe rust resistance QTLs. PAG XI. San Diego, CA. Jan. 11-15.

MATUS I., COREY A., FILICHKIN T., HAYES P.M., KLING J., POWELL W., RIERA-LIZARAZU O., SATO K., VALES M.I., WAUGH R. (2003): Development and

characterization of recombinant chromosome substitution lines (RCSLs) using *Hordeum vulgare* subsp. s*pontaneum* as a source of donor alleles in a *Hordeum vulgare* subsp. *vulgare* background. Genome, **46**: 1010-1023.

FRANCIA E., RIZZA F., CATTIVELLI L., STANCA A.M., GALIBA G., TÓTH B., HAYES P.M., SKINNER J.S., PECCHIONI N. (2003): Two loci on chromosome 5H determine low temperature tolerance in the new 'winter' x 'spring' ('Nure' x 'Tremois') barley map. Theor. Appl. Genet., **108**: 670-680.

HAYES P.M., CASTRO A., MARQUEZ-CEDILLO L., COREY A., HENSON C., JONES B.L., KLING J., MATHER D., MATUS I., ROSSI C., SATO K. (2003): Genetic diversity for quantitatively inherited agronomic and malting quality traits. *In* R. Von Bothmer, H. Knupffer, T. van Hintum, and K. Sato (ed.). Diversity in Barley. Elsevier Science Publishers, Amsterdam.

KLEINHOFS A. (2000): The future of barley genetics. *In* LOGUE, S. (ed.). Barley Genetics VII. Proc. Intl. Symp. Oct. 22-27, 2000. Adelaide, South Australia, Australia. p. 6-12.

NILAN R. (1970): Introduction. *In* NILAN, R. (ed.). Barley Genetics II. Proc. Intl. Symp. July 6-11, 1969. Pullman, Washington, USA.p. i.

SMITH S. (2000): International outlook for barley breeding. *In* LOGUE, S. (ed.). Barley Genetics VII. Proc. Intl. Symp. Oct. 22-27, 2000. Adelaide, South Australia, Australia. p. 3-5.

VON BOTHMER R. (1992): Opening address to the sixth international barley genetics symposium. *In* MUNCK, L. (ed.) Barley Genetics VI, Vol. 2. Proc. Intl. Symp. July 22-27, 1991. Helsingborg, Sweden. p. 655-666.

YAN L., LOUKOIANOV A., TRANQUILLI G., HELGUERA M., FAHIMA T., DUBCOVSKY J. (2003): Positional cloning of the wheat vernalization gene *Vrn1*. Proc. Nat. Acad. Sci. (USA), 100: 6263-6268.

YAN L., LOUKOIANOV A., BLECHL A., TRANQUILLI G., RAMAKRISHNA W., SANMIGUEL P., BENNETZEN J., ECHENIQUE V., DUBCOVSKY J. (2004): The wheat *Vrn2* gene is a flowering repressor downregulated by vernalization. Science. In press.

S 1 - GERMPLASM AND GENETIC RESOURCES

Global Inventory of Barley Genetic Resources

J. Valkoun and J. Konopka

Genetic Resources Unit, International Center for Agricultural Research in the Dry Areas (ICARDA), P.O. Box 5466, Aleppo, Syria

Abstract
The FAO estimates that more than 280,000 accessions of barley genetic resources are conserved in numerous *ex situ* collections. There is no doubt that there is significant overlap of collections but the extent of this overlap is not known. There is no global "one-stop shop" for scientists wishing to find particular germplasm although many genebanks offer Internet access to information on their collections. Genetic Resources Unit (GRU) of ICARDA in collaboration with European Barley Data Base (EBDB) and major barley collections has compiled a Global Inventory of Barley Genetic Resources. The project was supported by the System-wide Information Network for Genetic Resources (SINGER) and is to be published on the Internet in 2004. The Inventory lists more than 176,000 accessions from 42 institutes/genebanks. Approximately 40% of a 'global collection' refers to landraces, collected in the field, or selections from landraces. The inventory identified over 300 collection missions to 57 countries during the period 1921-2001. Whenever the collection site data were sufficiently detailed, the collection sites were geo-referenced to facilitate production of distribution maps and links with GIS. As expected, a significant part of conserved material is the result of breeding efforts and we attempted to cross-reference accessions using standardized names. For large part of breeding material the system also records pedigree, developer and date of release.
Keywords: barley; genetic resources; global inventory; Data Base

Introduction

Barley is a globally important crop that is adapted to marginal and stress-affected environments and is the fourth in importance after wheat, rice and maize,. It is, therefore, of high importance to resource-poor farmers in many developing countries. The breeding strategy for barley crop improvement in the developing world is targeted to low-input stress-affected, mostly subsistence farming systems in highly diverse environments. This differs to that in developed countries where barley may be grown for different purposes using high-input technologies.

In some developing and transitional countries barley is still an important food grain. The consumption per capita in 2001 was 42.1 kg in Morocco, 29.8 kg in Estonia, 21.0 kg in Moldova, 16.7 kg in Latvia, 13.5 kg in Lithuania and Azerbaijan, 12.8 kg in Ethiopia and Libya and 12.4 in Algeria (FAOSTAT 2004). It is also essential food in the highlands of Himalayas and Andes. However, the majority of the world's barley grain production is utilized as animal feed. Barley straw is a valuable feed for small ruminants in the Central and West Asia and North Africa (CWANA) region, which, in dry years, may have a higher price than barley grain. Barley landraces from those countries have soft and highly palatable straw, but this trait is rare or lacking in the modern lodging-resistant varieties.

The International Center for Agricultural Research in the Dry Areas (ICARDA), one of the Centers of the CGIAR system, has a global responsibility for barley improvement with a focus on resource-poor farmers in the developing world. The Center recognized the role of indigenous genetic resources in barley improvement for stress-affected low-input farming systems in the semi-arid regions of CWANA and other parts of the world. ICARDA's breeding for unfavorable environments is based on selection in the target environments. This usually involves selections in farmers' fields, with their participation, using their agronomic practices. This approach revealed that locally adapted landraces could be a useful source of breeding material that would have been missed had the evaluation taken place only in high-yielding environments. The presence of useful diversity within barley landraces has been documented CECCARELLI *et al.* (1987, 1995), VAN LEUR *et al.* and WELTZIEN and FISCHBECK (1990). The wild progenitor of barley, *Hordeum vulgare* L. subsp. *spontaneum* (C. Koch) was particularly useful in breeding for plants that remain tall in very dry years, a trait essential for farmers to enable mechanized harvesting VALKOUN *et al.* (1997).

Detailed information on available genetic resources is particularly important for barley breeding programs for and in developing countries, where selection from locally adapted landraces may be the first logical step. To facilitate the access and encourage use of genetic resources, ICARDA developed the Global Inventory of Barley Genetic Resources, which can serve as a basis for its further improvement through international collaboration of barley documentation specialists, collection curators, breeders and others who are interested.

Material and Methods

This project was done in collaboration with the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany who maintains the European Barley Data Base (EBDB), Information was gathered by direct contact with institutions holding major barley collections such as the USDA, VIR, and the AWCC. In addition to this, during 2004, the database established by the recently completed EURISCO project was consulted and web-enabled databases were interrogated on Internet. The project succeeded in obtaining passport data for 176,509 accessions conserved in 42 collections. The barley Core Collections for Europe, West Asia and North Africa (WANA), Americas, East Asia, and Oceania (total 1,126 accessions) were also included. Six major collections that maintain more than 10,000 accessions are listed below.

Table 1. Major barley collections

Collection/institute	Number of accessions
USDA, USA	30,979
ICARDA, Syria	25,202
VIR, Russia	20,245
IPK, Germany	13,150
AWCC, Australia	12,328
John Innes Center, UK	10,828

The assembled data was standardized in conformity with the Multi Crop Passport Descriptors (MCPD) proposed by IBPGR/FAO. In particular, priority was given to standardization of names and/or numbers identifying accessions and to geo-referencing collection sites.

There is significant overlap between the collections due to independent acquisition of material from the original collectors and breeders and exchange of accessions between collections. It is

imperative for the global inventory to detect such overlap and to indicate to the users in which collections the same or similar material is available. Accessions were cross-referenced using two inter-related approaches:

(i) For material originally collected in the field, the collection sites were assigned unique site codes and accessions were linked to the normalized collection site records. Currently the inventory registers 302 collection missions, which yielded 29,255 accessions from 14,200 sites. An additional 50,000 accessions have collection site data (over 7,800 different sites) but the mission could not be identified due to lack of data (collection date and/or collectors). Whenever sufficient information was available, the collection sites were geo-referenced and thus can be linked to Geographic Information Systems (GIS) for further processing.

(ii) For accessions that originated from crop improvement programs, the combined data on accession names, type of material, breeding organization and pedigree were used to identify same or closely related accessions. It should be noted that the term "accession name" is frequently ambiguous and therefore the project attempted to clarify the meaning of "names" in addition to applying several rules to standardize the names (like use consistent transliteration to Latin, convert to upper case, etc.).

(iii) A register of cultivars, landraces and breeding lines was created and currently contains 43,770 entries linked to over 92,000 accessions. The register lists 8,850 cultivars linked to nearly 40,000 accessions. Breeding lines are less frequently replicated in collections as 23,700 accessions refer to 17,000 research and breeding lines.

Global Inventory of Barley Genetic Resources is constructed as a relational database composed of 10 major inter-related tables:

ACCESSION	main file registering accessions in each collection
IDENTIFIER	file storing all identifying names and numbers for accessions
COLSITE	register of collection sites
COLLECTOR	file with name of collector(s) of each accession
MISSION	register of collecting missions: country(ies), dates and collecting organization(s)
CULTIVAR	register of named accessions: cultivars, landraces, breeding and research lines. Year of release of material, developer and pedigree information is included here
PEDIGREE	file with pedigree information of each accession as documented in source collection
NOTES	remarks as documented in institutes conserving accessions
TAXON	register of Latin names
COOPERATOR	names and addresses of people and organizations involved in collecting, breeding or conserving barley genetic resources

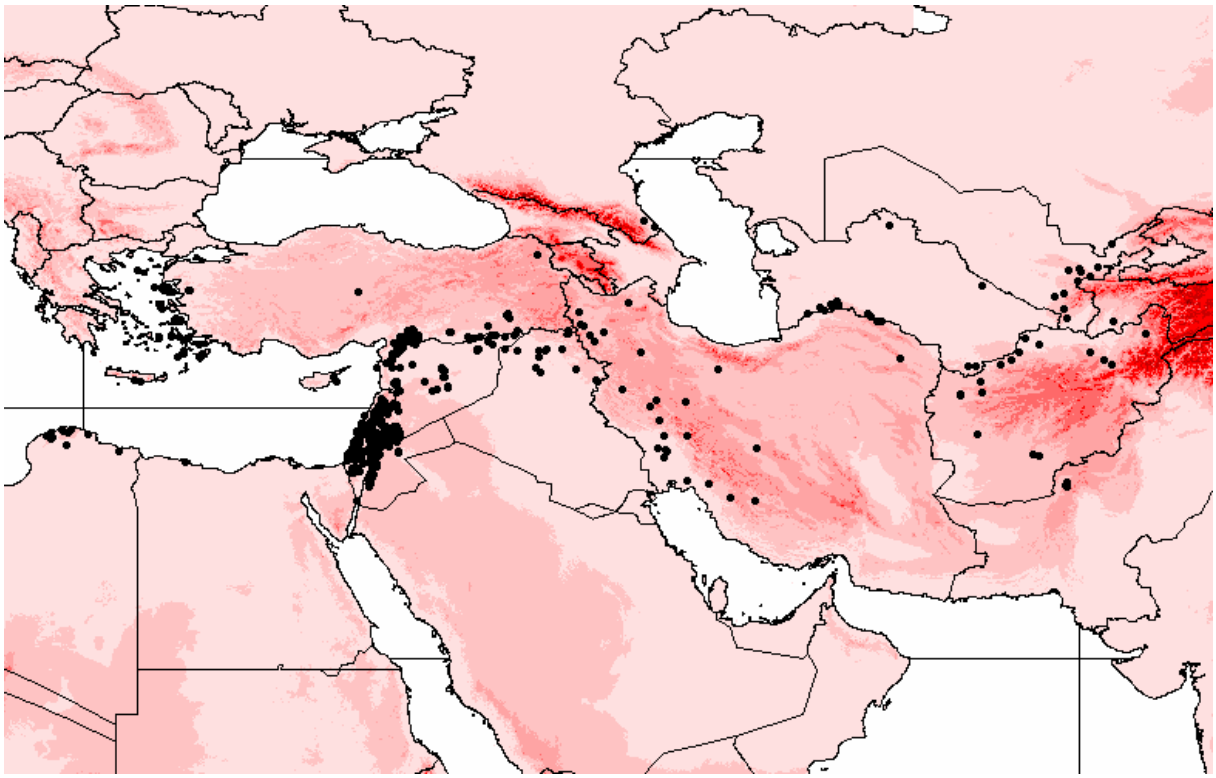
A fully searchable database was developed and is available on CD-ROMs and an internet based version is under development. A number of query forms and reports are available for users to interrogate the database.

Results and Discussion

The barley gene pool, held in ex situ genebank collections worldwide, is the second largest after wheat, with 486,724 accessions (FAO, 1996). However, an earlier estimate of 280,000 accessions by PLUCKNETT et al. (1987) is similar to more recent data reported for 2002 by the FAO's World Information and Early Warning System (WIEWS) on Plant Genetic Resources for Food and Agriculture (PGRFA) that estimates 284,408 barley gene pool accessions are conserved (WIEWS 2004). If we consider the WIEWS number as a more realistic estimate of the barley genetic resources global holdings, then accessions included in the Global Inventory of Barley Genetic Resources represent a significant 62 % of the total. However PLUCKNETT et al. (1987) estimate that only 20% of the barley global collections may be unique accessions.

HARLAN and DE WETT (1971) classified the crop gene pool into primary, secondary and tertiary pools, according to their relationships with cultivated species. According to BOTHMER *et al.* (1991), the primary barley gene pool has three major components: cultivates and breeding lines, landraces, and the barley wild progenitor, *Hordeum vulgare* L. subsp. *spontaneum* (C. Koch) Thell. As there are no crossability barriers within the primary gene pool, gene transfer from its three components to adapted cultivars is feasible. A single species, *Hordeum bulbosum* L. belongs to the secondary gene pool. All other *Hordeum* species belong to the secondary pool. The results below are discussed with a focus on the primary gene pool components.

Figure 1. Geographical distribution of *H. vulgare* subsp. *spontaneum* collection sites



The Wild Progenitor

The Global Inventory includes nearly 12,500 accessions of *H. vulgare* subs. *spontaneum* originating from 25 countries. Its geographical distribution is concentrated in the western part of the Fertile Crescent (Figure 1). Some 11,000 accessions were collected from more than more than 1300 sites, of which 789 are geo-referenced.

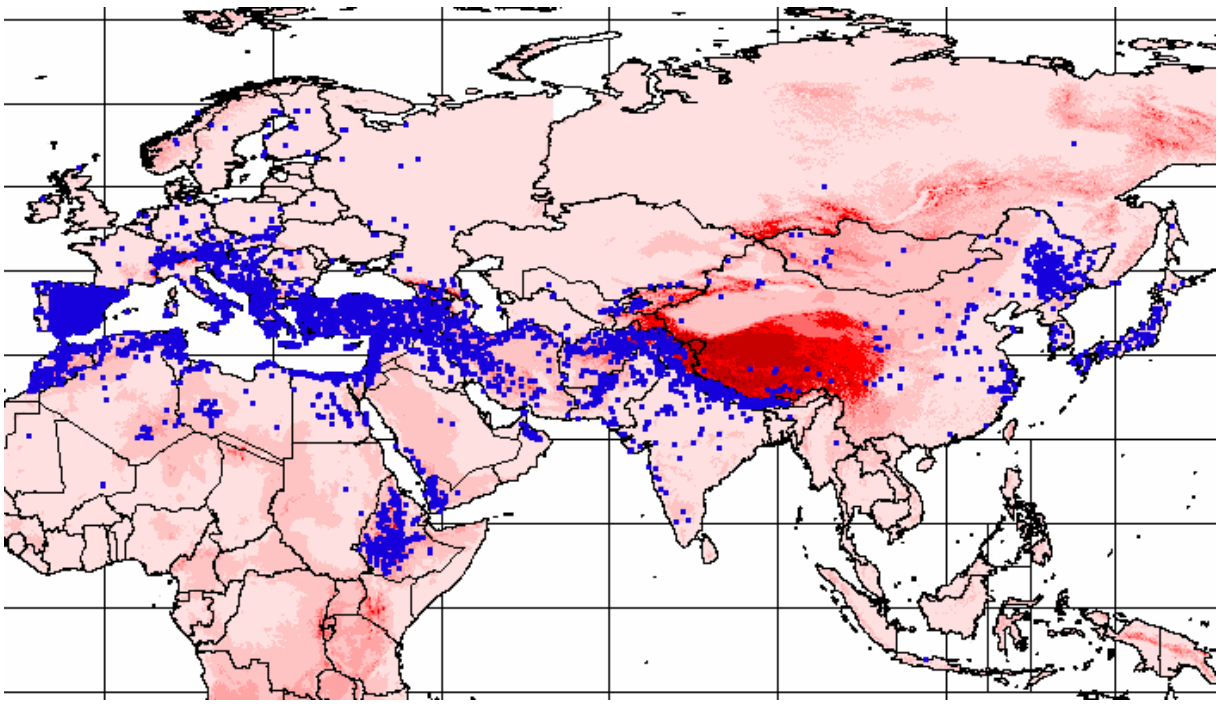
Landraces

Of the total of 129,000 accessions with germplasm type information, 66,000 accessions (51%) are landraces and mostly originate from a developing part of the world (Table 2 and Figure 2).

Table 2. Most frequent origin of barley landraces

Origin	***No. accessions***
Ethiopia	*15353*
China	*5966*
Turkey	*5884*
Nepal	*3162*
Switzerland	*2964*
India	*2629*
Pakistan	*2575*
Russia	*2387*
Afghanistan	*1582*
Iran	*1509*
Ukraine	*1275*
Morocco	*1263*
Total	***46549***

Figure 2. Geographical distribution of cultivated barley (mostly landraces) collection sites



For more than 50,000 accessions site information is available and some 30,000 geo-referenced accessions were sampled from nearly 16,000 sites. A total of 13,500 accessions have more than 5,000 names. This could indicate that only 38% are unique, but many of the names just relate to geographical origin and germplasm type. Collection number data show that a high proportion (61%) of landrace accessions may be unique.

Cultivars
In total, 36,173 accessions are classified as cultivars of which 25,291 have pedigree information. Since only 3,774 different pedigrees were identified in the Global Inventory, the percentage of unique accessions for the cultivar category is only 15%, indicating a high

Table 3. Most frequent cultivar accessions origin

Country	***Country code***	***No. accs.***
Germany	*DEU*	*6494*
United States	*USA*	*3644*
Great Britain	*GBR*	*2919*
France	*FRA*	*2598*
Japan	*JPN*	*1909*
Sweden	*SWE*	*1802*
Netherlands	*NLD*	*1286*
Czechoslovakia[1]	*CSK*	*1260*
Denmark	*DNK*	*1238*
Russia	*RUS*	*1137*
Austria	*AUT*	*1113*
	Total	***25400***

[1] Includes former Czechoslovakia, Czech Republic and Slovakia

redundancy in the genebanks. If only accession names are considered, then more than 91 % of 36,173 cultivar accessions are duplications or multiplications. Contrary to landraces, improved cultivars have their origin in developed countries with a long history of barley breeding (Table 3). The twelve countries listed in the Table 3 developed more than 70% of cultivar accessions held in genebanks worldwide. As expected, many cultivar accessions have moved from one genebank to another and, as a result, there are many multiple copies held in genebank collections (Table 4).

Table 4. The top cultivar multiplications

Name	***Pedigree***	***Country code***	***No. accs.***
Isaria	*Danubia/Bavaria*	*DEU*	*48*
Kenia	*Binder/Gull*	*DNK*	*46*
Binder	*Selection from Hanna*	*DNK*	*44*
Tschermaks	*(Kirsches Winter/Kirsches 2-Row)/(4-Row Winter/Heines Giant Winter)*	*AUT*	*41*
Wisa	*(Weihenst.MR I/Breun IN 2511)/Isaria*	*DEU*	*39*
Triumpf	*Diamant/Hadm. 14029/64/6 ((Alsa/S3170/Abyss)/11719/59)/Union*	*DEU*	*38*
Union	*(Weihenst.MR II/Donaria)/Firl. 621*	*DEU*	*38*

Breeding (Unfinished) Material
19,000 accessions, which represent a significant part of the barley global holdings, are breeding (unfinished) materials. According to their pedigrees, the level of multiplications is much lower than in cultivars, since 66 % of accessions of this category are unique.

Other Wild Barley Species
1,351 accessions of wild barley species of the secondary and tertiary gene pool are represented in the Global Inventory. Among them, *Hordeum bulbosum* L., *Hordeum murinum* L. and *Hordeum marinum* Huds were the most frequent with 328, 223 and 137 accessions respectively.

Future Developments
The Global Inventory of Barley Genetic Resources brings more insight into the extensive barley collections held in 42 major genebank worldwide. However, it needs to be extended to cover other major barley collections from Canada, Brazil (EMBRAPA), China, Iran and Korea. The database needs to be periodically updated – the facility to upload the fresh data by Internet is planned. Ideally, future developments should be guided by the International Barley Genetic Resources Network and be fully coordinated with other global systems for barley, mainly the International Barley Information System (IBIS). IBIS is being developed at ICARDA and targets barley germplasm in breeding programs.

Acknowledgements
The authors gratefully acknowledge the financial support provided to the project by the CGIAR System-wide Genetic Resources Program (SGRP) through a small grant. They also wish to thank Drs. D. Enneking and H. Knüpffer, IPK, Gatersleben, Germany for their collaboration during the development of this Global Inventory. The contribution of all genebank data donors is also gratefully acknowledged. Finally, we thank Dr. Ken Street, ICARDA, for the critical reading of this manuscript.

References

BOTHMER R. VON, JACOBSEN N., BADEN C., JORGENSEN R.B., LINDE-LAURSEN I. (1991): An ecogeographical study of the genus . Systematic and ecogeographic studies on crop genepools No. 7. International Board for Plant Genetic Resources, Rome, Italy.

CECCARELLI S., GRANDO S., VAN LEUR, J.A.G. (1987): Genetic diversity in barley landraces from Syria and Jordan. Euphytica, **36**: 389-405.

CECCARELLI S., GRANDO S., VAN LEUR, J.A.G. (1995): Understanding landraces: The Fertile Crescent's barley provide lessons to plant breeders. Diversity, **11**: 112-113.

FAOSTAT. (2004): http://faostat.fao.org/default.jsp?language=EN

HARLAN J.R., DE WETT J.M.J. (1971): Towards a rational classification of cultivated plants. Taxon, **20**: 509-517.

PLUCKNETT D.L., SMITH N.J.H., WILLIAMS J.T., MURTI-ANISHETTY N. (1987): Genebanks and the world's food. Princeton University Press, Princeton, NJ, USA.

VALKOUN J., CECCARELLI S., KONOPKA J. (1997): Barley. In: FUCILLO D., SEARS L. STAPLETON P. (eds.): Biodiversity in trust. Cambridge University Press, Cambridge: 191-212.

VAN LEUR, J.A.G., CECARELLI S., GRANDO S. (1989): Diversity for disease resistance in barley landraces from Syria and Jordan. Plant. Breed., **103**: 324-335.

WELTZIEN E., FISCHBECK G. (1990): Performance and variability of local barley landraces in near-eastern environments. Plant. Breed., **104**: 58-67.

WIEWS. (2004): http://apps3.fao.org/wiews/

Assessment of Diversity of Czech Barley Cultivars as Revealed by PCR Markers, SSR, AFLP and DNA Assays

J. Ovesná[1], L. Kučera[1], K. Poláková[1], K. Vaculová[2] and J. Milotová[2]

[1]Research Institute of Crop Production, Drnovská 507, 161 06 Prague 6 – Ruzyně, Czech Republic, e-mail ovesna@vurv.cz;
[2]Agricultural Research Institute Kroměříž, Ltd., Havlíčkova 2787, 76701 Kroměříž, Czech Republic

Abstract
We used a set of DNA markers to characterise malting barley cultivars used in the Czech Republic in the last century. SSR and AFLP markers revealed a shift in the genetic basis of currently used cultivars in comparison with obsolete cultivars. Principal component analysis showed that the gene-pool in use has narrower genetic basis. The results are supported by the analysis of the data released from repetitive field trials. TDI-FRET based analysis of β-amylase alleles has led to discovery of highly thermostable allele Sd2H in modern local adopted malting barley cultivar ´Malvaz´, which can be used further in breeding programmes. Other thermostable alleles Sd3 were found in old genetic resources. The results show that molecular analysis can reveal increased value of genetic resources.
Keywords: barley; genetic resources; malting quality; DNA markers; SSR; AFLP

Introduction

Barley (*Hordeum vulgare* L.) belongs to the most important small grain cereals. It is used for animal feeding, human nutrition and namely for malt production. High malting varieties are required by breweries and distilling industry. Barley improvement and deliberated breeding has a long history world-wide. Generally, it is possible to speak about breeding programmes oriented to individual agro-ecological regions combining good adaptability, yield stability and end-use quality.

Several different micro-regions are found in the Czech Republic with variable soil-climatic conditions in which specific landraces were developed (LEKEŠ 1961). Malting quality, however, has been the most important trait along with adaptability. To reach superior quality and good yield potential, usually genotypes of domestic origin have been combined with highly yielding foreign lines or donors of resistance against pathogens by Czech breeders.

Already at the end of 19th century a Moravian centre led by E. Proskowetz was formed producing first registered spring malting barley cultivars, e.g. highly malting cv. ´Proskowetzův Haná pedigree´ registered in 1884. Cultivars descending from this group of cultivars representing so called "Starohanácký" type of malting barley affected Czech and in some extent European barley breeding in the last century (LEKEŠ 1997). Selection and multiplication of best performing genotypes were used by breeders followed afterwards by individual selection. The use of deliberate crossing on the basis of Mendel laws resulted in release of superior malting cultivar ´Opavský Kneiflův´ in 1926. ´Opavský Kneiflův´ was used in Czech region and in Germany as desirable germplasm. A new generation of cultivars was released after World War II with the introduction of improved breeding technologies including mutational breeding. ´Valtický´ and its short-straw mutant ´Diamant´ are the most known examples.

In the following period domestic breeding mostly used local germplasm which was crossed with lines/cultivars carrying resistance genes to improve malting barley.

We used DNA technology based marker to follow the effect of breeding process and its impact on Czech malting barley gene pool diversity. It was shown that DNA markers can be successfully used for such task (MATUS & HAYES 2002). Namely SSR and AFLP markers reveal polymorphism on DNA level efficiently (ALLABY & BROWN 2003). Other marker types monitoring incidence of certain alleles are available as well (HOFFMANN & DAHLEEN 2002; PARIS *et al*. 2002; WENZEL *et al.* in press). We aimed to find correlation between marker incidence in genotype and presence of selected traits in phenotypes.

Material and Methods

Plant Material

80 barley obsolete and registered cultivars mostly of Czech origin were tested. Seeds were obtained from the Genebank of the Research Institute of Crop Production Prague–Ruzyně, Czech Republic. Data from field evaluation of biological, morphological and agronomic important traits, obtained from barley gene resources database IS-EVIGEZ, were collected during the last years for spring barley at the Agricultural Research Institute Kromeriz, Ltd. and for winter barley at Prague and Kromeriz (vegetation period in days = SOW_RIPP, vegetation period sowing-heading in days=SOWHEAD, plant height in cm = HEIGHT, resistance to leaf diseases in scale 9 = best to 1=worst – mildew = BLUMGRAM, net blotch = PYRENTER, leaf rust=PUCCHORD, grain yield in $t.ha^{-1}$=GRAINYIE, thousand grain weight in g = TGW, number of spikes = PRODTILL, number of grains per spike = GRAINEAR, grain weight per spike in g = GWEAR, seeds over sieve 2.5 mm = GRADING, crude protein content in %=N, malt extract content in % = EXT_MALT, chemical substances measured at NIR device: protein content = N_NIR, starch content = STARCH_NIR, fat content = FAT_NIR, fiber content = FIBER_NIR, extract content = EXT_NIR). List of the barley materials tested is given in Table 1.

Table 1. List of investigated barley materials

Cultivar	Years of registration*	Country of origin	Cultivar	Years of registration	Country of origin
Akcent	1992	CSK	Lumar	1995	CZE
Ametyst	1972-1979	CSK	Luran	1998	CZE
Amulet	1995	CZE	Luxor	1996	CZE
Atlas	1976-1981	CSK	Malvaz	1989-2004	CSK
Atribut	1996	CZE	Nitranský Export	1936-?	CSK
Branišovický C	1959-1970	CSK	Nolčův Bohemia	1900-?	CSK
Bučianský (Kneifl)	1946-1956	CSK	Novum	1988	CSK
Ćelechovický Hanácký	1956-1963	CSK	Okal	1992	CSK
Denár	1969-1976	CSK	Olbram	1996	CZE
Dětenický	1940-?	CSK	Opál	1980-1986	CSK
Diamant	1965-1976	CSK	Opavský Kneifl	1926-1960	CSK
Diosecký 102	1930-?	CSK	Perry	?	USA
Diosecký Kneifl	1938-?	CSK	Perun	1987-2003	CSK
Diosecký Sprinter	1946-?	CSK	Primus	1995	CZE
Dregerův	1900-?		Profit	1988	CSK
Dukát	1971-1976	CSK	**Proskowetzův Haná pedigree**	**1884-1958**	CSK
Ekonom	1960-1972	CSK	Radošínský Sladár	1934	CSK
Fatran	1980-1989	CSK	Rapid	1976-1983	CSK
Forum	1993	CSK	Ratbořský	1926-?	CSK
Hana	1973-1979	CSK	RTG Valtický	1965	CSK
Hana II	1904-?		Rubin	1982-2003	CSK

Hanácký Export	1935-?	CSK	Scarlett	1997	DEU
Hanácký Staroveský	1900-?		Selekční Hanácký	1926-?	CSK
Hanácký Jubilejní	1938-1956	CSK	Semčický pivovar	1920-?	CSK
Harrington	1983	CAN	Sladár	1967-1976	CSK
Horal	1982-1997	CSK	Sladko	1992	CSK
Hořický	1929-?	CSK	Slovenský 802	1942-1967	CSK
Chlumecký	1904-?		Slovenský Dunajský Trh	1946-1969	CSK
Jantar	1966-1973	CSK	Stabil	1993	SVK
Jarek	1987	CSK	Stupický Hanácky	1926-1962	CSK
Jaspis	1986-1997	CSK	Šumavský	1920-?	CSK
Jindřichovický K 64	1937-?	CSK	Tepelský 421	1930-?	CSK
Jubilant	1991	CSK	Terno	1991	CSK
KM 1192	not regist.	CSK	Tolar	1997	CZE
Kompakt	1995	SVK	Triumf	1938-1960	CSK
Krajová Starý Hrozenkov	1900-?		Valtický	1930-1976	CSK
Krajová z Orlové	not regist.		Viglašský Polojemný	40‘s	CSK
Kredit	1984-1995	CSK	Viktor	1994	CZE
Krona	1996	DEU	Zborovský Kargyn	1919-?	CSK
Krystal	1981-2003	CSK	Zefír	1981-1988	CSK

Legend:

*- ? – end of registration is not known,
- not regist. – landraces from the barley collection or new breeding lines not registered yet (namely beginning with KM),
- CSK - Czechoslovakia, CZE - Czech Republic, SVK - Slovakia, DEU - Germany

DNA Isolation

DNAs were isolated from 14 days old plants, according to SAGHAI-MAROOF *et al*. (1984), at least 30 plants were pooled per sample.

Microsatelite Analysis

27 microsatellites primer pairs were used in our investigation (BECKER & HEUN 1995; LIU *et al.* 1996). The forward primers were fluorescently labelled. PCR was carried our as described by authors. The amplification products were separated using Applied Biosystem ABI PRISM 310 Genetic Analyzer.

AFLP

Restriction and pre-selective amplification were performed according to Perkin-Elmer Protocol (Part number 402083, Rev.A, 1995) using EcoRI and MseI restriction enzymes. Altogether 21 primer combinations with 3/3 selective nucleotides were finally used for genotyping. Amplification products were separated using Applied Biosystem Generic Analyzer ABI PRISM 310. Results were evaluated using GeneScan and Genotyper software.

Data Analysis

For each accession, a binary matrix reflecting specific band presence (1) or absence (0) was generated. Pairwise distances between the accessions based on Hamman similarity metrics were calculated (KUČERA *et al.* 2004). UPGMA-clustering and principal component analysis were conducted using the statistical software package STATISTICA (StatSoft, Inc.). Diversity index was calculated as proposed by DAHLEEN (1997).

β-Amylase-1 SNP Analysis
Analysis was performed as described by POLÁKOVÁ *et al.* (2003) using the TDI-FRET technology (CHEN *et al.* 1997) based on ABI PRISM 7700 platform for cSNP genotyping assay. For sequence differences see Table 2.

Table 2. The cSNPs distinguishing the four allelic forms of *Bmy*1 identified by PARIS *et al.* (2002)

SNP^{495}	SNP^{698}	Allele	Thermostability
C	T	Sd2L	Low
G	T	Sd1	Intermediate
C	C	Sd2H	High
G	C	Sd3	High

Amplicon QTL-Specific PCR Primers
Primers published by LEE and PENNER (1997) were used for characterisation of genetic resources. For exact procedure see OVESNA *et al*. (2001).

DArT Technology
For details and procedure refer to WENZEL *et al*. (in press).

Results and Discussion
It is known that selection and breeding have led to changes in the genetic basis of currently used cultivars in comparison with those used earlier. Modern cultivars of malting barley are highly yielding, many malting quality associated traits have been improved as it has been shown in comparative field trials and malting quality tests (data not presented here). To trace the underlying changes on DNA level a set of 80 accessions representing cultivars and breeding materials grown in Czech region in the last century was investigated.
We have generated data on microsatellite (SSR) and AFLP polymorphism. Out of 47 tested microsatellite primer pairs 27 were found suitable for cultivar identification showing sufficient level of polymorphism. Using these primer pairs, 82 different polymorphic products were recorded across the 80 accessions. Cultivar specific DNA profiles were generated using these data.
Subsequently we surveyed 123 AFLP primer combinations. Out of them 21 were highly polymorphic (more than 8 polymorphic products per primer combination across a core set of 12 cultivars representing different periods). Using these 21 selective primer pairs 146 different products were recorded across the 80 accessions.
We observed that several amplification products, which may represent certain alleles, were typical for cultivars used in certain periods. Some amplification products were found in modern cultivars, others were amplified only in obsolete cultivars.
To assess degree of diversity and association among cultivars cluster analysis and principal component analysis were used. As input data for statistic analysis Hamman metrics was used as it was shown that Hamman metrics is the best for resemblance coefficient calculations (KUČERA *et al*. (in preparation).
SSR and AFLP data were used separately. In both cases, winter cultivars, which were used as out-layers, formed a separate cluster. Spring cultivars were divided into several groups, modern cultivars possess higher degree of similarity to each other in comparison with obsolete cultivars. SSR data based clustering and AFLP based clustering differed in some extend reflecting probably the nature of marker types. DNA markers are not always well dispersed throughout the genome. They are often clustered in some regions of chromosome. The statistical power of divergence measures estimates may be therefore reduced.

We selected afterward markers with a better fit with genetic proximity calculations that brought better characterisation of analysed genetic resources. If we excluded inconsistent markers – e.g. 24 markers out of 146 in case of AFLP - (KUČERA *et al.* (in preparation) better fit between SSR data based clustering and AFLP based clustering was observed. Inconsistent markers elimination increased the product moment correlation coefficients r = 0.420. Field data from three years trials (data not present here) representing twelve traits were analysed and association among 41 cultivars (Fig. 1) demonstrate differences between obsolete and currently used cultivars. This clustering is in compliance with clustering based on DNA markers.

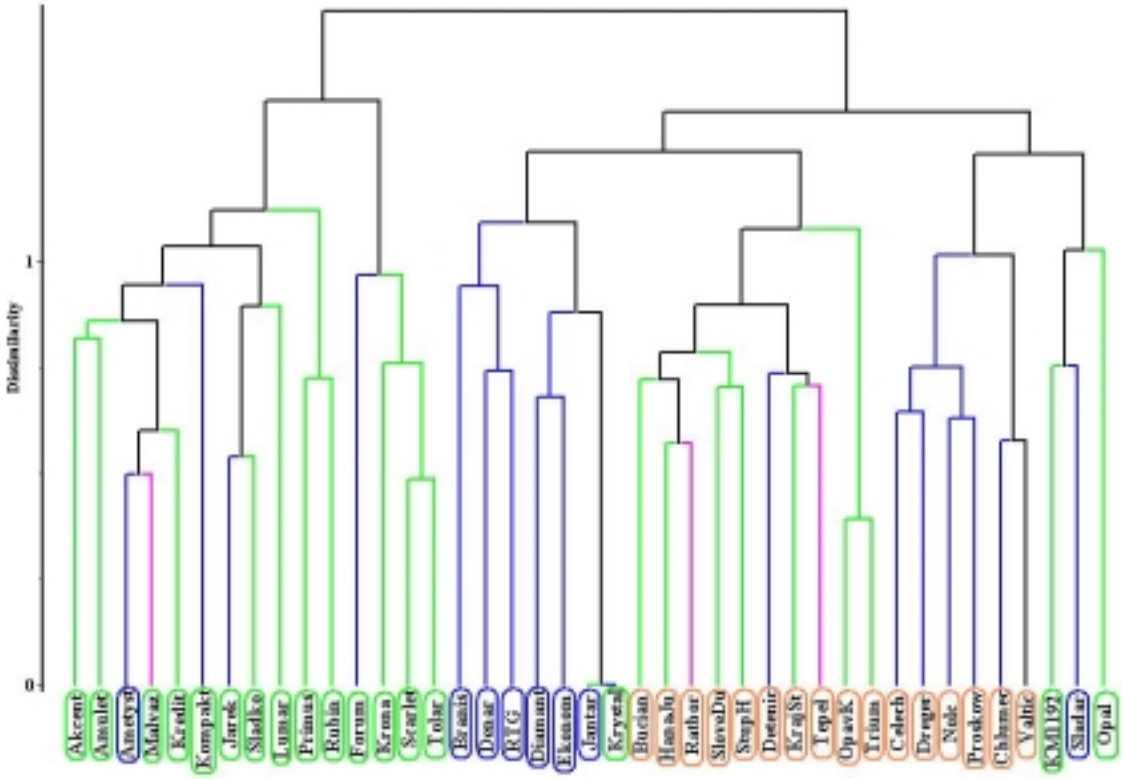


Figure 1. Dendrogram showing association among barley cultivars as reveal by analysis of field trials data. Cultivars used in the 90s and 80s are marked by green , 70s and 60s are marked blue circle and older cultivars are marked by orange. Lines colours in the dendrogram represent presence of Sd1 *Bmy1* allele (green line), Sd2L *Bmy1* allele (blue line) or Sd2H and Sd3 (purple line).

We decided also to use another technology which probably should be more powerful for cultivars profilling - DArT technology. It uses genome complexity reduction alike AFLP. Slightly different clustering of analysed genotypes was observed also in this case.
Even if approaches of diversity measures based on different marker system are not fully consistent, it is apparent that genetic basis of cultivars has changed over time also in the Czech Republic and genetic pool of modern cultivars is narrower. According to the pedigree information, two thirds of the evaluated accessions have cv. ´Diamant´ in their pedigrees. ´Diamant´, a short-straw mutant of cv. ´Valtický´, has shown a significant impact also on the European barley breeding (GRANNER et al. 2000). Inbreeding and combination of certain cultivars and breeding lines were frequently explored in Czechoslovakia. Moreover a special system of cross-examinations of the breeding lines among breeding station was in place. These are the facts, which should be taken into consideration. The effect of selection for end-

use quality on genetic diversity was revealed also by other authors who investigate barley germplasm in other regions (GRANER *et al.* 2003).

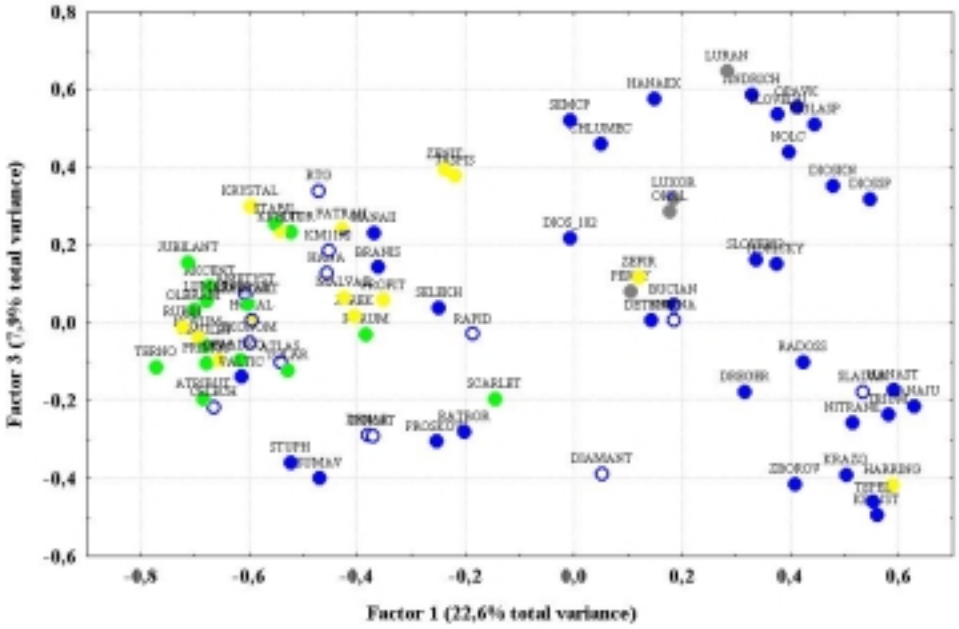


Figure 2. Association among analysed genotypes based on SSR and AFLP markers illustrate narrow genetic basis of modern Czech cultivars

- Cultivars released in the 90s
- Cultivars released in the 80s
- Cultivars released after World War II
- Cultivars released before World War II
- Winter cultivars

Brewing industry uses certain barley cultivars for malt production. Excellent malting quality is underlined by several genes and QTLs. We were interested, whether it is possible to trace association between known DNA markers and desirable traits in Czech malting barleys and whether the markers can be used for monitoring of the breeding process. Identification of markers of alleles which confer improved quality would facilitate its manipulation through contemporary improvement schemes.

Thermostability of β-amylase is one of the important traits. High thermostability in relation with high malt fermentability (the yield of fermentable sugars produced from the hydrolysis of starch). Fermentability affects the level of alcohol produced and is a critical parameter for brewing. Thus the ability to select for barley β-amylase with enhanced thermostability would be highly desirable (BARR *et al.* 1999). Even for such a simple trait, the best marker should detect the actual mutation that confers higher thermostability. Using TDE-FRET assay we found 48% analysed accessions to posses Sd1 allele (intermediate thermostability), 39% analysed accessions to posses Sd2L alleles (low thermostability), both alleles were found in 5% of analysed accessions. It is important to note that Sd2H allele (high thermostability) was found in adopted malting cv. ´Malvaz´. This cultivar can be therefore recommended to breeders as a suitable donor of Sd2H allele instead of e.g. Japanese cultivar ´Haruna Nijo´ with many undesirable agronomic traits. Cv. ´Malvaz´ is more over medium resistant to BYDV (OVESNA *et al.* 2000). Following pedigree of ´Malvaz´, exploitation of exotic germplasm was detected. The leaf disease resitance of variety Malvaz (especially against *Blumeria graminis*) was adopted from two primitive gene resources of barley indicated as ´CI

9588´ *(H. vulgare*, subsp. *distichon*, var. nigrescens Korn) and ´CI 7672´ (*H. vulgare*, susp. *vulgare*, var. *breviaristatum*, Mansf.) from Ethiopia and India respectively (BRÜCKNER 1990). The Sd2H allele with high β-amylase termostability could be transferred from these parents. Beside that we found also other high thermostable allele Sd3 in older Czech cultivars. The allele can be thus more easily introduced into new breeding lines.
Incidence of low, intermediate and high thermostable alleles in cultivars released in different period is shown in Fig. 3. Sd1 allele (intermedium thermostability) was detected namely in cultivars with very good malting quality, especially those with cvs. ´Valtický´ and ´Diamant´ in the pedigree. Incidence of Sd1 allele and Sd2L with cultivar clustering according to field data was proved (Fig. 1).
We have proved in our investigation, that amplicon specific primers (STS-PCR) developed for evaluation of Harrington/TR303 progenies and related genotypes can differentiate also among lines and cultivars used in our region. Namely *Hor2* and α-*Amy1* markers were informative (Fig. 3). Incidence of different alleles in cultivars grown in different periods is changing.

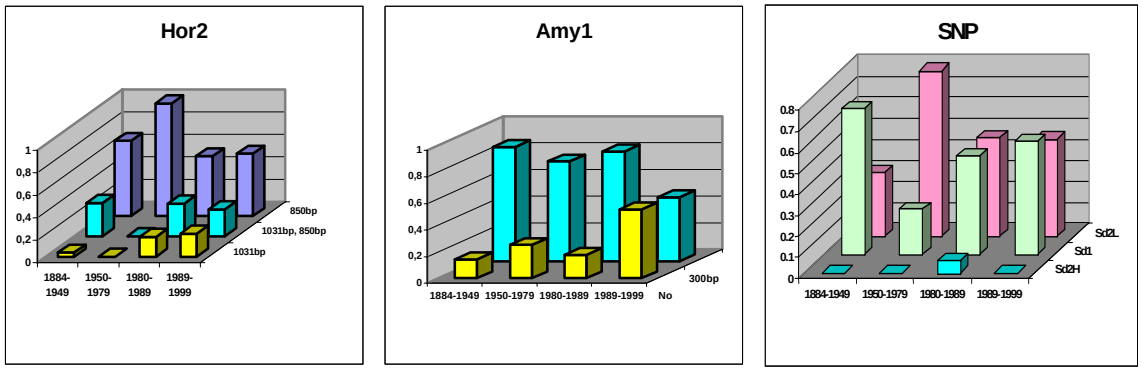


Figure 3. Incidence of different Hordein (*Hor2*), α-amylase (*Amy*1) and β-*amylase 1* (SNP) allele in malting cultivars released in several decades of the last century in the Czech Republic

We evaluated further possible coincidence between several marker allele (e.g size of AFLP or SSR amplification product) appearance and trait values using two-way join analysis. Two-way join analysis and its graphical representation is one of clustering methods. It is not frequently used. Two-way joining is useful in circumstances when one expects that both cases and variables will simultaneously contribute to the uncovering of meaningful patterns of clusters. For example, the researcher may want to identify clusters of individuals that are similar with regard to particular clusters of similar measures of selected traits.
Several marker alleles were found to be really associated with certain trait values to some extends. E.g. presence of AFLP marker allele named NewVar51 is typical for modern cultivars represented by ´Akcent´, ´Ametyst´, ´Rubín´, ´Amulet´, ´Lumar´, ´Kompakt´, ´Sladko´ (Figure 4 and Table 2.). These cultivars are shorter, with improved yield components represent other examples (e.g. ´TGW´, ´GRADING´, Fig. 4). We can conclude that indeed it is possible to reveal trends in the breeding by DNA markers.
Conservation biology emphasizes the negative impact of modern agriculture on genetic variability of the gene pool under cultivation. During the breeding process undesired alleles are removed from the gene-pool. However many alleles are present in old adapted local germplasm. Alleles need to be discovered and genetic resources offered to local breeders sometime instead of exotic locally non adapted germplasm. Thus the value of genetic resources can be explored.

Figure 4. Two-way joining analysis show coincidence between marker and trait forms in some analysed cultivars

Table 3. Analysis of variance between marker NewVar51 and studied traits

Traits	Analysis of Variance							
	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
SOW_RIPP	11.12	1	11.12	44.66	39	1.15	9.71	0.00
SOWHEAD	15.59	1	15.59	122.10	35	3.49	4.47	0.04
HEIGHT	641.17	1	641.17	2051.54	39	52.60	12.19	0.00
LODGING	35.39	1	35.39	169.94	39	4.36	8.12	0.01
BLUMGRAM	0.83	1	0.83	78.86	39	2.02	0.41	0.53
PYRENTER	1.32	1	1.32	30.85	39	0.79	1.67	0.20
PUCCHORD	0.94	1	0.94	15.07	39	0.39	2.44	0.13
GRAINYIE	12.12	1	12.12	28.68	39	0.74	16.48	0.00
TGW	81.27	1	81.27	299.21	39	7.67	10.59	0.00
PRODTILL	609.63	1	609.63	338079.16	39	8668.70	0.07	0.79
GRAINEAR	2.23	1	2.23	124.89	39	3.20	0.70	0.41
GWEAR	0.07	1	0.07	0.85	39	0.02	3.20	0.08
GRADING	1469.19	1	1469.19	5235.70	39	134.25	10.94	0.00
N	5.13	1	5.13	31.32	35	0.89	5.73	0.02
EXT_MALT	8.35	1	8.35	94.64	35	2.70	3.09	0.09
N_NIR	2.01	1	2.01	24.84	35	0.71	2.83	0.10
STARCH_NIR	62.88	1	62.88	284.63	35	8.13	7.73	0.01
FAT_NIR	0.09	1	0.09	1.23	35	0.04	2.66	0.11
FIBER_NIR	0.01	1	0.01	7.11	35	0.20	0.05	0.83
EXT_NIR	0.04	1	0.04	251.18	35	7.18	0.01	0.94

Marked effects are significant at $p < 0.05$

Acknowledgements
The work was supported by project of the Ministry of Agriculture of Czech Republic, NAZV No. QC1336, QD1335 and QD1345.

References

ALLABY R.G. and BROWN T.A. (2003): AFLP data and the origins of domesticated crops, Genome, **46:** 448-453.

BARR A.R ROUMELIOTISN S., LOGUE S. J. AND JEFFERIES S.P. (1999): The heritability of malt extract predicted using NIR spectroscopy ed. LOGUE S: Proc. 9th Australian Barley Technical Symposium.

BRÜCKNER F. (1990): Vyšlechtění sladovnického ječmene odrůdy Malvaz s rezistencí proti chorobám metodou vzdálené hybridizace. Genetika a šlechtění, **43:** 135.

DRESCHER A., PLEISSNER K.-P., ORDON F. and W. MICHALEK (2000): Genome analysis in barley and its impact on the utilization of genetic resources. *In:* integration of biodiversity and genome technology for crop improvement (K. OONO, T. KOMATSUDA, K. KADOWAKI., D. VAUGHAN, (eds.). National Institute of Agrobiological Resources, Tsukuba, Japan: 49-52.

CHEN X, ZEHNBAUER B, GNIRKE A, KWOK PY (1997): Flurescence energy transfer detection as a homogeneous DNA diagnostic method. Proc. Natl. Acad. Sci, **94:** 10756-10761.

DAHLEEN L.S. (1997): Mapped Clone Sequences Detecting Differences among 28 North American Barley Cultivars. Crop Sci., **37:** 952-957.

GRANER A.A. BJORNSTAD, T. KONISHI and F. ORDON (2003): Molecular diversity of the barley genome. In: R.v. Bothmer, T. v. Hintum, H. Knüpffer, K. Sato (eds.) Diversity in Barley (*Hordeum vulgare* L.), ElsevierScience B.V., Amsterdam, NL: 121-141.

HOFFMAN D and DAHLEEN L. (2002): Markers polymorphic among malting barley (*Hordeum vulgare L.*) cultivars of a narrow gene pool associated with key QTLs. Theor. Appl. Genet., **105:** 544-554.

KUČERA L., POLÁKOVÁ K., LEIŠOVÁ L., OVESNÁ J. (2004): Assessing the utility of SSR and AFLP markers for measures of genetic proximity in barley and wheat collections *manuscript in preparation.*

LEKEŠ J. (1961): Origin of Czech malting barley cultivars and their impact on breeding abroad (*in Czech*). Slezský ústav ČSAV, Opava, Czechoslovakia: 57.

LEKEŠ J. (1997): Breeding of cereals on the territory of Czechoslovakia (*in Czech*). Plant Select, s.r.o., Brázda publ.

LEE S.J., PENNER G.A. (1997): The conversion of RFLP markers to allele specific amplicons linked to QTLs governing malting quality in barley. Mol. Breed., **3:** 457-462.

MATUS I.A. and HAYES P.M. (2003): Genetic diversity in three groups of barley germplasm assessed by Simple Sequence Repeats. Genome, **45:** 1095-1106.

OVESNÁ J., VACKE J., KUČERA L., CHRPOVÁ J., NOVÁKOVÁ I., JAHOOR A., ŠÍP V. (2000): Genetic analysis of resistance in barley to barley yellow dwarf virus. Plant Breeding, **119:** 481-486.

OVESNÁ J., NOVÁKOVÁ I., KUČERA L., SVOBODOVÁ S., DOTLAČIL L. (2001): Variability of storage proteins in some Czech landraces, modern and obsolete cultivars of wheat (Triticum aestivum L.). Plant Production, **47:** 82-90.

PARIS M, JONES M.G.K. AND EGLINTON J.K. (2002): Genotyping single nucleotide polymorphisms for selection of barley β-amylase alleles. Plant Mol Biol Rep, **20:** 149-159.

WENZEL P., CARLING J., JUDRA D., JACCOUD D., HUTTNER E., KLEINHOFS A., KLIAN A.(2004).: Diversity Arrays technology (DArT) for EWhole fenome profiling of barley. Proc.Natl.Acad.Sci, *in press.*

PARIS M., JONES M.G.K. and EGLINTON J.K. (2002): Genotyping single nucleotide polymorphisms for selection of barley β-amylase alleles. Plant Mol Biol Rep, **20:** 149-159.

S 2 – BREEDING METHODOLOGIES I – MAPPING

Molecular Mapping: Shifting from the Structural to the Functional Level

A. Graner, R. Kota, D. Perovic, E. Potokina, M. Prasad, U. Scholz, N. Stein, T. Thiel, R.K. Varshney and H. Zhang

Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstrasse 3, D06466, Gatersleben, Germany

Abstract
As a resource for structural and functional barley genome analysis, more than 140,000 ESTs (expressed sequence tags) were generated from 22 cDNA libraries that yielded 25,224 tentative unigenes. About 50% of these belong to gene families. The size of the complete transcriptome is estimated to comprise between 35,000 and 75,000 genes. The barley EST collection forms a rich source for the development of novel markers including SSRs (simple sequence repeats) and SNPs (single nucleotide polymorphisms). Several bioinformatic tools have been developed facilitating the computer assisted analysis of EST databases for the presence of either SNPs or SSRs and the development of SNP-derived CAPS (cleaved amplified polymorphic sequences) markers. In an attempt to systematically map barley genes a high-density transcript map is under construction and presently comprises more than 1000 markers. This map forms a gateway for comparative genomics with particular emphasis on the rice genome. 65% of the mapped ESTs showing a significant homology to rice ESTs were found to display a syntenic relationship between barley and rice. Thus, the barley EST resource provides a gateway for the rapid and systematic transfer of genetic information from rice to barley and other *Triticeae,* which can be readily exploited for marker saturation of defined chromosome regions and their detailed comparison to rice. In the context of a functional genomics study, the complex trait "malting quality" is being investigated using a barley cDNA array. By correlating the phenotypic malting trait data of selected barley lines with the corresponding expression profiles, a set of candidate genes was identified and further verified by genetic analysis.
Keywords: ESTs; functional genomics; malting quality; DNA-marker; rice; synteny

Introduction
In the past 25 years the availability of molecular marker maps has provided unprecedented insight into structural features of the barley genome. Several generations of selectable markers have been included in these maps and a large number of qualitative and quantitative traits were located in the genome some of which are being routinely selected in marker assisted breeding programs. The first generation of molecular marker maps mainly comprised genomic DNA fragments, since cloning of low molecular weight genomic DNA for marker development is a straightforward process. Moreover, genomic DNA fragments revealed higher DNA polymorphism than gene-derived fragments, although it could be shown, that many genomic RFLP-probes have been derived from genes (MICHALEK *et al.* 1999). With the establishment of large scale EST-programs in several laboratories around the world, a comprehensive resource has been created that provides direct access to genes. In the following, the application of this EST-resource will be described for the construction of a genome-wide barley transcript map, the marker saturation of sub-chromosomal target regions by exploiting synteny between the genomes of rice-barley and the identification of candidate genes from an EST-based functional association approach.

Facets of the Barley Transcriptome

The salient challenge of applied genetics and genomics is the correlation between genetic and phenotypic information and the subsequent identification of the genes underlying a trait of interest. Since most traits are only defined by a phenotype rather than by proteins or metabolites, map-based cloning has been the strategy of choice to isolate genes of interest. However, positional cloning is a time and resource intense approach that has to be restarted from scratch for any novel trait/gene. Consequently, there is a quest for more systematic approaches for gene identification. In the best case, this results in deciphering the complete sequence of a genome as it has been done for Arabidopsis and rice (TAGI 2000; YU *et al.* 2001; GOFF *et al.* 2002). Along with the progress in the field of bioinformatics, the availability of a whole genome sequence will greatly accelerate the identification of trait-related genes. Until now, the relative size of the barley genome has hindered any serious effort of systematic sequencing. The available sequence data on selected subgenomic regions show that, despite of very variable gene densities, any genome-wide sequencing effort in barley will likely result in sequencing endless stretches of repetitive DNA. Therefore, the pre-selection of expressed sequences will avoid the issue of genome complexity in barley. As a result of a barley EST project that was initiated at the IPK in 1998 about 140,000 sequences originating from 22 libraries have been released into the public domain. Together with the ESTs released by other groups more than 380,000 ESTs have been deposited until now in the public EST database of the NCBI (http://www.ncbi.nlm.nih.gov/dbEST/). These are derived from more than 80 cDNA libraries covering virtually any tissue and growth stage as well as a series of physiological conditions. Since ESTs reflect the transcriptional status of the tissue they were derived from, the sequences are inherently redundant. EST clustering is applied to remove the redundancy and to sort the sequences into singletons and sequence clusters. The sum of the numbers of singletons and clusters yields the number of tentative unigenes (Tentative Unigene Consensi, TUCs). In this regard, cluster analysis of 330,000 ESTs that were available in 2003 resulted in the definition ca. 33,000 tentative unigenes. Evidently the outcome of this kind of analysis depends on a series of parameters including the average sequence length of an EST, the quality of the sequences and the contamination of EST data with sequences from other organisms, such as microbes or fungi. Moreover, the result of the analysis is influenced by the stringency of the cluster algorithm. The higher the stringency, the more singletons (which may be due to sequencing errors only) and thus the more unigenes will be defined. Using appropriate software and proper settings meaningful results can be obtained as has been shown for the differentiation of the individual members of the transcription elongation factor 1-alpha (eEF1A) gene family (MICHALEK *et al.* 2002).

A comparison of the available sequence data to 254 well characterized barley genes from the SWISSPROT database and to 1.2 Mb of annotated BAC-sequence originating from several regions of the barley genome revealed an EST coverage of 87% for the SWISSPROT dataset and 45% for the genomic sequences. Thus, a preliminary estimate of the gene repertoire of barley will lie between 38,000 and 72,000 genes (ZHANG *et al*. submitted for publication). However, the complexity of a genome is not only defined by the number of its genes, but also by the number of its proteins. The latter may be influenced by alternative splicing, which is a common feature of the human transcriptome. In higher plants alternative splicing may be much more infrequent, since so far only a few cases have been described. Our EST data revealed that about 4% of the barley genes show alternatively spliced isoforms, a similar figure as was recently reported for Arabidopsis (BRETT *et al.* 2002).

From ESTs to Markers

RFLPs: The barley unigene-set represents a comprehensive source for the development of gene-derived markers. A BLASTN analysis of the barley unigene set to itself revealed that

about 50% of the genes are putative single copy genes, while the remaining genes are members of gene families. Of these, the majority comprises only 2-5 copies (Fig. 1). This is in accordance with the fact that cDNAs represent a good source for the development of RFLP markers or conversely that genes readily can be placed as RFLPs on the genetic map. Despite the experimental efforts required for RFLP mapping more than 600 EST-derived RFLP markers have been placed to date on a consensus map consisting of 3 mapping populations (Igri/Franka; Steptoe/Morex; Oregon $Wolfe_{rec}$/Oregon $Wolfe_{dom}$).

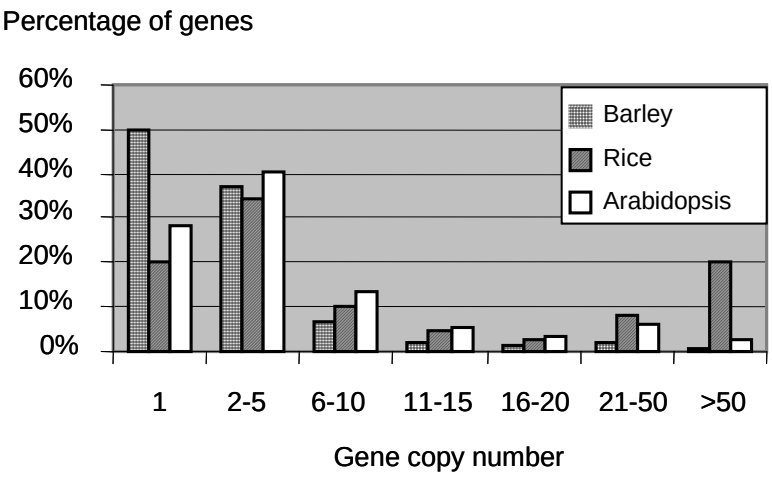


Figure 1. Distribution of the size of gene families. The copy number of each barley unigene was estimated by counting the number of BLASTN matches against the unigene data set itself with an E-value threshold of less than 10^{-20}.

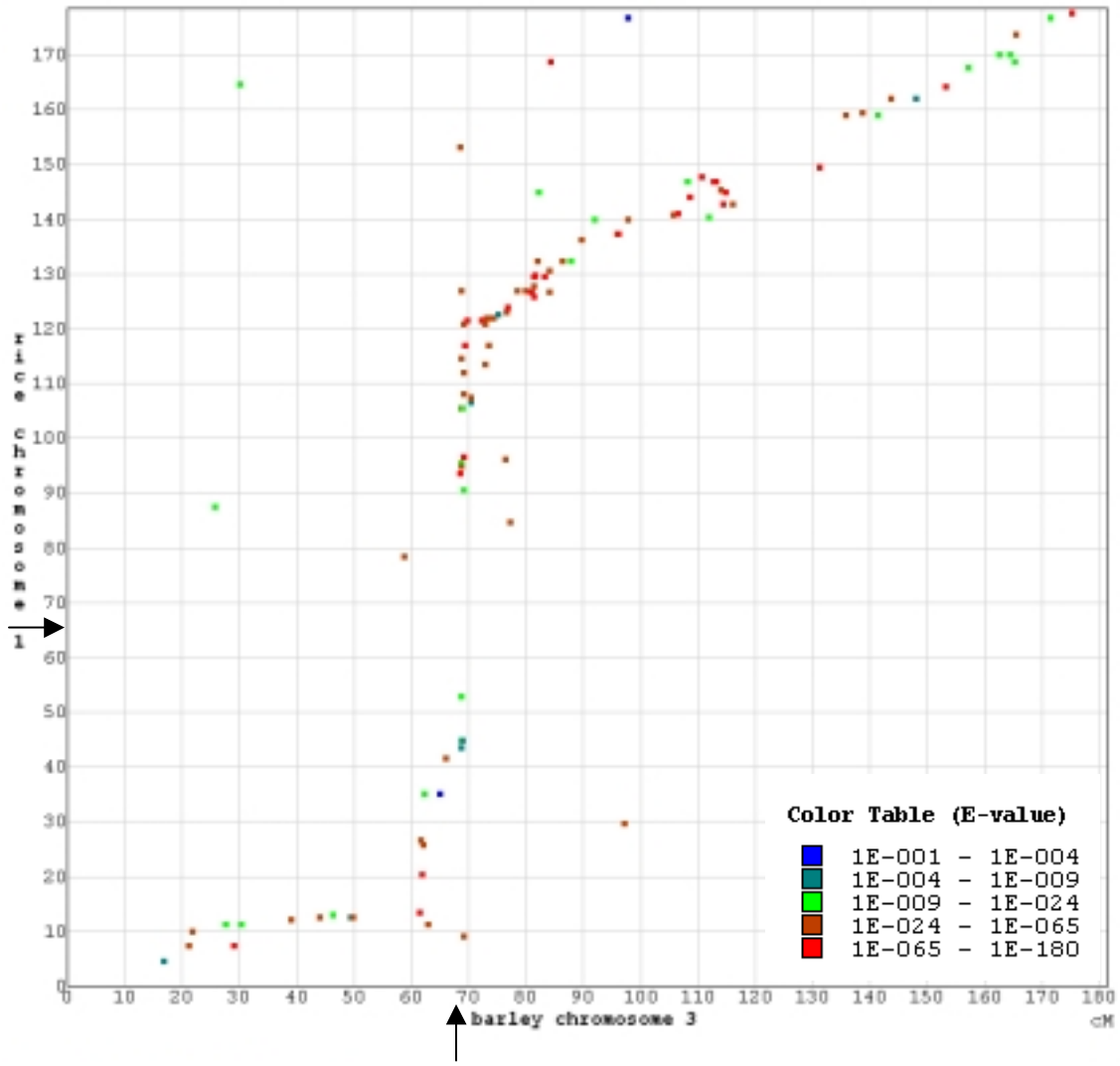
SSRs: Data from several plant species showed that, compared to non-coding regions, genes are enriched for microsatellites (SSRs) (MORGANTE *et al.* 2002). Analysis of the barley EST resource revealed similarities to other grass genomes: the barley transcriptome contains on average 1 SSR every 7.5 kb. This frequency of EST-SSRs is similar to that found in maize, wheat and sorghum, while the frequency in rice is 1/3.9 kb (VARSHNEY *et al.* 2002). Trimeric SSRs represent the largest class of repeat motifs in barley accounting for 56%. This may be explained by the suppression of non-trimeric SSRs in coding regions due to the ensuing frameshift mutations. Monomeric and dimeric repeats were observed at frequencies of 19% and 18%, respectively (THIEL *et al.* 2003). For SSR identification and primer development a software tool has been developed (MISA, http://pgrc.ipk-gatersleben.de/misa/), which allows a widely automated search of EST databases for SSR-containing sequences.
SNPs: Similar tools have been developed for the computer assisted identification of single nucleotide polymorphisms (SNPs). SNPs represent the most common class of genetic variation encountered and have rapidly become the marker of choice because of their high frequency in the genome and low mutation rates, when compared with other marker types. A survey for barley based on the comparison of EST-derived sequence tags in 7 divergent barley cultivars and one *H. spontaneum* accession revealed a mean nucleotide diversity of 3.2×10^{-3} with values ranging from 0 (no SNP) to 3×10^{-2}. A marked increase in SNP frequency could be obtained by the computer assisted pre-selection of polymorphic EST-sequences from public databases. This approach is based on the fact that the ESTs deposited in dbEST originated from different genotypes. Thus, sequence alignments can be searched for the presence of SNPs. By this approach it is evidently possible to identify genes or regions within genes that show a higher frequency of polymorphisms. Using a software tool that has been designed to automatically perform this task, 4,329 high scoring and putatively cultivar-specific SNPs were identified. Further experimental verification of the results obtained from the data mining approach resulted in the confirmation of 86 % of the SNPs detected. The average nucleotide diversity of the SNPs identified in this way amounted to 9×10^{-3}. This increase was mainly due to the reduced frequency of EST-alleles that were monomorphic in the genotype panel

analyzed, since this portion dropped from 34% in the random samples to only 9% after employing the data mining approach (KOTA *et al.* 2003).
With the influx of a plethora of SNP genotyping assays in recent years, there has been an imminent need for an assay that is robust, yet cost effective, and could be performed using standard gel-based procedures. In this context, CAPS (cleaved amplified polymorphic sequences) markers have been shown to meet these criteria. However, converting SNPs to CAPS markers can be a laborious process, if done manually. Therefore, a computer programme (SNP2CAPS, http://pgrc.ipk-gatersleben.de/snp2caps/), was developed that facilitates the computational conversion of SNPs into CAPS makers (THIEL *et al.* 2004). To investigate the number of potential CAPS markers present in our EST-allele database, 413 multiple aligned sequences derived from barley ESTs were analysed for the presence of polymorphisms in 235 distinct restriction sites. 282 (90%) of 314 alignments that contain sequence variation due to SNPs and InDels revealed at least one polymorphic restriction site. After reducing the number of restriction enzymes from 235 to a set of 10 common restriction enzymes, still 31% of the polymorphic sites could be detected. Thus, a significant portion of the available barley SNPs can be assayed as CAPS markers, which might be an option for laboratories that cannot afford or do not need the establishment high throughput SNP-detection platforms.

The Barley Transcript Map
The major challenge of genomics is the identification of candidate genes for a given trait. On the structural level this requires the identification of all possible genes residing on a chromosomal segment to which a trait has been assigned. In the best of all cases, each point on a genetic trait map can be connected to a defined region in a fully sequenced genome – so far a privilege of Arabidopsis and rice only. Although the barley genome is far from being fully sequenced, strategies are emerging for sequencing the gene rich regions of a genome. To relate these partial physical maps to the genetic map(s) of barley a high density transcript map is being developed that presently comprises 1023 ESTs and extends over 1131 cM. The corresponding loci represent potential anchor points for subgenomic physical maps of barley and the mapped markers, in particular the 255 SNPs and 185 SSRs further complete existing resources for trait mapping and marker assisted selection. Most importantly, the EST-loci form the connecting points to relate the genomes of barley and rice. The presence of extensive marker colinearity between these two genomes, which have diverged about 60 million years ago, provides the opportunity to tap into the wealth of information and resources available for rice. To explore the sequence similarity between barley and rice, the barley ESTs were compared to both rice ESTs and rice genomic sequences. Of the 1023 barley ESTs, 769 share a significant match with rice sequences (<1E-5, >80% sequence identity) and out of these 505 displayed a syntenic relationship at the chromosomal level as is shown in Fig. 2 for rice chromosome 1 and a consensus map of barley chromosome 3. The apparent discrepancy between the numbers of syntenic and colinear markers is mainly attributable to inaccuracies of a few cM in the present consensus map, which destroy the linear marker order. An increase in the number of anchor markers is expected to alleviate this problem.

Application of Marker Colinearity between Rice and Barley
Evidently, it is most tempting to use information from the rice genome for the immediate identification of orthologous genes in barley. But colinearity observed at the level of genetic resolution may provide too optimistic a picture. In several cases, the presence of the

Figure 2. Comparison of the genetic maps of barley chromosome 3 (x-axis) and rice



chromosome 1 (y-axis) (HARUSHIMA *et al.* 1998). The data for barley chromosome 3 are based on a consensus map of three populations). ESTs in common to both chromosomes are represented as dots with a colour code indicating the BLASTN similarity between homologous ESTs of the two species. The positions of the centromeres are indicated by arrows. Given similar map distances in both species, colinear markers would be ordered along the bisecting line. However, due to the reduced recombination observed in the proximal regions of the 7 barley chromosomes, colinear markers form a sigmoid curve. Moreover, because of the inaccuracies still present in the barley consensus map underlying this scatter plot, many dots deviate from an intended line that connects the colinear markers.

orthologue from barley is no longer present in rice, due to small scale genomic rearrangements or due to the rapid or the divergent evolution of genes that may prevent the detection of their orthologues (KILIAN *et al.* 1995; LEISTER *et al.* 1998). But even in the absence of the orthologous candidate gene, the information from rice can be applied to increase the marker saturation of a defined chromosome region in barley, as it may be required for the identification of a gene by positional cloning. A systematic approach for the EST based marker saturation of a target region around the *Rph16* rust resistance locus based on sequence information from rice has been described recently (PEROVIC *et al.* 2004). In this study, 309

non-redundant candidate syntenic clones have been identified for this region out of a collection of over 320,000 public barley ESTs in a two-step *in silico* selection procedure. For mapping, initially fifty-four barley cDNA-clones were selected due to the even distribution of their homologues on a putatively colinear 3 Mb rice BAC-contig. Out of these, 97 % (30) of the polymorphic markers could be genetically assigned in colinearity to the target region in barley and a set of eleven markers was integrated into a *rph16* high-resolution map. Although, the colinear target region of rice does not contain an obvious candidate gene for *rph16* the results demonstrated the potential of the procedure to efficiently utilize EST resources for synteny-based marker saturation. A similar approach was successfully employed to saturate the chromosomal regions harboring the GA-insensitive dwarfing gene *sdw3* which is located on the same chromosome (GOTTWALD *et al.* 2004). The systematic genome-wide exploitation of the increasing sequence data resources will strongly improve our current view of genome conservation and likely facilitate a synteny-based isolation of genes conserved across cereal species.

Candidate Gene Identification by Functional Association

The availability of a comprehensive EST resource for barley has also set the stage for the development of functional genomics-based strategies for the identification of trait-related genes. It could be shown that germination of a barley grain is based on the orchestrated, spatio-temporal expression of a large number of genes in embryo, scutellum and endosperm tissue (POTOKINA *et al.* 2002). In addition to tissue dependent gene expression, cDNA-array experiments also revealed that gene expression within a given tissue varies between different

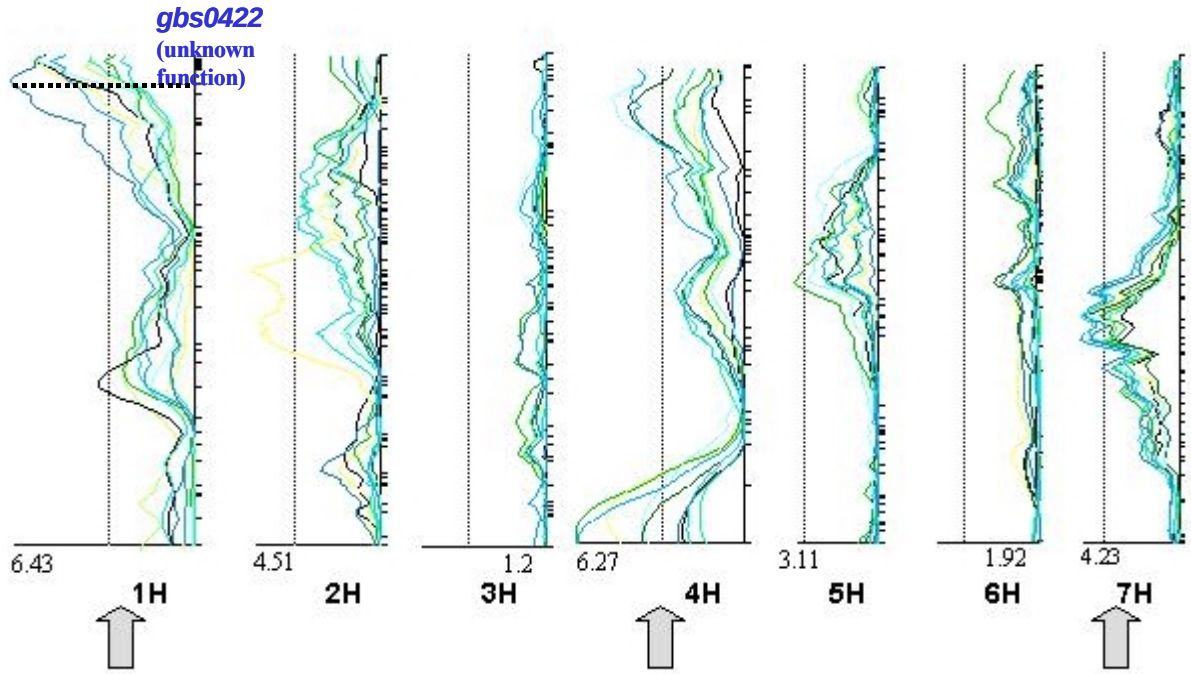


Figure 3. QTL-analysis of the trait diastatic power in the Steptoe/Morex progeny. Each LOD curve represents one of 7 environments analyzed. Chromosomes are shown as vertical lines with the skeleton markers used for QTL mapping represented as horizontal bars. The dashed line indicates a LOD of 3.0. The maximum LOD that has been reached on each chromosome is indicated. SNP-marker GBS0422 corresponding to the barley EST HY01D13, has been identified by the functional association approach as a candidate gene for malting quality (for details see POTOKINA *et al.* 2004).

genotypes. Under the hypothesis that differences in the expression of a quantitative trait are based on differences in the expression of the genes underlying this trait, a DNA-array based "functional association" approach has been devised to identify genes, whose expression is related to the manifestation of the trait malting quality (POTOKINA *et al.* 2004). As a result, 19 genes were identified in a pilot study that was based on a cDNA array comprising 1,400 genes, whose expression levels in a set of 10 European barley cultivars significantly correlated with the expression of the 7 malt parameters measured. This set of candidate genes contained genes that were already previously supposed to be related to malting quality(e.g. cysteine proteinase 1), genes hitherto unknown to be related to this trait (e.g. 70 kd heat shock protein) and genes of unknown function. Genetic mapping of these candidate genes in the Steptoe/Morex cross revealed in several cases a congruency between the map location of the candidate gene and the presence of a qtl for malting quality (Fig. 3).
Although these findings lend further strength to the validity of our functional association approach, it suffers from the limited resolution of QTL (quantitative trait locus) mapping. Hence, additional data from a larger set of genotypes are required for a final verification. In addition to the genetic mapping of candidate genes and subsequent QTL analysis in a biparental population, which inherently is confined to the segregation of two alleles only, the genetic association of candidate genes may be analyzed in a larger panel of genotypes using a case-control design.

Conclusions

While the development of molecular markers has shifted from anonymous DNA fragments to genes, the development of a comprehensive transcript map of the barley genome is still in its beginning. ESTs are presently being used at large scale for the systematic development of SSR and SNP markers and it will be important to integrate the maps generated in different laboratories into a robust consensus map, which needs to be curated in a coordinated and sustainable way. The same applies to the integration of trait data.
As long as there is no comprehensive sequence information available for barley, many attempts of positional cloning will benefit from the availability of sequence information from rice. In this concern, mapped barley ESTs provide an excellent resource to quickly identify the orthologous target region in the rice genome and to exploit the rice sequence for an EST-based marker saturation in barley.
In addition to gene isolation by positional cloning, the availability of comprehensive cDNA and oligonucleotide arrays now provides an option for the systematic investigation of quantitatively inherited traits, using a functional association strategy. Undoubtedly the verification of the candidate genes obtained by this approach presents further challenges for the future. Complementary to the genetic mapping of the candidate genes, genetical genomics studies are required to investigate whether candidate genes identified from the array analyses are regulated in cis or in trans, since only the cis-regulated genes will be amenable to marker assisted selection in plant breeding.
Despite the many issues that still await a solution, the availability of a large collection of barley ESTs has set the stage for a systematic dissection of the genetic basis of agronomic traits in barley. The identification of the corresponding genes is expected to lead to the development of improved strategies to identify novel and useful alleles from the vast number of genetic resources that rest on the shelves of the genebanks or that are thriving *in situ*. In this way genome research can deliver a significant contribution to the future use of biodiversity for the adaptation of barley to the future needs of mankind.

References

BRETT D., POSPISIL H., VALCARCEL J., REICH J., BORK, P. (2002): Alternative splicing and genome complexity. Nat. Genet., **30**: 29-30.

GOFF S.A., RICKE D., LAN T.H., PRESTING G., WANG R.L. et al. (2002): A draft sequence of the rice genome (*Oryza sativa* L. ssp *japonica*). Science, **296**: 92-100.

GOTTWALD S., BÖRNER A., STEIN N., SASAKI T., GRANER A. (2004): The Gibberellic-acid insensitive dwarfing gene *sdw3* of barley is located on chromosome 2HS in a region that shows high colinearity with rice chromosome 7L. Mol. Gen. Genomics (in press, DOI: 10.1007/s00438-004-0993-9).

HARUSHIMA Y., YANO M., SHOMURA A., SATO M., SHIMANO T. et al. (1998): A high-density rice genetic linkage map with 2275 markers using a single F_2 population. Genetics, **148**: 479-494.

KILIAN A., KUDRNA D.A., KLEINHOFS A., YANO M., KURATA N., STEFFENSON B., SASAKI T (1995): Rice-barley synteny and its application to saturation mapping of the barley *Rpg1* region. Nucl. Acids Res., **23**: 2729-2733.

KOTA R., RUDD S., FACIUS A., KOLESOV G., THIEL T., ZHANG H., STEIN N., MAYER K., GRANER A. (2003): Snipping polymorphisms from large EST collections in barley (*Hordeum vulgare* L.). Mol. Genet. Genomics, **270**: 224-233.

LEISTER D., KURTH J., LAURIE D.A., YANO M., SASAKI T. et al. (1998): Rapid reorganization of resistance gene homologues in cereal genomes. Proc. Natl. Acad. Sci. (USA), **95**: 370-375.

MICHALEK W., KÜNZEL G., GRANER A. (1999): Sequence analysis and gene identification in a set of mapped RFLP markers in barley (*Hordeum vulgare*). Genome, **42**: 849-853.

MICHALEK W., WESCHKE W., PLEISSNER K.P., GRANER A. (2002): EST analysis in barley defines a unigene set comprising 4,000 genes. Theor. Appl. Genet., **104**: 97-103.

MORGANTE M., HANAFEY M., POWELL W. (2002): Microsatellites are preferentially associated with nonrepetitive DNA in plant genomes. Nat. Genet., **30**: 194-200.

PEROVIC D., STEIN N., ZHANG H., DRESCHER A., PRASAD M. KOTA R., KOPAHNKE D., GRANER A. (2004): An integrated approach for comparative mapping in rice and barley with special reference to the *Rph16* resistance locus. Funct. Integr. Genomics (in press, DOI: 10.1007/s10142-003-0100-z).

POTOKINA E., SREENIVASULU N., ALTSCHMIED L., MICHALEK W., GRANER A. (2002): Differential gene expression during seed germination in barley (*Hordeum vulgare* L.). Funct. Integr. Genomics, **2**: 28-39.

POTOKINA E., CASPERS M., PRASAD M., KOTA R., ZHANG H., SREENIVASULU N., WANG M., GRANER A. (2004): Functional association between malting quality trait components and cDNA array based expression patterns in barley (*Hordeum vulgare* L.). Mol. Breed. (in press).

TAGI, The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature, **408**: 796-815.

THIEL T., KOTA R., GROSSE I., STEIN N., GRANER A. (2004): SNP2CAPS: a SNP and InDel analysis tool for CAPS marker development. Nucl. Acids Res., **32**: No. 1 e5.

THIEL T., MICHALEK W., VARSHNEY R.K., GRANER A. (2003): Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (Hordeum vulgare L.). Theor. Appl. Genet., **106**: 411-422.

VARSHNEY R.K., THIEL T., STEIN N., LANGRIDGE P., GRANER A. (2002): *In silico* analyses on frequency and distribution of microsatellites and ESTs of some cereal species. Cell. Mol. Biol. Lett., **7**: 537-546.

YU J., HU S. N., WANG J., LI S.G., WONG K.S.G. et al. (2001): A draft sequence of the rice (*Oryza sativa* ssp *indica*) genome. Chinese Science Bulletin, **46**: 1937-1942.

QTL for Malting Quality - a 25 Piece Puzzle

A.R. Barr[1], J.K. Eglinton[2], H.M. Collins[2], E. Vassos[2] and S. Roumeliotis[2]

[1]Australian Grain Technologies, PMB 1, Glen Osmond, South Australia, 5064;
[2]University of Adelaide, Waite Campus, Glen Osmond, South Australia, 5064.

Abstract
Over the past decade, great progress has been made in identifying and characterising at least 25 QTL associated with the key malting quality parameters of malt extract, diastatic power and cell and protein modification. This review examines the structural, biochemical and genetic basis of malt quality, concentrating on malt extract. It attempts to link malt extract QTL with the structural or biochemical factors, which contribute to malt extract allowing the breeder to better understand the trait under selection. Marker-assisted selection for malt quality is examined in a general sense, but more specifically in several current breeding projects including simple introgression of a single allele, pyramiding of malt quality QTL from several sources and attempts to "convert" a feed barley variety cv Keel (with low malt extract, low diastatic power and high wort β- glucan) to a malting barley through marker assisted introgression of key QTL from three donor parents with excellent malting quality. Preliminary results are presented for all of these breeding strategies.
Keywords: barley; malt; quantitative trait loci; mapping population

Introduction

Over the past decade, great progress has been made in identifying and characterising at least 25 QTL associated with the key malting quality parameters of malt extract, diastatic power and cell and protein modification (Figure 1). Does this knowledge greatly enhance the barley breeders' efforts to breed better malting barley varieties faster ? This review will examine the structural, biochemical and genetic basis of malt quality, concentrating on malt extract. This will lead into a discussion of marker-assisted selection for malt extract in a general sense, starting with simple introgression of a single locus leading to more elaborate schemes including attempts to "convert" a feed barley variety cv Keel (with low malt extract, low diastatic power and high wort β- glucan) to a malting barley through marker assisted introgression of key QTL from 3 donor parents with excellent malting quality.

The Relationship between the Biochemical, Structural and Genetic Basis of HWE

In a recent Ph D study, COLLINS (unpublished thesis, University of Adelaide) examined the relationship between malt extract and a range of biochemical and structural traits measured in the barley, malt, wort and spent grain produced from 12 barley varieties grown in South Australia in 1997 and 1998. The 12 varieties included locally adapted feed and malting varieties and elite malting varieties from the UK, Europe, Japan and North America. Malt extract was influenced by 13 different parameters (Table 1). A number of these traits have been studied in barley mapping populations and regions of the barley genome have been identified that are associated with them (HAYES *et al.* 1993; HAN *et al.* 1995; OBERTHUR *et al.* 1995; THOMAS *et al.* 1995; HAYES *et al.* 1996; LI *et al.* 1996; OZIEL *et al.* 1996; ZWICKERT-MENTEUR *et al.* 1996; BEZANT *et al.* 1997a; HAN *et al.* 1997; HAYES *et al.* 1997; LARSON *et al.* 1997; MANO & TAKEDA 1997; MATHER *et al.* 1997; POWELL *et al.* 1997; ULLRICH *et al.* 1997; BOREM *et al.* 1999; MARQUEZ_CEDILLO *et al.* 2000; ZALE *et al.* 2000; HAYES *et al.* 2001**;** BARR *et al*. 2003a; BARR *et al.* 2003b; KARAKOUSIS *et al.* 2003a; PALLOTTA *et al.* 2003; ASAYAMA, unpublished; PANOZZO *et al.,* in

preparation, COLLINS *et al*. 2004, EDNEY *et al*. 2004). HWE and the 13 parameters are likely to be linked genetically by either the pleiotropic effects of a single gene or the effects of gene 'clusters' where individual genes controlling each trait are closely linked and therefore identified through QTL mapping as a single region. The following discussion investigates the linkage between HWE and the 13 parameters.

Table 1. Characteristics of the grain, malt, wort and spent grain of varieties of high and low malt extract grown in South Australia in 1997 and 1998 (COLLINS 2003, unpublished thesis) and possible genetic control elucidated from various mapping populations

Malting & Brewing Stage	Trait	Malting class	Malting class	Mapping pop.	QTL associated With trait and extract
		High	Low		
Grain	SKSC Weight (mg)	<39	>44	Not mapped	
	Barley 1000 GW (g)	<36	<38	Alexis x *Sloop* *Blenheim x Kym*	3H 2H (bin 2-3, 12-14)
	grain hydration 72 hrs	>140	<120	Chebec x *Harrington*	5H
	barley husk content %	<9.5	>10.3	Galleon x *Haruna nijo*	2H
Malt	Malt 1000 GW (g)	<30	<34	Not mapped	
	malt beta glucan %	<0.4	>0.75	Steptoe x *Morex*	7H, 4H
	limit dextrinase (U/kg)	>500	<400	Galleon x *Haruna nijo*	2H
	Diastatic power µm/min/g	>500	<400	Many	Many
	Alpha amylase µm/min/g	>110	<80	Many	Many
	large starch granule size (m)	>18	<17	Steptoe x *Morex*	2H, 5H
	RVA peak viscosity (RVA units)	<100	>130	Not mapped	
wort, protein mod.	soluble protein (%)	>4.9	<4.0	Sloop x *Alexis* Chebec x *Harrington*	2H, 5H
	FAN (mg/L)	>170	<130	Arapiles x Franklin Sloop x *Alexis*	5H 1H
	KI (%)	>47	<40	Arapiles x *Franklin* Sloop x *Alexis* Dicktoo x Morex Arapiles x Franklin	1H 2H 5HS 5HL
wort, cell wall mod.	ß-Glucan (mg/L)	<150	>250	*Sloop x Alexis*	1H, 2H
	Arabinoxylan (mg/L)	<40	>70	Not mapped	
	Viscosity (cP)	<1.58	>1.65	*Sloop x Alexis*	1H, 2H
	glucose (mmols/L)	>50	<40	Arapiles x *Franklin* Sloop x *Alexis*	1H, 5H, 7H
	Fructose (mmols/L)	>10.5	<7	Arapiles x *Franklin* Sloop x *Alexis*	3H (denso), 1H
spent grain	% of original grain	<29.5	>31	Not mapped	
	Starch (%)	<1.5	>2.0	Not mapped	
	ß-Glucan (%)	<0.55	>1.0	Not mapped	

Barley husk content and HWE were found to be associated with a region on the short arm of chromosome 2H. It was found that husk content influenced HWE both directly, by diluting the amount of starch in the grain, and indirectly, by providing a physical or chemical barrier to water uptake, thereby lowering modification and hence HWE. The following discussion investigates the linkage between HWE and the 13 parameters.

Grain weight, in both barley and malt, was shown to negatively influence HWE. The selection of varieties used by COLLINS (unpublished thesis) would have intensified this relationship. The European, Canadian and Japanese high HWE varieties were poorly adapted to the Southern Australian growing conditions, which would have resulted in small grain size. However, in general, low extract feed varieties tend to have a larger grain size than higher

extract malting varieties. Large grain size is an important industry target for new barley varieties and there is a need therefore, to breed varieties with both high HWE and large grain size. Three regions of the barley genome have favourable alleles for the expression of both HWE and barley 1000 grain weight (the most commonly used assessment method for grain size). Two regions were found to be associated with HWE and barley 1000 grain weight on chromosome 2H (bins 2-3 and 12-14) in the mapping population Blenheim/ Kym (BEZANT *et al.* 1997a; BEZANT *et al.* 1997b; HAYES *et al.* 2001). One of these had Kym donating the favourable alleles for both traits and the other had Blenheim. Additionally a region on the long arm of chromosome 3H was found to be associated with the Sloop or Sloop-sib allele for both traits in the mapping populations Sloop/Alexis and Sloop-sib/Alexis (COVENTRY *et al.* 2003a; COLLINS *et al.* 2003). The exploitation of these regions by breeders may lead to the production of barley varieties with a combination of high HWE and larger grain size.

A high level of cell wall modification in both the malt, as indicated by low malt β-glucan content, and in the wort, as indicated by low wort β-glucan content and wort viscosity, were found to be positively associated with HWE. Malt β-glucan was measured in the Steptoe/ Morex population (HAN *et al.* 1995; HAN *et al.* 1997; ULLRICH *et al.* 1997; HAYES *et al.* 2001) and three regions of the barley genome, donated from Morex, were found to be associated with increased levels of HWE and decreased levels of malt β-glucan on chromosomes 7H and 4H (HAN *et al.* 1995; ULLRICH *et al.* 1997). Additionally, the regions of the genome found to be associated with HWE on chromosomes 1H and 2H in the Sloop/ Alexis and Sloop-sib/ Alexis populations (COLLINS *et al.* 2003) were found to be associated with traits relating to the level of modification of the cell wall material, wort β-glucan and wort viscosity. The manipulation of genes to increase the level of degradation of the cell walls is a viable method of improving HWE. Due to the problems associated with high levels of cell wall material in the wort during the lautering and filtration processes, increased modification of the cell walls is an important breeding target. Regions of the genome that influence the modification and degradation of the cell walls are an important source of genes for overall malting quality.

The diameter of the large starch granules is positively associated with HWE (COLLINS 2003, unpublished thesis). QTL influencing the diameter of the large starch granules were found in the Steptoe/ Morex population on chromosomes 2H and 5H (BOREM *et al.* 1999; HAYES *et al.* 2001). Whilst no QTL for HWE was found in these regions in the Steptoe/ Morex population, QTL for HWE were found on chromosome 2H in the Dicktoo/ Morex population (OZIEL *et al.* 1996; HAYES *et al.* 2001) and the Galleon/ Haruna Nijo population (COLLINS *et al.* 2003) and on 5H in the Blenheim/ E224/3 population (THOMAS *et al.* 1995; POWELL *et al.* 1997; ZALE *et al.* 2000; HAYES *et al.* 2001) and the Sloop/ Alexis and Sloop-sib/ Alexis populations (COLLINS *et al*. 2003). The large starch granules make up approximately 90% of the total volume of the starch in the barley and therefore contribute the greatest proportion of carbohydrates to the wort. Large starch granules are hydrolysed preferentially to small starch granules during mashing (BATHGATE & PALMER 1973) and have a lower gelatinisation temperature (MACGREGOR 1980; MACGREGOR & BALANCE 1980a), making them more available to enzyme degradation during mashing. Whilst the measurement of large starch granule diameter is both difficult and time consuming, further investigation of this trait in other populations may lead to useful information for the improvement of malt quality in general.

The activities of the starch degrading enzymes were found to be associated with HWE (COLLINS 2003, unpublished thesis). In particular the limit dextrinase activity was found to

be strongly associated with HWE. Limit dextrinase has been investigated in only a limited number of mapping populations. Whilst the gene encoding limit dextrinase, *LD*, is on chromosome 7H (LI *et al.* 1999; HAYES & JONES 2000), a number of QTL for limit dextrinase activity have been found elsewhere on the barley genome (LI *et al.* 1996). One of these regions is associated with the marker *Xmwg503(b)* on chromosome 2H (LI *et al.* 1996) in the mapping population Galleon/ Haruna Nijo. This region was also putatively found to be associated with HWE in the Galleon/ Haruna Nijo population (COLLINS 2003, unpublished thesis). The attention given to the more abundant starch degrading enzymes, alpha and beta amylase, has often overshadowed the importance of limit dextrinase to overall malting quality. COLLINS (2003) argued that selecting new varieties with increased levels of limit dextrinase should lead to an increase in HWE. To assist barley breeders with selection strategies for high limit dextrinase, further mapping studies to locate regions conferring high limit dextrinase will be important.

Kolbach index (KI), soluble protein and free amino nitrogen (FAN), all give an indication of the level of the modification and degradation of proteins during the malting and mashing processes and were found to be positively associated with HWE. In the Sloop/ Alexis and Sloop-sib/ Alexis populations, two regions of the genome were found to be associated with HWE and FAN, and one region was found to be associated with HWE and KI (BARR *et al.* 2003b; PANOZZO *et al.* in preparation). All regions had Alexis donating the higher allele. The increase in the degradation of the proteins during malting and mashing leads to higher levels of HWE by increasing the accessibility of the starch granules to starch degrading enzymes. However, a large amount of soluble protein in the wort is not necessarily advantageous as it can lead to haze problems in the final beer, reducing the shelf life of the product. Most breweries have strict limits on the level of soluble protein remaining in the wort. The release of new malting varieties with improved levels of HWE combined with high levels of soluble protein may not acceptable to the end user.

To our knowledge, RVA peak viscosity and grain hydration have not been mapped, while genetic analysis of wort monosaccharides has only recently been pursued (EDNEY *et al.* this edition). Likewise, the properties of the spent grain have not been mapped. Few studies have investigated either the quantity or the components of the spent grain. Generally when spent grain has been investigated it has been in relation to the influence of the spent grain on the lautering process (KANO & KARAKAWA1979). In depth analysis of the starch and cell wall material in the spent grain may lead to a better understanding of the reasons these materials remain in the spent grain and hence, methods of improving their release earlier in the malting process.

Applying the Genetic, Structural and Biochemical Knowledge to Practical Plant Improvement

From these studies, we have decided to concentrate for MAS on the following loci which influence malt extract;

- 1H XEbmac501 - positive alleles come from many European malting varieties derived from Triumph including Alexis and also the Australian variety Franklin. This locus appears to influence malt extract by improving cell wall hydolysis. There are no known contra-indications for this locus other than many Australian varieties carry alternative alleles, raising the possibility of deleterious effects on adaptation of the European alleles (or, at least, linked loci)
- 2H Xpsr108 - positive alleles come from Japanese varieties like Haruna nijo. This locus leads to higher HWE via thinner husk. This locus is readily selected with two high

throughput technologies i.e. NIR (ROUMELIOTIS & BARR 2003) and MAS. We were concerned that selection for thin husk alleles would lead to unacceptably high skinning or peeling but it now appears that the two traits are not inextricably associated (ROUMELIOTIS *et al*. 2001).

- 2HL Xwmg503 – positive alleles come from European varieties like Alexis and Japanese varieties like Haruna nijo. It appears that this locus affects HWE via improved cell wall hydrolysis and / or increased diameter of the large starch granules. This locus is linked to the *Ha2* locus for CCN resistance (in repulsion for many Australian lines) and we have found that selection for the 5 H *Ha4* locus is a better alternative, which avoids any linkage problems. It should be noted however that some excellent malting varieties with high HWE carry *Ha2,* so this is not an insurmountable problem (e.g. cv. Monarch).
- 5HL cluster – there are a cluster of QTL influencing HWE with positive alles coming from many Canadian varieties including Harrington, Manly and AC Metcalfe. These loci influence protein modification, diastatic power, the speed of germination and mean diameter of the large starch granules. Selection for Canadian alleles in this region certainly improves HWE but can lead to pre-harvest sprouting and a tendency to produce overmodified malts. Modifying genes at as yet unknown loci ameliorate these problems but the number of genes and their nature in this region are still unclear.

So, having chosen the key target loci and identified sources of favourable alleles, how does the malting barley breeder set about using MAS to breed new varieties with higher HWE? In our case, the use of MAS includes pre-screening of donors for doubled haploid production, screening of donor plants from top cross and four way cross F_1s and, in a few elite crosses, selection of fixed lines in the breeding program. MAS has shifted substantially from its beginnings around 1996 where selection concentrated on single major genes for disease resistance to 2004 where QTL for quality traits are now heavily targeted (Table 2). Note the number included in this table exclude the assays conducted in the feed variety conversion project reported later in this paper (around 4000 assays at 4 extract loci and 1 diastatic power locus).

Table 2. Number of marker assays in University of Adelaide barley breeding programme in calendar year 2003 by trait

Trait	Number of marker assays	Number of loci for quality traits
Spot form net blotch	18	
Aluminium Tolerance	203	
Scald	244	
leaf rust	300	
Waxy endosperm	805	1
Cereal cyst nematode	919	
Miscellaneous	1248	
Diastatic power	1251	3
Boron tolerance	2001	
Extract	2633	3

The following sections describe four of strategies used in the South Australian Barley Improvement Program.

Single Region Introgression - Introgression of the 5H Region into VB9524

The 5H region of Canadian varieties such as Harrington carry genes which increase the rate of modification of malt, increase diastatic power and lead to increased malt extract. We identified a line from the Chebec x Harrington mapping population which carried the Harrington alleles in this region (and the Chebec allele *Ha2* for CCN resistance) and by marker assisted selection (MAS) introgressed this region into BC2 populations of the elite Victorian breeders line VB 9524. Table 2 shows the effect of the Harrington allele in a number of related BC2 lines. The effects of this region on malt quality are dramatic, especially when measured in early season malts where post harvest dormancy remains (Table 3).

Table 3. Comparison of analysis of micromalted samples of recurrent parent VB 9524 and WI 3580, a BC2 derived line where VB 9524 was the recurrent parent and the Harrington 5HL segment was introgressed via MAS, in samples from Cummins, South Australia, 2001

Line	Grain Protein	Diastatic power	Alpha amylase	Beta amylase	Kolbach Index	Extract	Viscosity	Wort beta glucan	FAAN
VB 9524	8.6	424	143	282	36.3	81.2	1.94	825	107
WI 3580	8.2	581	232	348	52.4	81.6	1.63	110	168

Multiple Alleles from a Single Source

COLLINS *et al.* (2003) analysed the genotype and phenotype (for malt extract) of 85 lines in the Galleon x Haruna nijo mapping population during 1996 –1999 in South Australia. Galleon is a 2 row feed variety with poor malt extract (Table 4) whereas Haruna nijo is a high quality Japanese malting variety. Hence, these varieties differ greatly in extract and four QTL were identified which explained the genetic basis for this difference. When the allelic classes were separated at each of these loci, the contribution of each Haruna nijo allele to overall extract is apparent (Table 4). Several of these alleles, with the 2HL Xpsr108 most notable, have been used in MAS projects with positive results (COLLINS *et al*. 2003). Similar findings were also presented when alleles from Harrington and Alexis were used in MAS.

Table 4. Mean EBC HWE (1996-1999) for the Galleon/Haruna Nijo mapping population separated into groups based on the "alleles" at marker *Xpsr108* and, each of 3 marker loci - G, Galleon "allele"; H, Haruna Nijo "allele**"**

2HL *Xpsr108*	2HL *Xmwg503*	5HS *Xawbma32*	6HS *Xnar7*	No.	Group[A]	Mean EBC HWE (%db)
G				29-50		77.2***
H				15-33		79.2
G	G	G	G	6	1	75.2
G	G	G	H	5	2	76.9
G	G	H	G	2	3	75.0
G	H	G	G	3	4	76.6
H	G	G	G	3	5	77.7
G	G	H	H	0	6	-
G	H	G	H	9	7	77.5
H	G	G	H	0	8	-
G	H	H	G	10	9	77.4
H	G	H	G	0	10	-
H	H	G	G	3	11	79.3
G	H	H	H	10	12	78.4
H	G	H	H	1	13	78.2
H	H	G	H	7	14	79.4
H	H	H	G	3	15	79.3
H	H	H	H	7	16	80.7

*** The lines carrying the Galleon "allele" are significantly different to the lines carrying the Haruna Nijo "allele" at marker *Xpsr108* (P<0.05); [A]Group 1 is statistically different (P<0.05) from groups 7,9,11,12,14,15 and 16; Group 2 is statistically different (P<0.05) from groups 12,14 and 16; Group 3 is statistically different (P<0.05) from groups 12,14,15 and 16; Group 4 is statistically different (P<0.05) from group 16; Group 5 is statistically different (P<0.05) from group 16; Group 7 is statistically different (P<0.05) from groups 16 and 1; Group 9 is statistically different (P<0.05) from groups 16 and 1; Group 11 is statistically different (P<0.05) from group 1; Group 12 is statistically different (P<0.05) from groups 1,2 and 3; Group 14 is statistically different (P<0.05) from groups 1,2 and 3; Group 15 is statistically different (P<0.05) from group 1 and 3; Group 16 is statistically different (P<0.05) from groups 1,2,3,4,5,7 and 9.

Multiple Alleles from Multiple Sources – Pyramiding Alleles from 4 Mapping Populations

The characterisation of different malt quality QTL in a range of germplasm provides an opportunity to combine the desirable alleles in one genetic background. This has been pursued by selecting elite individuals from the Alexis/Sloop, Galleon/Haruna Nijo, Chebec/Harrington, and Amaji Nijo/WI2585 mapping populations for use as source material. A series of four-way crosses were developed and the progeny selected with MAS to maximise the frequency of preferred alleles in the subsequent populations. Preliminary malt quality analysis of the lines derived from this process has confirmed the presence of elite quality profiles in lines carrying multiple QTL. Further characterisation of this material will provide insight into the interactions between genomic regions influencing malt quality, and begin to define a molecular ideotype for elite quality. The concept of defining and specifically breeding for a molecular ideotype is also currently under investigation in a population derived from the cross Chieftan/Barque//Manley/VB9104 (McMICHAEL *et al*., this volume).

"Feed to Malt Conversion" – Marker Assisted Introgression of Malt Quality Alleles into Feed Varieties

Malting barley breeding programs have typically applied conservative strategies for the introgression of traits from non-malting germplasm, and this traditional approach can be considered as recognition for the genetic complexity of malt quality. However, these strategies

intrinsically limit the rate of genetic gain for adaptation and, in many water limited environments, the superior adaptation of feed varieties is well characterised.

This project aims to develop and test a new paradigm for breeding malting barley i.e. to introduce key malting quality genes via MAS from a range of elite international malting varieties into Keel (a high yielding and drought tolerant feed variety), while maintaining the superior adaptation and agronomic profile of the feed barley. This strategy implies that the genetics of malting quality is now better understood than the genetics of adaptation.

Keel was used as the recurrent parent to produce backcross lines containing the key quality genes from Alexis, Haruna nijo and AC Metcalfe. The BC_1F_1 generations were screened using molecular markers for a range of malt quality loci. A total of 1000 BC1F1 and BC2F1 individuals were marker screened, requiring over 3500 marker assays. Selected individuals were screened as BC_1F_2 single plants to identify individuals homozygous for the target loci. The subsequent populations were evaluated in a double row trial in 2002. Agronomic selection was applied to identify individuals exhibiting the Keel phenotype, the subsequent grain samples were evaluated for grain size and NIR predicted malt quality. Elite lines were identified and promoted for further agronomic evaluation in yield plots in the 2003 season. Selected BC_1F_2 individuals were used to develop BC_2 generations for each of the introgression streams. This germplasm is currently in a double row trial for agronomic and predicted malt quality evaluation. Populations derived from intercrosses between the BC_1F_2 individuals have also been developed to pyramid the malt quality genes from the three international varieties into a Keel genetic background. Thus far, this has required the production of over 1100 merged F1s and 700 marker assays.

Nine elite lines were identified from 89 Alexis/KeelBC_1F_3's, derived from one elite Alexis/KeelBC_1F_1. Unfortunately 29 lines from this population were unable to be analysed by NIR, because of their low yield due to drought effects. Only 25% of the population had the maturity of Keel, however over 60% had maturity in the range Keel to Barque. 18% had screenings of or lower than Keel, 76% had a malt extract value of or higher than Schooner (a commercial malting variety) and 23% diastatic power (DP) higher than Schooner. The promising malt extract values from this population are significantly greater than Mendelian expectation, demonstrating the effect of marker assisted selection.

Over half of the 91 WI3284/KeelBC_1F_3's derived from an elite WI3284/KeelBC_1F_1, have the same maturity as Keel and almost a quarter have screening percentages similar to Keel or lower. An impressive 90% of this population have extract values greater than Schooner, with 56% having values greater than Sloop, possibly reflecting the impact of the thin husk trait from Haruna nijo on NIR predicted malt extract. The DP values however are less impressive, with 10% having a DP greater than Sloop.
This approach aims for a complete quality conversion, from feed to malting quality, while retaining the agronomic advantage of the feed variety. The preliminary results suggest this strategy has successfully introgressed a range of malt quality QTL and largely retained the characteristics of the recurrent parent. Whilst it is unlikely that lines from any one of these 3 streams will be of sufficient merit to be released as a malting variety, especially since Keel is approximately 3 EBC percentage units of HWE lower than Schooner and 4.5% lower than more current targets, it seems quite likely that merges of 2 or more of the streams may achieve our aim. The project has proven very intensive on crossing and marker resources yet we believe this strategy still represents a very efficient approach compared to our mainstream breeding methods. However a more detailed characterisation of the malt quality, yield potential

and disease resistance profiles of the germplasm over ensuing seasons will be required to fully assess the merits of the alternative breeding strategy.

Conclusions

Mapping studies have not only defined key genomic regions influencing malting quality, but have also contributed to our knowledge of their mode of action through the use of QTL based trait dissection. These QTL are now routinely used in Australian malting barley breeding in both conservative and speculative breeding strategies. The improved genetic knowledge and application of MAS for malting quality has facilitated the development of novel breeding strategies such as complete quality conversion and molecular ideotype breeding. These alternative approaches may offer advantages to breeders attempting to manage the complex genome of malting barley.

References

ASAMAYA, unpublished

BARR, A. R., JEFFERIES, S. P., Broughton, S., CHALMERS, K. J., KRETSCHMER, J. M., COLLINS, H. M., ROUMELIOTIS, S., LOGUE, S. J., COVENTRY, S. J., MOODY, D., READ, B., POULSEN, D., LANCE, R., PLATZ, G., Park, R., PANOZZO, J., KARAKOUSIS, A., LIM, P., VERBYLA, A., ECKERMAN, P. (2003b): "Mapping and QTL analysis of the barley population Alexis x Sloop." Australian Journal of Agricultural Research, **54:** 1117-1124.

BARR, A. R., KARAKOUSIS, A., LANCE, R. C. M., LOGUE, S. J., MANNING, S., CHALMERS, K., KRETSCHMER, J. M., COLLINS, H. M., ROUMELIOTIS, S., COVENTRY, S., MOODY, D. B., READ, B. J., POULSEN, D., Li, C. D., PLATZ, G. J., INKERMAN, A., PANOZZO, J. F., CULLIS, B. R., SMITH, A. B., LIM, P., LANGRIDGE, P., BOYD, W. J. R. (2003a): "Mapping and QTL analysis of the barley population Chebec x Harrington." Australian Journal of Agricultural Research, **54:** 1125-1130.

BATHGATE, G. N., PALMER, G. H. (1973): "The In Vivo and IN Vitro Degradation of Barley and Malt Starch Granules." Journal of the Institute of Brewing, **79:** 402-406.

BEZANT, J. H., LAURIE, D. A., PRATCHETT, N., CHOJECKI, J., KEARSEY, M. J. (1997a): "Mapping of QTL Controlling NIR Predicted Hot Water Extract and Grain Nitrogen Content in a Spring Barley Cross Using Marker-Regression." Plant Breeding, **116**: 141-145.

BEZANT, J. H., LAURIE, D. A., PRATCHETT, N., CHOJECKI, J., KEARSEY, M. J. (1997b): "Mapping QTL controlling yield and yield components in a spring barley (*Hordeum vulgare* L.) cross using marker regression." Molecular Breeding, **3**: 29-38.

BOREM, A., MATHER, D. E., RASMUSSON, D. C., FULCHER, R. G., HAYES, P. M. (1999): "Mapping quantitative trait loci for starch granule traits in barley." Journal of Cereal Science, **29**: 153-160.

COLLINS , H.M. (2003) A study of the biochemical, physical and genetic factors influencing malt extract in barley (*Hordeum vulgare* L.) Unpublished thesis, University of Adelaide

COLLINS, H. M., PANOZZO, J., LOGUE, S. J., JEFFERIES, S. P., A.R., BARR. (2003): "Mapping and Validation of Chromosome Regions Associated with High Malt Extract in barley (*Hordeum vulgare* L.)." Aust. Journal of Agricultural Research, **54:** 1223-1240.

COLLINS, H.M., LOGUE, S.J., EGLINTON, J.K. (2004) Understanding the genetic basis of modification. This edition.

COVENTRY, S. J., COLLINS, H. M., BARR, A. R., JEFFERIES, S. P., CHALMERS, K. J., LOGUE, S. J., LANGRIDGE, P. (2003a): "Use of putative QTL and Structural genes in marker assisted selection for Diastatic Power in malting Barley (*Hordeum vulgare* L.)." Australian Journal of Agricultural Research, **54:** 1241-1250

EDNEY, M.J., EGLINTON, J.K., COLLINS, H.M., BARR, A.R. (2004): Biochemical basis of wort fermentability. This edition

HAN, F., ULLRICH, S. E., CHIRAT, S., MENTEUR, S., JESTIN, L., SARRAFI, A., HAYES, P. M., JONES, B. L., BLAKE, T. K., WESENBERG, D. M., KLEINHOFS, A., KILIAN, A. (1995): "Mapping of Beta-Glucan Content and Beta-Glucanase Activity Loci in Barley Grain and Malt." Theoretical & Applied Genetics, **91:** 921-927.

HAN, F., ULLRICH, S. E., KLEINHOFS, A., JONES, B. L., HAYES, P. M., WESENBERG, D. M. (1997): "Fine Structure Mapping of the Barley Chromosome-1 Centromere Region Containing Malting-Quality QTLs." Theoretical & Applied Genetics, **95**: 903-910.

HAYES, P. M., JONES, B. L. (2000): "Malting Quality from a QTL Perspective." 8th International Barley Genetic Symposium, **1**: 99-106.

HAYES, P. M., LIU, B. H., KNAPP, S. J., CHEN, F., JONES, B., BLAKE, T., FRANCKOWIAK, J., RASMUSSON, D., SORRELLS, M., ULLRICH, S. E., WESENBERG, D., KLEINHOFS, A. (1993): "Quantitative Trait Locus Effects and Environmental Interaction in a Sample of North American Barley Germ Plasm." Theoretical & Applied Genetics, **87**: 392-401.

HAYES, P. M., PREHN, D., VIVAR, H., BLAKE, T., COMEAU, A., HENRY, I., JOHNSTON, M., JONES, B., STEFFENSON, B., ST PIERRE, C. A., CHEN, F. (1996): "Multiple Disease Resistance Loci and Their Relationship to Agronomic and Quality Loci in a Spring Barley Population." Journal of Quantitative Trait Loci(2).

HAYES, P. M., CERONO, J., WITSENHOEr, H., KUIPER, M., ZABEAU, SATO, K., KLEINHOFS, A., KUDRNA, D., KILIAN, A., SAGHAI-MAROOF, M., HOFFMAN, D., North_American_Barley_Genome_Mapping_Project (1997): "Characterizing and Exploiting Genetic Diversity and Quantitative Traits in Barley (*Hordeum vulgare*) using AFLP Markers." Journal of Quantitative Trait Loci 2(3).

HAYES, P. M., CASTRO, A., MARQUEZ-CEDILLO, L., COREY, A., HENSON, C., JONES, B., KLING, J., MATHER, D., MATUS, I., ROSSI, C. and SATO, K. (2001): "A summary of published barley QTL reports.", http://www.css.orst.edu/barley/nabgmp/qtlsum.htm

KANO, Y., KARAKAWA, T. (1979): "Importance of the Residual Starch in the Spent Grains for Wort Run-off." Bulletin of Brewing Science, **25:** 1-7.

KARAKOUSIS, A., BARR, A. R., KRETSCHMER, J. M., MANNING, S., LOGUE, S. J., ROUMELIOTIS, S., COLLINS, H. M., CHALMERS, K. J., LI, C. D., LANCE, R. C. M., LANGRIDGE, P. (2003a): "Mapping and QTL analysis of the barley population Galleon x Haruna Nijo." Australian Journal of Agricultural Research, **54:** 1131-1136.

LARSON, S., BRYAN, G., DYER, W. E., BLAKE, T. (1996): "Evaluating Gene Effects of a Major Barley Seed Dormancy QTL in Reciprocal Backcross Populations." Journal of Agricultural Genomics 2.

Li, C. D., LANGRIDGE, P., LANCE, R. C. M., P., FINCHER, G. B. (1996): "Seven Members of the (1-3)-β-glucanase Gene Family in Barley (*Hordeum vulgare*) are Clustered on the Long Arm of Chromosome 3 (3HL)." Theor.& Appl. Gen., **92**: 791-796.

LI, C. D., ZHANG, X. Q., ECKSTEIN, P., ROSSNAGEL, B. G., SCOLES, G. J. (1999): "A polymorphic microsatellite in the LIMit dextrinase gene of barley (*Hordeum vulgare* L.)." Molecular Breeding, **5**: 569-577.

MACGREGOR, A. W. (1980): "Action of Malt a-Amylase on Barley Starch Granules." Master Brewing Association of America Technical Quarterly, **17**: 215-221.

MACGREGOR, A. W., BALLANCE, D. L. (1980a): "Hydrolysis of Large and Small Granules from Normal and Waxy Barley Cultivars by Alpha-Amylases from Barley Malt." Cereal Chemistry, **57:** 397-402.

MANO, Y., TAKEDA, K. (1997): "Mapping Quantitative Trait Loci for Salt Tolerance at Germination and the Seedling Stage in Barley (*Hordeum Vulgare* L)." Euphytica, **94**: 263-272.

MARQUEZ-CEDILLO, L. A., HAYES, P. M., JONES, B. L., KLEINHOFS, A., LEGGE, W. G., ROSSNAGEL, B. G., SATO, K., ULLRICH, E., WESENBERG, D. M. (2000): "QTL analysis of malting quality in barley based on the doubled-haploid progeny of two elite North American varieties representing different germplasm groups." Theoretical & Applied Genetics, **101**: 173-184.

MATHER, D. E., TINKER, N. A., LABERGE, D. E., EDNEY, M., JONES, B. L., ROSSNAGEL, B. G., LEGGE, W. G., BRIGGS, K. G., IRVINE, R. B., FALK, D. E., KASHA, K. J. (1997): "Regions of the Genome That Affect Grain and Malt Quality in a North American Two-Row Barley Cross." Crop Science, **37**: 544-554.

MCMICHAEl, G.L., EGLINTON, J.K., BARR, A.R., CHALMERS, K.J. (2004): Identification of Marker Trait Associations in a Barley Four Way Cross. This edition.

OBERTHUR, L., BLAKE, T., DYER, W. E., ULLRICH, S. E. (1995): "Genetic Analysis of Seed Dormancy in Barley (*Hordeum valugare L.*)." J. of Agric. Genomics, **1:** Paper 5.

OZIEL, A., HAYES, P. M., CHEN, F. Q., JONES, B. (1996): "Application of Quantitative Trait Locus Mapping to the Development of Winter-Habit Malting Barley." Plant Breeding, **115**: 43-51.

PALLOTTA, M., ASAYAMA, S., REINHEIMER, J., DAVIES, P., BARR, A., JEFFERIES, S., CHALMERS, K., LEWIS, J., COLLINS, H., ROUMELIOTIS, S., LOGUE, S., COVENTRY, S., LANCE, R., KARAKOUSIS, A., LIM, P., VERBYLA, A., ECKERMAN, P. (2003): "Mapping and QTL analysis of the barley population Amagi Nijo x WI2585." Australian Journal of Agricultural Research, **54:** 1141-1144.

PANOZZO, JF; P. LIM; P.J. ECKERMAN; D.B. MOODY; A.R. BARR; B.J. READ; B.R. CULLIS (2004): Quantitative Trait Locus Analysis of Malting Quality Barley (*Hordeum Vulgare L.*) in Elite Progeny from the Australian National Barley Molecular Marker Program. (in preparation)

POWELL, W., THOMAS, W. T. B., BAIRD, E., LAWRENCE, P., BOOTH, A., HARROWER, B., MCNICOL, J. W., WAUGH, R. (1997): "Analysis of Quantitative Traits in Barley By the Use of Amplified Fragment Length Polymorphisms." Heredity, **79**(Part 1): 48-59.

ROUMELIOTIS S., S.J. LOGUE, C. HUNT, A.R. BARR (2001): Pre-release characterisation

of the malting profile of WI-3102. Proc Australian Barley Technical Symposium, Canberra

ROUMELIOTIS S., BARR A.R. (2003): The Use of NIR Spectroscopy to Predict Husk Content and Percent Skinnings in Barley. In: Near Infrared Spectroscopy: Proceedings of the 11th International Conference, NIR publications UK.

THOMAS, W. T. B., POWELL, W., WAUGH, R., CHALMERS, K. J., BARUA, U. M., JACK, P., LEA, V., FORSTER, B. P., SWANSTON, J. S., ELLIS, R. P., HANSON, P. R. LANCE, R. C. M. (1995): "Detection of Quantitative Trait Loci For Agronomic, Yield, Grain and Disease Characters in Spring Barley (*Hordeum Vulgare* L)." Theoretical & Applied Genetics, **91**: 1037-1047.

ULLRICH, S. E., HAN, F., JONES, B. L. (1997): "Genetic Complexity of the Malt Extract Trait in Barley Suggested By Qtl Analysis." Journal of the American Society of Brewing Chemists, **55**: 1-4.

ZALE, J. M., CLANCY, J. A., ULLRICH, S. E., JONES, B. L., HAYES, P. M., North_American_Barley_Genome_Mapping_Project (2000): "Summary of Barley Malting Quality QTLs Mapped in Various Populations." Barley Gen. Newsl., **30**: 44-54.

ZWICKERT-MENTEUR, S., JESTIN, L., BRANLARD, G. (1996): "Amy2 Polymorphism As a Possible Marker of Beta-Glucanase Activity in Barley (Hordeum Vulgare L)." Journal of Cereal Science, 24: 55-63

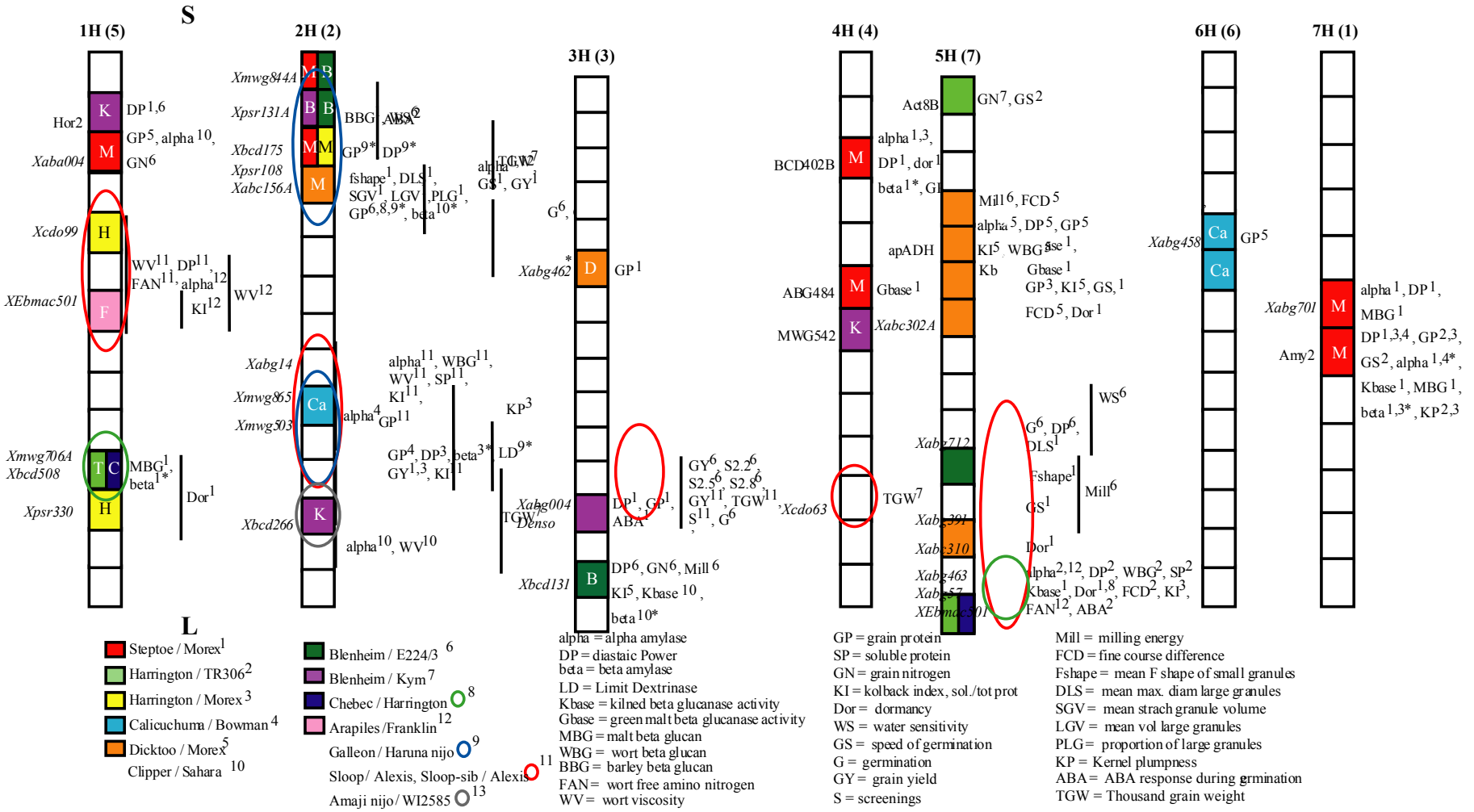

S
1H (5)
2H (2)
3H (3)
4H (4)
5H (7)
6H (6)
7H (1)
Hor2
Xaba004
Xcdo99
XEbmac501
Xmwg706A
Xbcd508
Xpsr330
Xmwg844A
Xpsr131A
Xbcd175
Xpsr108
Xabc156A
Xabg14
Xmwg865
Xmwg503
Xbcd266
Xabg462*
Xabg004
Denso
Xbcd131
BCD402B
ABG484
MWG542
Xabc302A
Xcdo63
Act8B
apADH
Xabg712
Xabg391
Xabc310
Xabg463
Xabg457
XEbmac301
Xabg458
Xabg701
Amy2
L
Steptoe / Morex 1
Harrington / TR306 2
Harrington / Morex 3
Calicuchuma / Bowman 4
Dicktoo / Morex 5
Clipper / Sahara 10
Blenheim / E224/3 6
Blenheim / Kym 7
Chebec / Harrington 8
Arapiles /Franklin 12
Galleon / Haruna nijo 9
Sloop/ Alexis, Sloop-sib / Alexis 11
Amaji nijo / WI2585 13
alpha = alpha amylase
DP = diastaic Power
beta = beta amylase
LD = Limit Dextrinase
Kbase = kilned beta glucanase activity
Gbase = green malt beta glucanase activity
MBG = malt beta glucan
WBG = wort beta glucan
BBG = barley beta glucan
FAN = wort free amino nitrogen
WV = wort viscosity
GP = grain protein
SP = soluble protein
GN = grain nitrogen
KI = kolback index, sol./tot prot
Dor = dormancy
WS = water sensitivity
GS = speed of germination
G = germination
GY = grain yield
S = screenings
Mill = milling energy
FCD = fine course difference
Fshape = mean F shape of small granules
DLS = mean max. diam large granules
SGV = mean strach granule volume
LGV = mean vol large granules
PLG = proportion of large granules
KP = Kernel plumpness
ABA = ABA response during grmination
TGW = Thousand grain weight

Figure 1: A schematic diagram of a number of regions found to be associated with HWE in Australian mapping populations. Each chromosome is separated into a number of regions called Bins as described by Kleinhofs and Han (2002). Coloured squares represent regions found in mapping populations around the world. Circles represent regions investigated in this study. Markers are listed left of each chromosome. Listed right of the chromosome are other traits found to be associated with each region.

References

1. Hayes et al., 1993; Han et al., 1995; Oberthur et al., 1995; Zwickert-Menteur et al., 1996; Han et al., 1997; Larson et al., 1996; Mano and Takeda, 1997; Ullrich et al., 1997; Borem et al., 1999; Zale et al., 2000; Hayes et al., 2001
2. Mano and Takeda, 1997; Mather et al., 1997; Zale et al., 2000; Hayes et al., 2001
3. Marquez-Cedillo et al., 2000; Zale et al., 2000; Hayes et al., 2001
4. Hayes et al., 1996; Hayes et al., 1997; Zale et al., 2000; Hayes et al., 2001
5. Oziel et al., 1996; Zale et al., 2000; Hayes et al., 2001
6. Thomas et al., 1995; Thomas et al., 1996; Powell et al., 1997; Zale et al., 2000; Hayes et al., 2001
7. Bezant et al., 1997a; Bezant et al., 1997b; Zale et al., 2000; Hayes et al., 2001
8. Li et al., 1996; Zale et al., 2000; Barr et al., 2003a; Collins et al., 2003
9. Li et al., 1996; Zale et al., 2000; Karakousis et al., 2003a
10. Zale et al., 2000; Karakousis et al., 2003c
11. Barr et al., 2003b; Panozzo et al., in preparation
12. Moody et al., unpublished; Panozzo et al., in preparation
13. Asamaya, unpublished; Pallotta et al., 2003

Experiences with Marker-Assisted Selection for Quantitative Traits in Barley

S.E. Ullrich[1], A. Kleinhofs[1], I. Romagosa[2], F. Han[1,3], W. Gao[1,4] and D. Schmierer[1]

[1]Crop & Soil Sci., Washington State Univ., Pullman, WA 99164-6420, USA;
[2]Centre Univ. de Lleida –IRTA, 25198, Lleida, Spain;
[3]Currently, Pioneer Hi-Bred Int'l., Inc., Johnston, IA 50131, USA;
[4]Currently, Dept. of Genetics, Univ. of Georgia, Athens, GA 30602, USA

Abstract
Molecular marker-assisted selection (MMAS) is a proven breeding and genetics research strategy for simply inherited traits. MMAS for complexly inherited true quantitative traits is less certain. Our research through the North American Barley Genome Project concentrated on malting quality and grain yield traits. These traits are difficult to evaluate and select due to number of genes, genotype (G), environment (E), GxG, and GxE effects, and cost. For malting quality, major quantitative trait locus (QTL) regions on chromosomes 1 (7H) (QTL1) and 4H (QTL2) with multi-trait QTLs (malt extract, alpha-amylase, diastatic power, beta-glucan) were MMAS targets in 'Steptoe' / 'Morex' (S/M) crosses. MMAS for QTL1 was effective but not for QTL2. Fine mapping of QTL2 improved its selectability. For yield in S/M, MMAS for QTLs on chromosomes 3H and 6H was more effective than for QTLs on chromosomes 2H and 7 (5H). Another S/M MMAS study resulted in lines with improved yield-related traits but not yield per se. MMAS resulted in improved yield and malting quality in 'Harrington' / 'Baronesse' backcross lines. Lessons learned include: 1) Quantitative traits are still complex, 2) genotypic or tandem genotypic-phenotypic selection can be as good as or better than phenotypic selection, 3) response of individual QTLs can be complicated by epistasis and cross-over GXE, 4) Optimum QTL allele composition is difficult to predict, 5) QTL fine mapping can improve MMAS, 6) MMAS can help maintain high malting quality in progeny from feed/malting type crosses for malting barley agronomic improvement.
Keywords: *Hordeum vulgare* L.; molecular markers; molecular breeding; QTL; epistasis

Introduction

Molecular genetic study of barley (*Hordeum vulgare* L.) and other crop species directed at construction of comprehensive molecular marker chromosome maps and molecular genetic analyses of agronomically and other economically important traits have led to a revolution in plant breeding. Relatively accurate chromosome locations of relevant genes or markers closely linked to relevant genes or quantitative trait loci (QTLs) allows for molecular marker-assisted selection (MMAS) in breeding and genetic study. This is particularly important for breeding difficult or expensive to select traits, including such traits as disease and insect resistance and quantitatively inherited agronomic and quality traits. Working with these traits is difficult because of the number of genes involved, genotype (G), environment (E), GxG, and GxE effects, and/or analysis costs. MMAS is a more proven breeding strategy for simply inherited traits, but MMAS for complexly inherited true quantitative traits is less certain. THOMAS (2002) presented a recent review of MMAS in barley. Considerable molecular genetics and breeding research and practice has occurred in barley over the past 25 years with major mapping and/or breeding programs, for example, in North America (http://www.barleyworld.org/NABGP.html), Europe (e.g.,http://pgrc.ipk-gatersleben.de; http://www.scri.sari.ac.uk/), Japan (http://www.rib.okayama-u.ac.jp/), and Australia (http://www.agwine.adelaide.edu.au/research/plant/plant/cb/).

Molecular breeding in barley for single gene traits has been particularly successful for major disease resistance genes. Examples include molecular marker-assisted introgression of the barley mild mosaic / barley yellow mosaic virus resistance gene, *rym4* (TUVESSON *et al*. 1998) and molecular marker-assisted backcross breeding of the barley yellow dwarf virus resistance gene *ryd2* (JEFFERIES *et al*. 2003). Barley stripe rust (caused by *Puccinia sriiformis* Westend. f. sp. *hordei*) resistance can be conditioned by major race-specific genes and/or by horizontal or quantitative resistance genes. The later genes tend to condition more durable resistance. Recent research has uncovered over 30 resistance genes both race-specific and quantitative (QTLs) across the chromosomes of barley (CHEN *et al*. 1994; TOOJINDA *et al*. 2000; CHEN & LINE 2003). Apparently, as few as two QTLs can condition durable resistance to barley stripe rust as evidenced by the MMAS developed and released cultivars Orca (HAYES *et al*. 2000) and Tango (HAYES *et al*. 2003).

Seed dormancy is another trait in barley that is quantitatively inherited. It is conditioned by a range of environmentally stable and unstable QTLs (27+). However, perhaps only four consistently expressed QTLs with allele effects of 5-50% are amenable to MMAS (ULLRICH *et al*. 1993; HAN *et al*. 1996a). MMAS of one of the two major-effect QTLs (15 and 50%) on chromosome 7 (5H) had a significant effect on the level of dormancy (HAN *et al*. 1996a; HAN *et al*. 1999; GAO *et al*. 2003). Barley stripe rust resistance and seed dormancy level are examples of situations in which traits can be very complexly inherited, but can be selected for or affected by one or two of the potentially many QTLs involved. This can simplify the practical molecular breeding process.

This paper is a reflection of experiences working with MMAS of two of the most difficult trait complexes in barley, grain yield and malting quality. The molecular genetic study and breeding application of these two trait complexes have been research priorities over the past 10+ years. Research performed directly or through collaboration will be primarily considered and cited.

Material and Methods

The research was part of the North American Barley Genome Project (NABGP) and involved the NABGP six-row 'Steptoe' / 'Morex' (S/M) doubled haploid (DH) mapping population (n = 150), a DH selection population (n = 92) not used in mapping, and molecular marker-assisted backcross derivatives of individual DH lines. Steptoe is a widely adapted, high yielding, feed type that dominated production in the Pacific Northwest, USA for almost 20 yr. Morex is a North American six-row malting quality standard. The basis for the research described herein was the original molecular map published by KLEINHOFS *et al*. (1993) and the original QTL analyses published by HAYES *et al*. (1993) and HAYES and IYAMABO (1996). Other research involved molecular marker-assisted backcross lines from 'Harrington' / 'Baronesse' (H/B) crosses. Harrington is a North American two-row malting quality standard. Harrington has been molecularly and phenotypically characterized as a parent in the NABGP mapping populations, Harrington / TR306 (H/T) (MATHER *et al*. 1997) and Harrington / Morex (MARQUEZ-CEDILLO *et al*. 2000). Baronesse is a German two-row variety that is widely adapted, high yielding, and currently dominates Pacific Northwest production. Current map and QTL information and data can be found on the NABGP website http://www.barleyworld.org/NABGP.html or links to it such as the USDA-ARS GrainGenes (http://wheat.pw.usda.gov/) and Washington State University Barley Genomics (http://barleygenomics.wsu.edu/) websites.

Traits for this presentation that were measured, mapped and selected were grain yield and the associated sub-traits, plant height, lodging, flowering time, shattering, kernel weight, and nodes/spike; and the malting quality traits, malt extract content (ME), diastatic power (DP), alpha-amylase activity (AA), and malt beta-glucan content (BG) The barley populations or backcross progeny, and parents were grown under multiple field test sites and years using standard production practices in the Pacific Northwest, USA. Malting quality was determined using micro-malting and malt analysis techniques using standard procedures at the USDA-ARS Cereal Crops Research Unit, Madison, WI, USA and usually according to the American Society of Brewing Chemists procedures (KNEEN 1976 with updates). Selection methods included genotypic (MMAS), phenotypic, and tandem genotypic – phenotypic schemes.

Results and Discussion

Malting Quality

The effectiveness of MMAS for malting quality was tested with an S/M DH population not used in the construction of the NABGP map on two chromosome regions containing major QTLs for ME, DP, AA, and BG; one on chromosome 1 (7H) flanked by Brz and Amy2 of ~27 cM (QTL1) and the other on chromosome 4S (4H S) flanked by WG622 and BCD402B of ~29 cM (QTL2) initially determined by HAYES *et al.* (1993) and HAN *et al*. (1995). Total variation explained for the traits in QTL1 and QTL2 ranged from 40-54% and 27-38%, respectively. Subsequent study indicated that these two QTL regions act additively without apparent epistasis (HAN *et al.* 1996b). HAN *et al*. (1997) found that QTL1 was stable in selection response using MMAS, but QTL2 was not due to a lack of effect in the selection population. In general a tandem genotypic – phenotypic selection scheme was more effective than either genotypic or phenotypic selection alone. A study conducted in a similar manner with an H/T population produced similar results both in terms of variable QTL selection response and selection scheme effectiveness (IGARTUA *et al*. 2000).

There were two problems with MMAS in the S/M malting quality study. One was the non-response of QTL2 in the selection scheme. However, subsequent fine mapping of this chromosome 4S region perhaps revealed the reason. Fine mapping was done on an expanded interval (by ~13 cM) thinking that the original interval was too small and the active region was missed. Indeed, fine mapping using reciprocal and duplicate molecular marker-assisted backcross isolines revealed that QTL effects for all four traits extended beyond the originally designated flanking markers (GAO *et al.* 2004). Fine mapping would likely improve the effectiveness of MMAS in that smaller and more accurate chromosome segments could be involved in chromosome introgression. QTL1 (HAN et al. 2004) as well as QTL2 have been fine-mapped revealing not only a complex of multiple traits but also multiple QTLs in these chromosome regions affecting malting quality. This kind of information can lead to additional study as well as refined MMAS. The second problem revealed in this study was that among the marker selected lines were several that did not have top quality and among the rejected lines were several that had top quality. Of course this is expected in essentially any selection scheme, certainly with phenotypic selection. A potential reason for this phenomenon is that the function of the major QTLs is regulated by or these QTLs interact with the allelic state of other minor QTLs in the genome. Certainly there are other known QTLs and structural genes for these traits in the genome.

Grain Yield

Comprehensive interval mapping QTL analyses of the S/M mapping population for yield and yield related traits identified QTLs on all seven barley chromosomes (HAYES *et al*. 1993; HAYES & IYAMABO 1996). In these initial studies, QTLs on Chromosome 2 (2H) and 3

(3H) were detected in 11 and 10 of 16 test environments, respectively, across the USA and Canada. All other QTLs were detected in only one or two environments. All yield QTLs except the one on chromosome 5 (1H) were variously coincident with yield related traits including lodging, plant height, shattering, heading date, kernel weight, and/or nodes/spike (HAYES *et al.* 1993; HAYES & IYAMABO 1996; KANDEMIR *et al.* 2000). The chromosome 3 yield QTL, considered to be the most significant QTL, was associated with all the above sub-traits, while the chromosome 2 yield QTL was associated with lodging, plant height, and heading date.

Four yield QTLs, one each on chromosomes 2, 3, 6 (6H), and 7 (5H) were MMAS targets within an S/M population of 92 DH lines not used for construction of the NABGP map (ROMAGOSA *et al. 1999*). The population was field-evaluated in three environments over two years. Positive selection response was greatest with the chromosome 3 S allele and combined chromosome 3 S and chromosome 6 M alleles. The QTLs on chromosomes 2 and 7 displayed qualitative or crossover QTL x E interactions. It was concluded that genotypic and tandem genotypic – phenotypic selection were superior or as good as phenotypic selection in individual environments.

Pyramiding all or most of the yield QTLs in the S/M background in F_1 derived DH lines from a cross of two selected S/M DH mapping lines did not result in lines with superior yields (ZHU *et al*. 1999). Field tests were conducted in five environments over two years. All QTLs showed QTL x E interactions, and the chromosome 2 QTL interaction was qualitative. Digenic epistasis among the QTLs was also detected. This study concluded that selecting optimum QTL combinations would be more effective than accumulating all positive QTL alleles into one genotype.

In another S/M study, the objective was to transfer high yielding QTL alleles on chromosomes 2, 3, and/or 5 from Steptoe into Morex to increase yield and maintain high malting quality (KANDEMIR *et al*. 2000). Three S/M DH mapping lines were selected for marker-assisted backcrossing (BC_3 or BC_4) to Morex to transfer the Steptoe high yield QTL alleles. Based on testing in four environments, none of the three QTLs alone or in combination altered the yield of the Morex isolines above Morex. However, the chromosome 2 QTL increased flowering duration, and the chromosome 3 QTL reduced plant height, lodging, and head shattering in some isolines. It was concluded that these yield QTLs must interact with other genes for full expression of yield per se. High malting quality was maintained in the Morex isolines indicating that none of the Steptoe yield QTL alleles negatively impacted quality. LARSON *et al*. (1997) reported a similar finding studying the effect of the chromosome 3 Steptoe yield QTL allele in S/M BC_1 derived lines.

The transfer of high yield QTL alleles on chromosomes 2L and 3L from the widely adapted cultivar Baronesse to the high malting quality cultivar Harrington involves another marker-assisted backcross scheme. Whereas this study is still in progress, a number of results and lessons have been gained (SCHMIERER *et al.* 2004). Several BC_3 Harrington isolines have displayed yields equal to Baronesse across test sites and years. And in most cases the malting quality of Harrington has been maintained or improved. The chromosome 3L QTL seems to be more effective than the 2L QTL. One line equaled the yield of Baronesse across 22 site-yr, but yielded less in the dry 2003 season. None of the isolines tested have both Baronesse yield QTL alleles. One line has yielded similarly to Baronesse but has low quality. It does not have either of the targeted yield QTL regions from Baronesse nor does it have a major malting quality chromosome 7L QTL region form Harrington (MATHER *et al.* 1997). Results thus far

indicate that there are other yield QTL regions of importance in Baronesse with portions of chromosomes 2S, 5L, and 6L as leading candidates, and the targeted yield QTL alleles from Baronesse do not negatively impact the malting quality of Harrington.

Conclusions

A number of conclusions or lessons have been gained from our experience with MMAS of quantitative traits in barley, which is an extension and practical breeding application of QTL identification and mapping:

1. Quantitative traits are still complex with much to learn yet even with the advances and advantages of using molecular genetics tools.
2. Genotypic (MMAS) or tandem genotypic – phenotypic selection can be as effective or more effective than phenotypic selection alone. Breeding effectiveness and potentially cost advantages of a tandem molecular marker – phenotypic selection scheme are (1) genotypes with potentially good malting quality or high yield can be identified in seedlings as early as the F_2, (2) no grain is needed for malt analyses or yield trials at early stages of a breeding program, and (3) after MMAS, the number of lines for malt analyses or yield trials at later stages is substantially reduced.
3. Selection responses of individual QTLs can be complicated by epistasis and QTL x E interaction, especially the QTL x E crossover type.
4. Optimum QTL allele composition is difficult to predict. For example, single QTL introgression may not be effective and total QTL introgression (pyramiding) may not be effective either.
5. Fine mapping of QTLs can improve MMAS. Conventional first order QTL analysis by interval mapping is rather crude. Refinement of map position or flanking marker linkage associations by any means can only improve transferring a target QTL with a minimum of linkage drag, since linkage drag can be detrimental. It is likely that microarray technology will improve or complement if not replace conventional MMAS in the near future.
6. Thus far in our MMAS experience with both six-rows and two-rows, when selecting for yield QTLs from feed barley in a malting barley background, the malting quality QTL complexes can be maintained by molecular marker monitoring. Conversely, when a line is selected with a significant malting quality QTL segment replaced by a donor feed barley segment, reduced malting quality is reflected in that line. Conventionally, malting barley breeders have avoided crosses that are so wide that malting quality trait complexes may be disrupted. MMAS can help overcome this concern and problem.

References

CHEN F.Q., PREHN D., HAYES P.M., MULROONEY D., COREY A., VIVAR H. (1994): Mapping genes for resistance to barley stripe rust (*Puccinia striiformis f.sp. hordei*). Theor. Appl. Genet., **88**: 215-219.

CHEN X.M., LINE R.F. (2003): Identification of genes for resistance to *Puccinia striiformis* f. sp. *hordei* in 18 barley genotypes. Euphytica, **129**: 127-145.

GAO W., CLANCY J.A., HAN F., JONES B.L., BUDDE A., WESENBERG D.M., KLEINHOFS A., ULLRICH S.E., NABGP (2004): Fine mapping of a malting quality QTL complex near the chromosome 4H S telomere in barley. Theor. Appl. Genet., In press.

GAO W., CLANCY J.A., HAN F., PRADA D., KLEINHOFS A., ULLRICH S.E. (2003): Molecular dissection of a dormancy QTL region near the chromosome 7 (5H) L telomere in barley. Theor. Appl. Genet., **107**: 552-559.

HAN F., CLANCY J.A., JONES B.L., WESENBERG D.M., KLEINHOFS A., ULLRICH S.E. (2004): Dissection of a malting quality QTL region on chromosome 1 (7H) of barley. Mol. Breed., In press.

HAN F., ROMAGOSA I., ULLRICH S.E., JONES B.L., HAYES P.M., WESENBERG D.M. (1997): Molecular marker-assisted selection for malting quality traits in barley. Mol. Breed., **3**: 427-437.

HAN F., ULLRICH S.E., CLANCY J.A., JITKOV V., KILIAN A., ROMAGOSA. I. (1996a): Verification of barley seed dormancy loci via linked molecular markers. Theor. Appl. Genet., **92**: 87-91.

HAN F., ULLRICH S.E., CHIRAT S., MENTEUR S., JESTIN L., SARRAFI A., HAYES P.M., JONES B.L., BLAKE T.K., WESENBERG D.M., KLEINHOFS A., KILIAN A. (1995): Mapping of beta-glucan content and beta-glucanase activity loci in barley grain and malt. Theor. Appl. Genet., **91**: 921-927.

HAN F., ULLRICH S.E., CLANCY J.A., ROMAGOSA I. (1999)**:** Inheritance and fine mapping of major barley seed dormancy QTL. Plant Sci., **143**: 113-118.

HAN F., ULLRICH S.E., ROMAGOSA I., JONESB.L., NABGMP (1996b): Genotype-phenotype associations at major malting quality QTL and implications for molecular marker assisted selection in barley. In: SlinkardA., Scoles G., Rossnagel B. (eds.): V Int'l. Oat Conf. & VII Int'l. Barley Genet. Symp. Poster Ses. Vol. 1. Univ. of Saskatchewan Ext. Press, Saskatoon, Canada: 259-261.

HAYES P.M., COREY A.E., DOVELL R., KAROW R., UNDI C., RHINART K.H., VIVAR H. (2000): Registration of Orca barley. Crop Sci., **40**:849-851.

HAYES P.M., COREY A.E, MUNDT C., TOOJINDA T., VIVAR H. (2003): Registration of Tango barley. Crop Sci., **43**:729-731.

HAYES P.M., IYAMABO, NABGMP (1996): Summary of QTL effects in the Steptoe X Morex population. Barley Genet. Newsl., **23**: 98-143.

HAYES P.M., LIU B.H., KNAPP S.J., CHEN, F., JONES B., BLAKE T., FRANCKOWIAK J., RASMUSSON D., SORRELLS M., ULLRICH S.E., WESENBERG D., KLEINHOFS A., NILAN R.A. (1993): Quantitative trait locus effects and environmental interaction in a sample of North American barley germplasm. Theor. Appl. Genet.*,* **87**: 392-401.

IGARTUA E., EDNEY, M., ROSSNAGEL, B.G., SPANER, D., LEGGE, W.G., SCOLES, G.J., ECKSTEIN, P.E., PENNER, G.A., TINKER, N.A., BRIGGS, K.G., FALK, D.E. (2000): Marker-based selection of QTL affecting grain and malt quality in two-row barley. Crop Sci., **40**: 1426-1433.

JEFFERIES S.P., KING B.J., BARR A.R., WARNER P., LOGUE S.J., LANGRIDGE P. (2003): Marker-assisted backcross introgression of the *Yd2* gene conferring resistance to barley yellow dwarf virus in barley. Plant Breed., **122**: 52-56.

KANDEMIR N., JONES B.L., WESENBERG D.M., ULLRICH S.E., KLEINHOFS A. (2000) Marker-assisted analysis of three grain yield QTL in barley (Hordeum vulgare L.) using near isogenic lines. Mol. Breed*.,* **6**: 157-167.

KLEINHOFS A., KILIAN A., SAGHAI MAROOF M.A., BIYASHEV R.M., HAYES P., CHEN F.Q., LAPITAN N., FENWICK A., BLAKE T.K., KANAZIN V., ANANIEV E., DAHLEEN L., KUDRNA D., BOLLINGER J., KNAPP S.J., LIU B., SORRELLS M., HEUN M., FRANCKOWIAK J.D., HOFFMAN D., SKADSEN R., STFFENSON B.J. (1993): A molecular, isozyme and morphological map of the barley (Hordeum vulgare) genome. Theor. Appl. Genet., **86**: 705-712.

KNEEN E. (ed) (1976): ASBC Methods of Analysis 7th Edition. Am. Soc. Brew. Chem., St Paul, MN, USA.

LARSON S.R., HABERNICHT D.K., BLAKE T.K., ADAMSON M. (1997): Backcross gains for six-rowed grain and malt qualities with introgression of a feed barley yield QTL. J. Amer. Soc. Brew. Chem., **55**: 52-57.

MARQUEZ-CEDILLO L.A., HAYES P.M., JONES B.L., KLEINHOFS A., LEGGE W.G., ROSSNAGEL B.G., SATO K., ULLRICH S.E., WESENBERG D.M. (2000): QTL analysis of malting quality in barley based on the doubled haploid progeny of two elite North American varieties representing different germplasm groups. Theor. Appl. Genet., **101**: 173-184.

MATHER D.E., TINKER N.A., LABERGE D.E., EDNEY M., JONES B.L., ROSSNAGEL B.G., LEGGE W.G., BRIGGS K.G., IRVINE R.B., FALK D.E., KASHA K.J. (1997): Regions of the genome that affect grain and malt quality in a North American in a two-row barley cross. Crop Sci., **37**: 544-554.

ROMAGOSA I., HAN F., ULLRICH S.E., HAYES P.M., WESENBERG D.M. (1999): Verification of yield QTL through realized molecular marker-assisted selection responses in a barley cross. Mol. Breed., **5**: 143-152.

SCHMIERER D.A., KANDEMIR N., KUDRNA D.A., JONES B.L., ULLRICH S.E., KLEINHOFS A. (2004): Molecular marker-assisted selection for enhanced yield in malting barley. Mol. Breed., Submitted.

THOMAS W.T.B. (2002): Molecular marker-assisted selection versus conventional selection in barley breeding. In: Slafer G.A., Molina-Cano J.L., Savin R, Araus J.L., Romagosa I. (eds.). Barley Science: Recent Advances from Molecular Biology to Agronomy of Yield and Quality. Haworth Press, Inc., New York: 177-204.

TOOJINDA T., BROERS L.H., CHEN X.M., HAYES P.M., KLEINHOFS A., KORTE J., KUDRNA D., LEUNG H., LINE R.F., POWELL W. (2000): Mapping quantitative and qualitative disease resistance genes in a doubled haploid population of barley (*Hordeum vulgare*). Theor. Appl. Genet., **101**: 580-589.

TUVESSON S., POST L., OHLUND R., HAGBERG P., GRANER A., SVITASHEV S., SCHEHR M., ELOVSSON R. (1998): Molecular breeding for the BaMMV/BaYMV resistance gene *ym4* in winter barley. Plant Breed., **117**: 19-22.

ULLRICH S.E., HAYES P.M. DYER W.E., BLAKE T.K., CLANCY J.A. (1993): Quantitative trait locus analysis of seed dormancy in 'Steptoe' barley. In: Walker-Simmons M.K, Reid J.L. (eds.): Preharvest Sprouting in Cereals 1992. Amer. Assoc. Cereal Chem., St. Paul, MN, USA: 136-145.

ZHU H., BRICENO G., DOVEL R., HAYES P.M., LIU B.H., LIU C.T., ULLRICH S.E. (1999): Molecular breeding for grain yield in barley: an evaluation of QTL effects in a spring barley cross. Theor. Appl. Genet., **98**: 772-779.

A Large Scale Mapping of ESTs on Barley Genome

K. Sato[1], N. Nankaku[1,2], Y. Motoi[1,2] and K. Takeda[1]

[1]Research Institute for Bioresources, Okayama University, Kurashiki, 710-0046, Japan;
[2]CREST, Japan Science and Technology Cooperation, Kawaguchi, 332-0012, Japan

Abstract
ESTs are the most informative sources of genetic markers on the linkage map in barley. We have generated ca. 60,000 3' end ESTs (Expressed Sequence Tags) from nine different cDNA libraries. Each EST sequence was base-called by phred, trimmed with vector sequences and quality controlled at the threshold QV (quality value)=20. Non-redundant sequences were developed by phrap (contigs 8,753, singlets 6,686) to develop primers by Primer3. Ca. 11,000 primer sets were synthesized. Our principal methods of mapping ESTs are (1) simple polymorphisms appear on agarose gel, (2) SNP (Single Nucleotide Polymorphism) analysis after sequencing of parental PCR amplicons either using PCR-RFLP or SNP typing system. In March 2004, more than 1,000 ESTs have been mapped by the simple polymorphisms on the agarose gel or PCR-RFLPs. The project aims to localize several thousands of non-redundant ESTs on the barley linkage map.

Introduction

The number of ESTs in barley has been increased dramatically in a recent few years and reached around 380,000 in March 2004 on the public databases collected by NCBI dbEST summary (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). EST has several advantages as a genetic marker as follows: (1) EST is a partial sequence of the gene and is mapped on the respective gene position on the genome; (2) By assembling the sequences of ESTs, especially 3' end ESTs, non-redundant set of genes can be assumed, and thus marker rich and poor regions on the map correspond to the gene rich and poor regions of the genome, respectively; (3) A cDNA clone where EST is derived can be used as an RFLP probe; (4) PCR based markers are easily developed from the EST sequences; (5) Markers can be directly used to identify positive BAC clones for gene isolation; (6) Genome wide gene expression analysis to estimate gene function is available from DNA array systems by PCR products of cDNA clones or synthesizing oligo-nucleotide from EST sequences (CLOSE *et al.* 2004). Several papers have already reported the abundant polymorphisms among barley ESTs by comparing different strains used to generate ESTs (KOTA *et al.* 2001, KOTA *et al.* 2003). Other than these polymorphisms, introns within genes may be rich source of polymorphisms if we can compare these sequences among different barley germplasms.

We have generated ESTs from barley including three representative strains of *Hordeum vulgare* L. collected in Okayama University. Since these strains have different genetic background from the point of evolution, geographical differentiation and uses, we assumed to identify polymorphisms by comparing EST sequences (SATO in preparation). For the development of high-resolution barley transcript map, we started mapping of ESTs by using the mapping population from two of the three EST donors as parents. The significant achievement by the mapping effort for the last one year is reported here.

Material and Methods

Source of ESTs

Three barley strains were used to construct cDNA libraries. H602 is a wild barley strain classified as *Hordeum vulgare* ssp. *spontaneum* (BOTHMER *et al.* 1995), which is assumed as a possible progenitor of cultivated barley (*Hordeum vulgare* ssp. *vulgare*). The both subspecies are classified

as *Hordeum vulgare* L. after BOTHMER *et al.* (1995). Akashinriki is a cultivated barley and a Japanese six-row hull-less landrace used for human food. Haruna Nijo is another cultivated barley and a two-row malting cultivar grown in Japan. Nine cDNA libraries were constructed by the procedure described in HarvEST (http://harvest.ucr.edu/; CLOSE and WANAMAKER 2004) or oligo capping method (MARUYAMA and SUGANO 1994). Cycle-sequencing was performed by ABI3700 or ABI3100 DNA sequencers with BigDye-terminator (Applied Biosystems Co.) from both 3' and 5' ends of cDNA clones.

EST Data Processing
Each read was base-called by the software package phred and vector trimmed. Poly A and Poly T stretches were also trimmed. By using phred base-calling quality score, high quality sequences were selected from each read, followed by the mapping of ABI base-calling results to confirm the identity of high quality sequences. The threshold of high quality sequence was set at QV=20. The resulted sequences were used for the clustering, multiple alignment and mostly published on the public DNA database.

Primer Development
Ca. 60,000 3' ESTs were assembled by the software package phrap and non-redundant set of sequences were developed (contigs 8,753, singlet 6,686). The EST with longest high quality sequence was selected from each contig to develop primer sets by the software package Primer3. Sequence from each singlet was also used to develop primer sets. The optimum conditions of primer development were Tm=60 ^{o}C (difference less than 2 ^{o}C between forward and reverse primers), GC content =50% (difference less than 20%), target size=400bp (ranged from 150 to 500bp). Ca. 11,000 primer sets were generated under these conditions.

Mapping Population
The pollen sample from F_1 plant derived from the cross between Haruna Nijo and H602 was harvested to develop haploids by microspore culture method (JAISER, personal communication). Plants naturally diploidized and matured were selected. Ninety-three doubled haploid lines were used for mapping.

Polymorphism Detection and Mapping
The genomic DNA samples of mapping parents (Haruna Nijo and H602) and their F_1 were amplified by PCR reaction to detect polymorphisms between parents. The PCR amplicons were electrophoresed on the 1.5% agarose gel and checked the amplification condition and polymorphisms on the agarose gel. If polymorphisms were detected between parental amplicons by presence/absence or size difference, they were used for the genotyping of mapping population (hereafter referred as 'simple polymorphism'). At the same time, each PCR amplicon from parents by all the developed primer sets was sequenced. The resulted sequences by the same primer set were aligned using the software package CLUSTALW and checked if they had polymorphisms between the sequences, which could be recognized by the respective restriction enzymes. If there is an enzyme recognition site between parents, it was used for the genotyping of mapping population as a CAPS (PCR-RFLP) marker. The SSR and STS markers which had known position of the chromosome by the previous reports (RAMSAY *et al.* 2000; MANO *et al.* 1999) were also used to screen polymorphisms between parents and to genotype mapping population. The linkage of overall marker data was analyzed by the software package MAPMAKER/EXP. The LOD threshold was 5.0 to declare the linkage on the map.

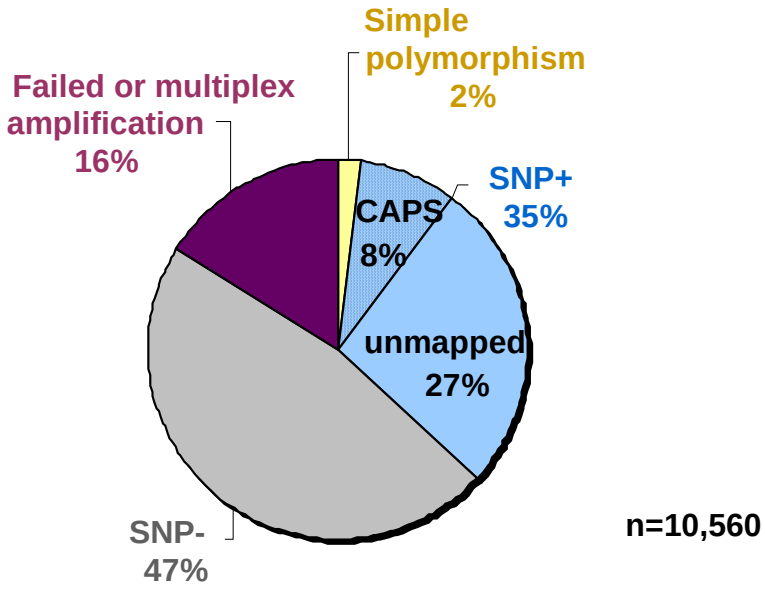


Figure 1. Categories of polymorphism for the PCR amplicons between mapping parents Haruna Nijo and H602 by the primer sets developed from the non-redundant 3' ESTs in Okayama University. SNP+ includes both single nucleotide substitutions and indels between parents.

Results and Discussion

Degree of Polymorphism

The agarose gel electrophoresis revealed that parental polymorphisms by the simple polymorphism are around 2% of the total primer sets used (n=10,560) in this study (Fig. 1). On the other hand, 16% of the primer sets could not be evaluated due to the failed or multiplex amplifications, which might come from the errors in primer development or inadequate PCR amplification conditions. After the analysis of sequences of PCR amplicons of parents, SNPs (Single Nucleotide Polymorphisms) and indels (insertions and deletions) were detected by the alignment of parental sequences. Ca. 35% of the EST markers showed polymorphisms between the mapping parents. The substitutions of SNPs were more frequent in transition types (A-G: 34.6%, C-T: 25.0%) than transversion types (other eight types). The polymorphic sites were further analyzed if they had recognition sites with 49 kinds of restriction enzymes. Ca. 8% of the markers had the recognition sites for the present mapping parents. There were 27% unmapped

Table 1. Number of markers assigned to the seven barley chromosomes. Markers form the previous reports were used as anchors for each linkage group.

Chromosome	Marker source				Total
	3' EST, Okayama Univ.		Previous reports		
	Simple polymor.	CAPS	SSR	STS	
1H	30	118	3	0	151
2H	34	137	4	4	179
3H	33	119	10	1	163
4H	21	97	4	2	124
5H	35	141	5	2	183
6H	18	114	3	3	138
7H	43	115	6	4	168
Total	214	841	35	16	1,106

SNP positive markers. These markers can be further mapped by using adequate methods of SNP typing. The restriction enzymes used were mainly chosen by the cost per marker, which must be cheaper than the analysis cost with SNP detection system. SNP negative markers are further checked if they have parental polymorphisms on other mapping populations. With these strategies, we assume almost half of the ESTs can be mapped by the current primer sets and other mapping populations such as the one by HORI *et al.* (2003).

Linkage Analysis and Map Development

Polymorphism data sets of 93 doubled haploid lines for simple polymorphism and CAPS from EST markers were analyzed with the anchor markers of SSR and STS. Most of the SSR and STS markers could be identified on the previously reported positions on each linkage group (RAMSAY *et al.* 2000; MANO *et al.* 1999). The map has 214 markers by simple polymorphism, 841 markers by CAPS in total (Table 1). Thirty-five SSR and 16 STS markers were also included as anchors on each linkage group. The total map length is 1362.7cM with 1,106 markers (Fig. 2). Since the population size for mapping was 93 lines, many markers located on the same position on the map, which did not indicate that these markers were derived from the same gene since all marker used were derived from non-redundant set of 3' EST sequences. According to the population size for mapping, the average marker density (1.23 cM/marker) might not be less than 1 cM. There were some regions with significantly distorted marker clusters on the linkage map. Since distorted markers were clustered, there might be some biological reasons such as lethal factors, pollen selection for viability during doubled haploid development process. However, these factors might not affect the mapping procedure since the linkage among these markers was robust enough (LOD threshold 5.0). For each chromosome, from 118 to 176 ESTs were mapped. The present map might be one of the highest density barley linkage maps reported.

Further Development and Application of EST Map

Since most of the markers available by simple polymorphism and CAPS has been mapped already on the population of Haruna Nijo x H602, the next mapping strategy may be to use the SNP typing system. A plenty of SNP typing system are available in the commercial market. We are now using fluorescent polarization SNP typing system (Perkin Elmer Co.), which is working nicely for our mapping project. An alternative strategy is to apply simple polymorphism and CAPS markers on other mapping populations. However, to merge several mapping population may cause minor errors in marker orders. The possible method to solve this problem may be to use rice genome information to search homology between mapped barley ESTs and rice genome sequences including ESTs, BACs and PACs (INE: http://rgp.dna.affrc.go.jp/giot/INE.html). We have already published the barley-rice homology map based on the rice genetic map (Fig. 3. http://www.shigen.nig.ac.jp/barley/). By comparing both barley linkage map and rice genome map, a certain amount of synteny and micro co-linearity relationship may be identified as well.

The present mapping strategy can be also expanded to merge other EST maps developed by other mapping projects (GRANER, personal communication). The consensus map development with reported core mapping populations with massive mapping information (HAYES *et al.* 2003) may be effective to combine trait and EST information. For this purpose, some core marker sets must be mapped on each population, although the map merging strategy needs be further refined.

Since there are some promising genes segregating in the present mapping population, these factors are mapped as QTLs. If these traits are mapped between EST markers on the map, the candidate gene can be estimated from the rice genome information. Since a BAC library from Haruna Nijo (SAISHO *et al*. in preparation) is available from the project in Okayama University, the candidate gene can be identified in combination with mapped cDNA information and the genome library. The present barley transcript map will play an important role to organize gene positions on the barley genome.

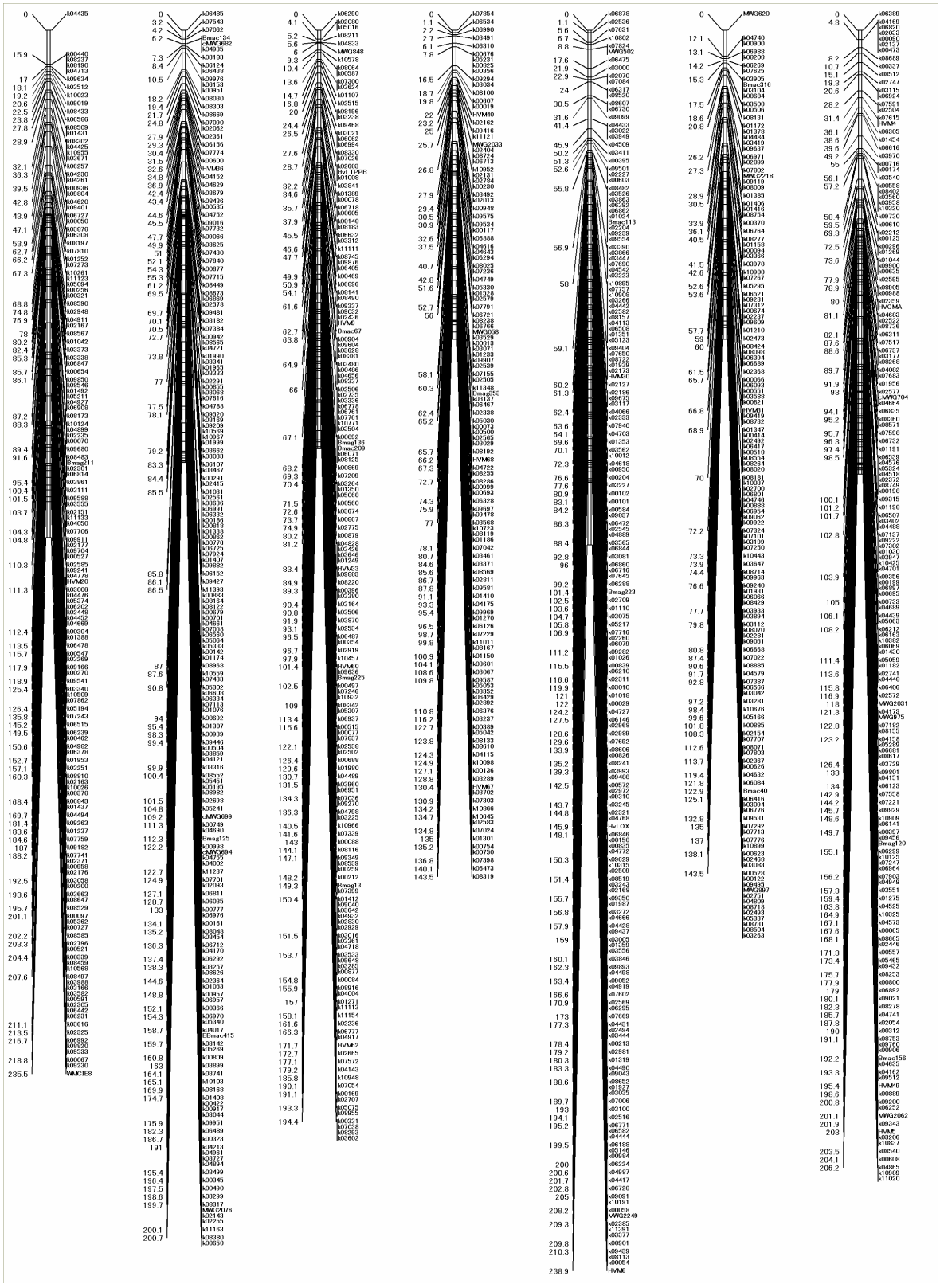


Figure 2. A transcript (EST) map of barley in the cross between Haruna Nijo and H602. SSR and STS markers were integrated as anchors for each chromosome. All the EST markers were PCR amplified by specifically developed primer pairs from the representative 3' ESTs, which were generated at Okayama University.

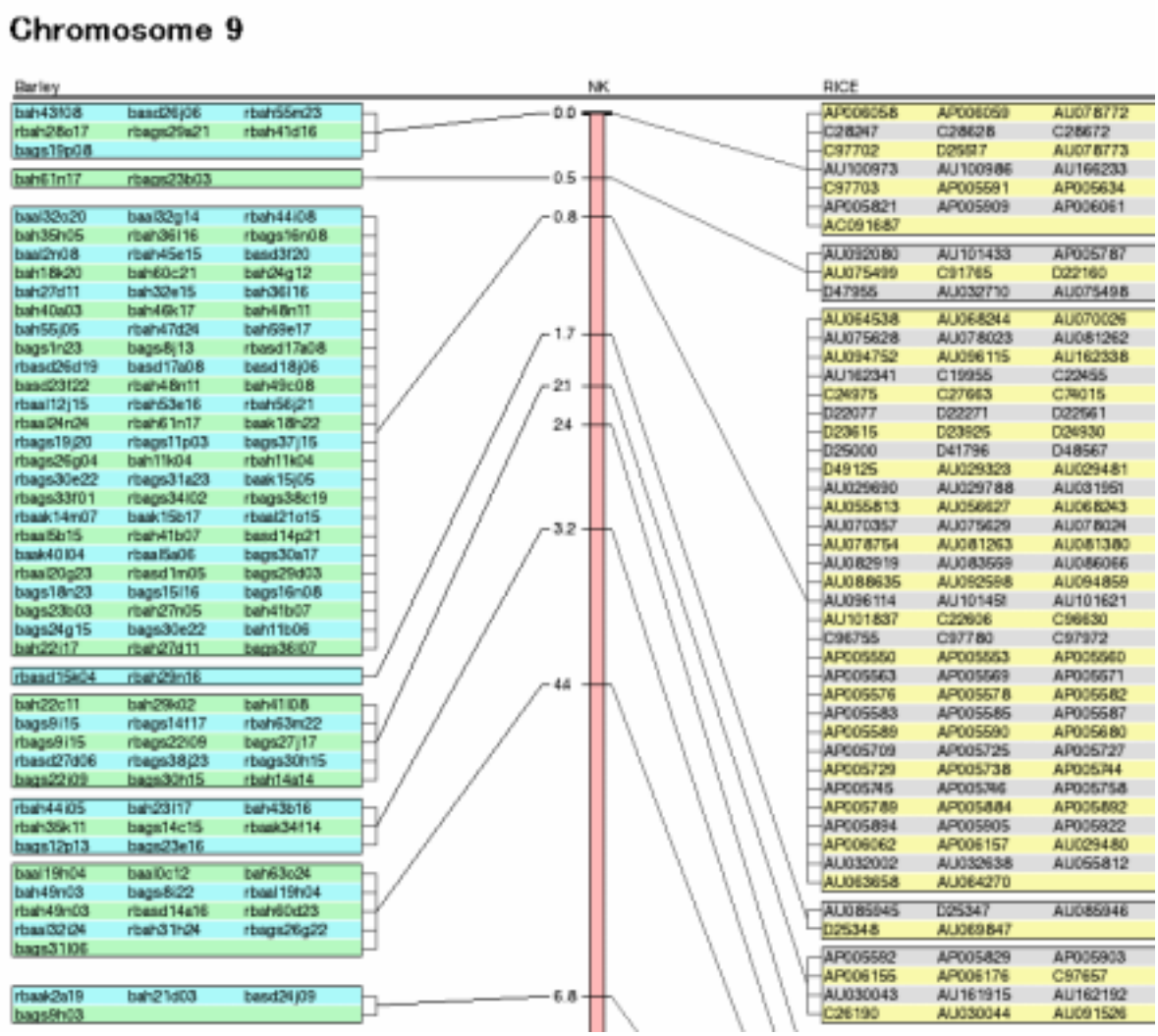


Figure 3. Barley-rice homology map on the genetic map of rice chromosome 9. Homologies among barley ESTs published from Okayama University were searched by blastn (e-30) against the mapped rice BAC, PAC, EST sequences. The homology information for all the rice chromosomes is available at http://www.shigen.nig.ac.jp/barley/.

References

CLOSE T. J., WANAKAMER S., CALDO R. A., TURNER, S. M., ASHLOCK D. A., DICKERSON J. A., WING R. A., MUEHLBAUER G. J., KLEINHOHS A., WISE R. P. (2004): A new resource for cereal genomics: 22K barley GeneChip comes of age. Plant Physiol., (in press)

KOTA R., VARSHNEY R. K., THEIL T., DEHMER K. J., GRANER A. (2001): Generation and comparison of EST derived SSRs and SNPs in barley. Hereditas, **135**: 145-151.

KOTA R., RUDD S., FACIUS A., KOLESOV G., THEIL T., ZHANG H., STEIN N., MAYER K. and GRANER A. (2003): Snipping polymorphisms from large EST collections in barley Mol. Gen. Genom., **270**: 24 – 33.

RAMSAY L., MACAULAY M., IVANISSEVICH S. D., MACLEAN K., CARDLE L., FULLER J. D., EDWARDS K. J., TUVESSON S., MORGANTE M., MASSARI A., MESTRI E., MARMIROLI N., SJAKSTE T., GANAL M., POWELL W., WAUGH R. (2000): A simple

sequence repeat-based linkage map of barley. Genetics, **156**: 1997-2005.

HORI K., KOBAYASHI T., SHIMIZU A., SATO K., TAKEDA K., KAWASAKI S. (2003): Efficient construction of high-density linkage map and its application to QTL analysis in barley. Theor. Appl. Genet., **107**: 806-813.

MARUYAMA K., SUGANO S. (1994): Oligo-capping: a simple method to replace the cap structure of eukaryotic mRNAs with oligoribonucleotides. Gene, **138**: 171-174

BOTHMER R., JACOBSEN N., BADEN C., JØRGENSEN R. B., LINDE-LAURSEN I. (1995): An ecogeographical study of the genus *Hordeum*. Systematic and Ecogeographic Studies on Crop Genepools, 7. IPGRI, Rome, 2nd ed. 129 p.

MANO Y., SAYED-TABATABAEI B. E., GRANER A., BLAKE T., TAKAIWA F., OKA S., KOMATSUDA T. (1999): Map construction of sequence-tagged sites (STSs) in barley (*Hordeum vulgare* L.). Theor. Appl. Genet., **98**: 937-946

HAYES P. M., CASTRO A., MARQUEZ-CEDILLO L., COREY A., HENSON C., JONES B. L., KLING J., MATHER D., MATUS I., ROSSI C., SATO K. (2003): Genetic diversity for quantitatively inherited agronomic and malting quality traits. In: BOTHMER R., HINTUM TH., KNÜPFFER H., SATO K., (eds.): Diversity in Barley (*Hordeum vulgare*). Elsevier Science B.V., Amsterdam, The Netherlands: 201-226.

S 3 – BREEDING METHODOLOGIES II – GENOMICS

New Insights into the Organization and Evolution of Genomes in the Tribe Triticeae

J. Dvorak

Department of Agronomy and Range Science, University of California,
Davis, CA 95616 USA

Abstract
Comparative linkage mapping indicated a high conservation of synteny across Triticeae and the grass family, leading to suggestions that cereal genomes can be treated as a single, largely collinear entity. To investigate the organization of the gene repertoire in wheat and to obtain better assessment of synteny in cereal genomes, gene loci detected by approximately 8000 EST unigenes were mapped in a collaborative mapping project into 159 bins covering the entire length of the 21 wheat chromosomes. Recombination rate was shown to increase from the centromere to telomere along wheat chromosome arms. A number of genomic parameters correlated with recombination rate, suggesting that recombination rate has played the central role in genome evolution in the tribe. Synteny between collinear wheat homoeologous chromosomes correlated negatively with recombination rate; it was the highest in the proximal chromosome regions and declined in the distal direction. The rates of locus deletion and locus duplication along the chromosomes of wheat diploid ancestors were estimated. Both rates correlated highly with recombination rate. An unexpectedly high rate of turnover and divergence of gene repertoire in otherwise collinear wheat chromosomes were discovered, particularly in the high recombination regions of chromosomes. Caution is therefore advocated in assuming synteny between homoeologous chromosomes, particularly in high-recombination regions, across Triticeae or the grass family.
Keywords: gene deletion; gene duplication; genome evolution; wheat

Introduction
Barley and wheat have traditionally been classified into different subtribes of the tribe Triticeae, reflecting the general sense that they represent ancient evolutionary lineages in the tribe. Molecular studies substantiated this believe and timed the divergence of barley and wheat from 11 to 15 million years ago (MYA) (HUANG *et al.* 2002; RAMAKRISHNA *et al.* 2002; AKHUNOV & DVORAK 2004). In contrast to this antiquity of barley and wheat divergence, comparative linkage mapping of the barley and einkorn wheat genomes revealed only three paracentric inversions and one reciprocal translocation perturbing the collinearity of their chromosomes (DUBCOVSKY *et al.* 1996). Comparative linkage mapping indicated conservation of synteny across the entire grass family, leading to the suggestion that, for the purpose of gene discovery and gene isolation, cereal genomes can be treated as a single, largely collinear entity (DEVOS & GALE 1997). For example, a locus for resistance to a disease identified in the barley genome should be present in rice and could be isolated by cross-referencing the two genomes (KILIAN *et al.* 1997). The first attempt to exploit this idea and to isolate the barley rust resistance gene *Rpg1* by presumed barley-rice synteny was unsuccessful (HAN *et al.* 1999). While synteny was clearly apparent in the targeted chromosomal region, the resistance gene was not detected in the rice genome.

To obtain a more detailed picture of synteny in Triticeae and across the grass family, the wheat genetic community in the USA initiated a collaborative effort aiming at the development of wheat ESTs and mapping loci detected by them in each of the three wheat genomes. Over 100,000 ESTs were developed and assembled into contigs (unigenes), each representing a gene motif. Using a set of 159 overlapping deletions entirely covering the length of the 21 chromosomes of Chinese Spring wheat (*T. aestivum*) (QI *et al.* 2003) genes detected by approximately 8000 unigenes were mapped in the wheat A, B, and D genomes (http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi).

Material and Methods

EST Deletion Mapping and Synteny Assessment

A total of 101 *T. aestivum* (genomes AABBDD) 'Chinese Spring' deletion stocks, comprising 159 terminal deletions, and 24 ditelosomic stocks and 21 nullisomic tetrasomic stocks were used in EST mapping (QI *et al.* 2003). The breakpoint of each deletion was expressed as a fraction of the chromosome arm length. Overlapping deletions delimited a physical region on a chromosome arm called a bin. The most proximal bin in each arm was delimited by the most proximal breakpoint and by the centromeric breakpoint in the relevant Chinese Spring ditelosome. A total of 159 bins were delimited by the breakpoints in the 21 wheat chromosomes.

DNA was isolated from individual plants of the deletion stocks, nulli-tetrasomic stocks, and ditelosomic stocks, digested with *EcoR* I and fractionated in 1% agarose gels. Southern blots were hybridized with single cDNA (EST) clone per unigene and restriction fragments were allocated into bins (QI *et al.* 2003). Mapping data including images of autoradiograms and summaries of restriction fragment allocation into bins were deposited into the wEST public database (http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi).

Mapping data for 1,993 EST unigenes, for which most restriction fragments were mapped in the three wheat genomes, were extracted from this database. Mapping data for 3,206 loci in the wheat A and D genomes were examined for synteny (AKHUNOV & DVORAK 2004). For each locus showing perturbed synteny, it was determined if the perturbation was caused by locus deletion or duplication and at which point of Triticeae radiation the event originated (AKHUNOV & DVORAK 2004).

Recombination Rate

Recombination rates, expressed as coefficients of exchange (CEs, cM/Mb) were derived from estimates of genetic lengths of bins in cM divided by estimates of bin lengths in Mb (AKHUNOV *et al.* 2003a; AKHUNOV *et al.* 2003b). Briefly, the midpoints of 159 bins were grouped according to the relative physical distance of bin midpoint from the centromere into six equal intervals, each 0.17 of the average chromosome arm. The CEs of bins grouped into the same interval across all wheat chromosome arms were averaged to obtain an estimate of CE in the interval.

Divergence Time Estimation

The divergence time of barley and wheat, the progenitors of the wheat A and D genomes, *T. urartu* (DVORAK *et al.* 1993) and *Ae. tauschii* (KIHARA 1944; MCFADDEN & SEARS 1946), and the time of the origin of tetraploid wheat (*T. dicoccoides,* genomes AABB) were determined in the following manner (for details see (AKHUNOV & DVORAK 2004)). Divergence time of the *T. urartu* and *Ae. tauschii* lineages was estimated from intronic nucleotide sequences of ATP-dependent metalloprotease (ADM) and seed maturation protein (SMP) and the divergence time of barley and wheat was estimated from exonic sequences.

Divergence time of major grass subfamilies, set at 55 MYA (KELLOGG 2001), was used to calibrate the exonic clock.

Because estimates of divergence time for closely related species, such as *T. urartu* and *Ae. tauschii,* can be greatly biased by polymorphism, haplotype polymorphisms was considered in the estimates. A total of 1.2 to 1.5 kb of the intronic and exonic sequence were determined for ADM and 1.0 kb of the intronic and exonic sequence of SMP in *T. urartu*, *Ae. tauschii,* and *H. vulgare.* Genomic sequences were amplified by PCR and the products were directly sequenced using the PCR primers. Haplotype variation was investigated in 226 *Ae. tauschii* plants, 315 *T. urartu* plants, and 96 *H. vulgare* plants. The net nucleotide substitutions between haplotype group means were computed using the Kimura 2-parameter (MEGA v 2.1 software) using distance option considering only synonymous and intronic sites.

The time of wild emmer wheat origin was inferred from the number of locus duplications that originated at the polyploid level relative to the number of locus duplications that originated since the *T. urartu-Ae. tauschii* divergence (AKHUNOV & DVORAK 2004).

The number of locus deletion and duplication events detected in Chinese Spring wheat that originated during the divergence of the diploid ancestors of the wheat A and D genomes was expressed per locus MY^{-1}. For the A-genome loci, time period from the divergence of the A and D genomes (2.8 MYA) to the origin of wild emmer wheat (0.24 MYA) was used. For the D-genome loci, time period from the divergence of the A and D genome to the origin of hexaploid wheat (8,000 YA, NESBITT & SAMUEL 1996) was used.

Results and Discussion

Organization of Gene Repertoire in the Wheat Genomes

Earlier linkage mapping of the barley and wheat genomes by Southern hybridization of random genomic (*Pst*I) and cDNA clones indicated a high locus redundancy in both species (ANDERSON *et al.* 1992; KLEINHOFS *et al.* 1993; DUBCOVSKY *et al.* 1996). This was substantiated by wheat EST unigene mapping (AKHUNOV *et al.* 2003b). At least a quarter of all gene motifs in wheat genomes are represented by sets of duplicated loci (paralogous sets), which originated by duplication and dispersion of genes across genomes via a currently unknown process (AKHUNOV *et al.* 2003b).

Recombination rate (cM/Mb) was shown to increase along the centromere-telomere axis of the average wheat chromosome arm with the square of distance from the centromere (LUKASZEWSKI & CURTIS 1993; AKHUNOV *et al.* 2003b; DVORAK *et al.* 2003). Several genomic parameters were shown to correlate with recombination rate. Genes present only once in a genome were located largely in the proximal, low-recombination regions whereas multi-gene loci were located predominantly in distal, high-recombination regions (AKHUNOV *et al.* 2003b). Gene density along the average wheat chromosome arm increased about four-fold in the proximal-to-distal direction and the increase correlated with recombination rate (AKHUNOV *et al.* 2003b; DVORAK *et al.* 2003). Duplicated gene loci were distributed non-randomly; they were concentrated in distal chromosome regions, and their concentration was greater than could be accounted for by the general increase in gene density along the centromere-telomere axis of wheat chromosomes (AKHUNOV *et al.* 2003a; AKHUNOV *et al.* 2003b). These correlations led to the realization that variation in recombination rates along wheat chromosomes has played a critical role in the evolution of wheat genomes and has shaped the structure of wheat chromosomes (AKHUNOV *et al.* 2003b).

Synteny Erosion along Chromosomes

An insertion of a duplicated locus into a chromosome region perturbs the synteny in that region. Hence, the tendency of duplicated loci to accumulate in distal, high-recombination regions of wheat chromosomes should result in more frequent perturbations of synteny

between otherwise collinear homoeologous chromosomes in distal, high recombination regions than in proximal, low-recombination regions (AKHUNOV *et al.* 2003b). To test this hypothesis, mapping data for 1,993 EST unigenes, for which most restriction fragments were mapped in the three wheat genomes, were extracted from the wEST database and synteny between the wheat A, B, and D genomes was examined in a pair-wise fashion (AKHUNOV *et al.* 2003a). Synteny was close to 100% in proximal, low-recombination regions of wheat chromosomes. In distal, high-recombination regions, only about 92% of loci were syntenous among the three wheat genomes. The decline of synteny along the centromere-telomere axis of wheat chromosomes correlated with the increase in recombination rate along chromosomes. The level of synteny perturbations in distal, high recombination regions of wheat homoeologues was unexpectedly high, considering that these genomes diverged only about 2.8 MYA (AKHUNOV & DVORAK 2004).

Gene Deletion and Duplication Rates

To characterize the underlying causes of wheat chromosome synteny erosion, mapping data for 3,206 loci in the wheat A and D genomes were examined for synteny. It was determined for each locus showing perturbed synteny if the perturbation was caused by locus deletion or duplication and at which point of Triticeae radiation the deletion or duplication originated. Locus deletion and duplication rates per locus MY^{-1} were computed both for the diploid and polyploid levels (AKHUNOV & DVORAK 2004). Since rates obtain for the diploid level are more relevant for this conference than those obtained for the polyploid level, only rates for the diploid level will be discussed here.

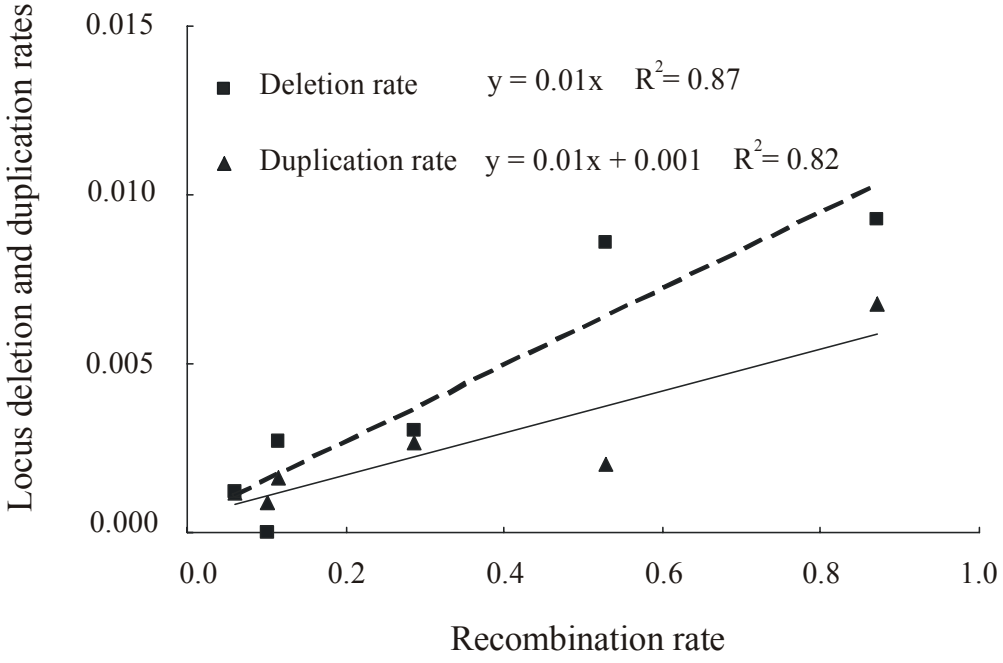


Fig. 1. Deletion and duplication rates per gene locus MY^{-1} relative to recombination rate in cM Mb^{-1} during the divergence of the *T. urartu* (A genome) and *Ae.tauschii* (D genome) lineages. The centromere of the average chromosome arm is at 0.0 and the telomere is near 1.0. A solid line represents a linear regression of locus duplication rate on recombination rate and a dashed line represents a linear regression of locus deletion rate on recombination rate. Linear regression equations and R^2s for each correlation are also shown. (Adapted from AKHUNOV & DVORAK 2004)

Both locus deletion rates and locus duplication rates correlated highly with recombination rates along the average chromosome arm (r = 0.93 for deletions and r = 0.9 for duplications). The regression of duplication rate on recombination rate expressed per locus MY^{-1} (Fig. 1) quantified the relationship between duplicated locus accumulation and recombination rate that was suggested earlier (AKHUNOV *et al.* 2003a; AKHUNOV *et al.* 2003b). Loci in distal, high-recombination chromosome regions were also subjected to greater deletion rates than loci in proximal, low-recombination regions. These relationships account for the greater erosion of synteny in distal chromosome regions than in proximal chromosome regions that was observed earlier in the A, B, and D genome chromosomes (AKHUNOV *et al.* 2003a).

Gene Repertoire Turnover

For additional quantitative analyses, the average wheat chromosome arm was divided into two intervals containing approximately equal numbers of gene loci, the proximal, low-recombination interval and the distal, high-recombination interval. The rates of locus deletion and locus duplication were estimated for each interval (AKHUNOV & DVORAK 2004). Using 12.5 MY for the barley-wheat divergence time, and the equation for exponential growth of gene repertoire by locus duplications, $G(t) = G_o e^{\gamma t}$, where $G(t)$ is the size of gene repertoire at time t, G_o is the initial size of gene repertoire, γ is a locus duplication rate constant, and t is time in MY, indicated that the distal, high-recombination interval of wheat and barley homoeologous chromosomes each acquired 6% new loci since barley-wheat divergence. Using an equation for exponential decline by locus deletions (AKHUNOV & DVORAK 2004), a total of 8% of all duplicated loci in this interval were deleted both from the wheat genomes and the barley genome. Even gene loci present only once per genome are being deleted with a high rate. A deletion rate constant of 5.3×10^{-3} locus MY^{-1} for these loci (AKHUNOV & DVORAK 2004) indicated that a total of 6% of these loci have been deleted from both the barley genome and the wheat genomes since the barley-wheat divergence. The function of these genes was examined using GenBank and other databases (AKHUNOV & DVORAK 2004). For 60% of the genes, no corresponding sequences were detected in the databases or their function was unknown. We speculate that most of these genes evolved recently by duplication and divergence. The remaining 40% of deleted gene motifs consisted of genes involved in biotic and abiotic stress responses and seed development.

Practical Considerations

Synteny between homoeologous chromosomes in Triticeae has been eroded by deletions and insertions of individual loci. Because this process is independent of structural chromosome evolution, homoeologous chromosomes may appear collinear even though their synteny may be poor. The quantification of locus deletion and duplication rates in the *Aegilops-Triticum* alliance showed that the rate of gene repertoire turnover by locus duplications and deletions is of such a high magnitude that, if the barley genome were evolving with the same rate as the genomes in the *Aegilops-Triticum* alliance, synteny in distal, high-recombination regions of barley and wheat homoeologous chromosomes would be perturbed for as many as a quarter of the loci. Yet, such homoeologous regions may appear entirely collinear. Because of selection constrains, deletions of loci present only once per genome involve mostly dispensable loci. Unfortunately, many economically important loci, such as those controlling disease resistance, abiotic stress responses, and grain quality traits fall into this category. Data obtained by analyses of wheat ESTs show that these loci are deleted with unexpectedly high rates. A great deal of caution should therefore be exercised in assuming synteny for economically important

genes across Triticeae and across the grass family, particularly for loci in distal, high-recombination regions of chromosomes.

Acknowledgements
This work was supported by the National Science Foundation of the United States of America under Plant Genome Research Program Contract Agreement No. DBI-9975989.

References

AKHUNOV E. D., AKHUNOV A. R., LINKIEWICZ A. M., DUBCOVSKY J., HUMMEL D., LAZO G., CHAO S., ANDERSON O. D., DAVID J., QI L. L., ECHALIER B., GILL B. S., MIFTAHUDIN, GUSTAFSON J. P., LA ROTA M., SORRELLS M. E., ZHANG D., NGUYEN H. T., KALAVACHARLA V., HOSSAIN K., KIANIAN S., PENG J., LAPITAN N. L. V., WENNERLIND E. J., NDUATI V., ANDERSON J. A., SIDHU D., GILL K., MCGUIRE P. E., QUALSET C. O. DVORAK J. (2003a): Synteny perturbations between wheat homoeologous chromosomes by locus duplications and deletions correlate with recombination rates along chromosome arms. Proc. Natl. Acad. Sci. USA, **100**: 10836-10841.

AKHUNOV E. D., DVORAK J. (2004): Recombination rate, gene deletion and duplication rates, and the evolution of gene repertoire along chromosomes. Science, submitted.

AKHUNOV E. D., GOODYEAR J. A., GENG S., QI L.-L., ECHALIER B., GILL B. S., MIFTAHUDIN, GUSTAFSON J. P., LAZO G., CHAO S., ANDERSON O. D., LINKIEWICZ A. M., DUBCOVSKY J., LA ROTA M., SORRELLS M. E., ZHANG D., NGUYEN H. T., KALAVACHARLA V., K. H., KIANIAN S. F., PENG J., LAPITAN N. L. V., GONZALEZ-HERNANDEZ J. L., ANDERSON J. A., CHOI D.-W., CLOSE T. J., DILBIRLIGI M., GILL K. S., WALKER-SIMMONS M. K., STEBER C., MCGUIRE P. E., QUALSET C. O. DVORAK J. (2003b): The organization and rate of evolution of the wheat genomes are correlated with recombination rates along chromosome arms. Genome Res., **13**: 753-763.

ANDERSON J. A., OGIHARA Y., SORRELLS M. E. TANKSLEY S. D. (1992): Development of a chromosomal arm map for wheat based on RFLP markers. Theor. Appl. Genet., **83**: 1035-1043.

DEVOS K. M., GALE M. D. (1997): Comparative genetics in the grasses. Plant Mol. Biol., **35**: 3-15.

DUBCOVSKY J., LUO M. C., ZHONG G. Y., BRANSTEITTER R., DESAI A., KILIAN A., KLEINHOFS A. DVORAK J. (1996): Genetic map of diploid wheat, Triticum monococcum L., and its comparison with maps of Hordeum vulgare L. Genetics, **143**: 983-999.

DVORAK J., AKHUNOV A. D., AKHUNOV A. R., LUO M. C., LINKIEWICZ A. M., DUBCOVSKY J., HUMMEL D., LAZO G., CHAO S., ANDERSON O. D., DAVID J., QI L. L., ECHALIER B., GILL B. S., MIFTAHUDIN, GUSTAFSON J. P., LA ROTA M., SORRELLS M. E., ZHANG D., NGUYEN H. T., KALAVACHARLA V., HOSSAIN K., KIANIAN S. F., PENG J. H., LAPITAN N. L. V., WENNERLIND E. J., NDUATI V., ANDERSON J. A., SIDHU D., GILL K. S., CHOI D.-W., CLOSE T. J., MCGUIRE P. E. QUALSET C. O. (2003): New insights into the organization and evolution of wheat genomes, in 10th Inernatl. Wheat Genet. Symp., edited by POGNA, N. E., ROMANO, M., POGNA, E. A. and GALTERIO, G. S.I.M.I., Rome, Italy, Paestum, Italy, 261-264.

DVORAK J., DI TERLIZZI P., ZHANG H. B. RESTA P. (1993): The evolution of polyploid wheats: Identification of the A genome donor species. Genome, **36**: 21-31.

HAN F., KILIAN A., CHEN J. P., KUDRNA D., STEFFENSON B., YAMAMOTO K., MATSUMOTO T., SASAKI T. KLEINHOFS A. (1999): Sequence analysis of a rice BAC covering the syntenous barley Rpg1 region. Genome, **42**: 1071-1076.

HUANG S., SIRIKHACHORNKIT A., SU X., FARIS J., GILL B. S., HASELKORN R. GORNICKI P. (2002): Genes encoding platid acetyl-CoA carboxylase and 3-phopshoglycerate kinase of the Triticum/Aegilops complex and the evolutionary history of polyploid wheat. Proc. Natl. Acad. Sci. USA, **99**: 8133-8138.

KELLOGG E. A. (2001): Evolutionary history of the grasses. Plant Physiol., **125**: 1198-1205.

KIHARA H. (1944): Discovery of the DD-analyser, one of the ancestors of Triticum vulgare (Japanese). Agric. and Hort. (Tokyo), **19**: 13-14.

KILIAN A., CHEN J. P., HAN F., STEFFENSON B. KLEINHOFFS A. (1997): Towards map-based cloning of the barley stem rust resistance genes Rpg1 and rpg4 using rice as an intergenomic cloning vehicle. Plant Mol. Biol., **35**: 187-195.

KLEINHOFS A., KILIAN A., MAROOF M. A. S., BIYASHEV R. M., HAYES P., CHEN F. Q., LAPITAN N., FENWICK A., BLAKE T. K., KANAZIN V., ANANIEV E., DAHLEEN L., KUDRNA D., BOLLINGER J., KNAPP S. J., LIU B., SORRELLS M., HEUN M., FRANCKOWIAK J. D., HOFFMAN D., SKADSEN R. STEFFENSON B. J. (1993): A molecular, isozyme and morphological map of the barley (Hordeum vulgare) genome. Theor. Appl. Genet., **86**: 705-712.

LUKASZEWSKI A. J., CURTIS C. A. (1993): Physical distribution of recombination in B-genome chromosomes of tetraploid wheat. Theor. Appl. Genet., **84**: 121-127.

MCFADDEN E. S., SEARS E. R. (1946): The origin of Triticum spelta and its free-threshing hexaploid relatives. J.Hered., **37**: 81-89, 107-116.

NESBITT M., SAMUEL D. (1996): From staple crop to extinction? The archaeology and history of hulled wheats, in Hulled Wheats. Promoting the conservation and use of underutilized and neglected crops. 4. Proc. 1st Internatl. Workshop on Hulled wheats., edited by PADULOSI, S., HAMMER, K. and HELLER, J. International Plant Genetic Resources Institute, Rome, Italy, Castelvecchio Pacoli, Tuscany, Italy, 41-100.

QI L. L., ECHALIER B., FRIEBE B. GILL B. S. (2003): Molecular characterization of a set of wheat deletion stocks for use in chromosome bin mapping of ESTs. Funct. Integr. Genomics, **3**: 39-55.

RAMAKRISHNA W., DUBCOVSKY J., PARK Y. J., BUSSO C., EMBERETON J., SANMIGUEL P. BENNETZEN J. L. (2002): Different types and rates of genome evolution detected by comparative sequence analysis of orthologus segments from four cereal genomes. Genetics, **162**: 1389-1400.

Current Perspectives in Barley Genomics (2004)

R. Waugh, D. Caldwell, N. Rostoks, A. Druka, I. Druka, D. Marshall, G. Muehlbauer, J. Russell and L. Ramsay

Genome Dynamics, Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, UK

(with contributions from Tim Close, Roger Wise, Andris Kleinhofs, Julie Dickerson and Pat Hayes (USA), Andreas Graner (IPK Gatersleben, Germany),Thomas Koprek (MPI-Koln, Germany), Alan Schulman (MTT & Uni. of Helsinki, Finland), Peter Langridge (Uni. of Adelaide, Australia) and Kaz Sato (Okayama Uni., Japan)

Introduction

In the proceedings of the 8th IBGS held in Adelaide in 2000 I presented a largely prospective view of the genomics tools and resources that had begun to be developed by a group of international laboratories that had received funding to seize the emerging 'genomics opportunity'. In this report I have the prospect of providing a retrospective update, current status and outcomes of this genomics resource development exercise and some preliminary examples of how these resources are already being exploited in my own and other laboratories. After reading, I hope you will agree that the progress has been impressive. I need to stress from the outset that it is my intention to extol the virtues of what in my view became a unique and effective collaboration on a global scale and congratulate those directly involved for having the vision to look outside their own project objectives and openly and unselfishly embrace the bigger picture.

The primary reason why the genomics resources developed by the global barley research community have emerged so quickly is the paucity of commercial research interest in barley as a high value commodity. Lack of commercial interest has led to a lack of activity in the genomics arena and provided an environment where collaborative efforts could be pursued openly in the public domain for the benefit of the entire research community and, paradoxically, those interested from the commercial sector. Because of this I conclude that barley research is somewhat unique within the major cereals. A true diploid inbreeder with seven pairs of chromosomes, it has had a long history of genetical research. Promoted by many as a model for its more complex and commercially important relative wheat, this proposal has not yet been embraced. It has a well-established classical gene map (FRANCKOWIAK *et al.* 1996). Over the last 13 or so years this has been supplemented with a large number of molecular marker-based maps that have been used to locate single gene and complex traits and provide diagnostics for marker assisted selection (MAS) that are currently being deployed in both publicly funded and commercial programs. Historically, chemical and irradiation mutagenesis procedures were widely adopted in barley. These have resulted in over 900 published descriptions of mutants with many more uncharacterized lines in private collections (see http://ace.untamo.net/). Prior to the Adelaide meeting, several large insert YAC and BAC libraries had been constructed and these have been used by forward genetics approaches to clone a number of target genes including *Mlo, Mla, Rpg1, Rpg5, Ror1* and *Rar1*. In addition, the sequencing of the rice genome has had and will increasingly have a major impact on barley genetics research, changing the way we plan and execute experiments,

realising the candidate gene approach and in many cases circumventing the time consuming and costly need for extensive genetic studies prior to gene identification.

In the following summary I have broken down the development of genomics tools and resources into major topic areas that I hope provide clarity. Some pieces of work I describe are effectively 'finished'. However, as they may be implicit for the development of other areas I cover I have included them for the sake of completeness. Much of what I describe are the deliverables from what was proposed four years ago. I will not attempt to cover the biological questions that these resources are currently being applied. I have however also included some emerging data and novel concepts, and would like to thank my colleagues throughout the community for providing access to exciting information in advance of publication.

Gene Discovery

High throughput single-pass sequencing of cDNA clones to generate collections of expressed sequence tags, or ESTs, remains the fastest route to gene discovery in large genome species. It circumvents the problems associated with sequencing extensive tracts of non-coding and repetitive DNA and focuses on transcribed and functional sequences that have direct biological relevance. Over the last four years, ESTs have become the foundation of barley genomics providing a substrate for the wide range of activities. Five major efforts in the USA, Finland, Germany, Japan and Scotland have contributed to the current (15.02.04) total of 377,074 ESTs available in dbEST (352,924 from *H. vulgare* ssp. *vulgare* and 24,150 from *H. vulgare* ssp. *spontaneum*; see http://www.ncbi.nlm.nih.gov/dbEST/ or http://www.harvest.ucr.edu/). These ESTs have been derived from 84 independent cDNA libraries covering most of the major barley tissue types, including those subjected to biotic or abiotic stress. Table 1 gives a comparison of this collection with those from other species. Bioinformatics based clustering and assembly of the entire EST collection (http://www.harvest.ucr.edu/, assembly 31, version 1.12) compresses the size of the collection to 26,747 contig (ie multiply represented or redundant) and 27,062 singleton sequences (Note: HarvEST V. 1.19 has major improvements in the annotations). It is difficult to estimate exactly what percentage of the total number of barley genes these figures represent. However, we can get an impression by comparison to Arabidopsis where 178,000 ESTs in dbEST revealed only 16,115 strong matches with annotated genes on the complete genome sequence (ie ~60%). A guestimate would therefore suggest that the current barley EST collection contains 50 – 80% of all barley genes assuming a gene content of approximately double that of Arabidopsis. Given the level of conserved synteny at the sequence and genome level the ESTs from related species such as wheat are a valuable extension to this collection. To date there are only a couple of reports that attempt to interpret the gene expression information inherent in these collections (MICHALEK *et al.* 2002; LIU *et al.* submitted).

As far as the authors know, there are no additional ongoing EST sequencing projects that will significantly alter these figures in the foreseeable future. We therefore predict that additional gene content information is more likely to come from projects funded to adopt the genome filtration based approaches (or similar) that are currently being pursued by the US Maize community (WHITELAW *et al.* 2003; PALMER *et al.* 2003) or on a gene by gene basis from targeted programs using the rice (or wheat if the IGROW proposal goes ahead) genomic sequence as a template for gene discovery.

Summary of Plant EST collections numbering over 100K entries (March 19, 2004)

Triticum aestivum (wheat)	549,915
Zea mays (maize)	391,145
***Hordeum vulgare* (barley)**	**380,998**
Glycine max (soybean)	344,524
Oryza sativa (rice)	266,949
Saccharum officinarum (sugarcane)	246,301
Arabidopsis thaliana (thale cress)	196,904
Medicago truncatula (barrel medic)	187,763
Sorghum bicolor (sorghum)	161,766
Lycopersicon esculentum (tomato)	150,410
Vitis vinifera (grapevine)	135,712
Solanum tuberosum (potato)	132,122
Pinus taeda (loblolly pine)	110,622

These barley (and indeed other Triticeae) EST sequences provide a firm foundation for much of the current efforts in genomics that I will attempt to cover in the following sections:

Gene-Based Marker Discovery and Comparative Genomics

Over the last decade the development of PCR-based molecular markers has had a profound impact on barley research and practical implementation. These have emerged largely in the popular form of functionally anonymous but easy to assay and highly informative single locus simple sequence repeats (SSRs). EST resources provide a valuable DNA sequence resource that is rich source of potential markers. Already the collection is brokering the change from anonymous to potentially 'functional' markers. In general the new markers fall into three distinct classes:

- *RFLPs:* RFLP continue to provide a robust and high value marker system that can be based on either random genomic or gene sequences (in the form of cDNAs). Mapping a large amount of sequenced cDNA based RFLP markers has been a focus of a two main groups, the IPK-Gatersleben and Washington State University Group in Pullman, USA. Currently over 1000 barley genes have been mapped in reference populations (Steptoe x Morex, OWBa x OWBb and Igri x Franka).
- *EST-SSRs*: SSRs are found at high frequency in EST sequences and are identifiable in between 5 and 10% of the entries in the complete cereal EST collections (Cardle et al, 2000). Several papers already describe the development of collections of EST-SSRs (PILLEN *et al.* 2000; KOTA *et al.* 2001; *Thiel et al* 2003) that supplement the existing collection of genomic SSRs (*Ramsay et al.* 2000). In general the former appear to be less polymorphic but more robust and easier to categorise alleles, perhaps because of the repeat size and conservation of the primer binding sites, which can frequently be embedded in coding sequences. Currently, over two hundred EST-SSRs have been located onto the barley genetic map.
- *Single nucleotide polymorphisms (SNPs):* SNP's are the most abundant form of polymorphism in both plant and animal genomes and importantly provide an opportunity to move away from gel based electrophoretic assays towards highly

multiplex non gel-based genotyping platforms. We are aware of at least four projects that are focussed on the discovery of a large number of SNPs in, or associated with, genes in the barley genome. Preliminary data have been published already by the IPK-Gatersleben group (KOTA *et al.* 2001, 2003) with additional data either in preparation or in press. However unpublished data from the IPK, University of California - Riverside / SCRI collaboration indicate that SNPs in approximately 400 and 1200 genes respectively have already been discovered and validated with a combined total of some 400 already mapped. An additional 18 EST-SNPs have been mapped at MTT. Finally in Okayama University, a PCR based EST map on the Haruna Nijo/H602 population with more than 1,000 markers from the Japanese barley unigene set has been developed.

The value of these markers is highly significant. They form a direct link to the rice genome sequence, which is figuring more and more in gene-focussed studies in barley such as the identification of candidate genes for either simple or complex traits. Thus, having mapped a trait in barley to a given location on the barley genetic map, with gene based markers it is relatively straightforward to identify the orthologous location in rice using user-friendly web based informatics tools such as those available at Okayama or at TIGR (http://www.shigen.nig.ac.jp/barley/, http://www.tigr.org/cgi-bin/htsearch). These bioinformatics resources can be exploited to identify a further source of candidate genes or additional gene-based markers for closing in on the gene in barley. As a warning this cross-species approach should proceed with caution as frequent genomic rearrangements can cause complications. Genome wide data from the IPK-Gatersleben group and saturation of a ca. 35 cM region by the Kleinhofs group suggested that only approximately 60% and 50%, respectively, map to the orthologous region in rice. Finally, innovative approaches using the new Affymetrix Barley1 GeneChip to detect single feature polymorphisms are also being explored which if successful will provide an unprecedented volume of data for both genetic or physical mapping exercises.

Integrating Genetic and Physical Maps

The development of a whole genome clone-based physical map of the barley genome has been discussed widely by the barley genomics community and several attempts have been made to obtain the level of funding required to pursue this task. Until recently, physical mapping has largely focused on models such as Arabidopsis *(Arabidopsis thaliana* L.) and rice (*Oryza sativa* L.) because they have relatively small genomes, 150 and 340 Mbp/1C, respectively. In large genome crop species like barley (5300 Mb), with most of the genome comprised of repeated sequences, clone-based physical mapping is a major challenge. However with technological advances, such as multiple fluorescence fingerprinting using commercially available SNaPshot technology (LUO *et al.* 2003), physical maps of other larger genome species are already under construction (e.g diploid wheat, maize, tomato, potato, brassicas etc). The Close lab at University of California Riverside has recently received funding to address this challenge in barley using the widely distributed cv. Morex BAC library and work is now underway. The outcome will be particularly valuable, allowing thousands of genes to be mapped rapidly and at high resolution by PCR or hybridization and circumventing the major requirement of meiotic mapping for polymorphic genetic markers. BACs hybridizing to thousands of 'overgo' probes designed to EST clones are being identified and "contigs" of overlapping clones assembled to (hopefully) span the entire gene rich regions of the barley genome. Intrinsically, clone based physical mapping is attractive because it is regarded as enhancing the molecular genetics of an organism as it serves as an archive of genomic information. Ultimately, construction of a 'joined up' whole genome physical map will almost

certainly involve integrating data generated from a number of approaches. For example, using a deletion mapping approach similar to that successfully used in wheat to map thousands of ESTs (NSF funded), the laboratory of Prof. T. Endo in Japan has used gametocidal chromosome 2C derived from *Aegilops cylindrica* in single barley chromosome addition lines of common wheat to generate panels of lines containing uniquely fragmented barley chromosomes which can be used to physically map barley genes to sub-chromosomal segments of the barley genome (MASOUDI-NEJAD and ENDO, personal communication). These lines are currently being used to physically map barley ESTs by simple PCR-based assays. Such approaches will serve to link independent BAC contigs that cannot be assigned an order by physically locating them at a greater physical scale. Integrated physical and genetic maps will be extremely valuable for map-based gene isolation, comparative genome analysis, and as sources of sequence ready clones for genome sequencing projects.

Platform Technologies for Assessing Gene Expression

A number of methodologies now make parallel assessment of the expression of thousands of genes in a cell, tissue, organ, timepoint or treatment possible in a single experiment. They fall into to two classes that I will term open and closed. Both rely to a greater or lesser extent on the extensive EST collections that have been developed. Micro-arrays are closed systems as it is only possible to monitor the expression of the features that are on the chip. In barley, microarrays have been developed both from spotting cDNA clones (SREENIVASULU *et al.* 2003, 2004) and latterly via the development of an Affymetrix GeneChip (Barley1) (CLOSE *et al.* 2004). I will only cover the latter as it represents – in my view – a major advance over all other crop plants (other than rice in the commercial sector) because it will facilitate global integration of data from a range of disparate experiments conducted in diverse locations. The Barley1 array was derived from the total collection of ESTs referred to above along with all 1,145 barley genes from the NCBI non-redundant database. It represents 22,792 barley genes comprised of 11 perfect match 25 mer oligos and 11 mis-match 25-mer oligos containing a base substitution at the 13th nucleotide position per gene - along with a host of controls. It also includes probes for commonly used transgene sequences. The sequences represented on the array can be viewed at http://barleypop.vrac.iastate.edu/BarleyBase/probealign.php (within http://barleybase.org/) and through HarvEST:Barley (see V.1.19). In the past 12 months, the Barley1 GeneChip has been validated by hybridization with labeled cRNA from over 100 treatments, involving at least ten laboratories on four continents. Treatments included contrasting developmental stage, tissues, genotype, abiotic and biotic stresses that reveal >22,000 probe sets corresponding to genes expressed above background in one or more conditions. The performance of the Barley1 GeneChip is consistent with other Affymetrix GeneChip probe arrays with respect to low false change rate (0.13-0.16%) for technical replicates and a 100,000-fold linear detection range (CLOSE *et al.* 2004). These experiments confirm its high technical specification and as with similar arrays in other species, illustrate the need for biological replication in array based experiments. In addition the array has been assessed for its ability to monitor gene expression in a range of related cereals (see Close et al. 2004 for details). To facilitate greater exploitation of the data from these experiments, BarleyBase (http://barleybase.org/) a public access functional genomics resource has been developed to 1) Archive expression data and 2) Allow individual researchers to remotely interrogate data from profiling experiments conducted by researchers worldwide. To facilitate the use of this resource the barley genomics community has co-funded a large experiment to profile gene expression in various tissues throughout barley development. It is envisaged that the data from this experiment will serve as a reference to assist in the interpretation of future array based research.

In contrast to microarrays, approaches such as SAGE and MPSS are open systems that effectively 'sample' the entire transcriptome irrespective of its composition of mRNAs. Both approaches are based on deriving and counting short sequence 'tags' from large libraries assembled from the total mRNA pool. These are both very powerful approaches, but are technically demanding and quite expensive (in comparison to microarrays which can themselves be expensive). They rely on extensive EST data (preferably genome sequence data) for interpretation and translation of 'tag to sequence'. To my knowledge MPSS has not been applied to barley. SAGE libraries have however been developed and the resulting data directly compared to data from the Barley1 GeneChip. In general terms the correlation between the approaches is high. However, even restricting the comparison to the 100 most abundant transcripts reveals that several messages are expressed that are not represented on the array. The conclusion is that, no matter how powerful, each technology has certain caveats that must be considered when interpreting the output data.

Functional Genomics

As an increasing array of genomics-based resources continue to be developed, the identification of candidate genes for a number of traits is already gathering pace. Consequently, rapid and high throughput methods for the confirmation and validation of gene function by targeted gene inactivation are now established priorities. Two complementary approaches for identifying mutations in target genes either have been or are being developed.

Chemically Induced Structured Mutant Populations

A long recognized large-scale approach for achieving random gene inactivation is to induce random mutations in a population of plants using chemical or physical mutagens. In barley, chemical and irradiation mutagenesis procedures have been widely adopted, resulting in over 900 published descriptions of mutants (von WETTSTEIN-KNOWLES 1992) and many more uncharacterized mutants in private collections. When coupled with recently emerging sensitive methods for the detection of 'aberrant' DNA fragments in complex PCR-derived mixtures, this previously random approach becomes amenable to the identification of mutations in targeted genes by reverse genetics. Recently, strategies deploying chemical mutagens to induce point mutations in DNA have been described in *Arabidopsis* and the Acronym TILLING (**T**argeted **I**nduced **L**ocal **L**esions **IN** **G**enomes) has been coined (McCALLUM *et al*. 2000). TILLING is based on a simple PCR-screen coupled with variants of heteroduplex analysis or mismatch cleavage to detect mutations in a specified target region.

At SCRI we have generated two structured, chemically-mutagenized populations of the barley cv. Optic which allow forward and, for the first time, reverse genetics in a large genome crop species like barley. We have demonstrated the use of this resource by identifying mutations in a 420 bp open reading frame of the *Hordoindoline-a* (*Hin-a*) gene and a number of others that cannot at this moment be disclosed. The wheat orthologue of *Hin-a* is *Puroindoline-a* (*Pin-a)*. Well-characterised mutations in Pin-a (*pin-a*) largely determine whether wheat endosperm is classified as hard or soft and thus specifies its end-user market. Sib analysis of the barley M3 family progenies is currently underway in order to determine whether mutations in *Hin-a* similarly influence grain hardness. We are currently screening the population for mutations in a host of other targets. To aid forward genetic screening, we have documented visible mutant phenotypes in a web accessible database (http://bioinf.scri.sari.ac.uk/distilling/distilling.html). The process involved in generating such a population is outlined in figure 1.

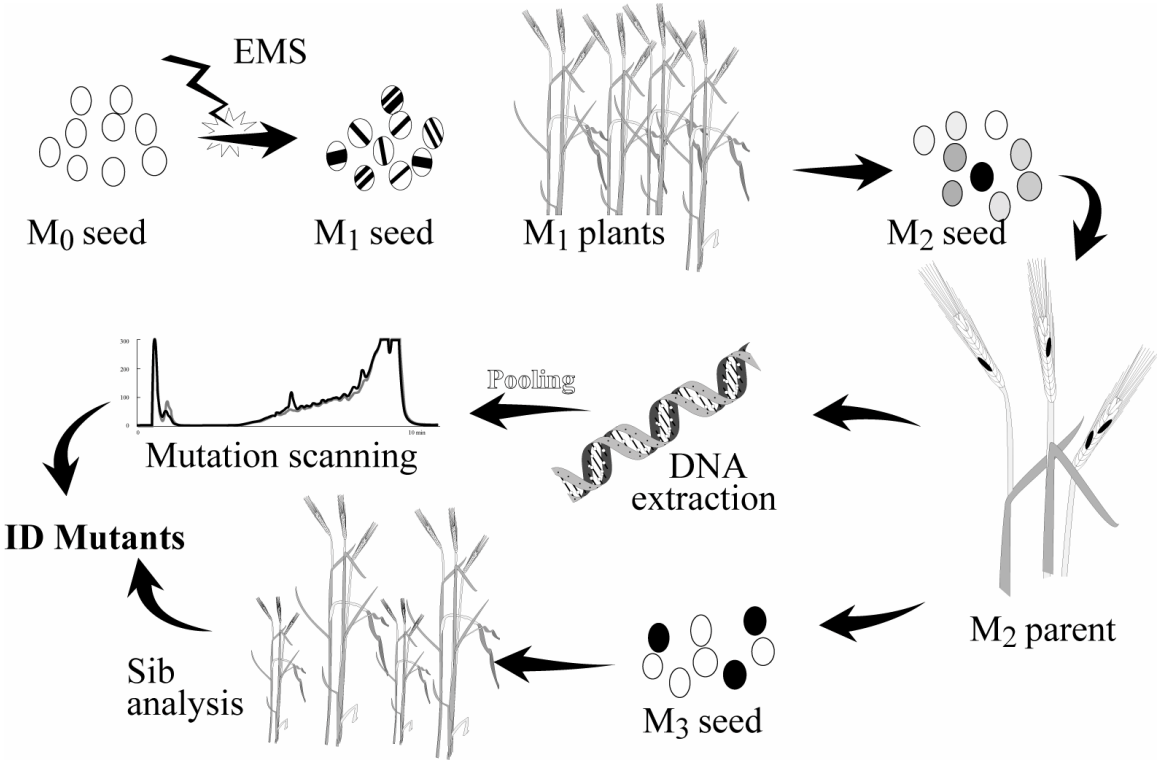


Figure 1. Creation of a structured mutant population. M_0 seed was mutated, propagated, and a single M_2 seed was taken forward from each chimeric M_1 plant. Genomic DNA was isolated from each M_2 plant and its M_3 seed was archived for sibling analysis and phenotyping. "Reverse Genetics" screening can be performed on the M_2 DNA pools or "Forward Genetics" screens can be performed on the archived M_3 seeds.

Transposon-Tagging Populations

The lack of facile and high throughput transformation systems for barley has hindered the development of large collections of transposon tagged or T-DNA insertion lines that have been so pivotal for functional genomics in model species. However this situation is changing. Two programs in the US (P. Lemaux and colleagues) and Europe (T. Koprek and colleagues) are focussed on the generation of transgenic barley lines containing single copies of a basta resistance gene (bar) located between the inverted repeats of the maize element Ds (KOPREK *et al*. 2001). These effectively dead elements can be brought back to life by crossing with plants expressing Ac transposase, which allows the elements to transpose in the barley genome and thus create new insertion mutants. Subsequent crosses allow the Ac and Ds elements to segregate away from each other and restore stability. 330 confirmed single copy independent Ds lines are now available in the assembled collection at MPI-Koln with others in the Lemaux lab in California. However because the Ds element tends to move to linked sites in the genome, for maximum utility as a generic resource, the genetic location of each of the Ds elements in each line needs to be established to target the identification of mutations in a genetically mapped target gene. This is currently the major challenge of this approach.

Reference Genotypes

Barley research – like that of many other crop plants has, in my view, suffered from a lack of focus by not adopting globally acceptable 'reference cultivars' for discovery-based research and genomic tool development. The power of reference biological material is clearly evidenced by the massive advances made by the Arabidopsis community by erecting Landsberg and Columbia ecotypes as globally accepted 'laboratory strains'. Within a group of

European organisations assembled under the banner of BarleyGenomeNet, the concept of establishing specific cultivars as 'reference genotypes' has already been accepted. Morex, the North American malting variety and Golden Promise, a gamma irradiation mutant of the old cultivar Maythorpe have been highlighted as the key material. The reasons: 1. Probably the largest collection of genomics tools and resources have been developed using the cv. Morex (ESTs, BAC library, Steptoe x Morex genetic map etc.) 2. The cultivar Golden Promise is the most widely used cultivar for genetic transformation (and thus for emerging transformation-based genomics resource development). A large doubled haploid population of a cross between Morex and Golden Promise (bi-directional) is currently under development and will be available for widespread distribution in the coming months. The objective is to fuel more rapid scientific advance by concentrating genomics resources (at least in the initial phase) on an integrated set of material.

Emerging Concepts

While there are many exciting emerging concepts, I would like to illustrate one that promises to embed the advances of high throughput genomics firmly back into a genetics framework. Recent reports have demonstrated that a significant portion of organismal gene expression is under genetic control and that quantitative differences in mRNA abundance may account for a major part of the phenotypic variation observed within and among species. A number of studies have now validated the potential of using mRNA abundance as a surrogate for classical quantitative phenotypes and demonstrated strong correlation between the two. For example, in mice, levels of gene expression determined by a 23,574 oligo array were quantified and the data for each treated as a quantitative trait. Of the 7,861 genes that were differentially expressed between the parental strains, standard interval mapping techniques allowed the authors to map 2,123 as quantitative traits at LOD >4.3. By combining this expression data with phenotypic data collected from the mice the authors were able to identify gene expression patterns that were strongly associated with obesity (SCHADT *et al.* 2003). The use of mRNA abundance as a surrogate phenotype is currently being explored in plants. In barley both individual mRNAs with Q-PCR, and populations of mRNAs with the Affymetrix array are being investigated. In the latter case, preliminary data has shown that ~10% of the 22K genes on the Barley1 array are differentially expressed in two different barley cultivars. The potential power of these experiments can be envisaged if it becomes possible to combine differential gene expression data with the volume of gene based marker segregation data that could be extracted from the same dataset in the form of SFPs.

In conclusion, I confidently predict that at the next IBGS, most of what I have covered above will be taken for granted and confined to history. However it will remain reasonable to reflect on its significance, how it was achieved and the impact it will ultimately make on our understanding of barley genetics.

References

CALDWELL, D.G., McCALLUM, N., SHAW, P., MUEHLBAUER, G.J. and WAUGH, R. (2004): A Structured Mutant Population for Forward and Reverse Genetics in Barley (*Hordeum vulgare* L.). The Plant Journal (in press).

CARDLE, L., RAMSAY, L., MILBOURNE, D., MACAULAY, M., MARSHALL, D.F. & WAUGH, R. 2000: Computational and experimental characterization of physically clustered simple sequence repeats in plants. Genetics, **156**: 847-854.

CLOSE, T.J., WANAMAKER, S., CALDO, R.A., TURNER, S. M., ASHLOCK, D.A., DICKERSON, J.A., WING, R.A., MUEHLBAUER, G.J., KLEINHOFS, A. and WISE, R.P. (2004): A new resource for cereal genomics: 22K barley GeneChip comes of age. Plant Physiology, **134:** 960-968.

FRANCKOWIAK, J. (1996): Revised Linkage maps for morphological markers in barley, *Hordeum vulgare*. BGN, **26:** 9-21.

KOPREK, T., RANGEL, S., McELROY, D., LOUWERSE, J.D., WILLIAMS-CARRIER, R.E. and LEMAUX, P.G. (2001): Transposon Mediated single-copy gene delivery leads to increased transgene expression stability in barley. Plant Physiology, **125:** 1354 – 1362.

KOTA, R., VARSHNEY, R.K., THEIL, T., DEHMER, K.J., and GRANER, A. (2001): Generation and comparison of EST derived SSRs and SNPs in barley. Heriditas, **135:** 145-151.

KOTA, R., RUDD, S, FACIUS, A, KOLESOV, G., THEIL, T., ZHANG, H., STEIN, N., MAYER, K. and GRANER, A. (2003): Snipping polymorphisms from large EST collections in barley Mol. Gen. Genom., **270:** 24 – 33.

LIU, H., HEDLEY, P., CARDLE, L., MARSHALL, D.F. and WAUGH, R. (2004): Developmental and functional analysis of genes expressed during the development of the barley grain. Submitted to Functional and Integrative Genomics.

LUO, M.C., THOMAS, C., YOU, F.M., HSIAO, J., SHU, O.Y., BUELL, C.R, MALANDRO, M., McGUIRE, P.E., ANDERSON, O.D., DVORAK, J. (2003): High-throughput fingerprinting of bacterial artificial chromosomes using the SNaPshot labeling kit and sizing of restriction fragments by capillary electrophoresis GENOMICS, **82 (3):** 378-389.

MICHALEK, W., WESCHKE, W., PLEISSNER, K.P. and GRANER, A. (2002): EST analysis in barley defines a unigene set comprising 4000 genes. Theor. Appl. Genet., **104**: 97-103.

PALMER, L.E., RABINOWICZ, P.D., O'SHAUGHNESSY, A.L., BALIJA, V.S., NASCIMENTO, L.U., DIKE, S., de la BASTIDE, M., MARTIENSSEN, R.A. and McCOMBIE, W.R. (2003): Maize genome sequencing by methylation filtrations. Science, **302** (5653): 2115-2117.

PILLEN, K., BINDER, A., KREUZKAM, B., RAMSAY, L., WAUGH, R., FORSTER, J. & LEON, J. (2000): Mapping new EMBL-derived barley microsatellites and their use in differentiating German barley cultivars. Theoretical and Applied Genetics, **101:** 652-660.

RAMSAY, L., MACAULAY, M., IVANISSEVICH, S.D., MacLEAN, K., CARDLE, L., FULLER, J.D., EDWARDS, K.J., TUVESSON, S., MORGANTE, M., MASSARI, A., MAESTRI, E., MARMIROLI, N., SJAKSTE, T., GANAL, M., POWELL, W. & WAUGH, R. (2000): A simple sequence repeat-based linkage map of barley. Genetics**, 156:** 1997-2005.

SCHADT, E.E., MONKS, S.A., DRAKE, T.A., LUSIS, A.J., CHE, N., COLINAYO, V., RUFF, T.G., MILLIGAN, S.B., LAMB, J.R., CAVET, G., LINSLEY, P.S., MAO, M., STOUGHTON, R.B., FRIEND, S.H. (2003): Genetics of gene expression surveyed in maize, mouse and man. Nature, **422** (6929): 297-302.

SREENIVASULU, N., ALTSCHMIED, L., RADCHUK, V., GUBATZ, S., WOBUS, U. and WESCHKE, W. (2004): Transcript profiles and deduced changes of metabolic pathways in maternal and filial tissues of developing barley grains. Plant Journal, **37** (4): 539-553.

SREENIVASULU, N., ALTSCHMIED, L., PANITZ, R., HAHNEL, U., MICHALEK, W., WESCHKE, W, and WOBUS, U. (2003): Identification of genes specifically expressed in maternal and filial tissues of barley caryopses: A cDNA array analysis. Mol. Gen . Gen., **266** (5): 758-767.

THIEL, T, MICHALEK, W., VARSHNEY, R.K. and GRANER, A. (2003): Exploiting EST Databases for the development of gene-derived SSR-markers in barley. Theor. Appl. Genet., **106:** 411-422.

VARSHNEY, R.K., THEIL, T., STEIN, N., LANGRIDGE, P. and GRANER, A. (2002): In silico analysis of the frequency and distribution of microsatellites in ESTs of some cereal species. Cellular and Molecular Biology Letters, **7:** 537 – 546.

WHITELAW, C.A., BARBAZUK, W.B., PERTEA, G., CHAN, A.P., CHEUNG, F., LEE, Y., ZHENG, L., van HEERINGEN, S., KARAMYCHEVA, S., BENNETZEN, J.L., SanMIGUEL, P., LAKEY, N., BEDELL, J., YUAN, Y., BUDIMAN, M.A., RESNICK, A., Van AKEN, S., UTTERBACK, T., RIEDMULLER, S., WILLIAMS, M., FELDBLYUM, T., SCHUBERT, K., BEACHY, R., FRASER, C.M. and QUACKENBUSH, J. (2003): Enrichment of gene-coding sequences in maize by genome filtration. Science, **302** (5653): 2118-2120

Map-Based Cloning in Barley: Coming of Age

A. Kleinhofs

Dept. Crop and Soil Sciences & School of Molecular Biosciences, Washington State University, Pullman, WA 99164-6420 USA

Abstract
Map-based cloning in barley has a brief and recent history. The published work will be reviewed. In my laboratory, the traditional high-resolution genetic mapping and BAC contig development resulted in cloning of the stem rust resistance gene *Rpg1* and the candidate genes for *rpg4* and *RpgQ*. Synteny with rice provided valuable markers, but chromosome walking was required to close the BAC contig. A saturation mapping approach, employing low-resolution genetic mapping combined with physical mapping, was attempted. Synteny with rice was again used. While the region of interest was highly saturated with genetic markers, the identified BAC clones failed to form a contig around the gene of interest, in this case the spot blotch resistance gene *Rcs5*. Chromosome walking was employed to close the contig. The examples of barley genes cloned by the map-based approach indicate feasibility, but required extensive work and a bit of luck. They also illustrate the usefulness and limitations of rice synteny. A third approach was opened by the development of the 22,700-gene Affymetrix Barley1 gene chip. We used the Barley1 chip to compare gene expression levels in a fast neutron induced mutant and its parent cultivar Morex. Two candidate genes were identified by highly down-regulated gene expression. Low-resolution mapping should differentiate among genes down-regulated because of limited transcription of the gene of interest and chance mutations elsewhere in the genome or down-stream effects. Future prospects and limitations of phenotype-based cloning in barley will be discussed.
Keywords: saturation mapping; physical mapping; disease resistance genes; gene cloning

Introduction

There are many different ways of cloning genes of interest, but if the gene is known only by its phenotype, then map-based cloning or insertional mutagenesis via transposons or T-DNA, currently provide the only viable approaches. Gene cloning by insertional mutagenesis has yet to be demonstrated in barley, although recent progress in developing a transposon based system has been reported (KOPREK *et al.* 2001; KOPREK *et al.* 2000). Map-based cloning in barley is also a relatively recent development. With its approximately 5 billion base pair genome, barley is not an ideal organism for map-based cloning. Nevertheless, it is essential if we are to isolate and work with the genes that are important and unique to barley. Barley can also serve as a model organism for other, even more difficult genome Triticeae, such as wheat and rye.

In principle, map-based cloning is straight-forward. One simply needs to have a high resolution map of the gene of interest and a large insert clone, such as Bacterial Artificial Chromosome (BAC), physical contig of the region (Fig. 1). Candidate genes are identified via sequencing of the region encompassing the gene of interest. Difficulty sometimes arises in proving that the candidate gene is the actual gene of interest. Two general approaches can be used, 1) transformation to complement a mutant or to silence the gene in a wild-type via interference RNA (TIJSTERMAN *et al.* 2002) and 2) sequencing of mutant and wild-type alleles to associate sequence differences with the phenotype. Other approaches are more specific to a particular situation as described below.

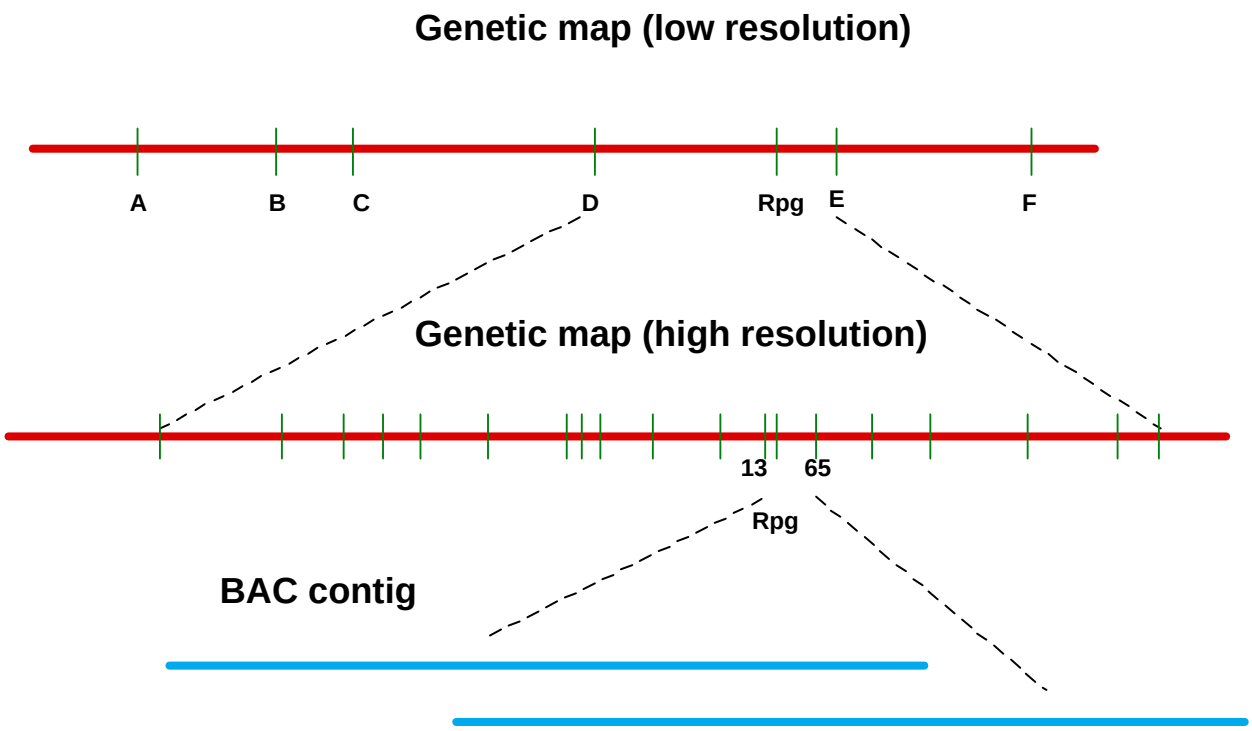


Figure 1. A simplified approach to map-based cloning. Starting with a low-resolution map, a high-resolution map and a physical contig are developed. The region between the markers flanking the gene of choice is sequenced and analyzed for candidate genes. Candidate gene are confirmed or rejected based on sequencing of wild-type and mutant alleles or transformation to complement a mutant allele or silence a wild-type allele.

The first barley gene to be cloned by the map-based approach was the recessive *mlo* gene conferring broad spectrum resistance to *Blumeria graminis* f. sp. *hordei* (previously *Erysiphe graminis* f. sp. *hordei*) (BUSCHGES *et al*. 1997). Starting with an RFLP defined region of approximately 2.7 cM, the authors saturated the region with markers using the Amplified Fragment Length Polymorphism (AFLP) technology (VOS *et al*. 1995) to generate a high resolution genetic map to approximately 0.05 cM. This genetic resolution and the availability of a large insert Yeast Artificial Chromosome (YAC) library, allowed them to identify a single YAC clone containing Mlo, an approach described as chromosome landing (TANKSLEY *et al.* 1995). The candidate gene was identified by sequencing and mRNA analyses and confirmed by sequencing of 11 mutagen-induced alleles and seven wild-type alleles from different barley cultivars. Nucleotide substitutions or deletions leading to amino acid changes or frame shifts were identified in all mutant alleles. The wild-type alleles were all identical except for a few nucleotide changes in one. These data are sufficient to conclude that the candidate gene is the *Mlo* gene, however, the authors went further. They performed inter-mutant crosses with alleles separated by the fullest extent of the gene. The hypothesis that recombination would occur between these mutant alleles and generate a wild-type gene sequence that would confer susceptibility to mildew, was confirmed. This demonstrated a clever way to confirm the nature of the candidate gene without the need for transgenic complementation experiments. However, this approach is not available in most cases were the mutants are fairly rare and are not the allele that confers resistance.

A somewhat different approach was taken to clone the *Rar1* (Required for *Mla* resistance) gene (SHIRASU *et al.* 1999). A YAC clone contig covering approximately 630 kb was established between flanking RFLP markers. The YAC clones were then used to develop a BAC library and additional markers to delimit the region to 300 kb. A contiguous DNA sequence of approximately 66 kb covering most of the markers co-segregating with Rar1 was established and analyzed for candidate genes. Three regions representing possible coding regions were identified. Comparative sequencing of wild-type and the rar1-1 and rar1-2 alleles identified one region with one single nucleotide change in each mutant. The G to A substitution in *rar1-1* resulted in Cys to Tyr conversion while the same base substitution in *rar1-2* changed the invariant 3' splice site of intron 2 from G to A. This resulted in a dramatic reduction of the mRNA quantity and production of a truncated message confirming the candidate gene as *Rar1*. These experiments demonstrate the ability to clone genes even in a relatively low recombination region.

The development of a high resolution genetic and physical map of the *Mla* region (WEI *et al.* 1999) led to cloning of multiple genes that are found at this locus and confer resistance to a large number of different pathotypes of the mildew pathogen *Blumeria graminis* f. sp. *hordei* (JORGENSEN 1994). The mapping and physical contig development was a major effort using RAPD, AFLP, and RFLP markers and a YAC and BAC library to extend the physical contig to 240 kb including the *Mla* locus (WEI *et al.* 1999). This work identified many potential candidate genes, but the identification of individual genes responsible for resistance to specific fungal pathotypes was facilitated by the development of a transient assay (reviewed in (PANSTRUGA 2004). This procedure was used to clone and identify the genes *Mla1* (ZHOU *et al.* 2001), *Mla6* (HALTERMAN *et al*. 2001), *Mla12* (SHEN *et al*. 2003), *Mla13* (HALTERMAN *et al.* 2003), *Mla7* and *Mla10* (HALTERMAN & WISE 2004).

The barley gene *Rpg1* conferring resistance to the stem rust pathogen *Puccinia graminis* f. sp. *tritici* was cloned in my laboratory (BRUEGGEMAN *et al*. 2002). Initially, we employed barley-rice synteny to saturate the region with markers and to delimit the corresponding rice genomic region to 33 kb. However, sequencing and analysis of this region failed to identify candidate genes suggesting that the barley genome had a large insertion at this point that was not present in the syntenous rice region (HAN *et al.* 1999). Consequently, a tedious chromosome walk was undertaken to develop a physical contig of the region. The identity of the candidate gene was first identified by a cross-over within the gene and allele sequencing (BRUEGGEMAN *et al.* 2002) and later by stable transformation (HORVATH *et al*. 2003).

Barley-rice synteny was successfully used to clone the *Ror2* gene (COLLINS *et al.* 2003). In this case the synteny between barley and rice was nearly perfect in the region of interest and the authors were able to isolate a candidate gene with a relatively small mapping population. The identity of the candidate gene was confirmed by demonstrating that the *ror2-1* mutant line had a 31 aa in-frame deletion. Additional confirmation was obtained by transient complementation of the *ror2-1* mutation by the wild-type *Ror2* genomic clone driven by the native promoter in barley leaf epidermal cells (COLLINS *et al.* 2003).

Work in Progress

"Traditional" map-based cloning of barley genes is continuing in spite of the tedious work and relatively slow progress. In my laboratory, we have developed a BAC clone physical contig for the *rpg4* and *Rpg5* genes conferring resistance to *Puccinia graminis* f. sp. *tritici* and *Puccinia graminis* f. sp. *secalis*, respectively (DRUKA *et al*. 2000). This work has uncovered a number of new problems that were not encountered in our previous experience with map-based cloning. A significant problem, although one that can be overcome, is the absence of the gene of interest from the cultivar used to construct the large insert clone library. In this

particular case, however, the resistant cultivar Q21861 genome has a significant insertion/deletion with respect to the susceptible cultivar Morex that was used to construct the BAC library. The second problem occurs when a phenotype is dependent on two or more complementary genes and both are segregating in the mapping population used to construct the high-resolution genetic map. In spite of these difficulties a candidate *Rpg5* gene with a novel structure containing NBS-LRR-S/TPK domains was identified (BRUEGGEMAN *et al.* unpublished). The proof of this candidate gene identity, however, remains to be confirmed. Identifying the *rpg4* candidate gene has become problematic due to the probable requirement of two interacting genes for this resistance. An actin depolymerizing factor gene was suspected as being involved, but at least one other gene, as yet unidentified, may be required.

Other map-based cloning efforts are nearing completion. A 200 kb BAC contig was established for the barley leaf rust resistance gene *Rph7* and candidate gene verification is in progress (SCHERRER *et al.* 2004). Similarly, the barley yellow mosaic virus resistance genes *rym4/5* have been localized to a 450 kb region 200 kb of which co-segregates with the gene in over 5,000 meiotic events (STEIN *et al.* 2004). This work illustrates how extremely suppressed recombination can be in some regions of the barley genome.

Another gene that should be cloned in the near future is the leaf stripe resistance gene *Rdg2a*. A high-resolution map has been completed and several Resistance Gene Analogs (RGAs) map very close to the resistance gene (BULGARELLI *et al.* 2004).

Other map-based cloning projects are targeting such barley specific genes as *Vrs1* (2 vs. 6 rowed spike), *Btr 1* and *2* (brittle rachis), and *Nud* (naked vs covered seed) (KOMATSUDA, personal communications).

Looking for an easier route to map-based (phenotype-based) cloning, we attempted barley-rice synteny cloning of the spot blotch resistance gene *Rcs5* (JOHNSON *et al.* 2004). Synteny with rice provided many markers and facilitated a start of a BAC contig. However, chromosome walking was required to close the gaps in the contig. A candidate gene has not been identified to date. This may be due to difficulty in mapping the phenotype or due to the small gap in the rice sequence. Barley-rice synteny is a highly valuable tool, particularly with the emerging rice genomic sequence. It can always be counted on to provide useful markers, but the synteny is not always good enough to produce the gene of interest. If it does work, it is clearly the most straight-forward way to clone barley genes by map-based cloning.

We have also been experimenting with phenotype based cloning of genes by expression pattern profiling of mutant vs. wild-type lines (ZHANG *et al.* 2004). A complete lack of two mRNAs was identified in a fast neutron induced mutant that suppressed the stem rust resistance gene *Rpg1* function. Unfortunately the deletion seems to be fairly large involving at least two genes and thus complicating the identification of the gene responsible for the phenotype. This procedure may be very useful and applicable to many situations if the mutant is a deletion or mRNA deficient and the profiling is done with a high density microarray such as the Affymetrix Barley 1 chip (CLOSE *et al.* 2004).

Discussion

The history of map-based cloning in barley is brief and the number of genes cloned still relatively few. Nevertheless the uniqueness and value of the cloned genes have clearly demonstrated the importance and need for being able to do this in barley. For example, the *Rar1* gene, first identified and cloned from barley, has become known as an important disease resistance gene interactor in Arabidopsis and involved in the control of development in other eukaryotes. It is difficult to prescribe a "recipe" for map-based cloning in barley, but certain elements are essential.

High-Resolution Mapping – is relatively easily achieved in barley. What exactly is high-resolution is more difficult to answer. In the successful efforts described above, the number of gametes examined has varied from 1,522 for the rice synteny facilitated cloning of *Ror2* (COLLINS *et al.* 2003) to 8,620 for the cloning of the *Rar1* gene (SHIRASU *et al.* 1999) (Table 1). These numbers are easily achieved by selecting recombinants between two markers flanking the gene of interest from an F2 population followed by selection of the homozygous recombinant among the progeny of the recombinants.

Marker Availability – can be a limiting factor. The need for marker density is determined by the insert size of the libraries available for physical contig development. Since the currently most readily available large insert BAC library has inserts averaging about 100 kb, the markers must be more closely spaced than that. A variety of techniques, including AFLP, RAPD, rice synteny, and BAC or YAC subcloning, have been used to saturate specific chromosome regions with markers. In our hands, selection of markers based on rice synteny has worked well to identify new RFLP probes. The rice BAC or PAC clone sequence from the syntenous region is searched against a Triticeae EST database using BLASTn at the NCBI BLAST home page http://www.ncbi.nlm.nih.gov/BLAST/. Barley or wheat ESTs with reasonably high homology to the rice sequence are used for mapping. About 50% of them map to the expected region. The rest may be probes that give multiple bands and the wrong band is mapped or a wrong homolog is identified and some represent genes that have moved out of synteny.

Once a physical contig has been started, the large insert clones can be used to develop new markers. We have tried a number of approaches, but the most reliable has been to develop small insert plasmid libraries from the BAC clone and search them for low copy probes by hybridizing with total genomic DNA. The clones that do not hybridize represent low copy and sometimes artifact clones. Although tedious, this procedure has allowed us to construct physical BAC contigs for all regions we have attempted so far.

Physical Contig Development – depends on the availability and completeness of large insert libraries. The libraries used to date include a YAC library (KLEINE *et al.* 1993; KLEINE *et al.* 1997) and a cv. Morex BAC library (YU *et al.* 2000). Although the YAC library has been used with success, it is not readily available in the public domain making the Morex BAC library the primary source of large insert clones for public domain researchers. Both libraries also have limitations. The YAC library apparently was not saturated enough to complete the *Mla* locus physical contig (WEI *et al.* 1999) and the Morex BAC library has limited size inserts (106 kb) and was constructed with a single enzyme (YU *et al*. 2000). Additional libraries may soon become available. The cv. Haruna Nijo BAC library has been completed (K. Sato, personal communications). The 2-rowed cv. Haruna Nijo BAC library will be a good complement to the 6-rowed Morex BAC library. A BAC library from cv. Cebada Capa was developed to facilitate isolation of the *Rph7* gene (SCHERRER *et al.* 2004).

Candidate Gene Identification – depends on the ability to sequence the physical region between the flanking markers that segregate away from the gene of interest. With today's automated sequencing, that is not a problem. Sequence analysis with gene prediction programs and searching against EST databases will usually identify some (hopefully not too many) candidate genes. The better the high-resolution mapping the smaller the potential number of candidate genes that one may have to deal with. However, there are regions of the barley genome with exceedingly low recombination rates that may be difficult. These regions probably have low gene density and are rich in repeat sequences.

Table 1. Barley genes clones by map-based cloning

Gene	Reference	Gametes	Confirmation Method
mlo	Buschges et al., 1997	4,044	Allele sequence; Mutant recombination
Rar1	Shirasu et al., 1999	8,620	Allele sequence
Mla	locus; Wei et al., 1999 *Mla1*; Zhou et al., 2001 *Mla6*; Halterman et al., 2001 *Mla12*; Shen et al., 2003 *Mla13*; Halterman et al., 2003 *Mla7* & *Mla10*; Halterman & Wise, 2004	3,600	 Transient expression Transient expression Transient expression Transient expression Transient expression
Rpg1	Brueggeman et al., 2002	8,512	Allele sequence; Stable transformation
Ror2	Collins et al., 2003	1,522	Allele sequence; Transient expression

Candidate Gene Identification – depends on the ability to sequence the physical region between the flanking markers that segregate away from the gene of interest. With today's automated sequencing, that is not a problem. Sequence analysis with gene prediction programs and searching against EST databases will usually identify some (hopefully not too many) candidate genes. The better the high-resolution mapping the smaller the potential number of candidate genes that one may have to deal with. However, there are regions of the barley genome with exceedingly low recombination rates that may be difficult. These regions probably have low gene density and are rich in repeat sequences.

Candidate Gene Confirmation – can present difficulties. Two major methods are allele sequencing and transformation. Allele sequencing is straightforward, particularly if a good collection of induced mutants is available. If one has to rely on alleles available in the barley collections then subtle mutations such as a single or few amino acid changes become hard to interpret if they are responsible for the phenotype or just random changes that have nothing to do with the phenotype. The solution is to sequence a large number of wild-type and mutant alleles.

Transformation can be used to stably or transiently introduce genes to complement a mutant allele or silence a wild-type allele. Stable transformation in barley is possible, but a slow process. Consequently it has not been extensively used to confirm candidate genes and certainly one would not want to use it to examine many candidate genes. A very clever transient transformation assay has been developed and used to confirm the various Mla specificities (PANSTRUGA 2004). This assay, however appears to be limited to the mildew

resistance genes and may not have wide applicability. Similarly, the mutant recombination assay employed by Buschges et al. (1997) is limited to a special case where large number of mutants are available and the rare recombinants can be readily identified.

Future Prospects

Map-based cloning in barley has resulted in the isolation of unique and interesting genes that have accelerated science not only in barley or the Triticeae, but in all respects. The *mlo* gene is still unique among disease resistance genes in spite of the extensive cloning of genes from the model plant Arabidopsis, as is the *Rpg1* gene with its tandem protein kinase domains. Similarly, the *Rar1* gene, first cloned from barley, has become an important player for understanding plant pathogen interactions in all species. Cloning of the *Mla* locus combined with the transient asaay to test for protein interactions has resulted in one of the best systems for studying fungal pathogen plant interactions.

However, the cloning of these genes has been tedious long-term effort. What does the future hold? Is it possible that in the not too distant future cloning based on phenotype will become relatively easy and maybe even possible for complex QTL traits? Availability of a complete barley genome sequence would greatly facilitate map-based cloning in barley. However, with the approximately 5 billion base pair genome, barley is not a likely candidate for complete genome sequencing in the near future. Contiging and sequencing of gene-rich regions, however, may be realistic. Other developments that would facilitate map-based cloning include availability of 1) high density maps, or at least the ability to develop such maps with ease 2) large insert clone physical contigs or at least the ability to develop such contigs and 3) availability of deletion mutants. Many exciting new developments are taking place in barley genomics that will advance our ability to efficiently clone genes by phenotype. These are described by others at this symposium and will not be covered here (see Current Perspectives in Barley Genomics (2004) by Robbie WAUGH *et al.,* in this symposium proceedings).

References

BRUEGGEMAN, R., N. ROSTOKS, D. KUDRNA, A. KILIAN, F. HAN, J. CHEN, A. DRUKA, B. STEFFENSON, A. KLEINHOFS (2002): The barley stem rust-resistance gene Rpg1 is a novel disease-resistance gene with homology to receptor kinases. Proc. natl. Acad. Sci. USA, **99:** 9328-9333.

BULGARELLI, D., N.C. COLLINS, G. TACCONI, E. DELLAGLIO, R. BRUEGGEMAN, A. J. KLEINHOFS, A.M. STANCA, G. VALE (2004): High-resolution genetic mapping of the leaf stripe resistance gene Rdg2a in barley. Theor. Appl. Genet. DOI 10.1007/s00122-003-1557-9.

BUSCHGES, R., K. HOLLRICHER, R. PANSTRUGA, G. SIMONS, M. WOLTER, A. FRIJTERS, R. van DAELEN, T. van der LEE, P. DIERGAARDE, J. GROENENDIJK, S. TOPSCH, P. VOS, F. SALAMINI, P. SCHULZE-LEFERT (1997): The barley *Mlo* gene: A novel control element of plant pathogen resistance. Cell, **88:**695-705.

CLOSE, T.J., S. WANAMAKER, R.A. CALDO, S.M. TURNER, D.A. ASHLOCK, J.A. DICKERSON, R.A. WING, G.J. MUEHLBAUER, A. KLEINHOFS, R.P. WISE (2004): A new resource for cereal genomics: 22K barley GeneCip comes of age. Plant Physiol., **134**: 960-968.

COLLINS, N.C., H. THORDAL-CHRISTENSEN, V. LIPKA, S. BAU, E. KOMBRINK, J.-L. QIU, R. HUCKELHOVEN, M. STEIN, A. FREIALDENHOVEN, S.C. SOMMERVILLE, P. SCHULZE-LEFERT (2003): SNARE-protein-mediated disease resistance at the plant cell wall. Nature, **425:** 973-977.

DRUKA, A., D. KUDRNA, F. HAN, A. KILIAN, B. STEFFENSON, D. FRISCH, J. TOMKINS, R. WING, A. KLEINHOFS (2000): Physical mapping of barley stem rust resistance gene *rpg4*. Mol Gen Genet, **264:** 283-290.

HALTERMAN, D., F. ZHOU, F. WEI, R.P. WISE, P. SCHULZE-LEFERT (2001). The MLA6 coiled-coil, NBS-LRR protein confers *AvrMla6*-depedent resistance specificity to *Blumeria graminis* f. sp. *hordei* in barley and wheat. Plant Journal, **25:** 335-348.

HALTERMAN, D.A., R.P. WISE (2004): A single-amino acid substitution in the sixth leucine-rich repeat of barley MLA6 and MLA13 alleviates dependence on RAR1 for disease resistance signaling. The Plant Journal.

HALTERMAN, D.A., F. WEI, R.P. WISE (2003): Powdery mildew-induced *Mla* mRNAs are alternatively spliced and contain multiple upstream open reading frames. Plant Physiol., **131:** 558-567.

HAN, F., A. KILIAN, J.P. CHEN, D. KUDRNA, B. STEFFENSON, K. YAMAMOTO, T. MATSUMOTO, T. SASAKI, A. KLEINHOFS (1999): Sequence analysis of a rice BAC covering the syntenous barley *Rpg1* region. Genome, **42:** 1071-1076.

HORVATH, H., N. ROSTOKS, R. BRUEGGEMAN, B. STEFFENSON, D. von WETTSTEIN, A. KLEINHOFS (2003): Genetically engineered stem rust resistance in barley using the *Rpg1* gene. Proc. Natl. Acad. Sci. USA, **100:** 364-369.

JOHNSON, K.A., B.J. STEFFENSON, R.S. BRUEGGEMAN, D.A. KUDRNA, A. KLEINHOFS (2004): Genetic and physical mapping of the barley spot blotch resistance gene Rcs5. Plant and Animal Genome XII Abstract P160: p112.

JORGENSEN, J.H. (1994): Genetics of powdery mildew resistance in barley. Crit. Rev. Plant Sci., **13**: 97-119.

KLEINE, M., W. MICHALEK, A. GRANER, R.G. HERRMAN, C. JUNG (1993): Construction of a barley (Hordeum vulgare L.) YAC library and isolation of a Hor1-specific clone. Mol. Gen. Genet. 240:265-272.

KLEINE, M., W. MICHALEK, T. DIEFENTHAL, H. DARGATZ, C. JUNG (1997): Construction of a MluI-YAC library from barley (Hordeum vulgare L.) and analysis of YAC insert terminal regions. Genome, **40:** 896-902.

KOPREK, T., S. RANGEL, D. MCELROY, J.D. LOUWERSE, R.E. WILLIAMS-CARRIER, P.G. LEMAUX (2001): Transposon-mediated single-copy gene delivery leads to increased transgene expression stability in barley. Plant Physiol., **125:** 1354-1362.

KOPREK, T.K., D. MCELROY, J. LOUWERSE, R. WILLIAMS-CARRIER, P.G. LEMAUX (2000): An efficient method for dispersing Ds elements in the barley genome as a tool for determining gene function. Plant J., **24:** 253-263.

PANSTRUGA, R. (2004): A golden shot: how ballistic single cell transformation boosts the molecular analysis of cereal-mildew interactions. Molec. Plant Pathology, **5**: 141-148.

SCHERRER, B., S. BRUNNER, E. ISIDORE, B. CHALHOUB, B. KELLER, C. FEUILLET (2004): The barley Rph7 leaf rust resistance gene: a new type of R gene? Plant and Animal Genome XII abstract W34: p13.

SHEN, Q.-H., F. ZHOU, S. BIERI, T. HAIZEL, K. SHIRASU, P. SCHULZE-LEFERT (2003). Recognition specificity and RAR1/SGT1 dependence in barley *Mla* disease resistance genes to the powdery mildew fungus. The Plant Cell, **15:** 732-744.

SHIRASU, K., T. LAHAVE, M.-W. TAN, F. ZHOU, C. AZEVEDO, P. SCHULZE-LEFERT (1999): A novel class of eukaryotic zinc-binding proteins is required for disease resistance signaling in barley and development in *C. elegans*. Cell, **99:** 355-366.

STEIN, N., D. PEROVIC, B. PELLIO, S. STRACKE, F. ORDON, A. GRANER (2004): Chromosome walking reveals a candidate gene for barley mild/barley yellow mosaic virus resistance at the locus rym4/5. Plant and Animal Genome XII Abstract P162: p112.

TANKSLEY, S.D., M.W. GANAL, G.B. MARTIN (1995): Chromosome landing: a paradigm for map-based gene cloning in plants with large genomes. Trends in Genet., **11:** 63-68.

TIJSTERMAN, M., R.F. KETTING, R.H.A. PLASTERK (2002): The genetics of RNA silencing. Annu. Rev. Genet., **36:** 489-519.

VOS, P., R. HOGERS, M. BLEEKER, M. REIJANS, T. van de LEE, M. HORNES, A. FRIJTERS, J. POT, J. PELEMAN, M. KUIPER, M. ZABEAU (1995): AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research, **23:** 4407-4414.

WEI, F., K. GOBELMAN-WERNER, S.M. MORROLL, J. KURTH, L. MAO, R. WING, D. LEISTER, P. SCHULZE-LEFERT, R.P. WISE (1999): The Mla (powdery mildew) resistance cluster is associated with three NBS-LRR gene families and suppressed recombination within a 240-kb DNA interval on chromosome 5S (1HS) of barley. Genetics, **153:** 1929-1948.

YU, Y., J.P. TOMKINS, R. WAUGH, D.R. FRISCH, D. KUDRNA, A. KLEINHOFS, R.S. BRUEGGEMAN, G.J. MUEHLBAUER, R.P. WISE, R.A. WING (2000): A bacterial artificial chromosome library from barley (Hordeum vulgare L.) and identification of clones containing putative resistance genes. Theor. Appl. Genet., **101:** 1093-1099.

ZHANG, L., R. BRUEGGEMAN, N. ROSTOKS, T. FETCH, B. STEFFENSON, A. KLEINHOFS (2004): Expression profiling of a barley stem rust resistance gene Rpg1 suppressor. Plant and Animal Genome XII Abstract P795: p269.

ZHOU, F., J. KURTH, F. WEI, C. ELLIOT, G. VALE, N. YAHIAOUI, B. KELLER, S. SOMERVILLE, R.P. WISE, and P. SCHULZE-LEFERT (2001): Cell-autonomous expression of barley Mla1 confers race-specific resistance to the powdery mildew fungus via Rar1-independent signaling pathway. The Plant Cell, **13:** 337-350.

S 4 – BIOCHEMISTRY, CYTOGENETICS, TRANSGENIC BARLEY

Genetic Transformation of Barley: Improved Technology, Safety Assessment and Future Potential

W.A. Harwood, L.J. Bilham, S. Travella[1], V. Bourdon[2], H. Salvo[3], J. Harden, M. Perry and J.W. Snape

John Innes Centre, Norwich Research Park, Colney, Norwich, NR4 7UH, U.K.
Current addresses:
[1]Institute of Plant Biology, University of Zurich, 8008 Zurich, Switzerland;
[2]University of Oxford, Department of Plant Sciences, Oxford, OX1 3RB, U.K.;
[3]Biotechnology Unit, INIA, Carillanca, P.O. Box 58 D, Temuco, Chile

Abstract
Genetic transformation offers the exciting possibility of producing barley lines with improved traits of significant benefit to the consumer, producer and processor. One of the main goals of plant genetic engineering is to achieve stable transgenic events that give predictable and reproducible levels of expression of a transgene, and that are fully characterized in terms of the effect and implications of the transgene insertion for the plant. In practise this goal is still rather difficult to achieve.
Reliable methodologies are now available for the genetic transformation of barley using either direct DNA delivery by particle bombardment, or *Agrobacterium*-mediated gene delivery. Although the *Agrobacterium*-mediated transformation offers a number of advantages, methods are still genotype dependent and transgene expression is unpredictable. Some variation in expression may be attributed to so called 'position effects' that are due to the genomic environment of the transgene. This source of variation may be further understood by examining the genomic environment of the transgene both by physical and genetic mapping and by detailed molecular analysis.
For the safety assessment of transgenic barley lines, we need to ask whether the transgene insertion leads to any unintended consequences for the plant. Here we discuss how our recent work on the transgene insertion site and control of transgene expression, contributes to our understanding of unintended consequences of transgene insertions in barley plants grown under glasshouse and field conditions.
Keywords: barley; genetic modification; transgene expression; transgene insertion; *Agrobacterium*; GM field trial

Introduction
Reliable methodology for the transformation of cereal species is of enormous value both in answering fundamental questions on gene action and in the production of transgenic lines with a range of improved traits. Since the first reports of the production of fertile transgenic barley plants (WAN & LEMAUX 1994), considerable improvements have been made in the technology available for the production of transgenic cereals. However, methodology for the genetic transformation of barley lags behind that of other cereals in terms of efficiency and

genotype independence. Perhaps one of the most significant advances was the development of *Agrobacterium*-mediated techniques for the genetic modification of barley (TINGAY *et al*. 1997). This method offers a number of advantages over particle bombardment techniques, for example, less complex transgene insertion sites. In addition, the *Agrobacterium*-mediated technique offers the possibility of producing transgenic barley lines where the selectable marker gene can be segregated away from the gene of interest (MATTHEWS *et al*. 2001). This is a particularly important safety consideration in lines developed for field release.

Despite improvements in transformation technology and in our understanding of the transformation event, a number of challenges still exist. One of the most important challenges is the production of transgenic lines where the transgene shows stable inheritance and expression over a number of generations. The problems of instability of transgene inheritance and expression are well documented in barley. BREGITZER and TONKS (2003) found that the majority of transgenic barley lines examined showed some form of instability including failure to transmit transgenes to the progeny and transgene silencing. Transgene silencing has been attributed to a number of causes but so called 'position effects' are often thought to be involved, where the genomic environment of the transgene influences its expression and stability. For this reason, a better understanding of the nature of the transgene insertion site in barley is desirable as this may, in turn, lead to a better understanding of the insertion site characteristics that are desirable for stable expression and inheritance. While some features of the genetic transformation process can now be more accurately controlled, for example targeting transgene expression to particular plant tissues, other features, in particular the exact insertion site of the transgene, cannot at present be controlled. In practise, it is currently necessary to produce and examine, a large number of independent transgenic lines for any particular gene of interest, through several generations. Lines with stable patterns of expression and inheritance can then be selected. Another approach might be to only select lines that have transgene insertions of a type previously shown to be associated with stable expression patterns. A third possibility is to engineer the transformation vector in such a way as to improve the chances of obtaining stable expression. In this paper we discuss work that aims to increase our understanding of the transgene insertion site together with possible methods for improving transgene stability over generations. We also consider the safety assessment of GM barley lines, linking this to the study of the transgene insertion site. Despite the problems of transgene stability described above, we are able to demonstrate stable transgene expression and inheritance in lines grown under field conditions over five years.

Barley Transformation Methodology

The most popular method for the production of transgenic barley plants to date has been the particle bombardment of immature embryos. In addition, most work has been carried out with the model spring variety Golden Promise (HARWOOD *et al*. 2002). Recently, *Agrobacterium*-mediated techniques have been improved such that this method is now the method of choice both in terms of efficiency and in terms of the quality of transgenic plants obtained. We have made a comparison of transgenic barley lines of the variety Golden Promise produced using the two different methods. Immature embryos were used as the target tissue for both methods and the same tissue culture and selection procedures employed. Full details of the tissue culture procedures and *Agrobacterium*-mediated transformation method are included on the Biotechnology Resources for Arable Crop Transformation (BRACT) web-site (**www.bract.org**).

Our study showed a number of advantages of the *Agrobacterium*-mediated system. Firstly, the transformation efficiency was approximately double that obtained using particle bombardment. Particle bombardment gave just over 1% transformation efficiency when all experiments were considered. *Agrobacterium*-mediated transformation gave over 2% efficiency with individual experiments giving up to 11%. Transgene silencing was far less frequent in the *Agrobacterium*-derived lines although cases of transgene instability were still seen. Transgene copy number was also significantly different in the *Agrobacterium*-mediated and particle bombardment derived lines. *Agrobacterium*-derived lines usually had between one and three copies of the transgene whereas 60% of the particle bombardment derived lines had more than eight copies of the transgenes. Southern analysis showed that the *Agrobacterium*-derived lines had predominantly simple transgene insertion patterns whereas many of the particle bombardment-derived lines had complex insertion patterns showing evidence of extensive rearrangement of the introduced sequences. This comparison clearly demonstrates the advantages of the *Agrobacterium*-mediated technique in barley but also shows that transgene instability can still be a problem in *Agrobacterium*-derived lines.

Transgene Insertion

The site of transgene insertion in the barley genome cannot at present be controlled. For the safety assessment of transgenic barley lines it is useful to obtain information about the location of the transgene in the barley genome and detail of the surrounding genomic DNA. In order to further understand transgene insertion in barley we have determined the position of transgenes in the barley genome both by physical mapping using fluorescence *in situ* hybridisation (SALVO-GARRIDO *et al*. 2001) and by genetic mapping. In addition we have isolated the genomic sequences adjacent to the transgenes in barley lines and compared these sequences to those held in databases (SALVO-GARRIDO *et al*. 2004).

The physical location of the transgenes was determined in 19 independent barley lines using fluorescence *in situ* hybridisation. Transgene insertions were only found on five of the seven barley chromosomes and evidence was seen for clustering of independent transgene insertion sites within the barley genome. This indicated that transgene insertion was not random. There was also a tendency for transgene insertions to be more frequently found in telomeric or sub-telomeric locations. For 11 of the barley lines, the location of the transgene was also determined by genetic mapping. This was done by using a Bulk Segregant Analysis (BSA) approach on F_2 plants derived from crosses between transgenic plants and non-transgenic varieties. In all cases the transgene locations agreed with those determined by physical mapping. On chromosome 4H, three independent transgene insertions were found within a distance of 4cM suggesting a preference for this region of the genome.

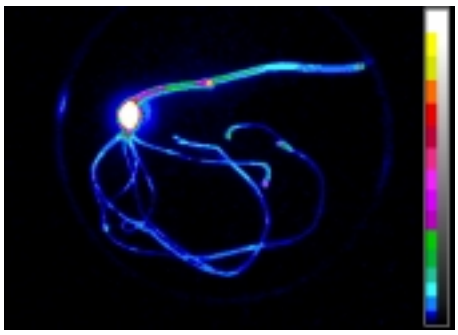
Genomic regions flanking the transgenes were obtained using PCR based procedures. These sequences were compared to available databases and it was seen that the isolated genomic DNA corresponded to recognised coding regions in six out of seven cases. The results suggest that transgenes are more likely to insert into gene rich regions of the genome. This finding might explain the non-random pattern of transgene insertion seen in barley and is in agreement with studies of transgene insertion in rice (SHA *et al*. 2004) and *Arabidopsis* (QIN *et al*. 2003).

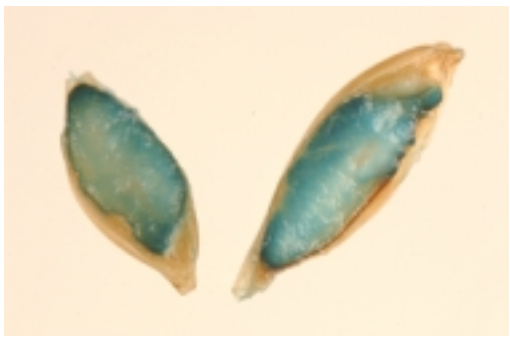
Transgene Expression

Instability of transgene expression has been widely reported in a range of transgenic plants. In some cases instability is particularly obvious in lines homozygous for the transgene and with

high levels of transgene expression, for example in wheat (BOURDON *et al.* 2002). In barley, we have observed large variation in transgene expression from one generation to the next by assaying for expression of the firefly luciferase reporter gene (Fig. 1a). In some cases, clonal lines derived from the same transformation event exhibited very different patterns of transgene expression showing that factors other than position effects were involved. Examples were seen where a line that had maintained high levels of expression for four generations then showed very low expression in the T_5 generation. Levels of transgene expression can be influenced in a number of ways, from the choice of promoter to the arrangement of the expression cassettes in the transformation vector. For many applications, use of a tissue specific promoter is desirable, for example the seed specific low molecular weight glutenin promoter (Fig. 1b). Targeting transgene expression to the required site only may help to reduce instability problems.

Many attempts have been made to improve transgene expression stability, for example by flanking transgenes with matrix attachment regions (MARs). In barley MARs were shown to reduce variability in transgene expression in callus lines (PETERSEN *et al.* 2002) while in rice, MARs increased the stability of transgene expression in transgenic plants (VAIN *et al*. 1999). An alternative to the use of MARs is to include additional introns within the coding region of transgenes (BOURDON *et al*. 2001). We have shown that, in wheat, additional introns have a significant effect on the stability of expression of the luciferase transgene between generations. Lines containing additional introns are more likely to maintain or increase the level of transgene expression between the T_1 and T_2 generations. It might be expected that similar effects would be seen in barley.





A **B**

Figure 1. A: Expression of the firefly luciferase gene driven by the maize ubiquitin promoter in a barley seedling. B: Expression of the β-glucuronidase (*gus*) gene driven by the low molecular weight glutenin promoter in barley grain.

Safety Assessment and Field Evaluation of GM Barley Lines

As stated above, the exact insertion site of transgenes cannot be controlled and therefore it is possible that some unintended effects of the transgene insertion may occur. This could be due, either, to direct disruption of native genes by the transgene insertion or to some form of interaction with native genes. The tissue culture process necessary for genetic transformation is well known to cause somaclonal variation in the regenerated plants (BREGITZER *et al*. 2002). It is therefore necessary to distinguish variation caused by the tissue culture and transformation

process from that caused by the transgene insertion. This can be done by examining each independent transformation event together with its null-segregant control, that is, a progeny line derived from the original transformation event that no longer contains the transgene due to segregation. Such control lines would still be expected to show somaclonal variation due to the tissue culture and selection process but not due to the transgene itself.

In barley, any variation in the grain is of particular importance because of its use in human nutrition and animal feed. We have examined the seed storage proteins of barley lines together with their null-segregant controls to look for such variation. In many cases it was possible to detect differences between the lines that had been through tissue culture and the seed derived controls. However, such differences were not due to the transformation itself. In some cases a clear difference could be seen between the transgenic plants and the null-segregant controls. An example is shown in Fig.2 where barley line HC12 shows down regulation of one of the hordeins in the transgenic compared to the null-segregant plants.

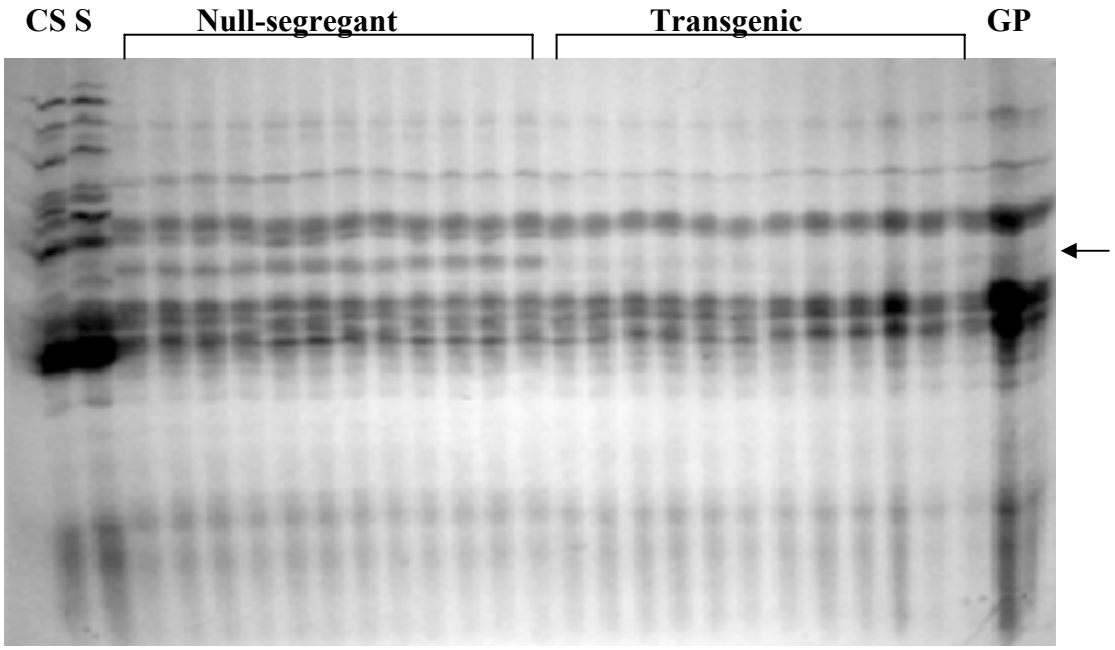


Figure 2. SDS-PAGE gel showing the seed storage proteins in barley line HC12. Arrow indicates a difference between the transgenic plants and the null-segregants. CS= Chinese spring, S=Sicco, GP=Golden Promise controls.

By combining the results of such studies with detailed molecular analysis, metabolite profiling, and expression profiling it will be possible to more fully address the question of unintended consequences of transgene insertions.

Studies of transgenic barley that rely on glasshouse-derived material to examine questions of safety are limited because plants cannot be exposed to the range of environmental conditions expected in the field. We have grown representative lines from three independent transformation events over five years in the field. Variation was observed from year to year as illustrated in Figure 3 showing differences in plant height. The plant height data also shows that some plants were very stunted in phenotype. These stunted plants were observed in both

the transgenic populations and in the null-segregants indicating that this phenotype was probably due to the tissue culture and selection procedures and not to the transgene insertion. The transgenic lines grown under field conditions, despite showing some variation in height and other characteristics, have shown stable expression of the transgenes over five years of field trials.

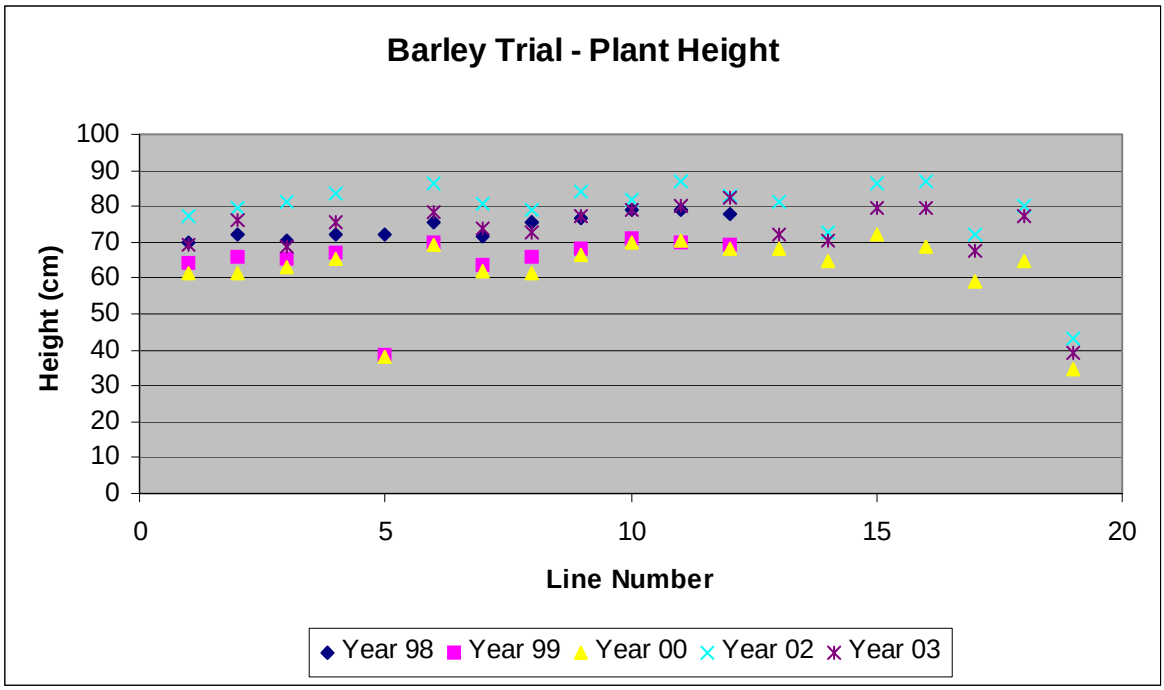


Figure 3. Plant height data from barley field trials over five years. Lines 1-9 are transgenic, line 10 is a tissue culture derived control, lines 11-12 are seed derived controls and 13-19 are null segregant lines. Null-segregant lines were only grown in three of the five years.

Conclusions

In barley transgenic research, as with other crop species, one of the main goals is the precise and stable integration of genes of interest, as well as the accurate control of transgene expression. Considerable progress has been made in understanding the transgene integration event. It is likely that future improvements in transformation methodology will allow the site of transgene integration to be controlled through targeting of transgenes to particular regions of the genome. In addition, new technology allowing greater control of transgene expression will mean that the next generation of transgenic plants will be more efficiently produced and better understood. This in turn will aid the safety assessment process by reducing further the risk of unexpected consequences of transgene insertion.

References

BOURDON V., HARVEY A., LONSDALE D.M. (2001): Introns and their positions affect the translational activity of mRNA in plant cells. EMBO reports, **2**: 394-398.

BOURDON V., LADBROOKE Z., WICKHAM A., LONSDALE D., HARWOOD W.A. (2002): Homozygous transgenic wheat plants with increased luciferase activity do not maintain their high level of expression in the nest generation. Plant Science, **163**: 297-305.

BREGITZER P., ZHANG S., CHO M.J., LEMAUX P.G. (2002): Reduced somaclonal variation in barley is associated with culturing highly differentiated, meristematic tissues. Crop Sci., **42**: 1303-1308.

BREGITZER P., TONKS D. (2003): Inheritance and expression of transgenes in barley. Crop Sci.. **43**: 4-12.

HARWOOD W.A., ROSS S.M., BULLEY S.M., TRAVELLA S., BUSCH B., HARDEN J., SNAPE J.W. (2002): Use of the firefly luciferase gene in a barley (*Hordeum vulgare*) transformation system. Plant Cell Rep., **21**: 320-326.

MATTHEWS P., WANG M.B., WATERHOUSE P.M., THORNTON S., FIEG S.J, GUBLER F., JACOBSEN J.V. (2001): Marker gene elimination from transgenic barley, using co-transformation with adjacent 'twin T-DNAs' on a standard *Agrobacterium* transformation vector. Molecular Breeding, **7**: 195-202.

QIN G., KANG D., DONG Y., SHEN Y., ZHANG L. (2003): Obtaining and analysis of flanking sequences from T-DNA transformants of *Arabidopsis*. Plant Science, **165**: 941-949.

SALVO-GARRIDO H., TRAVELLA S., SCHWARZACHER T., HARWOOD W.A., SNAPE J.W. (2001): An efficient method for the physical mapping of transgenes in barley using *in situ* hybridisation. Genome, **44**: 104-110

SALVO-G H., TRAVELLA S., BILHAM L.J., HARWOOD W.A., SNAPE J.W. (2004): The distribution of transgene insertion sites in barley determined by physical and genetic mapping. Genetics, *in press.*

SHA Y., LI S., PEI Z., LUO L., TIAN Y., HE C. (2004): Generation and flanking sequence analysis of a rice T-DNA tagged population. Theor. Appl. Genet., **108**: 306-314.

TINGAY S., MCELROY D., KALLA R., FIEG S., WANG M., THORNTON S., BRETTELL R. (1997): *Agrobacterium tumefaciens*-mediated barley transformation. The Plant Journal, **11**: 1369-1376.

VAIN P., WORLAND B., KOHLI A., SNAPE J.W., CHRISTOU P., ALLEN G.C., THOMPSON W.F. (1999): Matrix attachment regions increase transgene expression levels and stability in transgenic rice plants and their progeny. The Plant Journal, **18**: 233-242.

WAN Y., LEMAUX P.G. (1994): Generation of large numbers of independently transformed fertile barley plants. Plant Physiol., **104**: 37-48.

Dissecting Large Genomes of Triticeae by Chromosome Sorting

J. Doležel, M. Kubaláková, P. Suchánková, J. Šafář, J. Janda, P. Kovářová, J. Bartoš, J. Číhalíková, and H. Šimková

Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Sokolovská 6, CZ-77200 Olomouc, Czech Republic; e-mail: dolezel@ueb.cas.cz

Abstract

Nuclear genomes of some *Triticeae* species are characterized by large size and prevalence of repetitive DNA sequences. These features hamper their physical mapping and gene cloning. Purification of individual chromosomes by flow cytometry can simplify these tasks by providing small and defined genome fractions. This lecture reviews the development of the methodology and its potential for genome mapping in barley, rye and wheat. Due to small differences in relative DNA content, only one chromosome type can be discriminated and sorted in each of the three species. Cytogenetic stocks facilitate separation of other parts of the genomes as individual chromosomes, translocation chromosomes and chromosome arms. Chromosome analysis by flow cytometry permits quantitative detection of structural and numerical chromosome changes. Chromosomes sorted onto microscopic slides have been used for discovery of rare structural changes and for high-resolution cytogenetic mapping using FISH. The use of sorted chromosomes for HAPPY mapping, targeted isolation of low copy "genic" sequences, and high-throughput physical mapping of ESTs on microarrays are attractive options. As millions of chromosomes with intact DNA may be sorted, construction of BAC libraries is possible. Subgenomic, chromosome-specific and chromosome arm-specific BAC libraries have already been produced in wheat and represent unique resources for genomics of cereals.

Introduction

Although some species of *Triticeae* possess small genomes, the genomes of barley (~ 5100 Mb / 1C), rye (~ 7900 Mb / 1C) and wheat (~ 16 900 Mb / 1C) are complex, consisting mainly of various classes of repetitive DNA sequences. In addition, the recent evolution of wheat involved two episodes of polyploidization giving rise to progenitors of allotetraploid durum wheat and allohexaploid bread wheat. These features hamper physical mapping and gene cloning. Purification of individual chromosomes by flow cytometry can simplify these tasks by providing small and defined genome fractions (DOLEŽEL *et al.* 2004a).

The methods for chromosome analysis and sorting using flow cytometry have been termed flow cytogenetics (DOLEŽEL *et al.* 2004b). Chromosome analysis by flow cytometry is based on the ordered flow of mitotic metaphase chromosomes at high speed in a narrow liquid stream through a beam of intense light. Prior to analysis, the chromosomes in suspension are stained with a DNA-binding fluorochrome. The fluorescence, which is emitted during the short time each chromosome is in the light beam, is collected and the result of analysis is displayed as a histogram of relative fluorescence intensity (flow karyotype). Ideally, each chromosome is represented by a single peak on a flow karyotype. Individual chromosomes can be purified by breaking the liquid stream into droplets and deflecting electrically charged droplets containing chromosomes of interest by a passage through an electrostatic field.

We have been developing flow cytogenetics for cereals since the late 1990s. Barley, with only seven chromosomes, seemed to be a logical choice for a species to start with (LYSÁK *et al.* 1999). Subsequently, we showed that protocols developed for barley were generally applicable and could be modified to other cereals like rye and wheat (VRÁNA *et al.* 2000, KUBALÁKOVÁ *et al.* 2003a). The main problem identified in the course of this work was a difficulty in discriminating and sorting all chromosomes from a karyotype. Even in diploid barley and rye, only one chromosome could be sorted due to small differences in relative DNA content among the chromosomes.

Various strategies were evaluated, including dual staining with AT- and GC-binding fluorochromes as described by LUCRETTI & DOLEŽEL (1997) and fluorescent labelling of repetitive DNA sequences prior to cytometric analysis according to MACAS *et al.* (1995). However, these approaches failed and only the use of cytogenetic stocks such as deletions, translocations and additions facilitated sorting of single chromosome types. Interestingly, alien chromosome additions in wheat appeared to be one of the most useful materials for sorting chromosomes of diploid barley and rye (KUBALÁKOVÁ *et al.* 2003a, SUCHÁNKOVÁ *et al.*, in preparation). Hence, flow cytogenetics of cereals should be considered an integrated system where sorting of chromosomes from one species relies on the availability of corresponding protocols for other species.

Material and Methods

Chromosome suspensions were prepared from synchronized root tips of cultivars and various cytogenetic stocks of barley (*Hordeum vulgare* L., $2n = 2x = 14$), rye (*Secale cereale* L., $2n = 2x = 14$), tetraploid (*Triticum durum* L., $2n = 4x = 28$), and hexaploid wheat *Triticum aestivum* L., $2n = 6x = 42$) according to LYSÁK *et al.* (1999), VRÁNA *et al.* (2000) and KUBALÁKOVÁ *et al.* (2003a, b). Suspensions of isolated chromosomes were stained with a DNA-specific fluorochrome DAPI and their fluorescence was analysed using the Becton Dickinson FACSVantage SE flow cytometer and sorter at rates of 200 – 500/sec. At least ten thousand chromosomes were analysed in each sample and the results were displayed as histograms of relative fluorescence intensity (flow karyotypes). Chromosomes were sorted at rates of 5 – 20/sec as described by VRÁNA *et al.* (2000). PCR on flow-sorted chromosomes with primers for chromosome-specific markers was performed according to LYSÁK *et al.* (1999); fluorescence *in situ* hybridization (FISH) with various probes was done on chromosomes sorted onto microscope slides according to KUBALÁKOVÁ *et al.* (2002). High molecular weight DNA was prepared from flow-sorted chromosomes according to ŠIMKOVÁ *et al.* (2003) and BAC libraries were constructed from chromosomal DNA according to ŠAFÁŘ *et al.* (2004).

Results and Discussion

Chromosome Analysis and Sorting in Hexaploid Wheat

Flow cytometric analysis of chromosomes isolated from most of hexaploid wheat cultivars resulted in flow karyotypes consisting of three composite peaks (labelled I, II, and III), which represented various chromosomes, and a well separated peak corresponding to chromosome 3B (Figure 1a) (VRÁNA *et al.* 2000). The identity of sorted wheat chromosomes could be determined by PCR with specific primers. However, quantification of contaminating particles was done best by microscopic observation of sorted chromosomes after fluorescent labelling GAA microsatellites using FISH (Figure 1a, insert). As the D-group chromosomes do not exhibit the GAA banding, their identification was done after fluorescent labelling *Afa* repeats (KUBALÁKOVÁ *et al.* 2002).

The analysis of various cytogenetic stocks demonstrated that flow karyotyping facilitated detection of deletion and translocation chromosomes with altered DNA content. For example, flow karyotypes of cultivars carrying the 5BL·7BL translocation were characterized by the presence of an additional peak, which represented the translocation, and which appeared on the right of the peak of chromosome 3B (VRÁNA *et al.* 2000). Ditelosomic lines were characterized by the presence of peaks on the left of the composite peak I, which represented chromosomes 1D, 4D and 6D. For all short-arm telosomes and for most of long-arm telosomes, peaks representing telosomes were well discriminated (Figure 1b). Peaks of 3BL and 5BL overlapped with the composite peak I. However, these arms could be sorted as isochromosomes, as their peaks were localized on the right of the peak of chromosome 3B (KUBALÁKOVÁ *et al.* 2002). Alternatively, they could be sorted from telosomic lines of durum wheat (KUBALÁKOVÁ *et al.* 2003b).

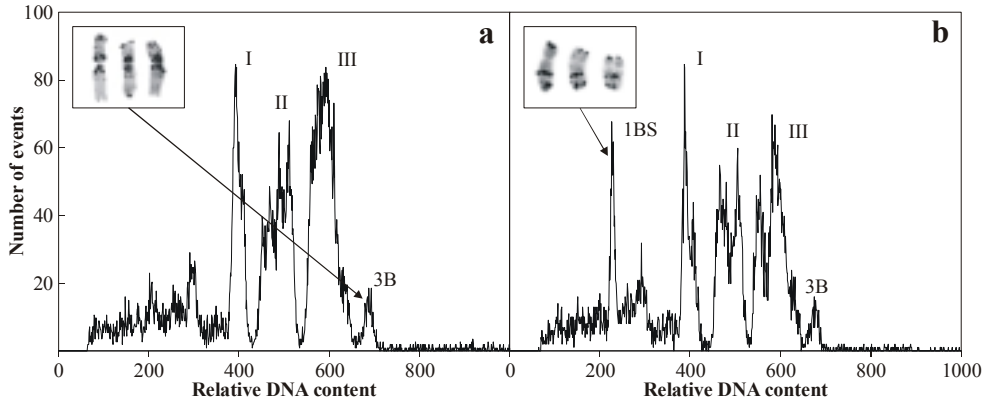


Figure 1. Flow karyotypes obtained after the analysis of DAPI-stained chromosome suspensions prepared from two wheat lines. (**a**) Flow karyotype of 'Chinese Spring' consists of three composite peaks (I – III) representing groups of chromosomes and a peak representing chromosome 3B (insert: chromosome 3B after GAA banding); (**b**) Flow karyotype of 'Pavon' carrying short arm of chromosome 1B shows three composite peaks (I – III), peak of 3B and a clearly discriminated peak of 1BS (insert: GAA-banded 1BS).

Chromosome Analysis and Sorting in Durum Wheat

The analysis of DAPI-stained chromosome suspensions prepared from durum wheat resulted in flow karyotypes with two composite peaks (labelled II and III) representing specific groups of chromosomes and a peak representing chromosome 3B (Figure 2a). Thus, similar to hexaploid wheat, only chromosome 3B could be sorted individually in durum wheat. When compared with hexaploid wheat, the flow karyotype reflected the absence of group D chromosomes. However, while the peak II corresponded to chromosomes 1A, 4A and 6A in the hexaploid wheat, peak II of durum wheat contained only chromosomes 1A and 6A, pointing to differences in chromosome 4A size between both species.

Flow karyotyping of double ditelosomic lines of durum wheat carrying chromosome arms of hexaploid wheat 'Chinese Spring' showed that all A- and B-genome chromosome arms could be easily discriminated and sorted. As an example, a flow karyotype of a double ditelosomic line 1A is shown in Figure 2b. The identity and purity of sorted chromosome arm fractions was determined by double FISH with probes for GAA microsatellite and telomeric repeat. Although most of the arms could be sorted from telosomic lines of hexaploid wheat

(KUBALÁKOVÁ *et al.* 2002), the durum wheat system offered an attractive alternative (KUBALÁKOVÁ *et al.* 2003b).

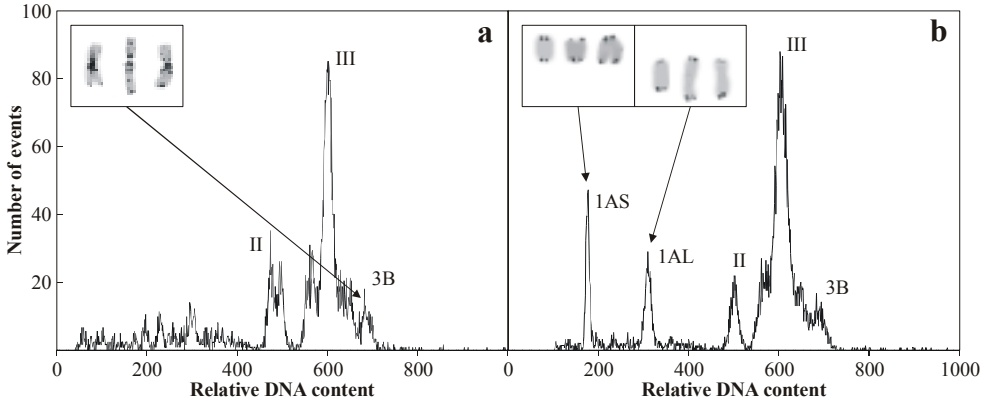


Figure 2. Flow karyotypes obtained after analysis of DAPI-stained chromosome suspensions prepared from two lines of durum wheat. (**a**) Flow karyotype of durum wheat 'Langdon'. The karyotype consists of two composite peaks (II, III) representing specific groups of chromosomes and a peak representing chromosome 3B (insert: chromosome 3B after GAA banding); (**b**) Flow karyotype of a double ditelosomic line 1A of 'Langdon' with clearly discriminated peaks representing short and long arms of chromosome 1A (insert: chromosomes 1AS and 1AL after FISH with probe for telomeric repeat).

Chromosome Analysis and Sorting in Rye

The flow karyotype obtained after the analysis of a rye cultivar 'Imperial' comprised a large composite peak and a small peak to the left of the main composite peak (Figure 3a). Chromosome assignment to the two peaks was done after double-FISH on sorted chromosomes with various combinations of probes for pSc119.2 and pSc250 repeats, GAA microsatellite, and 5S rDNA. This analysis revealed that the minor peak represented chromosome 1R, and that the composite peak represented the remaining rye chromosomes (2R - 7R) (KUBALÁKOVÁ *et al.* 2003a). Chromosome 1R could be sorted with purities reaching 95% (Figure 3a, insert). Subsequent analysis of other rye cultivars resulted in flow karyotypes with poorly resolved peak of 1R. Hence, the chromosome cannot be readily sorted from any rye cultivar.

With the aim to develop a protocol for sorting other wildtype rye chromosomes in addition to 1R, wheat-rye chromosome addition lines were flow-karyotyped (KUBALÁKOVÁ *et al.* 2003a). The analysis showed that the remaining chromosomes (2R – 7R) could be easily discriminated as their peaks appeared to the right of the peak of the largest wheat chromosome 3B (Figure 3b). Interestingly, the peak representing chromosome 1R was localized between the composite peak III of wheat and a peak of wheat chromosome 3B. Thus, unlike the chromosomes 2R – 7R, chromosome 1R could not be sorted from the addition line with a sufficient purity (KUBALÁKOVÁ *et al.* 2003a).

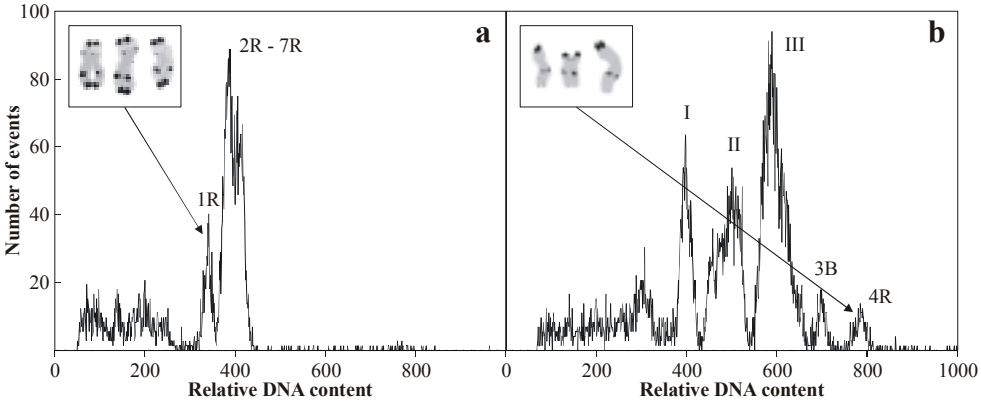


Figure 3. Flow karyotypes obtained after analysis of DAPI-stained chromosome suspensions prepared from rye and from a wheat-rye chromosome addition line. (**a**) Flow karyotype of rye 'Selgo' showing composite peak corresponding to chromosomes 2R - 7R and a peak representing chromosome 1R (insert: chromosome 1R after FISH with a probe for the pSc119.2 repeat); (**b**) Flow karyotype of a wheat-rye chromosome addition line with four peaks containing wheat chromosomes (I – III, 3B) and a peak representing chromosome 4R (insert: chromosome 4R labelled with a probe for the pSc119.2 repeat).

Chromosome Analysis and Sorting in Barley

Flow cytometric analysis of chromosome suspensions prepared from various cultivars of barley resulted in flow karyotypes with one minor peak, which represented the smallest chromosome 5(1H), and one dominant peak, which represented the six remaining barley chromosomes (Figure 4a). Inability to sort other chromosomes than 5(1H) stimulated a survey of available cytogenetic stocks. The use of barley chromosome translocation lines turned out to be a fruitful approach (Figure 4b). As shown by LYSÁK *et al.* (1999), the number of chromosomes that could be discriminated and sorted, depended on the nature of a translocation and ranged from one in 'Elgina' (T1-7an) to three in 'Bonus' (T2-6y) and 'St. 13559' (T2-6an). Any barley chromosome whose peak could be clearly discriminated on a flow karyotype could also be sorted at high purity (~95%).

Although the availability of purified fractions of translocation chromosomes was shown useful for mapping DNA sequences to subchromosomal regions (LYSÁK *et al.* 1999), many applications require DNA prepared from a specific wildtype chromosome or its segment. In barley, this seemed to be an insurmountable problem until recently, when we have demonstrated that the use of wheat-barley telosome addition lines facilitated sorting of any barley chromosome arm with purities exceeding 90% (SUCHÁNKOVÁ *et al.*, in preparation). Thus, flow cytometry may be used to dissect the genome of barley into small and defined fractions representing only 5 % to 9 % of the whole genome.

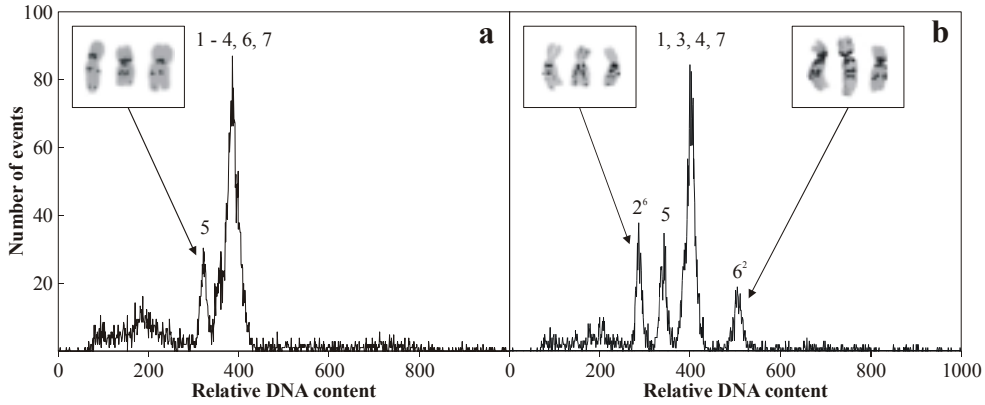


Figure 4. Flow karyotypes obtained after the analysis of DAPI-stained chromosome suspensions prepared from two barley lines. (**a**) Flow karyotype of 'Akcent' showing composite peak corresponding to chromosomes 1 – 4, 6 and 7 (2H – 7H) and a peak representing chromosome 5(1H) (insert: chromosome 5(1H) after GAA banding); (**b**) Flow karyotype of barley translocation line 'St. 13559' in which three chromosomes 2^6, 5 and 6^2 could be sorted (inserts: chromosome 2^6 and 6^2 after GAA banding).

Applications of Flow Karyotyping

The analysis of isolated chromosomes demonstrated suitability of flow karyotyping for quantitative detection of numerical and structural chromosome changes. Any change in relative chromosome frequency and size was reflected by a change in peak area and position, respectively. Thus, chromosome translocations, deletions and additions could be identified easily (LYSÁK *et al.* 1999, KUBALÁKOVÁ *et al.* 2002, 2003a). For example, 5BL·7BL chromosome was detected for the first time in some wheat cultivars (KUBALÁKOVÁ *et al.* 2002), and supernumerary B chromosomes were discovered in a rye cultivar in which their presence has not been reported before (KUBALÁKOVÁ *et al.* 2003a). Flow karyotyping was also found sensitive enough to detect polymorphism in chromosome DNA content in wheat and rye (KUBALÁKOVÁ *et al.* 2002, 2003a). It is interesting to note that the "fingerprint" patterns of flow karyotypes were found heritable and their transmission from parental to new varieties could be followed, indicating stability of chromosome variants.

Quantitative analysis of numerical chromosome changes involves comparison of peak areas of a chromosome with known frequency and chromosome whose frequency is not known. Because chromosomes from large numbers of individuals are analysed, the frequency of a chromosome in plant population can be estimated. A suitability of flow karyotyping to evaluate the frequency of alien rye chromosomes in wheat-rye chromosome addition lines was demonstrated by KUBALÁKOVÁ *et al.* (2003a). While the peak area of chromosome 6R was comparable to that of wheat chromosome 3B, indicating a stable transmission of 6R, the analysis of chromosome 7R peak area suggested that the chromosome was present in only about 30% of seeds.

The Use of Sorted Chromosomes

Any chromosome and chromosome arm that can be discriminated on a flow karyotype can be sorted at high purities (90 – 100%). Sort rates of 5 – 20 / sec, which can be routinely achieved, allow sorting of chromosomes in large numbers. This offers a range of applications that may greatly simplify genome analysis in cereals.

Chromosomes sorted onto microscope slides are ideal targets for high resolution FISH, as they are free of cytoplasmic remnants and their chromatin is more relaxed compared to metaphase spreads. Furthermore, thousands of chromosomes can be sorted onto one slide, which speeds up the analysis and facilitates detection of rare structural chromosome changes. Using this approach, KUBALÁKOVÁ *et al.* (2003a) discovered two rare translocations between A and B chromosomes of rye, occurring at frequencies of only about 0.5%. A conventional analysis would require screening of several hundred metaphase spreads. Furthermore, flow-sorted chromosomes can be stretched longitudinally up to 100-fold their original length to achieve considerable increase in the spatial resolution and sensitivity of FISH (VALÁRIK *et al.* 2004). In addition to a possibility of detecting sequences as short as 1kb, cytogenetic mapping using super-stretched chromosomes offers greatly improved spatial resolution of 70 kb compared to 5 – 10 Mb after FISH on mitotic chromosomes.

Chromosomes sorted into PCR tubes can be used as template for PCR with sequence-specific primers. This facilitates rapid localization of DNA sequences to specific chromosomes (VRÁNA *et al.* 2000). Sorting of several hundred chromosomes or chromosomes arms, which are sufficient for PCR, requires only few minutes, thus large numbers of fractions may be produced in a short time and used for large-scale physical mapping. Sorted deletion and translocation chromosomes enable subchromosomal localization of DNA sequences (LYSÁK *et al.* 1999, KUBALÁKOVÁ *et al.* 2003a).

Construction of chromosome-specific BAC (Bacterial Artificial Chromosome) DNA libraries is one of the most attractive applications of sorted chromosomes. Recently, protocols for preparation of high molecular weight DNA from flow-sorted chromosomes of cereals were reported (ŠIMKOVÁ *et al.* 2003). These methodological advances facilitated preparation of a BAC library specific for wheat chromosome 3B (ŠAFÁŘ *et al.* 2004). Subsequently, a subgenomic BAC library specific for wheat chromosomes 1D, 4D and 6D was produced as well as a BAC library specific for the short arm of wheat chromosome 1B (JANDA *et al.*, in preparation). The latter result confirms that it is possible to prepare BAC libraries for individual chromosome arms. It is worth noting that all three libraries have excellent genome coverage and large average insert size.

Several other applications of flow-sorted chromosomes have not been explored yet. These include rapid physical mapping of ESTs on DNA microarrays, HAPPY mapping and targeted isolation of low-copy ("genic") DNA sequences. While the ability to detect structural and numerical chromosome aberrations may not have far reaching impacts, a capability to discriminate and sort single chromosome arms offers a unique tool to dissect complex genomes of cereals into manageable fractions representing only few per cent of a whole genome. In rye, all seven chromosomes may be sorted; in wheat and barley, every chromosome arm can be purified from cytogenetic stocks. To conclude, flow cytogenetics has been fully developed for cereals and may be readily integrated into projects aiming at comparative genome analysis, physical mapping and molecular cloning of genes of interest.

Acknowledgements
Cytogenetic stocks were kindly provided by B.S. Gill and B. Friebe (Manhattan, USA), G. Künzel (Gatersleben, Germany), S.F. Kianian (Fargo, USA), A.J. Lukaszewski, (Riverside, USA), N. Watanabe (Gifu, Japan). We thank Ms. Michaela Libosvárová, Bc. and Ms. Radka Tušková for excellent technical assistance. This work has been supported by the Grant Agency of the Czech Republic (grants 522/03/0354 and 521/04/0607), and the Ministry of Agriculture of the Czech Republic (grant QC1336).

References

DOLEŽEL J., KUBALÁKOVÁ M., BARTOŠ J., MACAS J. (2004a): Flow cytogenetics and plant genome mapping. Chrom. Res., **12**: 77–91.

DOLEŽEL J., KUBALÁKOVÁ M., VRÁNA J., BARTOŠ J. (2004b): Flow cytogenetics. In: GOODMAN R.M. (ed.): Encyclopedia of Plant and Crop Science. Marcel Dekker, Inc., New York, USA: 460–463.

KUBALÁKOVÁ M., VRÁNA J., ČÍHALÍKOVÁ J., ŠIMKOVÁ H., DOLEŽEL J. (2002): Flow karyotyping and chromosome sorting in bread wheat (*Triticum aestivum* L.). Theor. Appl. Genet., **104**: 1362–1372.

KUBALÁKOVÁ M., VALÁRIK M., BARTOŠ J., VRÁNA J., ČÍHALÍKOVÁ J., MOLNÁR-LÁNG M., DOLEŽEL J. (2003a): Analysis and sorting of rye (*Secale cereale* L.) chromosomes using flow cytometry. Genome, **46**: 893–905.

KUBALÁKOVÁ M., BARTOŠ J., KOVÁŘOVÁ P., ČÍHALÍKOVÁ J., WATANABE N., KIANIAN S.F., DOLEŽEL J. (2003b): Chromosome analysis and sorting in durum wheat and physical mapping of repetitive DNA sequences. In: Proceedings of the Tenth International Wheat Genetics Symposium. Istituto Sperimentale per la Cerealicoltura, Rome, Italy: 601–603.

LUCRETTI S., DOLEŽEL J. (1997): Bivariate flow karyotyping in broad bean (*Vicia faba*). Cytometry, **28**: 236–242.

LYSÁK M.A., ČÍHALÍKOVÁ J., KUBALÁKOVÁ M., ŠIMKOVÁ H., KÜNZEL G., DOLEŽEL J. (1999): Flow karyotyping and sorting of mitotic chromosomes of barley (*Hordeum vulgare* L.). Chrom. Res., **7**: 431–444.

MACAS J., DOLEŽEL J., GUALBERTI G., PICH U., SCHUBERT I., LUCRETTI S. (1995): Primer-induced labelling of pea and field bean chromosomes *in situ* and in suspension. BioTechniques, **19**: 402–408.

ŠAFÁŘ J., BARTOŠ J., JANDA J., BELLEC A., KUBALÁKOVÁ M., VALÁRIK M., PATEYRON S., WEISEROVÁ J., TUŠKOVÁ R., ČÍHALÍKOVÁ J., VRÁNA J., ŠIMKOVÁ H., FAIVRE-RAMPANT P., SOURDILLE P., CABOCHE M., BERNARD M., DOLEŽEL J., CHALHOUB B. (2004): Dissecting large and complex genomes: flow sorting and BAC cloning of individual chromosome types from bread wheat. Plant Journal (in press).

ŠIMKOVÁ H., ČÍHALÍKOVÁ J., VRÁNA J., LYSÁK M.A., DOLEŽEL J. (2003): Preparation of HMW DNA from plant nuclei and chromosomes isolated from root tips. Biol. Plant., **46**: 369–373.

VALÁRIK M., BARTOŠ J., KOVÁŘOVÁ P., KUBALÁKOVÁ M., DE JONG H., DOLEŽEL J. (2004): High-resolution FISH on super-stretched flow-sorted plant chromosomes. Plant J., **37**: 940–950.

VRÁNA J., KUBALÁKOVÁ M., ŠIMKOVÁ H., ČÍHALÍKOVÁ J., LYSÁK M.A., DOLEŽEL J. (2000): Flow-sorting of mitotic chromosomes in common wheat *(Triticum aestivum* L.). Genetics, **156**: 2033-2041.

SSR Markers in Barley Breeding and Genomics

P. Langridge[1], M. Pallotta[1] and M. Hayden[2]

[1]Australian Centre for Plant Functional Genomics;
[2]Molecular Plant Breeding CRC, School of Agriculture and Wine, University of Adelaide, Glen Osmond, SA 5064, Australia

Abstract

The use of molecular markers to track loci and genome regions in barley is routinely applied in many breeding programs. The location of major loci is now known for many disease resistance genes, tolerances to abiotic stresses and quality traits.

There are three aspects that are particularly significant. First, we now have markers closely linked to many traits of importance in the breeding programs. Indeed we have markers for more loci than we can screen in a conventional breeding program. Second, we have tools that allow marker scanning of the whole genome. SSR markers form the base for this analysis and highly multiplexed SSR screens have been developed for barley. This has improved our capacity for whole genome screens. Third, through association mapping projects we have or are in the process of developing, whole genome fingerprints for many key lines and varieties of importance in breeding.

Existing strategies for marker-assisted selection (MAS) were initiated with a view of markers as providing a rapid and cheap alternative to bioassays and they have largely been used in this role. While highly successful, this strategy does not fully utilize the technology. The key challenge of new work is to investigate strategies for whole genome breeding: that is, to see how we can use genome information to design optimal breeding strategies that integrate as much of the available information as possible.

Introduction

Molecular markers have now been available for many years and a large number of key traits have been effectively tagged with markers. Markers are available for many of the key malting quality traits for barley, disease resistance loci, loci conferring tolerance to abiotic stresses and several morphological and development traits, such as dwarfism and hulless grain. There have also been many analyses of the utility of markers and frequent reports on the various ways in which they can be applied to plant breeding programs. Not only can markers be used to track specific loci in segregating populations, they can also be used to monitor the entire genome. However, if we actually look at the use of markers within active breeding programs, we see a very different story with many programs not using molecular markers. Moreover, those that do use molecular markers tend to apply them as a direct replacement for bioassays and have made few changes to their overall breeding strategy. A key reason for the slow uptake of marker technology has been the expense and technical difficulty of many of the marker assays or the absence of polymorphic markers close to the traits of interest. These difficulties were particularly pronounced with the early form of markers that were based around the detection of restriction fragment length polymorphisms. These problems were largely resolved through the availability of microsatellite or simple sequence repeats (SSRs) markers. SSRs are short stretches of tandemly repeated units of 1 to 6 bases in length. They show considerable variation in the number of repeats and this forms the basis for detecting polymorphism between lines and individuals. SSRs are found in all eukaryote genomes and can be detected by using PCR primers that flank the tandem repeat block. In humans it has been estimated that there

are over 100,000 SSR loci (ELLENGREN 2002) making SSRs a valuable source of large numbers of markers. SSR markers usually map to a single locus and are co-dominant. In barley, SSR markers can show a large number of alleles, for example MAROOF *et al.* (1994) reported up to 37 alleles amongst wild and cultivated barleys. The variation in the number of repeats is believed to be due to unequal crossing-over or slip strand mispairing during DNA replication (ZHU *et al*. 2000; GOLDSTEIN & SCHOLOTTERER 1999). The frequency of changes in the number of repeat units has not been measured in plants and is believed to occur at a low rate. However SSR primer sets tend not to be transferable between species and SSRs developed for wheat have not be useful for barley (RODER *et al*. 1995)

Material and Methods

Generation of SSR Markers

Although SSR markers have been developed for nearly all major crop species their development has been costly and labour intensive. Two broad methods have been used to produce SSR markers for barley; screening genomic libraries enrich for SSRs or analysis of ESTs that contain SSRs. The use of genomic libraries enriched for SSRs has been the most widely used technique for SSR discovery. An example of the process used to scan through the clones and the yield of useful markers at each phase of the process is shown in Figure 1.

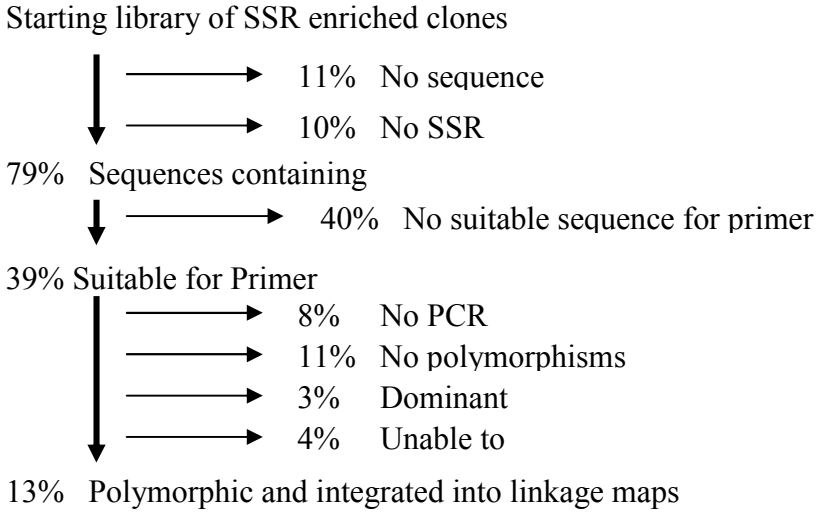


Figure 1. Yield of SSR markers from an enriched genomic library. The percentages refer to a barley library generated by the method of EDWARDS *et al.* (1996) and give the proportion of starting clones that survive each phase of analysis.

Over the past decade well over 700 SSR markers have been made available for barley through the efforts of several groups (BECKER & HUEN 1995; LIU *et al.* 1996; PILLEN *et al.* 2000; RAMSEY *et al.* 2000; RUSSELL *et al*. 1997; STRUSS & PLIESKE, 1998; KARAKOUSIS *et al*. 2003a). These now form the base for many genetic linkage maps and provide a key resource for marker assisted selection (MAS).

Although SSRs derived from genomic sequences have provided most markers to date, more recently SSRs have been identified from within the large barley EST (Expressed Sequence Tags) databases that have become available. Many of the EST-derived SSR projects are still underway but preliminary results on the screening of ESTs show that these will be a valuable source of additional SSRs and will probably double the number of SSRs in the public domain. An example of the use of ESTs for SSR discovery was shown by VARSHNEY *et al.* (2002) and the key results of the analysis are shown in Table 1.

Table 1. Yield of SSR markers from screens of EST databases (from VARSHNEY *et al.* 2002)

	Numbers of clones	*Percentage*
EST sequences screened	*87205*	*100*
Length of examined sequence	*55 Mbp*	
ESTs containing SSRs	*20135*	*23*
Different SSRs	*7001*	*8*
Unique primer pairs for total EST	*1839*	*2.1*

It is expected that the proportional yield of unique SSRs will decline as the EST databases expand. However, if we use the above number for percentage yield of useful SSRs (2.1%) and with the barley EST databases now approaching 500,000, we can expect to see several thousand new SSRs becoming available over then next few years. The EST derived SSRs tend to be less polymorphic than genomic sequence-derived markers but they show even genome distribution and may help fill gaps present in current SSR maps of barley. There are also initial indications that these types of SSRs may be more suitable for transfer between wheat and barley than the previous genomic SSRs.

Consensus Map with SSRs

Before SSR markers can be effectively applied to barley breeding programs several key tasks are required. First, SSR markers need to be integrated in genetic linkage maps. Most early molecular linkage maps were based around RFLP markers and more recently AFLP markers have been widely used to improve genome coverage and marker density. A problem with AFLP markers has been that they are not transferable across populations and this makes comparison and integration of linkage maps difficult. RFLP markers have formed the basis for comparing maps. SSR markers now provided an ideal replacement for RFLP markers in helping to tie multiple linkage maps together. Most recent linkage maps of barley have made extensive use of SSRs. The recent paper by ABLETT *et al.* (2003) describes the use of SSR markers in developing 12 barley genetic maps. The availability of linkage maps that have used common markers allows the direct comparison of maps and development of "consensus" maps. A recent barley consensus map, based around five separate linkage maps, contains 703 markers including 161 SSRs (KARAKOUSIS *et al*. 2003a). This map provides a framework for integration of additional maps and a reference map for identifying SSR markers close to target loci. A new consensus map including around 450 SSR markers and a total number of over 1100 markers is currently under development (data not presented).

Current Methods for Detecting SSRs

A number of methods have been described for the detection of microsatellite markers. These approaches typically involve the separation of SSR amplicons by electrophoresis in agarose or polyacrylamide gels followed by visualization using ethidium bromide, silver staining, radiography or fluorescence. However, methods based on polyacyrlamide gel electrophoresis and visualization with ethidium bromide or fluorescence are most commonly used. The choice of detection method depends primarily on the availability of instrumentation for fluorescent-based SSR detection, and the experimental work that is to be undertaken. Important factors that must be considered in the context of the experimental work are the number of alleles that are expected at each marker locus and the minimum allele size difference. Non-fluorescent SSR detection provides relatively rapid, low-cost genotyping and is widely used in cereal research for marker assisted selection and genetic mapping, where the segregation of only a small number of alleles is expected. The separation of SSR fragments is usually performed on an 8% polyacrylamide gel under non-denaturing conditions to enable the gel to be handled

during ethidium bromide staining. However, non-denaturing gel electrophoresis cannot reliably resolve small allele size differences (<5-bp). Moreover, non-denaturing gels cannot be loaded multiple times to increase the amount of information that can be obtained from each gel lane as heteroduplex structures typically form secondary banding patterns that extend along much of the length of the gel lane. In contrast, fluorescent-based SSR detection using gel or capillary electrophoresis under denaturing conditions permits an increase in genotyping throughput to a level comparable to multi-locus DNA fingerprint techniques. The absence of heteroduplex structures under denaturing conditions allows SSR fragments from individual PCR reactions to be post-amplification pooled and separated in a single gel lane or capillary. Typically, post-PCR pooling of up to five SSR markers can be comfortably accommodated with prior planning (ABLETT *et al*. 2003). The only prerequisite for successful SSR detection using pooling strategies is that the markers have sufficient spatial separation to prevent allele overlap. Many instruments for fluorescent-based SSR detection are also capable of simultaneously detecting up to 5 different fluorophores, and therefore permit the separation of SSR fragments on the basis of both size and fluorophore colour. With prior planning, post-PCR pooling strategies based on both spatial and spectral separation can allow the separation of up to 20 SSRs in a single gel lane or capillary, thereby reducing the costs of fluorescent-based SSR detection to levels that are comparable with non-fluorescent based methods. Moreover, fluorescent-based SSR detection using denaturing gel electrophoresis permits alleles with single base pair differences to be resolved. Consequently, fluorescent-based SSR analysis is ideal for the accurate sizing of alleles and is widely used for genetic diversity analysis. A drawback of fluorescent-based detection is the considerable cost of dye labelling a specific primer for each marker. However, a steady increase in the popularity of fluorescent-based SSR detection in recent years has seen the cost of synthesizing dye labelled primers steadily decrease. In addition, several strategies have been developed to further reduce the cost of deploying microsatellites on fluorescent-based detection platforms. A popular alternative to the dye labelling of a specific primer for each marker is to resynthesis the forward primer with a generic 5' tag (typically corresponding to the M13 universal sequencing primer), and performing PCR in the presence of a third fluorescently labelled version of the 5' tag primer. This approach has the advantage that only one dye labelled primer is required for microsatellite detection (SCHUELKE 2000; RAMPLING *et al*. 2001). Similarly, microsatellite detection using a reduced number of dye labelled primers has also been achieved through the design of novel types of microsatellite markers such as sequence tagged microsatellites (STMs), in which PCR amplification of a target locus is achieved using a primer specific to the conserved region flanking the microsatellite sequence in combination with a primer that anchors to the repeat motif itself (HAYDEN *et al*. 2004). As the microsatellite anchoring primer is common to all markers targeting the same type of repeat motif, fluorescent-based SSR detection requires only the common microsatellite anchoring primers to be dye labelled.

Multiplexing Markers for Genome Coverage and Specific Traits

The efficiency of SSR genotyping can be increased significantly by multi-loading and multiplexing, thereby lowering the cost barriers to the large scale use of SSRs as genetic markers. Post-amplification pooling of SSR fragments from individual PCR reactions ("multipooling") provides the simplest approach to increasing the information content contained in each gel lane or capillary. In contrast, multiplex PCR adds a further dimension to reducing the cost of SSR genotyping by permitting the co-amplification of several SSRs in the same reaction (MITCHELL *et al.* 1997). However, the development of multiplex PCR assays is not trivial and considerable time is often required to optimize individual reactions, especially when several markers are to be co-amplified. Consequently, a common approach to achieving high-throughput genotyping is to design microsatellite marker panels using a combination of

multi-pooling and multiplexing. SSR marker panels can be designed to provide whole genome coverage and to target chromosomal regions and genes of interest. Hence, SSR marker panels can greatly increase the efficiency of genetic diversity assessment, variety fingerprinting and identification, the genetic and physical mapping of genes and quantitative traits, and marker assisted selection during breeding. Of particular importance to increasing the efficiency of genotyping is the ability to develop SSR marker panels on the basis of both spatial separation for markers that are labelled with the same fluorophore, and spectral separation using different coloured fluorophores when markers overlap in size (ZIEGLE *et al*. 1992).

Results and Discussion

Application of SSR Markers

Direct Use in Marker Assisted Selection

RFLP markers were reliable and effective for MAS but they were technically demanding, labour intensive and slow compared to PCR-based assay systems. The availability of a large number of SSR markers showing good genome coverage has been of central importance in the application of markers to barley breeding programs. In establishing a good pool of SSR markers for MAS, several points need to be considered. First a set of markers are required that are within a defined distance from the target locus. Within the Australian marker programs we have aimed to use only markers that lie within 5cM of the target locus (KARAKOUSIS *et al*. 2003). Second, the SSR markers must be able to detect polymorphisms between key lines used a breeding program. The PIC or polymorphic information content is a useful criterion for selecting markers but ultimately one must screen germplasm within the breeding program to assess to value of each SSR. In developing a series of SSR markers for use in the South Australian barley improvement program a set of 40 genotypes were screened (KARAKOUSIS *et al*. 2003). The key objective in this analysis was to identify markers that could detect a polymorphism between the donor of the desirable allele at a particular locus and the germplasm being widely used in the breeding program. Based on these criteria, between 1 and 13 SSR markers were identified that could be used to track 21 loci controlling 17 traits. A summary of the traits and the number of SSR that could be used to track these traits in the South Australian barley program are shown in Table 3.

Whole Genome Screening

In addition to the simple tracking of a target locus described above, SSR markers have proved particularly valuable where broad genome coverage is required. This applies to wide range of techniques for MAS, assessing genetic diversity and for identifying marker/trait associations. Although AFLP markers still tend to be preferred for Bulked Segregant Analysis, SSR can also be used in this technique. The various marker applications where SSR can be used have been extensively covered elsewhere. However, it is important to note that the ease of assay, ability to multiplex and transferability across populations has made microsatellite markers highly valuable in screens and has opened many new options for breeding strategies. One of the new developments have been the use of SSR in developing graphical genotypes of lines and varieties. This permits a new view of barley genome structure and behaviour and has the potential to open new techniques for breeding and genetic analysis. Amongst the new breeding strategies that are becoming available, the identification and tracking of major linkage blocks is likely to be particularly important. This will provide breeders with the option of screening for specific recombination events to either break-up undesirable blocks, where key loci may be linked in repulsion, or to keep key adaptive gene blocks together. Those working on gene discovery are also finding SSR a powerful tool in measuring linkage disequilibria across the genome or in specific regions and identify candidate genes for traits based on marker

association. It is probable that this will be a key area of barley genetics research over the next few years.

Table 3. Summary of traits for which SSR markers are available in close linkage and capable of detecting polymorphisms between the donor or major recipient lines in the South Australian barley improvement program (data from Karakousis *et al.* 2003)

Trait	Chromosome	Source	Number of SSRs
Abiotic stress			
Aluminium tolerance	4HL	Brindabella	3
Boron tolerance	2HS	Sahara 3771	13
	3HL	Sahara 3771	6
	4HS	Sahara 3771	6
Manganese efficiency	4HS	Amaji Nijo	2
Quality			
Malt extract, DP, FAAN	1HS	Alexis	6
Malt extract	2HS	Haruno Nijo	3
	2HL	Amaji Nijo	2
Malt extract, FAAN	5HL	Harrington	2
Ant-28	3HL	Caminant	2
DP	4HL	Harrington	2
Disease and pest resistance			
Ha2	2HL	Chebec	2
Ha4	5HL	Galleon	3
Leaf rust	5HS	Sloop	2
	7HS	Sloop	1
mlo	4HS	Alexis	1
Scald	3HS	B87/14	6
Rpt4	7HL	Galleon	1
Yd2	3HL	Franklin	6
Other			
Denso	3HL	Alexis	1
Naked grain	7HS	SB85216	10

Map-Based Cloning – High Resolution Mapping

The availability of detailed linkage maps of barley with anchored SSR markers allows the rapid comparison between populations for markers and traits. This provides a valuable base for looking at the relationship between trait loci, including QTL, and candidate genes. As the density of trait data on maps accumulates and we also locate more and more genes of known function, the probability of candidates linked to important breeding traits increases. An example of this is shown in Figure 2. The map in Figure 2 shows the positions of genes encoding enzymes involved in cell wall biosynthesis. These are candidates for a range of quality related traits in barley, particular traits such as viscosity, fermentability and feed quality. The candidates were mapped by using five different barley populations and using SSR markers to locate each locus to specific bins.

Conclusions

SSR markers have played a key role in moving marker technology from theoretical studies of barley genetics through to application. They have also helped open the way to the development of several new, and potentially significant breeding strategies. The broad use of SSR markers and their integration into a large number of linkage maps now enhances our ability to use genetic information in gene discovery and genomics. It is probable that over the next few years, SSR marker will slowly be replaced by alternative marker systems that allow

higher degrees of multiplexing and reduce the overall costs of marker analysis. This will further enhance the use of markers in whole genome analysis and will make methods such as association mapping more attractive. In particular, new methods for SNP detection and the use of array based screening methods are likely to supersede SSRs over the next few years.

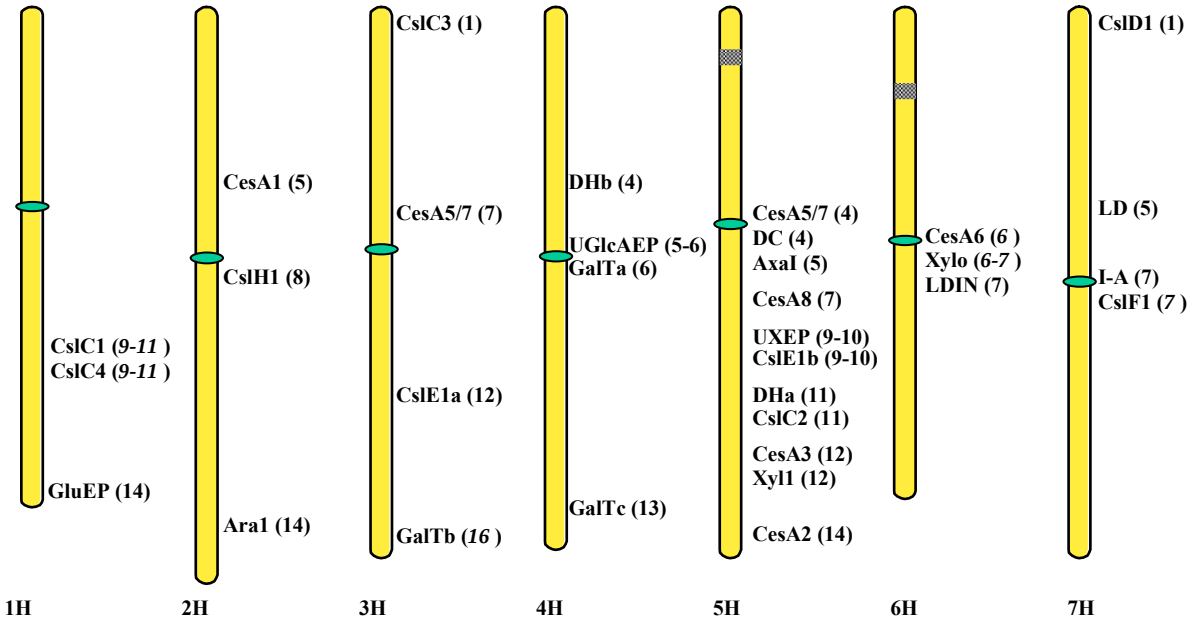


Figure 2. Mapped barley cell wall genes. Numbers in brackets indicate the Bin positions. AraI, alpha-L-arabinofuranosidase; AxaI, arabinxylan arabinofuranosidase; Ces, cellulose synthase; Csl, cellulose synthase-like; DC, UDP-glucuronic acid decarboxylase; DH, UDP-glucose dehydrogenase; GalT, galactosyl transferase; GluEP, UDP-glucose epimerase; I-A, iso-amylase; LD, limit dextrinase; LDIN, limit dextrinase inhibitor; UGlcAEP, UDP-glucuronic acid epimerase; UXEP, UDP-xylose epimerase; Xyl, xylanase isozyme X-I; Xylo, beta-D-xylosidase

References

ABLETT G.A., KARAKOUSIS A., BANBURY L., CAKIR M., HOLTON T.A., LANGRIDGE P., HENRY R.J. (2003): Application of SSR markers in the construction of Australian barley genetic maps. Aust J Agric Res, **54**: 1187-1195.

BECKER J., HUEN M. (1995): Barley microsatellites-allele variation and mapping. Plant Mol Biol, **27**: 835-845.

EDWARDS, K.J., BAKER J.H.A., DALY A., JONES C., KARP A. (1996): Microsatellite libraries enriched for several microsatellite sequences in plants. Biotechniques, **20**: 758.

ELLENGREN H. (2002): Microsatellite evolution: A battle between replication slippage and point mutation. Trends Genet., **18**: 70.

GOLDSTEIN D.B., SCHOLOTTERER C. (1999): Microsatellites: evolution and applications, Oxford Univ Press.

HAYDEN M.J., STEPHENSON P., LOGOJAN A.M., KHATKAR D., ROGERS C., KOEBNER R.M.D., SNAPE J.W., SHARP P.J. (2004): A new approach to extending the wheat marker pool by anchored PCR amplification of compound SSRs. Theor Appl Genet, **108**: 733-742.

KARAKOUSIS A., GUSTAFSON J.P., CHALMERS K.J., BARR A.R., LANGRIDGE P. (2003a): A consensus map of barley integrating SSR, RFLP and AFLP marker. Aust J Agric Res, **54**: 1173-1185.

KARAKOUSIS A., BARR A.R., CHALMERS K.J., ABLETT G.A., HOLTON T.A., HENRY R.J., LIM P., LANGRIDGE P. (2003b): Potential of SSR markers for plant breeding and variety identification in Australian barley germplasm. Aust J Agric Res, **54**: 1197-1210.

LIU Z.W., BIYASHEV R.M., MAROOF M.A.S. (1996): Development of simple sequence repeat DNA markers and their integration into a barley linkage map. Theor Appl Genet, **93**: 869-876.

MARROF M.A.S., BIYASHEV R.M., YANG G.P., ZHANG Q., ALLARD R.W. (1994): Extraordinarily polymorphic microsatellite DNA in barley: Species diversity, chromosomal locations and population dynamics. Proc Natl Acad Sci USA, **91**: 5466-5470.

MITCHELL S.E., KRESOVICH S., JESTER C.A., HERNANDEZ C.J., SZWEC-MCFADDEN A.K. (1997): Application of multiplex PCR and fluorescent-based, semi-automated allele sizing technology for genotyping plant genetic resources. Crop Science, **37**: 617-624.

PILLEN K., BINDER A., KREUZKAM B., RAMSEY L., WAUGH R., FORSTER J., LEON J. (2000): Mapping new EMBL-derived barley microsatellites and their use in differentiating German barley cultivars. Theor Appl Genet, **101**: 652-660.

RAMPLING L.R., HARKER N., SHARIFLOU M.R., MORELL M.K. (2001): Detection and analysis systems for microsatellite markers in wheat. Aust J Agric Res, **52**: 1131–1141.

RAMSEY L., MACAULAY M., MCLEAN K., FULLER J., EDWARDS K., TUVESSON S., MORGANTE M., DEGLI IVANISSEVICH S., MARMIROLI N., MAESTI E., MASSARI A., POWELL W., WAUGH R. (2000): A simple sequence repeat based linkage map of barley. Genetics, **156**: 1997-2005.

RUSSELL J.R., FULLER J.D., MACAULAY M., HATZ B.G., JAHOOR A., POWELL W., WAUGH R. (1997): Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs, and RAPDs. Theor Appl Genet, **95**: 714-722.

RÖDER M.S., PLASCHKE J., KONIG S.U., BORNER A., SORRELLS M.E., TANKSLEY S.D., GANAL M.W. (1995): Abundance, variability and chromosomal location of microsatellites in wheat. Mol Gen Genet, **246**: 327-33.

SCHUELKE M. (2000): An economic method for the fluorescent labelling of PCR fragments. Nature Biotech, **18**: 233–234.

STRUSS D., PLIESKE J. (1998): The use of microsatellite markers for detection of genetic diversity in barley populations. Theor Appl Genet, **97**: 308-315.

VARSHNEY R.K., THIEL T., STEIN N., LANGRIDGE P., GRANER A. (2002): In silico analysis on frequency and distribution of microsatellites in ESTs of some cereal species. Cell Mol Biol Lett, **7**: 537-546.

ZIEGLE J.S., SU Y., CORCORAN K.P., NIE L., MAYNARD P.E., HOFF L.B., MCBRIDE L.J., KRONIK M.N., DIEHL S.R. (1992): Application of automated DNA sizing technology for genotyping microsatellite loci. Genomics, **14**: 1026-1031.

ZHU Y., QUELLER D.C., STRASSMANN J.E. (2000): A phylogenetic perspective on sequence evolution in microsatellite loci. J Mol Evol, **50**: 324-338.

The Barley Microarray. A Community Vision and Application to Abiotic Stress

T.J. Close

Department of Botany & Plant Sciences, University of California, Riverside, CA, USA, 92521-0124

Abstract
A microarray chip representing approximately 20,000 barley unigenes was produced as part of a USA project entitled, "An integrated physical and expression map of barley for Triticeae improvement". The content of the chip was derived from more than 400,000 barley "Expressed Sequence Tag" (EST) sequences received from cooperators in USA, Germany, Japan, Scotland, and Finland, plus about 1,000 sequences retrieved from the GenBank nr database or GrainGenes. All EST sequences were trimmed to high quality regions, contaminants were identified and removed, and the remaining information was compiled using the CAP3 assembly program. A "stringent" assembly (paralogs separated) contained about 53,000 "unigenes" (the sum of contigs plus singletons), among which about 50% had reliable 3' ends and were therefore suitable for chip content. From these, probe sets were designed and the "Barley1" chip fabricated by Affymetrix. Complete details on the content of the barley microarray, and enhanced probe-set annotations, can be obtained using the software HarvEST:Barley, available from http://harvest.ucr.edu. The availability of an Affymetrix barley microarray has facilitated the study of gene expression on a large scale. Replicated experiments have revealed commonalities and differences between responses to abiotic stresses, and inherent differences between barley genotypes. The design and a brief summary of the results of drought stress experiments are stated.
Keywords: genomics; ESTs; microarray; abiotic stress

Introduction

The "Barley1" microarray continues a tradition of cooperation and sharing within the worldwide barley community. It is my pleasure to provide some insights about the contributions of many people who participated in the development of this new resource. I will also take this opportunity to provide some simple numerical assessments of the "Barley1" array, which is far from being a "whole-genome" device, so that we do not become complacent by falsely thinking that we have the ultimate expression profiling tool. Certainly we do not, but we can and will do better in the future by continuing to work together as a community. Finally, I will briefly describe some of the experiments that my group has conducted using the "Barley1" microarray to study the transcriptional response of barley to drought stress, one of several abiotic stresses including drought, low temperature and salinity that we have studied using the Barley1 microarray.

The period of 1998 to 2002 was an incubation time for the Barley1 microarray. Representatives of the international barley genomics community met formally and informally during this time at the annual Plant and Animal Genome (PAG) Conference in San Diego, California, where we exchanged information about progress and new initiatives in the development of barley genome resources, and to identify mechanisms to share them. During this period there was a consensus that a standard for parallel expression profiling in barley was needed. The diploid nature of barley, together with a tradition of cooperation in genetic

resource development, gave us the sense that barley held an advantageous position as a model for Triticeae plants. This team spirit helped to drive us forward.

There was considerable discussion about what would be the most appropriate first large-scale microarray for barley. If a barley-only array would be produced, then would barley become isolated from other Triticeae transcriptome research? If an all-Triticeae array (we used the term "Trit-chip") would be produced from A, B, D, H, R, S and possibly other Triticeae genomes, then would it adequately support any single Triticeae genome, including barley? We debated whether there should be a single barley genotype, or several, represented on the array. If just one, then which one, my favorite or yours? If more than one, then what would be the mechanism of representing different alleles? There was also considerable debate over the appropriate format for a microarray. Should it be composed of whole cDNAs, 3' ends of cDNAs, oligonucleotides (oligos) in the 60-70 length range, or smaller oligos? Whole cDNAs would hybridize across many Triticeae genomes and therefore a cDNA-based chip might be most broadly useful, but precision would be compromised by cross-hybridization between signals from paralogs within any single genome and by homoeologs in polyploid Triticeae. On the other extreme, multiple gene-specific 25-mers would provide excellent gene-specificity but require (at the time) some hundreds of thousands of dollars for the design fee alone. The literature of the period contained impressive research articles with data from each format. Images of thousands of green, red and yellow spots that could be scanned by the human eye with the assistance of image analysis software were popular in the late 1990's. Larger datasets and attention to statistical validity had become more prominent themes of microarray publications by 2002. Where would barley place its first community chips? The merits and limitations of each scenario were debated at PAG, every other venue that I can remember, and through waves of emails preceding various grant proposal deadlines. In retrospect, the discussion process was extremely useful as a mechanism of community education, preparing us to recognize and seize tangible opportunities. But, the decision to produce an Affymetrix chip for barley was ultimately a pragmatic choice made by a few rather than a declaration of worldwide consensus. Engagement in this plan by worldwide colleagues came naturally from our habits of collegiality. Without a doubt, this had a positive outcome on the quality of the final product.

A group of US investigator including Andris Kleinhofs (Principal Investigator, Washington State University), Gary Muehlbauer (University of Minnesota), Rod Wing (Clemson University), Roger Wise (Iowa State University) and me, received grant funds in 2001 from the United States Department of Agriculture Initiative for Future Agriculture and the Food Supply Program that allowed us to move forward. Roger Wise, in particular, deserves credit for spearheading the decision to place most of the resources from this grant on the development of a microarray and its immediate use. Within this group, we had quite some debate over chip format. Our initial plan was to use a commercial provider to spot 10,000 whole cDNAs, each representing a single "unigene". From my own perspective, as one who has an interest in a multigene family related to abiotic stress, the dehydrin gene family (CHOI *et al*. 2002), whole-cDNA arrays did not appear to be a satisfactory format. Just one or a few cDNAs from this family would represent them all. There would then be no possibility to examine the expression of each gene individually. This same concern applied to resistance gene analogs and all other multigene families. I advocated against the whole-cDNA format. For a brief time, our plan shifted to the use of 3' ends of unigenes (truncated cDNA clones), but this required a different databasing method and sequencing strategy than we had initiated. Our shift of plans in favor gene-specificity prompted Roger Wise to explore options to have commercial providers produce single 60- to 70-mer or multiple 25-mer arrays We nearly chose the single long oligo method but in the end were swayed by several apparent advantages of multiple short 25-mers. These advantages included: 1) a considerable cost savings in chip

fabrication, 2) representation of a larger number of unigenes, each with more probes per unigene, and 3) less chance of bias in signal detection by multiple oligos versus a single oligo. The latter has since been validated in the literature (for example, ROGOJINA *et al*. 2003).

Affymetrix (www.affymetrix.com) was the provider of the format that we chose. However, one catch was that we had to provide our own informatics up to the point of delivery of "exemplars" (trimmed unigene sequences). The separation of paralogs and determination of orientation of unigene sequences to support gene-specific oligo design demanded further changes in our databasing methods. Fortunately, my colleague Steve Wanamaker and I had already developed a relational database for barley and other Triticeae ESTs (HarvEST, http://harvest.ucr.edu) that could be readily adapted to accommodate these needs. HarvEST originated as an in-house tool for EST data management related to the design of oligos for PCR and BAC library probing. The HarvEST database became our informatics engine and has subsequently provided foundational data for other viewing tools, foremost among which is BarleyBase (http://www.barleybase.org/), an excellent product of colleagues at Iowa State University.

It was an enlightening and enjoyable experience to be deeply engaged in the development of content for the Barley1 microarray (CLOSE *et al*. 2004). It has also been gratifying to turn our attention back to abiotic stress, and to see many colleagues succeeding with their use of this new resource.

Material and Methods

ESTs and other sequences. The first objective in the design of a microarray was to create a considerable "Expressed Sequence Tag" (EST) resource, from which chip content principally could be drawn. From January to October 2002, each project transmitted their EST data to my group at University of California, Riverside. This included either a pair of matching sequence and quality value files generated using the base-calling software "phred" from each EST (EWING and GREEN 1998; http://www.phrap.org/) or the original chromatogram from which we then extracted the two phred files. By the end of 2002, worldwide projects had contributed approximately 350,000 high-quality barley ESTs originating from more than 400,000 raw sequences, constituting about 25,000 "unigenes" with satisfactory 3' ends for chip content. These came from 84 cDNA libraries representing various developmental stages, in addition to abiotic- and biotic-stress treatments (Table 1). Most of the EST data contributors and their barley EST projects are cited below.

- Clemson University Genomics Institute, Clemson, South Carolina, USA: Rod Wing and Dorrie Main, with EST production support from Dilara Begum, David Frisch, Michael Atkins, Yeisoo Yu, D. Henry, M. Palmer, T. Rambo, J. Simmons and R. Oates. Supported by the United States Department of Agriculture – National Research Initiative project, "Establishment of a Genetically and Physically Anchored EST Resource for Barley Genomics" (Andris Kleinhofs, PI; Rod Wing, Timothy Close, Roger Wise).
- Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany: Andreas Graner, Nils Stein and Winfriede Weschke with support from Hangning Zhang, Elena Potokina, Volodya Radchuck and Jelena Perovic. Barley ESTs from the project "Partial Sequencing of cDNAs for the Generation of Expressed Sequence Tags (ESTS) of Barley" funded by the state of Saxony-Anhalt.
- Okayama University, Japan: Kazuhiro Sato, Daisuke Saisho and Kazuyoshi Takeda. National Institute of Genetics, Japan: Yuji Kohara and Tadasu Shin-i. ESTs from the collaborative project between the Barley Germplasm Center, Okayama University and Center for Genetic Resource Information, National Institute of Genetics. This project has been supported by a Grant-in-aid for Scientific Research on Priority Areas C from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and by

CREST (Core Research for Evolutional Science and Technology) of JST (Japan Science and Technology Corporation).

- Scottish Crop Research Institute (SCRI), Invergowrie, Dundee, Scotland, UK: Robbie Waugh, Peter Hedley, H. Liu, D. Caldwell, Luke Ramsay, David Marshall, and Linda Cardle. Developed as part of the barley transcriptome resources of BBSRC/SEERAD funded cereal 'Investigating Gene Function' project.
- Hans Bohnert (USA), Dept. Biochemistry and Molecular Biophysics, University of Arizona, Tucson, AZ 85721, and Plant Sciences and Dept. Crop Sciences, University of Illinois, Urbana-Champaign, IL, 61801. Supported by NSF – Plant Genome Program (98-13360); data collected by: N.Z. Ozturk, C.B. Michalowski, S. Brazille, C. Borchert, C. Palacio, C. Normand, C. Murphy, R. Kelley, S.A. Sant, H. McLaughlin, and M.A. Fredricksen.
- Institute of Biotechnology, University of Helsinki, Helsinki, Finland and MTT Agrifood Research, Jokioinen, Finalnd: Alan Schulman, Ari-Matti Sarén, Jaakko Tanskanen, and Lars Paulin with support from Tanja Horko and Ursula Lönnqvist. ESTs from the project, "Production of EST tools for barley gene discovery and exploitation" funded by major contributions of TEKES, the National Technology Agency of Finland, and Boreal Plant Breeding Ltd., as well as by contributions from Polttimo Companies Ltd. and the Raisio Group, Ltd. Additional in-kind contributions from CSC- Scientific Computing Ltd. and Visipoint OY.

In addition, Dan Ashlock at Iowa State University extracted complete barley cDNA and gene sequences from the National Center for Biotechnology "nr" database, and David Matthews, head curator of GrainGenes at Cornell University, provided sequences from Gottfried Kuenzel (IPK Gatersleben) that were available only from the GrainGenes database (http://wheat.pw.usda.gov/cgi-bin/graingenes/browse.cgi). The final sequences represented on the chip included commonly used transgenes suggested by Peggy Lemaux and Shibo Zhang, University of California, Berkeley and several disease resistance genes and additional control sequences provided by Roger Wise, Stacey Turner and Rico Caldo at Iowa State University and by Andris Kleinhofs.

Several barley genotypes were the source of tissues for cDNA libraries and ESTs (Table 1). In general, each group chose a Spring malting barley popular in their own country as the main source of ESTs. Most of the US libraries were from Morex, the German from Barke, the Japanese from Haruna Nijo, the Scottish from Optic, and the Finnish from Saana. A few other genotypes also contributed to the EST dataset, including: Golden Promise, a two-row Spring barley popular for transformation; Kympii, a Finnish two-row Spring malting barley that was a convenient source of callus tissue; one wild barley accession *H. vulgare* ssp. *spontaneum* OUH602; Akashinriki, another Japanese barley; Tokak, a Turkish barley chosen for drought studies (OZTURK *et al*. 2002); Rolfi, a Finnish Spring feed barley studied for net blotch disease; and other genotypes containing resistance determinants in essentially a Morex or Ingrid background. The tissues spanned the range from roots to leaves to highly specialized reproductive structures. Stages of development covered the gamut from germinated seed to mature spike. Treatments included abiotic stresses and pathogen challenge in addition to normal growth. Further information on source materials is displayed within HarvEST:Barley and in GenBank accessions for each EST.

Table 1. cDNA Libraries and ESTs

Library	Genotype	Source	Tissue	Stage	Condition	Clones	ESTs	% Unique[a]
BaAK	Akashinriki	Japan	leaf	vegetative		10671	19912	7.8
BaAL	Haruna Nijo	Japan	leaf	heading		9720	16013	6.7
BaGS	Haruna Nijo *spont.*	Japan	shoot	seedling 5 d		10212	18069	9.2
BaH	OUH602	Japan	leaf	heading		13132	23752	14.1
BaSD	Haruna Nijo	Japan	leaf	seedling 2 wk		5081	8624	11.0
EBan01	Optic	Scotland	anther	pre-anth.yellow		1480	1778	21.8
EBca01	Optic	Scotland	carpel	pre-anthesis		1415	1415	7.8
EBed01	Optic	Scotland	endosperm	6 DPA		661	661	8.0
EBed02	Optic	Scotland	endosperm	8 DPA		646	646	7.4
EBed07	Optic	Scotland	endosperm	28 DPA		1071	1071	3.2
EBem04	Optic	Scotland	embryo	12 DPA		1152	1493	5.2
EBem05	Optic	Scotland	embryo	14 DPA		1347	1347	7.7
EBem06	Optic	Scotland	embryo	21 DPA		1198	1198	5.3
EBem07	Optic	Scotland	embryo	28 DPA		1191	1191	5.3
EBem08	Optic	Scotland	embryo	40 DPA		1173	1254	5.6
EBem09	Optic	Scotland	embryo	malted 1 d		1912	1912	8.2
EBem10	Optic	Scotland	embryo	malted 2 d		1327	1345	7.6
EBes01	Optic	Scotland	embryosac	4-6 DPA		1283	1283	8.3
EBma01	Optic	Scotland	maternal	4 DPA		1047	1047	7.4
EBma03	Optic	Scotland	maternal	8 DPA		963	963	7.4
EBma04	Optic	Scotland	maternal	10 DPA		767	767	4.6
EBma05	Optic	Scotland	maternal	12 DPA		879	879	4.6
EBma07	Optic	Scotland	maternal	21 DPA		270	270	3.3
EBma08	Optic	Scotland	maternal	28 DPA		1159	1159	4.5
EBpi01	Optic	Scotland	pistil	1 DPA		1447	1447	7.5
EBpi03	Optic	Scotland	pistil	4 DPA		1040	1040	6.5
EBpi05	Optic	Scotland	pistil	8 DPA		1227	1227	5.8
EBpi07	Optic	Scotland	pistil	12 DPA		631	631	7.6
EBro01	Optic	Scotland	root	seedling 3 wk		1403	1403	12.8
EBro02	Optic	Scotland	root	seedling 3 wk	lowN	2055	2055	12.6
EBro03	Optic	Scotland	root	seedling 3 wk	water-log	2462	2462	9.9
EBro04	Optic	Scotland	root	seedling 3 wk	salt	1184	1308	11.4
EBro05	Optic	Scotland	root & shoot	seedling 3 wk	etiolated	106	106	5.7
EBro06	Optic	Scotland	root	seedling 3 wk	drought	125	125	16.0
EBro07	Optic	Scotland	root & shoot	seedling 3 wk	etiolated	822	822	5.1
EBro08	Optic	Scotland	root	seedling 3 wk	drought	3759	3759	17.3
HB	Tokak	US (Bohnert)	leaf	seedling 3 wk	drought	523	523	9.0
HC	Tokak	US (Bohnert)	root	seedling 3 wk	drought	928	928	23.2
HV_CEa	CI16155	US (Wing)	leaf	seedling 8 d	*Blumeria*	4313	5515	15.0
HV_CEb	CI16151	US (Wing)	leaf	seedling 8 d	*Blumeria*	4298	5997	17.1
HVSMEa	Morex	US (Wing)	shoot	seedling 7 d	cold	4266	6587	16.9
HVSMEb	Morex	US (Wing)	shoot	seedling 6 d	drought	4712	6838	18.2
HVSMEc	Morex	US (Wing)	shoot	seedling 5 d	etiolated	2322	3139	29.0
HVSMEf	Morex	US (Wing)	root	seedling 5 d	etiolated	5089	7271	22.2
HVSMEg	Morex	US (Wing)	spike	pre-anthesis		4786	7455	17.2
HVSMEh	Morex	US (Wing)	spike	5-45 DAP		4630	5164	8.3
HVSMEi	Morex	US (Wing)	spike	20 DAP		4707	6214	14.4
HVSMEk	Morex	US (Wing)	testa/pericarp	seed		4248	6566	16.0
HVSMEl	Morex	US (Wing)	spike	spikedevel.	*Fusarium*	4515	6287	18.1
HVSMEm	Morex	US (Wing)	leaf	seedling 8-10 d	*Blumeria*	4491	6284	24.9
HVSMEn	Morex	US (Wing)	rachis	developing		4321	6152	28.6
IPK_HA	Barke	Germany	embryosac	0-7 DAP		9944	11270	7.4
IPK_HB	Barke	Germany	caryopsis	8-15 DAP		10779	10987	8.7
IPK_HD	Golden Promise	Germany	embryo	callus		4659	5361	15.1
IPK_HE	Barke	Germany	leaf	seedling 7 d	etiolated	312	511	6.7
IPK_HF	Barke	Germany	caryopsis	16-25 DAP		7489	7756	8.2
IPK_HG	Barke	Germany	leaf	seedling 7 d		324	535	3.4

Library	Genotype	Source	Tissue	Stage	Condition	Clones	ESTs	% Unique[a]
IPK_HI	Barke	Germany	fem. inflorescense	pre-anthesis 3 mm		4256	4936	10.7
IPK_HK	Barke	Germany	leaf	seedling 6 d	etiolated	1050	1436	17.8
IPK_HM	Barke	Germany	male inflorescense	pre-anthesis 2 mm		4204	4858	14.5
IPK_HO	Ingrid BC mlo5	Germany	epidermis	seedling 7 d	*Blumeria*	4796	4798	22.5
IPK_HP	Barke	Germany	epidermis	seedling 7 d		236	406	14.4
IPK_HR	Barke	Germany	root	seedling 2-3 d		330	534	12.1
IPK_HS	Barke	Germany	embryo	malted 0-16 h		4630	7238	16.3
IPK_HT	Barke	Germany	endosperm	malted 0-16 h		4920	7248	16.3
IPK_HU	Barke	Germany	seed	malted 16-4 8h		4609	7313	17.6
IPK_HV	Barke	Germany	seed	malted 48-96 h		4493	7122	16.2
IPK_HW	Barke	Germany	root	seedling 2 d		3165	5960	19.3
IPK_HX	Barke	Germany	apex 3-5 mm	adult		4876	5681	9.9
IPK_HY	Barke	Germany	caryopsis	3-29 DAP		3480	6298	11.6
IPK_HZ	Barke	Germany	pericarp	0-7 DAP		10067	10072	8.9
S00002	Saana	Finland	embryo	1 DAP		5094	5263	8.2
S00007	Saana	Finland	shoot	2,3,4 d		3461	3842	8.7
S00008	Kymppi	Finland	callus K19	callus		9903	10269	11.3
S00010	Saana	Finland	seed	1-9 DAP		1444	1444	9.2
S00011	Saana	Finland	seed	12-18 DAP		10401	10904	4.4
S00014	Rolfi	Finland	leaf	seedling 2 leaf	*Pyrenophora*	842	875	5.5
S0MISC	Saana	Finland	various	various		224	224	8.9
WHOLE	Genbank"nr"	US (Wise)	various	various	various	977	977	15.0
KUENZEL	Kuenzel probes	US (Matthews)	various	various		22	22	4.5
CPLAST	Chloroplast	various	various	various	various	44	44	100.0
MITO	Mitochondrion	various	various	various	various	44	44	100.0
BARLEY1X	Other	US (Wise/Kleinhofs)	various	various	various	127	127	100.0
Totals						**265,383**	**349,709**	

[a]% unique is defined as the number of contigs and singleton that are unique to the library divided by the number of clones sequenced from the same library.

Sequence processing. Briefly, sequence processing steps (CLOSE *et al.* 2004) were: 1) phred was applied to chromatograms to produce sequence and quality files, 2) cross_match (http://www.phrap.org/) was used to mask cloning system sequences, 3) an in-house script "qvtrim" was used to synchronously remove low quality regions outside of a sliding window with an average phred quality value of 17, reduce poly(T) or poly(A) ends to a maximum of 17 consecutive T's or A's, and remove residual cloning system sequences, 4) sequences less than 100 bases after steps 1-3 were discarded, 5) a filter based on frequency of four-nucleotide repeats was applied to remove additional ESTs that resulted from poor quality sequencing reactions, 6) orientations were determined using information on sequencing primer, high BLASTX orientation (default parameters), and presence of a poly(A) or poly(T), 7) BLASTN searches (ALTSCHUL *et al*. 1997; http://www.ncbi. nlm.nih.gov/BLAST/) were performed to identify contaminant sequences from *E. coli,* bacteriophage *lambda*, fungal sources, rRNA or the repetitive portion of Triticeae genomes (TREP; http://wheat.pw.usda.gov/ggpages/ITMI/Repeats/index.shtml), 8) several chimera filters, including searches for interior sequences from the cloning system or ESTs that both begin with poly(T) and end with poly(A), were applied to individual EST sequences, 9) assemblies were produced using "tgicl" from Geo Pertea at TIGR (http://www.tigr.org/tdb/tgi/software/) to manage the preclustering of ESTs using "megablast" (ZHANG *et al*. 2000) and final clustering using a special version of CAP3 (HUANG and MADAN 1999) kindly provided by Xioaqiu Huang at Iowa State University, 10) contig orientations were determined using the ratio of forward and reverse EST sequences and the orientation of each EST used by CAP3, 11) additional chimera filters, including searches for contigs whose overall orientation could

not be resolved or whose consensus sequence both begins with poly(T) and ends with poly(A), were applied to assembled ESTs, 12) assembly and chimera removal was repeated several times, 13) sequences with reliable 3' ends were determined, 14) reverse orientation unigene sequences were converted to forward-oriented sequences, 15) multiple poly(A) sites were trimmed to their first poly(A) site to define "exemplars" that were transmitted to Affymetrix for probe set (oligo) design, 16) several unigenes were designated as standards for labeling controls, 17) additional sequences were added as controls and for other anticipated research purposes. All information from these steps was recorded in a Visual FoxPro relational database, from which the HarvEST:Barley software is an extraction product. Annotations of the probe sets (rice gene model, etc.) can be exported from HarvEST:Barley using the "Search the Barley Chip" function.

Drought Stress. Morex barley seeds were sown in pots filled with a standard soil mixture at a density of 40 seeds per pot. Plants were grown in a growth chamber with 23/20°C day/night temperatures and 12 h photoperiod. Ten days after sowing, water was withheld from "stressed" material, while watering was continued for unstressed reference material. The pots were weighed at regular intervals for calculation of the soil water content (SWC). SWC was determined as the water content relative to the total soil water content 16 h after complete hydration. For each of three fully replicated experiments, samples were taken when the SWC was approximately 70, 35, 20, 12 and 8%. Leaf relative water content (RWC) was measured in two fully expanded leaves. Osmotic potential was measured in pressure-extruded leaf-sap using a vapor pressure osmometer (model 5100C, Wescor Inc. Logan, Utah) with sugar solutions as a calibration curve. Experiments were also conducted on low temperature, salinity and abscisic acid treatment (not shown). Total RNA was isolated from crown tissue using TRIzol Reagent following the procedure described in the Arabidopsis consortium web site (http://www.arabidopsis.org/info/2010_projects/comp_proj/AFGC/RevisedAFGC/site2RnaL.htm). Further purification was achieved using an RNeasy spin column (Qiagen, Chatsworth, CA) with on-column DNase treatment, following the manufacturer's instructions, to remove contaminating DNA and tRNAs, 5S RNA and most RNA less than 200 bases.

Results and Discussion

Barley Unigenes and Microarray Content. A previous description of the content and performance of the Barley1 microarray summarized categories of exemplar sequences and data related to error rates and applicability of the Barley1 microarray to other cereal plants (CLOSE *et al*. 2004). We stated in that publication that the Barley1 chip represents more than 21,000 non-redundant exemplar sequences. While this is correct in the context of the sequence assembly that we used, a more conservative estimate of the number of barley genes represented by the Barley1 chip is about 14,000, or about 25% of the barley genome if we assume that barley and rice have about the same number of genes. A BLASTX search of rice coding sequences available from TIGR (ftp://ftp.tigr.org/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_2.0/all_chrs/all.cds) revealed that 85.5% of 21,350 unigenes represented by Barley 1 probe sets (18,523) match a rice coding sequence at an e-score of -5 or better, but only 11,805 rice coding sequences account for all of these matches. If we assume that the remaining 14.5% of barley unigenes (those without a high match to rice) have a similar compression rate (11,805/18,523), then the Barley1 chip may represent about 13,200 barley genes. This would be an overly conservative estimate, however, since the rice genome has not yet been fully annotated and the numbers of barley unigenes that match rice genes is higher if the e-score threshold is relaxed. In addition, alternative polyadenylation sites and other splice variants are often represented by the Barley1 probe sets. A conservative estimate of the number of barley genes represented on the Barley1 microarray is therefore about 14,000. From the perspective of abiotic stress, it is intere sting to make an equivalent assessment considering 17 "osmotic

stress related" libraries including drought, low temperature, salinity, embryo dehydration and pollen maturation. Together, these 17 libraries contributed 8068 non-redundant unigenes (of about 21,400), among which 89.9% (7254) match rice version 2.0 coding sequences at an e value of -5 or better. Furthermore, these stress-related libraries account for 51.4% (6072/11,805) of rice coding sequences identified as highest BLASTX match with barley genes represented on the chip. The Barley1 microarray therefore seems to be an excellent tool for initial investigations of abiotic stress responses.

Drought Stress. In the experiments described in Materials and Methods and illustrated in Figure 1, it was observed (Edmundo Rodriguez, University of California, Riverside, unpublished) that there were clear, reproducible differences in the categories of genes that were expressed at different stages of the stress treatment. For example the dehydrin genes, which are a favorite topic in my laboratory (CHOI *et al.* 1999), were expressed mainly during the most severe stages of the stress treatment. In contrast, changes in the expression of transcription factors were a prominent theme of the early stage of the stress treatment. The location of some of these genes on the barley linkage map, and on the rice linkage map using orthology and synteny relationships between barley and rice, provide some intriguing examples of stress-regulated genes that are candidates for stress tolerance traits. For example, the drought-up-regulated dehydrin gene *Dhn6* is located within a region of chromosome 4H recently associated by SSR markers with drought tolerance (IVANDIC *et al*. 2003).

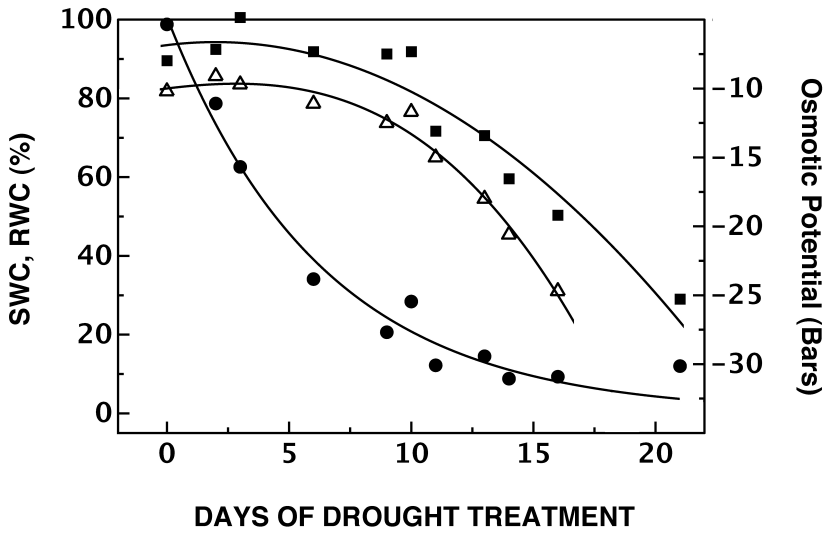


Figure 1. Time course of drought stress. SWC, circles; RWC, squares; leaf sap osmotic potential, triangles.

Summary. The Barley1 microarray provides an excellent starting point for global analysis of gene expression in abiotic stress and other aspects of barley, representing at least 25% of the genes in the barley genome. Major changes in transcriptional activity can readily be measured using this new device, and the standardized platform facilitates data sharing. It is now appropriate for the barley community to engage in the design of a microarray representing a larger number of barley genes.

Acknowledgements

Development of the barley microarray was supported by US Department of Agriculture Initiative for Future Agriculture and Food Systems grant #01-52100-11346. New features of HarvEST:Barley were supported by US Department of Agriculture Plant Genome grant # USDA-NRI 02-35300-12548. Excellent advice of the Affymetrix chip design team is also gratefully acknowledged.

References

ALTSCHUL S.F., MADDEN T.L., SCHÄFFER A.A., ZHANG J., ZHANG Z., MILLER W., LIPMAN D.J. (1997): Gapped BLAST and psi-BLAST: A new generation of protein database search programs. Nucl. Acids Res., **25:** 3389-3402.

CHOI D.W., ZHU B., CLOSE T.J. (1999): The barley (*Hordeum vulgare* L.) dehydrin multigene family: sequences, allelic variation, chromosome assignments, and expression characteristics of 11 *Dhn* genes of cv. Dicktoo. Theor. Appl. Genet., **98**: 1234-1247.

CLOSE T.J., WANAMAKER S., CALDO R., TURNER S.M., ASHLOCK D.A., DICKERSON J.A., WING R.A., MUEHLBAUER G.J., KLEINHOFS A., WISE R.P. (2004): Global expression profiling in cereals: 22K barley GeneChip comes of age. Plant Physiol., **134**: 960-968.

EWING B., GREEN P. (1998): Base-calling of automated sequencer traces using Phred II. Error probabilities. Genome Res., **8:** 186-194.

HUANG X., MADAN A. (1999): CAP3: a DNA sequence assembly program. Genome Res., **9:** 868-877.

IVANDIC V., THOMAS W.T.B., NEVO E., ZHANG Z., FORSTER B.P.. (2003): Associations of simple sequence repeats with quantitative variation including biotic and abiotic stress tolerance in *Hordeum spontaneum*. Plant Breed., **122:** 300-304.

OZTURK Z.N., TALAMÉ V., DEYHOLOS M., MICHALOWSKI C.B., GALBRAITH D.W., GOZUKIRMIZI N., TUBEROSA R., BOHNERT H.J. (2002): Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. Plant Mol. Biol., **48:** 551-573.

ROGOJINA A.T., ORR W.E., SONG B.K., GEISERT E.E. (2003): Comparing the use of Affymetrix to spotted oligonucleotides microarrays using two retinal pigment epithelium cell lines. Molec. Vision, **9:** 482-496.

ZHANG Z., SCHWARTZ S., WAGNER L., MILLER W. (2000): A greedy algorithm for aligning DNA sequences. J Comput. Biol., **7:** 203-14.

Transgenic Barley

D. von Wettstein

Department of Crop and Soil Sciences, School of Molecular Biosciences & Center of Integrated Biotechnology, Washington State University, Pullman WA 99164-6420, USA

Abstract
Genetic transformation of scutellum cells of immature zygotic barley embryos is routine using either particle bombardment or co-cultivation with *Agrobacterium tumefaciens*. In a few cultivars regeneration of the scutellum cells via callus and somatic embryos into plants has yielded frequencies of more than 50% transformants. For expression of desired genes during grain germination/malting alpha-amylase promoters are preferred together with the code of the transit peptide for secretion of the expressed protein into the endosperm. For deposition of microbial and human enzymes and growth factors in the storage protein bodies of the mature grain, the hordein gene promoters can deliver up to 1g of recombinant protein per kg of grain. The herbicide resistant gene required for selection of a transformant can be eliminated with double cassette vectors or by site directed recombination. The genetically stable transformants have generally decreased grain production and thousand-grain weights. As with spontaneous and induced mutations, decreased agronomic performance is rectified by marker assisted recombination breeding with elite cultivars. The transgene insertion site in the barley genome at the nucleotide sequence level is determined with a modified tail PCR procedure. Maize Ac/Ds transposons have been inserted into the barley genome for gene tagging. Greenhouse and field trials with genetically engineered stem rust and *Rhizoctonia* root rot resistant transformants are reviewed. Addition of 0.02% grain containing a protein engineered, thermotolerant (1,3-1,4)-β-glucanase to normal barley is sufficient to achieve in feeding trials equal weight gain of broilers as obtained with corn diets.
Keywords: transgenic barley; recombinant proteins; disease resistance; molecular farming

Introduction

Barley is a highly suitable crop for the production of recombinant proteins in the grain as well as during controlled germination in the malting process (HORVATH *et al*. 2002; HORVATH *et al.* 2000; HORVATH *et al.* 2001; VON WETTSTEIN *et al.* 2003). It is a self-pollinating small grain cereal, that unlike wheat or rice, does not hybridize with any wild species growing in the surrounding natural habitats of its areas of cultivation (VON BOTHMER 1992). Interspecific hybridization with the 31 annual and perennial wild species of *Hordeum* growing in the Mediterranean area, North or South America is characterized by strong incompatibility barriers, usually as F_1 sterility. So far, a significant breakthrough for breeding with these wild species, that would represent important germplasm resources, has not been obtained using conventional sexual crosses (VON BOTHMER 1992; FEDAK 1992). Cross-pollination among barley cultivars, especially with two-row spring barley, is low and can be controlled adequately by spatial separation.

Of today's world barley production about 85% is used for feeding animals, while the second important utilization is by the malting industry for beverage production (FISCHBECK 2002). While barley is used as ingredients in many human food products, it plays and is also likely to continue to play a minor role in food products (FISCHBECK 2002). Barley acreage has decreased worldwide by 14-20% since 1989/1991, a decrease that continues. A major reason is that low-priced corn with higher nutritional quality is a strong competitor of barley in the feed industry, ─ today also in areas, where grain corn for climatic reasons cannot mature. But barley is needed for

crop rotations in these areas and it is therefore desirable to keep barley competitive by breeding for improved nutritional and other value adding characteristics.
The losses of barley crop production to pests, pathogens and weeds may be as high as 20 to 40% and can locally be disastrous. Thus, the economic losses caused by *Fusarium* head blight epidemics during 1993-98 are estimated at $ 3 billion comprising $ 2.6 billion lost by wheat farmers and $ 400 million by barley producers in the Midwest of the US (WINDELS 2000). Barley yield dropped from 75.4 to 46.5 bushels per acre in North Dakota and from 76.2 to 60.2 in Minnesota. Application of transgenic resistance may be the only remedy when useful germplasm for resistance or tolerance against a disease cannot be identified.

Material and Methods
Detailed protocols and reviews on the production and identification of transgenic barley by particle bombardment or *Agrobacterium* mediated transformation are found in the following publications: WAN and LEMAUX (1994), LEMAUX *et al.* (1999), TINGAY *et al.* (1997), WANG *et al.* (2001), HORVATH *et al.* (2002), MURRAY *et al.* (2004).
Gene tagging has been established with the maize two element *Ac-Ds* transposon system (KROPEK *et al.* 2000, 2001).
Patterns and sites of T-DNA integrations into the barley genome from single and double cassette vectors are determined for the identification of cultivars with value-added properties as well as for the production of selection marker-free transgenic lines (STAHL *et al.* 2002; VAN FLEET 2001). T-DNA/Plant DNA junctions are captured as single stranded DNA with a biotinylated primer annealing to the vector adjacent to the border and an adaptor is ligated to a restriction site overhang in the flanking barley DNA. The captured junction is converted into a double strand and sequenced. Primers of 15-30 nucleotides designed from the genomic DNA at the insertion site can PCR amplify fragments that identify unequivocally any transformant. Adjacent transgene insertions with single cassette vectors are always in tandem direct repeat configuration. Twelve of the 46 integrations characterized by BLAST searches were within different regions of the *BARE-1* retrotransposon element occurring with a frequency of 2×10^5 copies in the barley genome. Transcription and translation of transgenes inserted in retrotransposon elements is of normal efficiency.

Results and Discussion
Converting Barley from a Low Energy Feed to a High Energy Feed
The low nutritional value of barley for poultry is due to the absence of an intestinal enzyme for efficient depolymerization of (1,3-1,4)-β-glucan, the major polysaccharide of the endosperm cell walls. This leads to high viscosity in the intestine, limited nutrient uptake, decreased growth rate, and unhygienic sticky droppings adhering to chickens and floors of the production cages. Consequently, the 7.5 billion broiler chickens produced annually in the United States are primarily raised on corn-soybean diets. If barley is used as non-ruminant animal feed the diet is supplied with enzymes from *Trichoderma*. For practical purposes the enzyme solutions are produced in fermenters, transferred to mixers with starch carrier material in the form of barley or wheat flour. The coating on to a carrier material is required to stabilize the enzyme. After mixing the material is dried in warm air and the pellets milled, homogenised and packaged for shipping (HAARASILTA *et al.* 1994). We have performed 4 broiler chicken trials with 240 Hubbard High Yield broilers and 200 Cornish Cross broiler chickens to investigate, if transgenic grain expressing a protein-engineered thermostable (1,3-1,4)-β-glucanase from *Bacillus* during malting (JENSEN *et al.* 1996, 1998) or in the endosperm during grain maturation (HORVATH *et al.* 2000) as feed additive can increase the nutritive value of barley based diets to that of maize (VON WETTSTEIN *et al.* 2000, 2003). In both cases equal weight gain, feed consumption and feed efficiency over a 21 day period could be achieved. The frequency of chicks with sticky droppings could be reduced to

the same level as observed with the maize diet. Analyses of the different parts of the gut and excreta revealed this to be due to the activity of the enzyme in β-glucan degradation in the duodenum, ileum, caeca and excreta. With a barley-soybean diet containing 620 g non-transgenic barley/kg diet, it was sufficient to add 0.2g (0.02%) transgenic grain/kg diet to achieve the high nutritive value while the commercial Avizyme 1100[@] is added at a concentration of 1g/kg diet. The addition of the transgenic grain in the ground diet compares to the amount of trace minerals added to standard diets. The transgene has been bred into modern barley varieties. Some data of the F_6 generations are given in Table 1.

Table 1. Yields of homozygous transgenic lines expressing heat-stable (1,3-1,4)-β-glucanase on the Spillman Farm in 2002. Also given are: amount of heat-stable β-glucanase for 2002 and 2003, soluble β-glucans and 1000 grain weights [g] for 2002. * proanthocyanidin-free

Pedigree and Phenotype	Enzyme $g \cdot kg^{-1}$	β-glucan $mg \cdot g^{-1}$	Grain weight	lbs/acre
271.0.4 × Galena –235, High nutans	702, 711	0.55	36.0	3673
271.0.4 × Galena –246, Low nutans	730, 781	0.70	37.1	5322
271.0.4 × Baronesse-268, Low nutans	816, 618	0.50	40.9	5022
271.0.4 × Baronesse-270,erectoides	702, 878	0.42	42.0	4348
271.0.4 × Baronesse-285, nutans	731, 758	0.32	41.5	4797
271.0.6 × CA2110-314, lax ert *	962, 833	0.39	34.9	4722
271.0.6 × CA2110-322, nutans *	693, 888	0.53	33.0	4947
271.0.7 × Baronesse-323, erectoides	530, 801	0.89	34.3	4197
271.0.7 × B1202-325, nutans	397, 788	0.89	35.7	5097
271.0.8 × CA-803803-329, erectoides *	1097, 749	0.70	41.5	3598
271.0.6 × Baronesse-307, nutans, def.	611, 692	0.12	41.2	5234
271.0.8 × Caminant-330, nutans *	639, 653	0.70	38.3	4192
Baronesse, Nutans, deficiens	**0**	**15**	-	**4487**

With these lines a yield has been achieved that compares favorably with that of Baronesse, the cultivar that is grown on 70% of the barley acreage in the State of Washington. The lines produce constant amounts of recombinant enzyme per kg of grain. The endosperm cell walls of the transgenic lines lack (1,3-1,4)-β-glucans and are not stainable with calcofluor. The recombinant β-glucanase is targeted from the lumen of the endoplasmic reticulum via the Golgi apparatus by vesicle transport into the storage vacuoles, where it is embedded in the D-hordein protein. The (1,3-1,4)-β-glucans are synthesized in the Golgi apparatus and apparently the newly synthesized β-glucans are depolymerized as the β-glucanase passes the Golgi apparatus. This is an interesting observation, as it demonstrates an efficient way of changing the composition (fiber content) of the cereal endosperm cell walls. It will thus not only be possible to produce recombinant xylanase in barley but also to eliminate the anti-nutrititive arabinoxylans leading to cell walls containing only cellulose. Transgenic barleys expressing a fungal or *Bacillus* xylanase gene in the endosperm have been generated (PATEL *et al.* 2000; KOHL 2003).

The use of transgenic grain containing recombinant (1,3-1,4)-β-glucanase as feed additive will boost the production of non-transgenic barley in areas where grain maize cannot be grown and thus has to be imported. Feeding 40 million broiler chicks with barley instead of imported maize will require 280 000 ton of non-transgenic barley but only 56 ton of transgenic barley containing 0.14mg/kg thermotolerant (1,3-1,4-β-glucanase), which could be produced on 25 acres of farmland. The barley feed with added transgenic grain or malt containing thermotolerant (1,3-1,4)-

β-glucanase provides an environmentally friendly alternative to enzyme additives, as it uses photosynthetic energy for production of the enzyme in the grain and thus avoids use of non-renewable energy for fermentations. The deposition of the enzyme in the protein bodies of the grain in the field makes coating procedures for stabilization of enzyme activity superfluous.

Converting Stem-Rust-Susceptible Barley into a Resistant One by Inserting the Rpg1 Gene

The *Rpg1* gene provides resistance to most pathotypes of *Puccinia graminis* f. sp. *tritici* and has been incorporated into North American barley cultivars to protect against stem rust epidemics that plagued the Northern Great Plains production region during the first half of the last century. *Rpg1* has protected barley cultivars from significant stem rust losses for over 60 years and is remarkable for its durability. *Rpg1* was cloned by a map-based approach (BRUEGGEMAN *et al*. 2002). The *in silico* translated *Rpg1* gene revealed a receptor-like protein of 837 amino acids with two tandem kinase domains. The nucleotide sequence of *Rpg1* from resistant cultivars Kindred, Chevron, Peatland, Q21861, Leger, Bowman, and 80-TT-29 is identical to that of Morex. The susceptible cultivar Golden Promise lacks the *Rpg1* gene; as demonstrated by the failure to PCR-amplify the gene with seven primer pairs spanning the length of the 4919 bp *Rpg1* gene in Morex (HORVATH *et al.* 2003). Genomic DNA of susceptible barley cultivars like Baronesse, Betzes, Harrington, Klages and others failed to hybridize with probe RSB228, which is diagnostic for *Rpg1*, and also could not serve for amplification of the *Rpg1* gene with the appropriate PCR primers. Thus the gene in these cultivars is either deleted or highly degenerate. In other susceptible cultivars the *Rpg1* gene contained stop codons at amino acid positions 192, 524 and 788 (BRUEGGEMAN *et al*. 2002). In order to determine, if the stem rust susceptible cv. Golden Promise can be converted into a resistant cultivar, it was transformed with the *Rpg1* genomic clone of Morex containing a 520-bp 5' promoter region, 4919-bp gene region, and 547-bp 3' non-transcribed sequence (HORVATH *et al.* 2003). Representatives of 42 transgenic barley lines obtained were characterized for their seedling infection response to pathotype Pgt-MCC of the stem rust fungus *Puccinia graminis* f. sp. *tritici.* Golden Promise was converted from a highly susceptible cultivar into a highly resistant one by transformation with the dominant *Rpg1* gene. A single copy of the gene was sufficient to confer resistance against stem rust and progenies from several transformants segregated in a 3:1 ratio for resistance:susceptibility as expected for Mendelian inheritance. One of the remarkable aspects about the transformants was that they exhibited at the seedling stage a higher level of resistance than the original sources of *Rpg1* (cvs. Chevron and Peatland).

T_2 and T_3 generations of 5 primary transformants with single, two or several copies of *Rpg1* were tested in a field nursery together with the cultivars Chevron, Morex, Beacon (resistant) and Golden Promise, Steptoe, Harrington (susceptible) at St. Paul MN (B. STEFFENSON, pers. commun.). The nursery was inoculated 5 times by spraying with freshly propagated urediniospores of *Puccinia graminis f.sp. tritici* isolate MCC at a concentration of 0.3-0.8 g/300-350ml Soltroil oil. Spreader rows were infected by injection with urediniospores at the 4th inoculation. The purpose of the study was to assess the level of adult plant stem rust resistance of the Golden Promise transgenics with the *Rpg1* gene. Severity was recorded as the visual percentage (0-100%) of tissue covered by uredinia and infection types were recorded using a letter scale, where **I** = Immune: no visible sign of infection; **HR** = Highly Resistant: hypersensitive flecks, but no sporulation; **R**= Resistant: minute uredinia with chlorosis; **MR**= Moderately Resistant: small uredinia with chlorosis; **MS** = Moderately Susceptible: medium-sized uredinia without chlorosis and **S** = Susceptible: large uredinia without chlorosis.

Stem rust infections with severities of up to 80% and infection types of **S-MS** were observed on the susceptible checks (Steptoe, 80-tt-30), while the resistant checks of Chevron and 80-TT-29 exhibited 0% severity and infection types of HR. The Golden Promise wild type with severity readings of 15-30% and infection types of MS-S was not as susceptible to stem rust as Steptoe and 80-tt-30. Plants of transgenic lines with one (G03-210 and G02-448B-3R), two (G02-448B-2R,

rep 1), and three (G02-448L-5R) copies of Rpg1 gave severities (0%) and infection responses (HR) similar to the resistant controls. Unlike in the seedling test, an enhanced level of resistance in the transgenic lines over that exhibited by the resistant controls was not observed with adult plants. Hypersensitive flecks were consistently present.

Towards Transgenic Barley with Resistance to Rhizoctonia Root Rot

Compared to traditional cropping systems that use tillage, direct-seed cropping systems place seed and fertilizer directly into undisturbed soil with all residues of the previous crops left on the soil surface. These systems are being increasingly adopted by farmers worldwide because of their potential to reduce input costs, increase yields where water is limiting, reduce soil erosion, and conserve energy (COOK & VESETH 1991; COOK 2000a). One of the main limiting factors to the adoption of direct-seeding for wheat and barley are root rot epidemics caused by *Rhizoctonia solani* anastomosis group 8 (AG8) and *R. oryzae* (WELLER *et al*. 1986; OGOSHI *et al.* 1990; MAZZOLA *et al*. 1996` BOCKUS & SHROYER 1998; COOK 2000a). Of the many practices tested to limit the severity of *Rhizoctonia* root rot, only two work consistently. These are 1) timely elimination of volunteer-cereal and grass-weed hosts of the pathogens (SMILEY *et al.* 1991), which minimizes the sources of inoculum; and 2) placement of fertilizer directly beneath each seed row (COOK 2000b), which makes nutrients more accessible to diseased roots. However these two practices combined only can elevate grain yields to 80-85% of potential yield without root disease (COOK *et al*. 2003). No useful resistance has been identified in germplasm of wheat and barley. Root rot caused by *R. solani* AG8 and *R. oryzae* can therefore be considered a logical candidate for control by transgenic resistance.

Two observations led to an investigation of the hypothesis that expression in barley of the 42-kDa endochitinase-encoding gene *ThEn42* of *Trichoderma harzianum* can confer resistance to one or both pathogens responsible for *Rhizoctonia* root rot. (1) ZEILINGER *et al.* (1999) monitored expression of this endochitinase during mycoparasitism with a fusion of the green fluorescent protein (GFP) to the 5' regulatory sequence of the chitinase as a reporter in *T. harzianum* P1 during confontration assays with *R. solani*. In contrast to other enzymes, this endochitinase was induced before *T. harzianum* is in physical contact with its host and was able as such to inhibit the growth of *R. solani* strain 1450. (2) In 1998, LORITO *et al*. (1998) successfully transferred the 42-kDa chitinase gene *ThEn42 (chit42)* into tobacco and potato and obtained high expression of this chitinase in different plant tissues. Selected transgenic lines were coordinately tolerant or highly resistant to *Alternaria alternata*, *A. solani*, *Botrytis cinerea*, and *R. solani*. Their research provides the first demonstration that plants transformed for expression of a fungal chitinase gene show high resistance to several fungal pathogens. Recently resistance to apple scab was conferred with the endochitinase in transgenic apples (BOLAR *et al.* 2000) and to *Alternaria brassicicola* in transgenic broccoli (MORA & EARLE 2001).

A cDNA encoding the 42 kDa endochitinase [*cThEn42(GC)*], GenBank accession L14614, was synthesized from 28 overlapping oligonucleotides. It included the nucleotide sequences of the fungal signal and activation peptides and of the signal peptide from the barley 26-kDa endochitinase HVChi26, GenBank accession M36989. The gene was expressed in *Pichia pastoris*. The produced and purified recombinant enzyme turned out to inhibit efficiently the hyphal growth of *R. solani* AG8 and *R. oryzae*. The enzyme was most effective against *R. oryzae* (ED_{50}=97µg/ml) followed by *R. solani*AG8 (ED_{50}=159.2 µg/ml) and *Gaeumannomyces graminis* var.*tritici* (ED_{50}=392µg/ml). The gene was accordingly provided with suitable gene promoters and also other signal peptide codes. With constructs for *Agrobacterium* mediated transformation 52 transformants were obtained in the barley cultivar Golden Promise. Expression of the endochitinase was demonstrated in leaves and roots of a considerable number of these transformants by Western blots and activity assays. Segregating T_1 plants displayed in seedling assays disease resistant phenotypes, when compared to infected control plants (WU 2003; WU, COOK, VON WETTSTEIN &

KANNANGARA, in prep.). This provides a large transgenic material for further investigation of the possibility to obtain transgenic resistance to these two necrotrophic fungi.

References

BOCKUS W.W., SHROYER J.P. (1998): The impact of reduced tillage on soilborne plant pathogens. Ann. Rev. Phytopathol., **36**: 485-500.

BOLAR J.P., NORELLI J.L., WONG K.W., HAYES C.K., HARMAN G.E., ALDWINCKLE H.B. (2000): Expression of endochitinase from *Trichoderma harzianum* in transgenic apple increases resistance to apple scab and reduces vigor. Phytopathology, **90**: 72-77.

BRUEGGEMAN R., ROSTOKS N., KUDRNA D., KILIAN A., HAN F., CHEN J., DRUKA A., STEFFENSON B. KLEINHOFS A. (2002): The barley stem rust-resistance gene *RPG1* is a novel disease-resistance gene with homology to receptor kinases. Proc. Natl. Acad. Sci. USA, **99**: 9328-9333.

COOK R. J. (2000a): Advances in plant health management in the twentieth century. Annu. Rev. Phytopathol., **38**: 95-116.

COOK R.J. (2000b): Influence of paired-row spacing and fertilizer placement on yield and root diseases of direct-seeded wheat. Crop Science, **40**: 1079-1087.

FEDAK G. (1992): Intergeneric hybrids with *Hordeum*. In: SHEWRY P.R. (ed.) Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology. CAB. Int. Willinford, UK.: 45-70.

FISCHBECK G. (2002): Contribution of barley to agriculture; A brief overview. In: SLAFER G.A., MOLINA-CANO J.L., SAVIN R., ARAUS J.L., ROMAGOSA J. (eds.): Barley Science. The Harworth Press, New York 2002: 1-14.

HAARASILTA A. N. O., RIIKONEN P.L.A., VUORENLINNA L. (1994): Enzyme premix for feed and method. *United States Patent* **5,314,692**: 1-10.

HORVATH H., HUANG J.,WONG O.T., KOHL E., OKITA, KANNANGARA C.G., VON WETTSTEIN D. (2000): The production of recombinant proteins in transgenic barley grains. Proc. Natl. Acad. Sci. USA, **97**: 1914-1919.

HORVATH H., JENSEN L.G., WONG O.T., KOHL E., ULLRICH S.E., COCHRAN J., KANNANGARA C.G., VON WETTSTEIN D. (2001): Stability of transgene expression, field performance and recombination breeding of transformed barley lines. Theor. Appl. Genet., **102**, 1-11.

HORVATH H., HUANG J., WONG O.T., VON WETTSTEIN D. (2002): Experiences with genetic transformation of barley and characteristics of transgenic plants. In: SLAFER G.A., MOLINA-CANO J.L., SAVIN R., ARAUS J.L., ROMAGOSA J. (eds.): Barley Science. The Harworth Press, New York 2002: 143-176.

HORVATH H., ROSTOKS N., BRUEGGEMAN R., STEFFENSON B., VON WETTSTEIN D., KLEINHOFS A. (2003): Genetically engineered stem rust resistance in barley using the *Rpg1* gene. Proc. Natl. Acad. Sci. USA, **100**: 364-369.

JENSEN L.G, OLSEN O., KOPS O, WOLF N., THOMSEN K.K., VON WETTSTEIN D. (1996) Transgenic barley expressing a protein-engineered, thermostable (1,3-1,4)-β-glucanase during germination. Proc. Nat.l Acad. Sci. USA, **93**: 3487-3491.

JENSEN, L.G., POLITZ, O., OLSEN O., THOMSEN, K.K & VON WETTSTEIN, D. (1998) Inheritance of a codon-optimized transgene expressing heat stable (1,3-1,4)-β-glucanase in scutellum and aleurone of germinating barley. Hereditas, **129**: 215-225.

KOHL E., A. (2003) Development of transgenic barley expressing (1,4)-β-xylanase. M.Sc. Thesis, Washington State University, December 2003, p.1-74.

KOPREK T., MCELROY D., LOUWERSE J., WILLIAMS-CARRIER R., LEMAUX P.G. (2000) An efficient method for dispersing *DS* elements in the barley genome as a tool for determining gene function. Plant J., **24**: 253-263.

KOPREK T., RANGEL S., MCELROY D., LOUWERSE J., WILLIAMS-CARRIER R.E., LEMAUX P.G. (2001) Transposon-mediated single-copy gene delivery leads to increased stability in barley. Plant Physiol., **125**: 1354-1362.

LEMAUX P.G., CHO M.J., ZHANG S., BREGITZER P. (1999): Transgenic cereals: *Hordeum vulgare* L. (barley). In: I.K. VASIL (ed.) Molecular Improvement of Cereal Crops. Kluver Academic Publishers, Great Britain: 255-316.

LORITO M., WOO S.L., FERNANDES I.G., COLUCCI G., HARMAN G.E., PINTOR-TORO J.A., FILIPPONE E., MUCCIFORA S., LAWRENCE C.B., ZOINA A., TUZUN S., Scala F. (1998) Gene from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. Proc. Natl. Acad. Sci. USA, **95**: 7860-7865.

MORA A., EARLE E.D. (2001): Resistance to *Alternaria brassicicola* in transgenic broccoli expressing a *Trichoderma harzianum* endochitinase gene. Molecular breeding 8:1-9.

MURRAY F., BRETTELL R., MATTHEWS P., BISHOP D., JACOBSEN J (2004): Comparison of *Agrobacterium*-mediated transformation of four barley cultivars using the GFP and GUS reporter genes. Plant Cell Rep., **22**: 397-402.

OGOSHI A. (1987): Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani* Kuhn. Ann. Rev. Phytopathol., **25**: 125-143.

PATEL M., JOHNSON J., S., BRETTELL R., I., S., JACOBSEN J., XUE G-P. (2000) Transgenic barley expressing a fungal xylanase gene in the endosperm of the developing grains. Molec. Breeding, **6**: 113-123.

SMILEY R.W., OGG A.G.Jr., Cook R.J. (1991): Influence of glyphosate on Rhizoctonia root rot, growth, and yield of barley. Plant Disease, **76**: 937-942.

STAHL R., HORVATH H., VAN FLEET J., VOETZ M., VON WETTSTEIN D., WOLF N. (2002): T-DNA integration into the barley genome from single and double cassette vectors. Proc. Natl. Acad. Sci. USA, **99**: 2146-2151.

TINGAY S., McELROY D., KALLA R., FIEG S., WANG M., THORNTON S., BRETELL R. (1997) *Agrobacterium tumefaciens*-mediated barley transformation. Plant J., **11**: 1369-1376.

VAN FLEET J. (2001): Production of recombinant human lysozyme and lactoferrin in transgenic barley grains. M.Sc. thesis. Washington State University. pp. 1-75.

VON BOTHMER R. (1992): The wild species of *Hordeum*: Releationships and potential use for improvement of cultivated barley. In: SHEWRY P.R.(ed.) Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology. CAB. Int. Willinford, UK: 3-18.

VON WETTSTEIN D., MIKHAYLENKO G., FROSETH J.A., KANNANGARA G., C., (2000): Improved barley broiler feed with transgenic malt containing heat-stable (1,3-1,4)-β-glucanase. Proc. Natl. Acad. Sci. USA, **97:** 13512-13517.

VON WETTSTEIN D., WARNER J., KANNANGARA C.G. (2003): Supplements of transgenic malt or grain containing (1,3-1,4)-β-glucanase to barley based broiler diets lift their nutritive value to that of corn. Brit. J. Poultry Sci., **44**: 438-449.

WAN Y., LEMAUX P.G. (1994): Generation of large numbers of independently transformed fertile barley plants. Plant Physiol., **104**: 37-48.

WANG M.B., ABBOTT DC., UPADHYAYA N.M., JACOBSEN J.V., WATERHOUSE P.M. (2001): *Agrobacterium tumefaciens*-mediated transformation of an elite Australian barley cultivar with virus resistance and reporter genes. Aust. J. Plant Physiol., **28**, 149-156.

WELLER D.M., COOK R.J., MACNISH G., BASSETT E.N., POWELSON R.L., PETERSEN, R.R. (1986): *Rhizoctonia* root rot of small cereals favored by reduced tillage in the Pacific Northwest. Plant Dis., **70**: 70-73.

WINDELS C.E. (2000): Economic and social impacts of Fusarium head blight: Changing farms and rural communities in the Northern Great Plains. Phytopath., **90**: 17-21.

WU Y. (2003): Transformation of barley for resistance to Rhizoctonia root rot. Ph.D. thesis. Washington StateUniversity, Department of Plant Pathology. P.1-97.

ZEILINGER S., GALHAUP C., PAYER K., WOO S.L., MACH R.L., FEKETE C., LORITO M., KUBICEK P. (1999): Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. Fung. Genet., **26**: 131-140.

S 5 – MALTING AND BREWING – TECHNOLOGICAL PARAMETERS

French Qualification of Malting Barley

P. Boivin[1], N. Ouarnier[1], P. Brignon[2] and J.-L. Delatte[3]

[1]Institut Français des Boissons de la Brasserie Malterie – 7 rue du Bois de la Champelle – 54512 VANDOEUVRE LES NANCY CEDEX – France
e-mail : patrick.boivin@ifbm-qualtech.com;
[2]TEPRAL-20 rue Jacob - 67200 STRASBOURG- France;
[3]MALTERIES SOUFFLET- 10 Quai du Général Sarrail – 10400 NOGENT SUR SEINE – France

Abstract
In France, the registration of a new barley variety is very structured. Three main organisations take part in the process. First, there is the CTPS (Permanent Technical Committee of Breeding) which is involved in the registration on the French catalogue and on the malting barley variety list. Then there is the CBMO (Beer Malt Barley Committee) which is involved in the testing for registration on the Malteurs and Brasseurs de France preferred variety list. Finally, IFBM is the technical tool for all tests (barley technical analyses, micromalting, pilot malting and brewing, and analyses on produced malts, worts and beers). IFBM is certified by BVQI for ISO9002 and Qualtech Laboratory is accredited for barley, malt and beer analyses by the French Accreditation Committee (COFRAC).
Such a system is aimed at improving barley varieties by selecting them according to measurable data. Choice criteria depend on the Maltster and Brewer needs taking expectations of all the barley-malt-beer chain into account.
The variety selection is based on barley technical analyses (moisture, proteins, extract, diastasic power and dormancy), Agronomic Index [AI] (yield, diseases…), Quality Index [QI] (extract, Kolbach Index, diastasic power, wort viscosity, final attenuation…), Functional Index [FI] (Modification, friability, β-glucans, α-amylase, FAN…), pilot productions from barley grown on the same location to beer (600 kg / 20 hl).
Each year an official list for malting barley is published by French brewers & Malsters associations to sum up the results of the work done.
The aim of this paper is to present the procedure to assess French malting barley.

Introduction
This text presents all the tests through which every new barley variety has to go to be registered on the "Preferred Variety List of Malteurs and Brasseurs de France".
A well structured system has been developed by all the barley-malt-beer chain partners in order to promote the cultivation of malting barley in France, to improve malting barley quality and to dispose of varieties meeting their expectations. Four years of testing are necessary. The main steps, which will be described in this text, are the following ones:

- registration on the French "catalogue"
- registration on the malting barley list
- functional evaluation
- technical evaluation
- commercial assessment

- registration on the "Preferred Variety List of Malteurs and Brasseurs de France".

Tests have to be simple, low time consuming, accurate and recognised by all the barley-malt-beer chain partners. All evaluations are based on objective criteria obtained by computations of reference analysis results. New barley varieties are compared to control varieties which were NEVADA and SCARLETT for spring barleys and ESTEREL and CLARINE for winter barleys for harvest 2002.

Three main organisations are involved in the system: CTPS, CBMO and IFBM.
CTPS is the permanent technical committee of breeding, which is an official instance. CBMO is the beer, malt and barley committee. This organisation comprised 16 members. There are 6 brewers representing the main French brewers (Kronenbourg, Heineken, Interbrew), 6 maltsters representing the main French maltsters (Cargill, Grandes Malteries Modernes, Malteries Franco-Suisses, Malteurop, Soufflet), 2 breeders and 2 members of IFBM. Breeders and IFBM are present as an advisory capacity. All decisions are taken by maltsters and brewers. CBMO is composed of 4 commissions. The agronomic commission evaluate agronomic criteria such as yield or disease resistances. Two technical commissions define malting and brewing processes. The technological commission evaluate analytical criteria assessed by IFBM. Other commissions can be created in necessary. A commission for beer sensory evaluation is in construction at the present time.
The figure 1 sums up the general running of a new barley variety evaluation. It reveals the relations between breeders, farmers, maltsters, brewers, Malteurs and Brasseurs de France and IFBM. IFBM is the technical instrument of the system. This institute performs micromaltings, pilot tests, analyses and index computations to give objective informations about new barley varieties to CTPS experts (barley analyses, quality index) and to CBMO members (functional index and pilot trials) who take decisions.

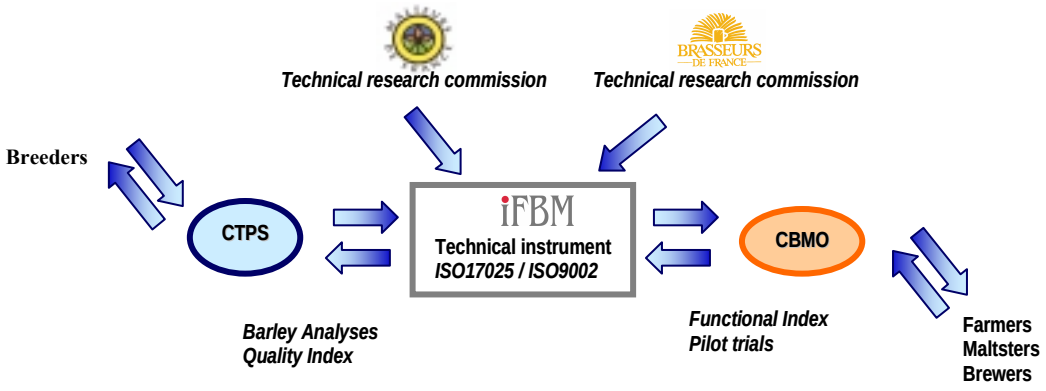


Figure 1 : General presentation

STEP 1: REGISTRATION ON THE FRENCH CATALOGUE LIST

The first requierement for a new barley variety is to be better than existing varieties. Tests are performed over two years. On one hand, DHS (Distinction, Homogenity, Stability) is evaluated to prove that the tested variety is different from existing varieties, homogeneous and constant. On the other hand, VAT (Agronomic and Technological Value) is assessed. The agronomic performance is measured by treated and non treated yield evaluation, disease resistance, precocity, or straw strenght asssessment. The technological performance is evaluated by analyses on harvested barley :

- protein content, sieving : 2 years on 8 experimental locations
- extract prediction, diastasic power : 1 year on 4 experimental locations
- dormancy (for winter barleys) : 2 years on 2 experimental locations.

STEP 2: REGISTRATION ON MALTING LIST

To test malting potentialities of a variety, a micromalting is performed on samples cultivated on 4 experimental locations over 2 years. Samples are the same as ones on which VAT analyses were performed. Produced malts are then analysed and Quality Index is assessed. Table 1 presents parameters taken into account for this index computation.

Table 1. Quality index parameters

Analyses	Contribution	Weight
Fine grind extract	+	0.40
Kolbach Index	+	0.15
Diastasic Power	+	0.15
Wort viscosity	-	0.15
Final attenuation	+	0.15

Contributions indicate the sign used in the computation formula. For example, a negative contribution is applied for wort viscosity. Indeed, the higher the viscosity, the worse the variety.

Too high kolbach indices are not better than too low kolbach indices. For this reason, before computation kolbach index is corrected. A variety with a kolbach index higher than the control mean will have a corrected kolbach index equal to the control mean minus a half point per each unit over the control mean.

The formula to assess quality index is given below :

$$QI = \frac{(ext - ext_{control}) \times 0.40}{1.24} + \frac{(KI_{corrected} - KI_{corrected-control}) \times 0.15}{4.19} + \frac{(DP - DP_{control}) \times 0.15}{52.30} - \frac{(visc - visc_{control}) \times 0.15}{0.10} + \frac{(FA - FA_{contrl}) \times 0.15}{1.50}$$

where :

ext = fine grind extract

$ext_{control}$ = fine grind extract of the control (s)

KI = kolbach index

DP = diastasic power

visc = wort viscosity

FA = final attenuation

The numbers 1.24, 4.19… can be considered as standard deviations calculated for numerous varieties, numerous experimental locations and numerous years.

In order to compare varieties to the control varieties, a statistical treatment is performed on the analytical results and on the quality indices.

Varieties are then classified into three classes :

Class A : QI significantly (α = 5 %) higher than QI of the controls

Class B : QI equivalent (α = 5 %) to the QI of the controls

Class C : QI significantly (α = 5 %) lower than QI of the controls

In addition, it exists eliminatory values for diastasic power (75 % controls for spring barleys – 85 % controls for winter barleys) and final attenuation (90 % controls).

Varieties of class A and B are registered on the malting barley list.

STEP 3: THE AGRONOMIC INDEX

Agronomic performances are also evaluated. Indeed, if a variety has a very good malting quality but a bad yield, farmers will not cultivate it.

Since barley is graded before malting, the criteria measuring agronomic performance is an economic yield taken sieving into account.

This economic yield (EcoY) is calculated as follows :

$$EcoY = TYgraded + 0.65 \times (TY - TYgraded)$$

Where

TY = treated yield

TYgraded = treated yield x grading > 2.5 mm

The term 0.65 x (TY – TYgraded) corresponds to the valorisation of small barley grains (< 2.2 mm).

The mean calculated for two years is compared to the control variety economic yield. A threshold is fixed which is around 105 % of the controls at the present time.

Another important parameter is the protein content. It has to be below 12 % and below 107 % of the control protein content. It is important to notice that the protein content should not be too low and a limit of 9 % is recommended.

Moreover the resistance to mosaic virus Y1 and M is also compulsory for winter barleys.

STEP 4: FUNCTIONAL EVALUATION

For varieties registered on the malting barley variety list, *ie* with QI of class A or B, and with good agronomic performances, a functional evaluation is then performed. Further analyses are carried out on malt produced for QI assessment. Table 2 sums up analyses performed, in regards to maltster and brewer expectations.

Tepral filtration test reveals very well malt behaviour in brewhouse. Indeed, temperatures are similar to those used in industrial brewing processes.

There are not indicators for foam and flavour stability because of a lack of analysis method for assessing such parameters in malt.

As for the quality index, a computation allows the assessment of the functional index. A statistical treatment of results is performed to compare varieties to controls for each parameter and for the functional index. Varieties are classified in three classes : A, B and C.

Table 2. Functional index parameters

Expectations - Analyses	Contribution	Weigth
Yield		
Malting yield		
Fine grind extract (QI)		
Modification		
Calcofluor modification	+	0.10
Calcofluor homogeneity	+	0.10
Friability	+	0.10
β-glucans	-	0.10
Kolbach index (QI)		
Saccharification		
Diastasic power (QI)		
α-amylase	+	0.10
Filtration		
TEPRAL filtration test		
- filtration speed	+	0.10
- time for filtering 350 g		
Viscosity (QI)		
Fermentation		
Free amino nitrogen	+	0.10
Final attenuation (QI)		
Colloidal stability		
Polyphenols	+	0.10
Hartong at 45 °C		

STEP 5: TECHNICAL EVALUATION

The technical evaluation is aimed at checking if a new malting barley does not cause process problems such as mash or beer filtration or fermentation difficulties. Moreover, since beer is produced, parameters such as foam, colloidal stability or organoleptic criteria can be assessed. Varieties undergoing this evaluation are chosen by the CBMO among varieties with good agronomic performance, quality index of class A or B and functional index of class A or B.

Each year 5 varieties are chosen. The repartition between spring and winter barleys is also decided by the CBMO.

Tests consists in pilot transformations. For spring barleys all the varieties under testing and a control are cultivated at the same location to avoid environmental effects. Winter barleys are supplied by breeders.

Each variety is malted three times at a 600 kg scale and brewed four times at a 17 hl scale. Transformations are carried out in the pilot plant of IFBM which is representative of what happens in industrial maltings and breweries. The facilities are certified ISO9002 and a technical audit is yearly performed by maltsters and brewers.

Analyses are performed on barleys, malts, worts and beers included sensorial analysis. Moreover, process parameters are measured : moisture control during malting, chitted grains, acrospire length, brewing yield, mash filtration time, fermentation speed, beer filtration parameters…

All the collected results are then analysed by the CBMO which decides to accept or to reject the variety.

If the variety is accepted by the commission, it is registered on the list of varieties under commercial observation.

The figure 2 sums up a new barley variety story from its born to its registration on the preferred barley variety list of Malteurs and Brasseurs de France.

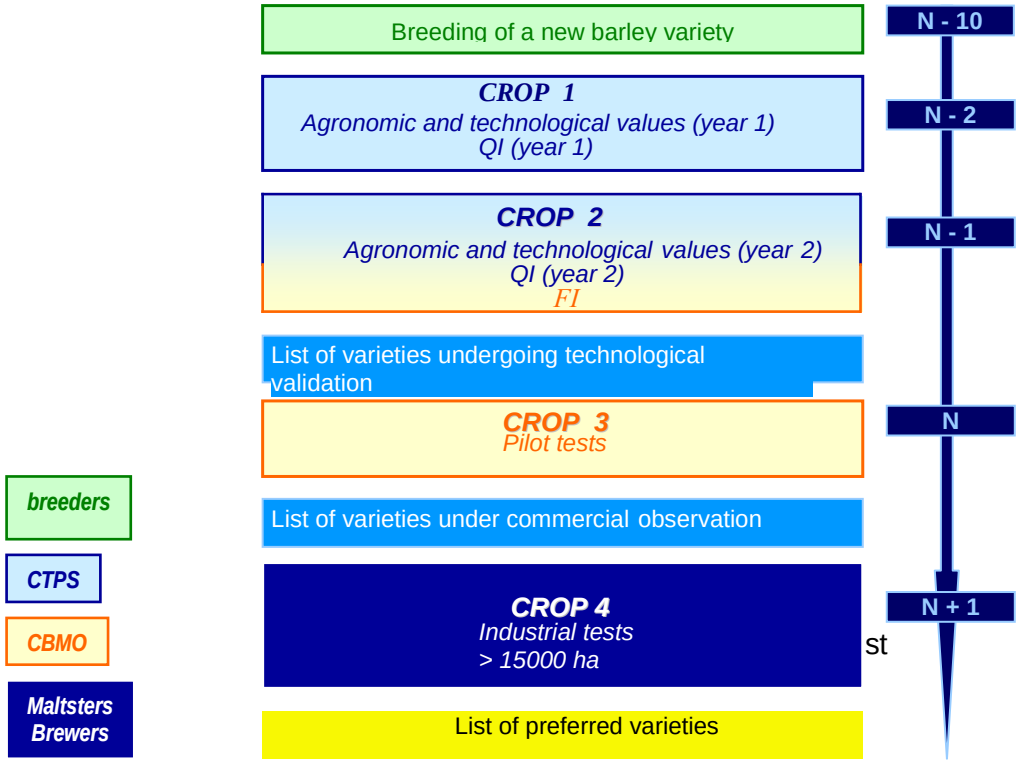


Figure 2. A new barley variety story

Conclusion

The French system is complete and takes most of farmer, maltster and brewer expectations into account. To be registered on the list of preferred varieties, a new barley has to pass lots of tests to prove its technological quality. For Harvest 2003, the list is presented in figure 3.

PREFERRED VARIETY LIST OF MALTEURS AND BRASSEURS DE FRANCE
MALTING BARLEY - HARVEST 2004

SPRING BARLEYS		WINTER BARLEYS	
		2 ROWS	6 ROWS
PREFERRED VARIETIES			
For multi purposes	For specific use	VANESSA	ESTEREL
ASPEN / CELLAR / SCARLETT	ASTORIA		
VARIETIES UNDER COMMERCIAL OBSERVATION			
CEYLON / COUNTY / PEWTER / PRESTIGE / RANGOON		BOREALE	
VARIETIES UNDERGOING TECHNOLOGICAL VALIDATION			
CARAFE / CHRISTINA / DOYEN / SEBASTIAN		NECTARIA / SUNBEAM	REGALIA

*** For multi purposes** : suitable for most brewer specifications.
*** For specific use** : suitable for some brewer specifications.
Varieties under commercial observation : Varieties that have passed IFBM pilot tests and evaluated on an industrial scale in order to verify that they meet all brewer production expectations.
This period should allow the variety commercial development.
Varieties undergoing technological validation : Varieties recently registered on the CTPS malting variety list and proposed by the CBMO to be tested on a pilot scale at IFBM.

MALTEURS DE France - 66, rue de La Boétie - 75008 PARIS - TEL. 01 43 59 44 93 - FAX. : 01 45 63 00 70 - malteursf.s.lecocq@wanadoo.fr

BRASSEURS DE FRANCE - 25, boulevard Malesherbes - 75008 PARIS - TEL. 01 42 66 29 27 - FAX. 01 42 66 07 66 - contact@brasseurs-de-france.com

Figure 3. Preferred variety list of Malteurs and Brasseurs de France

Environmental Modulation of Barley Malting Quality

J.L. Molina-Cano[1*], J.S. Swanston[2], M. Moralejo[1], J.P. Polo[1], A. Rubió[3] and A.W. MacGregor[4]

[1]Centre UdL-IRTA, Alcalde Rovira Roure 198, 25198 Lleida, Spain;
[2]Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, United Kingdom;
[3]Damm Brewing Company, Malthouse, Bell-lloc, Lleida, Spain;
[4]Retired from Grain Research Laboratory, Winnipeg, Manitoba, Canada
* E-mail: joseluis.molina@irta.es

Abstract
Experimental procedures aimed at maximising environmental differences in barley growing conditions in temperate regions were used to study their influence on malting quality. The mutant *TL43* and its parent genotype *Triumph* were grown in different seasons at Lleida (Spain) and Dundee (UK). The mutant showed higher protein and C- and D-hordein contents in all environments, but its B-hordein content showed crossover GE (genotype x environment) interaction, being higher than *Triumph* in Scotland and lower in Spain. When studying water uptake, it was concluded that although influenced by both genotype and environment, the environmental effect was higher, and this was linked to hordein composition. A reappraisal of the differences between Iberian- and Nordic-grown barleys was carried out with the malting cultivar *Scarlett*. Barley total and insoluble β-glucans were an effective barrier to modification in the Nordic-, but appeared to increase extract in the Iberian-grown barleys. This apparent positive effect of β-glucans was considered a consequence of their greater capacity to synthesise and release β-glucan hydrolases during germination, so increasing total soluble material. Another study was carried out to analyse the differences between Spanish- and Canadian-grown barleys, for which four cultivars were used. Canadian-grown barleys had significantly lower contents of grain protein and all three hordein fractions than the Spanish ones. They had also higher malt yield, wort β-glucan and viscosity but lower fine- and coarse-ground malt extract, friability, free amino nitrogen, Kolbach index, α-amylase and diastatic power. The higher extract of the Spanish-grown barleys was attributed to their capacity to produce higher quantities of endosperm-degrading enzymes during germination.

Introduction

Together with germination properties, barley protein content has been considered the main economic malting quality factor since the original work of BISHOP (1930). Recently, however, the relationship between protein and quality has come under scrutiny, when barley genotypes have been submitted to very contrasting environments, usually northern v southern Europe and, in particular, NE Spain v E Scotland (MOLINA-CANO *et al*. 1995; ELLIS *et al*. 1997; SWANSTON *et al*. 1997). It is the hordeins, major storage proteins accounting for up to 60% of total grain protein (BRENNAN *et al*. 1998), that are largely responsible for the negative correlation between malt extract and barley protein content. They are classified into four groups and encoded by the genes Hor 2 (B-fraction), Hor1 (C- fraction), Hor3 (D-fraction) and Hor5 (γ-fraction), located on barley chromosome 5(1H) (KREIS & SHEWRY 1992). The B- and C-fractions account for 70-80% and 10-20%, respectively, of the total hordein, while the D and γ groups are quantitatively minor components.

The precise role of these hordein components is subject to debate and this may reflect differences in germplasm and/or environments. PELTONEN *et al*. (1994) found that the B

fraction had some effect on malting quality by regulating diastatic power, which was decreased by a higher proportion of D-hordein. In Australia EAGLES *et al*. (1995) showed seasonal differences in malt extract levels that could not be entirely explained by differences in protein concentration, while HOWARD *et al*. (1996) found a negative correlation between D hordein and malting quality. However this was not supported by findings from SHEWRY *et al.* (1997), in the UK, using pairs of near-isogenic barley lines with and without D-hordeins.

It thus appeared that the differential effects of barley hordein fractions on malt extract were subject to strong environmental influence. This was investigated by MOLINA-CANO *et al*. (2000), who compared samples of the malting barley cultivar *Alexis* from Scandinavia and the Iberian Peninsula and found different mechanisms for extract development. When barley protein content, especially the B-hordein fraction, increased, the resultant decrease in malt extract was significantly greater in the Nordic samples, compared to those from the Iberian Peninsula. The use of total grain protein content as a predictor of malting quality was therefore considered inadequate to fully account for the different malting behaviour of northern and southern European barleys.

High levels of β-glucan have also been regarded as deleterious to malting quality, as they may reduce the rate of endosperm modification (MARTIN & BAMFORTH 1980), and residual cell walls are a barrier to the enzymes involved in protein and starch hydrolysis during malting and mashing. In addition, high levels of β-glucan in the wort cause increases in viscosity, which may lead to filtration problems (BAMFORTH & BARCLAY 1993). Barley β-glucan exists in two forms, classed according to solubility in water, which can be assessed separately by an enzymic method (AMAN & GRAHAM 1987). SWANSTON *et al*. (1997) found differences in the pattern of β-glucan deposition during grain filling between barleys grown in Spain and Scotland, with Spanish-grown samples having higher levels of total β-glucan but lower levels of the insoluble fraction.

In the present paper we report on results from three experiments carried out to improve knowledge of environmentally induced differences in barley quality. Comparisons of Spanish and Scottish growing conditions have continued, using the mutant *TL43* and its parent, *Triumph*, a high-quality malting cultivar from the former German Democratic Republic. MOLINA-CANO *et al*. (1999) induced this mutant with greatly reduced dormancy, compared to its parent, under Scottish conditions and less sensitivity to exogenous ABA. *TL43* also had higher levels of β-amylase activity than *Triumph*, due, in part, to higher grain nitrogen (SWANSTON & MOLINA-CANO 2001), but no data on storage proteins was presented. In addition, experimental procedures have been set up aimed at maximising the environmental differences to which barley can be subjected in temperate regions. Comparisons of Northern and Southern Europe were continued, using the cultivar *Scarlett*, while four further cultivars were utilised to compare Spanish and Canadian environments. The contrasting growing regimes of Northern and Southern Europe and of Canada were exemplified by the differences in sowing time (spring in Scandinavia and Canada and autumn/winter in the Iberian Peninsula). Consequently, heading time (early summer in Scandinavia and Canada and spring in the Iberian Peninsula) and maturity (late summer in the northern region and early summer in the South) also differed. The grain development period is thus more humid, with milder temperatures, in Scandinavia and Canada than in the Iberian Peninsula, although the extent of the variation within regions may be fairly large.

Material and Methods

The spring malting barley mutant *TL43* and its parent cultivar, *Triumph*, were grown in replicated field trials at Dundee, Scotland in 1997 and 1999, and Lleida, Spain, in 1997, 1998 and 1999. Kjeldahl grain protein (GP) and HPLC hordein analyses were carried out, assessing B-hordein (BH), C-hordein (CH) and D-hordein (DH) (MOLINA-CANO *et al.* 2001), as well as water uptake studies using starch gelatinisation and image analyses (MOLINA-CANO *et al.* 2002a). The ultra structure of the barley endosperm was studied on halved kernels with SEM (scanning electron microscopy) and on thin sections of the grains with silver staining used to enable immunolocalisation of monoclonal antibodies raised against B-hordein.

For the second experiment, 25 samples of the cv. *Scarlett* were collected in Scandinavia (Denmark 10, Finland 15) and 16 in the Iberian Peninsula (15 Spain, 1 Portugal) in 1998 and 1999. Grain protein and β-glucan analyses, and HPLC hordein fractionation were performed on barley grain, and then, micromalting and malt analyses were carried out (MOLINA-CANO *et al*. 2002b). Replicated trials were also laid out in Lleida, Spain and Saskatchewan, Canada in 1998 and 1999 for the third experiment, attempting to produce both high and low protein grain. Initially, four malting varieties were used, *Alexis* (European two-rowed), *Harrington* (Canadian two-rowed), *Stander* (US six-rowed) and the Spanish cultivar *Dobla* (six-rowed). *Dobla* was, however, eliminated from the experiment because its poor adaptation to Canadian growing conditions. Samples of barley taken from each field replication were micromalted. Barley and malt analyses, including, barley protein and hordein fractionation by HPLC, and fine- and coarse-grind extracts, wort soluble protein, wort β-glucan, viscosity, friability and levels of diastatic power and α-amylase, were carried out (MOLINA-CANO *et al.* 2004).

Results and Discussion

Differences between Spanish and Scottish Barleys

Analysis of variance (data not presented) showed significant G (genetic), E (environmental) and GE (genotype x environment) effects for all the measured parameters: GP, BH, CH and DH, except BH that did not show significant genetic effects. The E effect was always larger than the G effect, except for CH, while the largest GE was by far for BH, and it was of crossover type. The mutant TL43 showed about 2 per cent higher GP than Triumph across

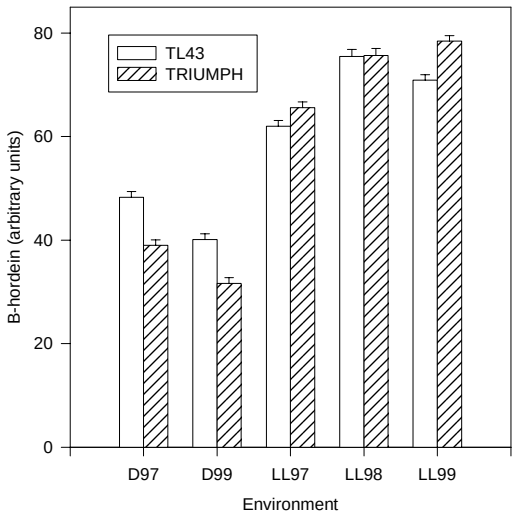


Figure 1. Means of B-hordein content (arbitrary units) of two barley genotypes, the malting cultivar Triumph and its mutant TL43, at five environments in Lleida (NE Spain) and Dundee (NE Scotland). Bars are SE values for $p<0.05$.

the five environments, and GP was always higher at the Spanish than at the Scottish site. The mutant also showed higher CH and DH than the parent variety in each environment, data that

paralleled those of GP in so far as the Spanish environments had always higher CH and DH than the Scottish ones. The most interesting result was, however, that of BH (Fig 1) because *TL43* had higher content than *Triumph* in Scotland but lower in Spain, i.e. there was crossover interaction. The Spanish environments always showed higher BH than their Scottish counterparts.

The endosperm ultra-structure, as assessed by SEM, showed that the mutant had a more compacted sub-aleurone and inner endosperm than *Triumph*, and this might reflect its higher GP content. Moreover, *TL43* had tighter packing of the starch granules into a more dense protein matrix. The immunolocalisation showed that the sub-aleurone layer of *TL43* had less BH deposition than its counterpart in *Triumph*. Water uptake studies showed that in 1997 *TL43* absorbed more water than *Triumph* at both sites despite having higher GP and hordein content, however it had lower BH content. With the different steeping regime of 1999, incorporating longer air rests, water uptake of both *TL43* and *Triumph*, grown in Spain, was very high. Conversely, Dundee-grown *Triumph* showed greater water uptake than *TL43*, but poorer distribution in the endosperm. This could be attributed to the high BH in the sub-aleurone region of *Triumph*, which created an effective barrier to initial hydration of the endosperm. However, subsequent hydration was more effective in *Triumph* than *TL43*, due to the less compacted structure of its inner endosperm. NMR imaging showed that hydration in the mutant spread throughout the sub-aleurone layer before moving to the inner endosperm, but in *Triumph* proceeded on a broader front from the scutellum towards the distal parts of the grain. A general conclusion was that water uptake was more dependent on the environment, though it had a clear genetic influence. The most important structural factor seemed to be BH content, whereas β-glucans, previously implicated in water uptake, had seemingly little effect.

Differences between Iberian and Nordic Barleys

Equivalent mean levels were attained in both regions for most of the barley and malt quality parameters. However, the Scandinavian samples, when compared with their Mediterranean counterparts, had slightly higher malt extract (82.46 vs. 81.62%), higher levels of total barley (4.48 vs. 4.12%) and malt (1.53 vs. 1.06%) β-glucans, but the relative proportion of total barley β-glucans digested during malting, was significantly smaller (66.92 vs. 75.79%). The incomplete digestion of β-glucans led to a higher viscosity in the Nordic worts (1.60 vs. 1.51 mPa.s). These results suggest a higher β-glucanase activity in the Iberian barleys, and, thus, a more thorough cell wall modification. The protein degradation of the Nordic malts was more limited than in the Iberian ones, with lower wort soluble protein (4.94 vs. 5.30%) and Kolbach index (44.71 vs. 45.57). Despite the higher proportion of proteinaceous material in the Iberian worts, Nordic samples also showed lower fermentabilities, as apparent final attenuation was lower (82.51 vs. 83.73%). These data suggest that differences in wort dextrin profile may result from more limited amylolytic activity in the samples from northern Europe.

The above results suggest that similar extract levels may be obtained by different mechanisms in the two groups of barleys. The environment may thus induce differences in the metabolic pathways that affect germination, these differences being of a quantitative type, i.e. linked to the speed of germination and, therefore, to the quantity of enzyme produced at the end of it. To explore the origin of these differences, in Fig 2 the principal component analysis graphs are presented. In both cases, higher malt extract yields were associated with the negative direction of Component 1, while barley protein increased in the opposite direction. Malt extract increased with increases in Kolbach index, apparent final attenuation, B/C hordein ratio and β-glucans degradation. Conversely, it decreased when barley protein, D-hordein, malt total β-glucans and viscosity increased.

There is, however, a very clear difference between Scandinavian and Iberian barleys in Fig 2, i.e. the opposite way in which total barley β-glucans and its insoluble fraction affect malt

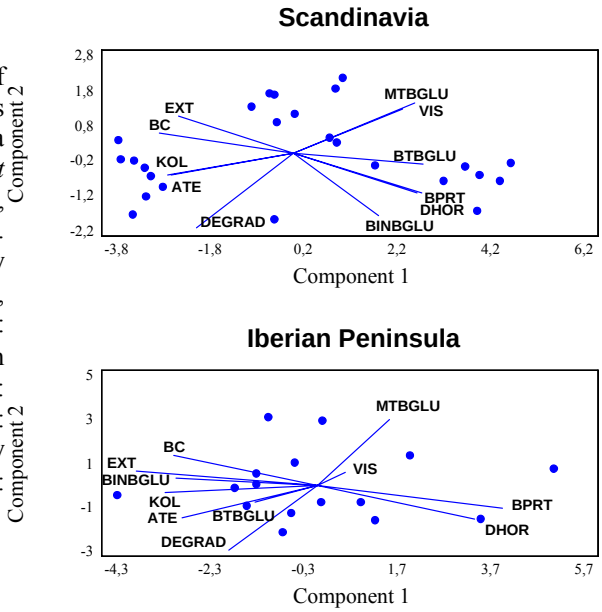


Figure 2. Principal component analysis byplots of several quality parameters in *Scarlett* barley samples from Scandinavia (top) and the Iberian Peninsula (bottom). The points represent the different *Scarlett* samples, and the lines are the principal vectors, whose projection on each axis is its loading on it. EXT: malt extract yield (%), BINBGLU, barley insoluble β-glucans (%), KOL: Kolbach index (%), ATE: apparent final attenuation (%), BTBGLU: barley total β-glucans (%), DEGRAD: β-glucan degradation (BTBGLU-MTBGLU, %), DHOR: barley D-hordein content (arbitrary units), BPRT: total barley protein (%), VIS: wort viscosity (mPa.s), MTBGLU: malt total β-glucans (%), BC: ratio of barley B to C hordein.

extract. They are positively associated with increases in extract in the Iberian samples, but cause decreases in Scandinavia. Additionally, there is a quantitative difference between these barleys with regard to wort viscosity. It had a greater negative effect on malt extract in the North than in the South, indicating less complete β-glucan hydrolysis there. These results may be explained by the enzymatic differences between these groups of barleys. Thus, in the South, the higher total and, particularly, insoluble barley β-glucan content may have contributed positively to increases in extract because they are degraded to a large extent and add fermentable material to the wort. Barley β-glucan content would not, however, be a positive trait unless it would be linked to a high β-glucanase activity, but depending on cultivar, the environment may modify extract through the quantity of β-glucans synthesised and the differential development of enzymes able to degrade them into fermentable sugars. The products of cell wall modification could thus enhance the final extract.

Differences between Spanish and Canadian Barleys

The barley grain grown in Canada (data not presented) had significantly lower grain protein and hordein levels as well as higher malt yield (MY), wort β-glucans (WBG) and viscosity (VIS) but lower fine-ground extract (FEX), coarse-ground extract (CEX), friability (FRI), free amino nitrogen (FAN), Kolbach index (KOL), α-amylase (AMY) and diastatic power (DP). Overall, the Spanish-grown barleys, despite having higher protein and hordein levels, produced higher quantities of starch degrading enzymes during germination, thus being able to attain higher FEX levels, better modification and higher FRI and CEX, although this could be limited by their sugar and starch contents. (MOLINA-CANO *et al.* 2004). This is clearly shown by their higher levels of FAN, KOL, AMY and DP although the KOL and FAN levels are somewhat high.

These differences have been explored in more detail with the aid of principal component analysis (PCA). Plots of all of these data on the Spanish-Canadian barley quality differences are presented in Figs 3a and b. In Fig 3a, projection of the points are shown, each representing a variety x country value. On PCA axes 1 and 2, the arrow represents the direction of increase in: FEX, CEX, FRI, FAN, KOL, AMY (on PCA axis 1), and BPT, CH, FAN and DP (on PCA axis 2). PC1 measures the result of germination in terms of endosperm degradation and

extract development, i.e. protein and carbohydrate solubilisation. Both extract estimates (FEX and CEX) increase toward the negative side of the axis, as do FRI, FAN, KOL, and AMY, whereas MY, WBG, and VISC increase in the positive side. PC2 is related to barley protein and hordein C, and their relationship with endosperm degradation (friability, FRI), amino acids available for yeast growth during fermentation (free amino nitrogen, FAN) and diastatic power. Parameters BPT, CH, FAN and DP lay on the negative side of this axis, while FRI and hordein ratio BH/CH lay on the positive side. The loadings of BPT and CH were much larger than those of the other parameters.

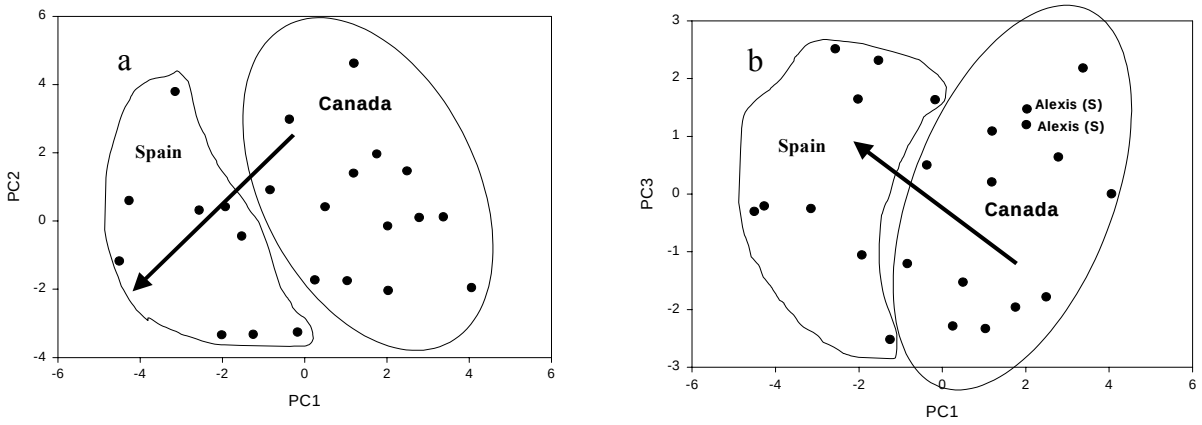


Figure 3. Principal component analysis (PCA) graph. (a): Projection on the plane determined by the first two PCA axes, which account for 60.8% of the total variance, of the points representing the variety x country data that are clustered by country and (b) by the first and third PCA axes, which account by 48.5% of total variance .

The meaning of the graph is that the Spanish-grown samples, albeit with higher BPT and CH than their Canadian counterparts, also have higher FEX, CEX, FRI, FAN, KOL, AMY and DP. It should be pointed out, though, that *Alexis* has behaved in a somewhat inconsistent way across the experiment. In Fig 3b, we show the projection of the points on PCA axes 1 and 3, where the arrow marks the increase of: FEX, CEX, FRI, FAN, KOL, AMY (on PCA axis 1), and BH, DH and FRI (on PCA axis 3). PC3 is almost a pure hordein axis, related to hordein B and D and, again, friability. Parameters BH/DH and CH/DH lay on the negative side of this axis; BH, DH and also FRI lay on the positive side but FRI has only half the loading of the hordein fractions. The results show that the Spanish-grown barleys, despite having higher BH and DH content (on PCA axis 3), showed higher FEX, CEX, FRI, FAN, KOL and AMY (on PCA axis 1) than the Canadian barley samples.

Genetic and Environmental Effects on Malting Quality

The experiments reported here reveal both similarities and differences with previous work and reinforce the view that good malting quality may be reached by several routes. The data obtained with cv. *Scarlett* did not support the previous view (MOLINA-CANO *et al*. 2000) that differences between Nordic and Iberian malting barleys lay in their response to protein content, in particular B-hordein, indicating, instead, that cell wall breakdown could be a limiting step in the Nordic-grown material. This is similar to earlier results comparing Scottish and Spanish-grown barley (ELLIS *et al*. 1997), so may reflect genotypic differences between cvs. *Scarlett* and *Alexis*. The particular characteristics of *Alexis* may also explain its apparently anomalous performance in the comparison between Spanish and Canadian grown barley. The division of the two sites along the PC1 axis (Figs. 3a and b) suggests that the higher extracts of the Spanish-grown material may relate to more extensive modification, so again cell wall breakdown may be a limiting factor in the genotypes other than *Alexis*. The

association of hordein fractions B and D with PC3 (Fig. 3b) suggests that high levels of B hordein are again the limiting factor for cv. *Alexis*.

Comparison of Spanish-grown *Triumph* and *TL43* (above) indicated differences in water uptake and distribution through the endosperm. High levels of B-hordein were postulated as a reason for slow, initial hydration in *Triumph*, so a similar problem may exist in *Alexis*. The work with *Triumph* and its mutant gave different results to previous comparisons between Scottish and Spanish growing conditions (MOLINA-CANO *et al.* 1995) in that β-glucan was not implicated in effects on water uptake. It is clear, however, that in all cases Spanish-grown material exhibits fast and extensive enzyme production. It is therefore possible that extract levels may be enhanced by the products of cell wall breakdown (MOLINA-CANO *et al.* 2002), although it may be that higher β-glucan levels are necessary to reduce a tendency to excessive modification of the endosperm during malting.

Genotypic and environmental factors both play a significant role in defining malting quality and this is potentially problematic in selecting cultivars suited to a wide range of environments. Selecting optimal genotypes for a given location requires knowledge of how that location will affect grain composition and modification during malting and understanding of which genotypic features will enable these effects to be best exploited.

References

AMAN P., GRAHAM H. (1987): Analysis of total and insoluble mixed-linked (1-3),(1-4) β-D-glucan in barley and oats. J. Agric. Food Chem., **35**:704-709.

BAMFORTH C.W., BARCLAY A.H.P. (1993): Malting technology and the use of malt. In: MacGregor A.W., Bhatty R.S. (eds.): Barley, Chemistry and Technology. AACC, St Paul, MN: 297-354.

BISHOP L.R. (1930): The Institute of Brewing Research Scheme. I. The prediction of extract. J. Ins. Brew., **36**: 421-434.

BRENNAN C.S., SMITH D.B., HARRIS N., SHEWRY P.R. (1998): The production and characterisation of Hor 3 null lines of barley provides new information on the relationship of D hordein to malting performance. J. Cereal Sci., **28**:291-299.

EAGLES H.A., BEDGGOOD A.G., PANOZZO J.F., MARTIN P.J. (1995): Cultivar and environmental effects on malting quality in barley. Australian J. Agric. Res., **46**: 831-844.

ELLIS R.P., SWANSTON J.S., RUBIÓ A., PÉREZ-VENDRELL A.M., ROMAGOSA I., MOLINA-CANO J.L. (1997): The development of β-glucanase and degradation of β-glucan in barley grown in Scotland and Spain. J. Cereal Sci., **26**:75-82.

HOWARD K.A., GAYLER K.R., EAGLES H.A., HALLORAN G.M. (1996): The relationship between D hordein and malting quality in barley. J. Cereal Sci., **24**: 47-53.

KREIS M, SHEWRY P.R. (1992): The control of protein synthesis in developing barley seeds. In. SHEWRY P.R. (ed): Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology. CAB International. Wallingford.:319-334.

MARTIN H.L., BAMFORTH C.W. (1980): The relationship between β-glucan solubilase, barley autolysis and malting potential. J. Ins. Brew., **86**: 216-221.

MOLINA-CANO J.L., RAMO T., ELLIS R.P., SWANSTON J.S. BAIN H., URIBE-ECHEVERRÍA T., PÉREZ-VENDRELL A.M. (1995): Effect of grain composition on water uptake by malting barley: a genetic and environmental study. J. Ins. Brew., **101**: 79-83.

MOLINA-CANO, J.L., SOPENA, A., SWANSTON, J.S., CASAS, A.M., MORALEJO, M.A., UBIETO, A., LARA, I., PÉREZ-VENDRELL, A.M., ROMAGOSA, I. (1999): A mutant induced in the malting barley cv. Triumph with reduced dormancy and ABA response. Theor. Appl. Genet., **98**: 347-355.

MOLINA-CANO J.L., POLO J.P., SOPENA A., VOLTAS J., PÉREZ-VENDRELL A.M., ROMAGOSA I. (2000): Mechanisms of malt extract development in barleys from different European regions: II. Effect of barley hordein fractions on malt extract yield. J. Ins. Brew., **106**:117-123.

MOLINA-CANO J.L., POLO J.P., ROMERA E, ARAUS J.L., ZARCO J., SWANSTON J.S. (2001): Relationships between barley hordeins and malting quality in a mutant of cv. Triumph. I. Genotype by environment interaction of hordein content. J. Cereal Sci., **34**: 285-294.

MOLINA-CANO J. L., SOPENA A., POLO J.P., BERGARECHE C., MORALEJO M.A., SWANSTON J.S., GLIDEWELL S.M. (2002a): Relationships between barley hordeins and malting quality in a mutant of cv. Triumph. II. Genetic and environmental effects on water uptake. J. Cereal Sci., **36**:39-50.

MOLINA-CANO J.L., ROMERA E., AIKASALO R., PÉREZ-VENDRELL A.M., LARSEN J., RUBIÓ A. (2002b): A reappraisal of the differences between Scandinavian and Spanish barleys: Contribution of β-glucan degradation to malt extract yield in the cv. Scarlett. J. Ins. Brew., **108**: 221-226.

MOLINA-CANO, J.-L., POLO, J.P., ROMAGOSA, I., MACGREGOR, A.W. (2004): Malting behaviour of barleys grown in Canada and Spain as influenced by hordein and enzyme content. J. Ins. Brew., **110** (in press).

PELTONEN J., RITA H., AIKASALO R., HOME S. (1994): Hordein and malting quality in northern barleys. Hereditas, **120**: 231-239.

SHEWRY P.R., BRENNAN C., TATHAN A.S., WARBURTON T., FIDO R., SMITH D., GRIGGS D., CANTRELL I., HARRIS N. (1997): The development, structure and composition of the barley grain in relation to its end use properties. In: WRIGLEY C.W. (ed) Proceedings of the 46th Australian Cereal Chemistry Conference. Cereal Chemistry Division, Royal Australian Chemical Institute, North Melbourne: 158-162.

SWANSTON J.S., ELLIS R.P., PÉREZ-VENDRELL A., VOLTAS J., MOLINA-CANO J.L. (1997): Patterns of barley grain development in Spain and Scotland and their implications for malting quality. Cereal Chem., **74**: 456-461.

SWANSTON J.S., MOLINA-CANO J.-L. (2001): Beta-amylase activity and thermostability in two mutants derived from the malting barley cv. Triumph. J. Cereal Sci., **33**: 155-161.

Barley Varieties Suitable for the Production of a Czech Type Beer

K. Kosař, V. Psota and A. Mikyška

Research Institute of Brewing and Malting, PLC, Malting Institute, Brno,
Mostecká 7, 614 00 Brno, Czech Republic

Abstract
In the course of the 20th century the production of beer was substantially changed. Economic pressure led to concentration, modernization and automation of the beer and malt production. These changes were reflected in a new view of the malting barley varieties' quality. In two last decades varieties with a strong enzymatic activity, high extract content and high final attenuation have been promoted. Due to historical reasons the traditional production of pale lager has been preserved in the Czech brewing. For the production of a Czech type beer, those varieties of malting spring barley are suitable that enable the production of beer with a higher level of residual extract, strong palatefulness, excellent foaming power and relatively lower alcohol content.
Keywords: barley; malt; Czech type beer

The tradition of barley growing in the region of the Czech Republic is connected with the tradition of brewing which was documented in this country already in 993. Brewing and barley growing has, however, older roots as in our region beer was brewed before the arrival of the Slavs by our predecessors, by the Gaelic Boii and German Markomans.

From the European and world point of view, contribution of our barley-growing can be seen mainly in the targeted and historically very early breeding. In its scope genetically unique regional materials from Bohemia and Moravia were used. Systematic breeding in our countries was introduced by Emanuel Proskowetz, who in the 70s of the 19th century was improving the regional barley of old-haná agroecotype. As a result a world-known variety Proskowetz Haná pedigree was formed. Other varieties that achieved the world reputation were Kneifl's barley, Valtice and first of all the variety Diamant that became a world donor of short-stems at high and balanced malting quality.

The current world assortment of beers includes a much varied set of drinks. Local customs decide on the type of beer drunk in the given region. Nevertheless certain general dependencies related to the quantity of beer consumed in the given country or region per person can be traced. At the low annual beer consumption when beer serves only for extinguishing physiological thirst, the type of beer does not principally matter. High annual consumption of this drink per person, however, relates to certain specific features of beer. Even old Czech maltsters claimed that good beer must after consummation urge to another drink. Though it is evident that the amount of beer consumption is influenced by a number of factors, influence of a type of consumed beer in no case can be eliminated.

In the 19th century, in the area of the today's Czech Republic, a type of beer indicated as Czech or Pilsen was developed. Its success-local and all over the world-cannot be connected only with business abilities of salesmen and efficiency of advertisement. Today in days of mass media a consumer can be temporarily influenced by advertisement, in the nineteenth century, however, this influence was evidently lower and reputation of a Czech type beer must be greatly ascribed to its ability to urge a consumer to have another drink. This type of

beer still prevails in this country and high consumption of beer per person and year corresponds to it.

Majority of lagers produced in Western Europe and North America are originally derived from a Czech type beer and in numerous cases also indication "pils" is used, in reality however, it differed basically from this type. The principal representative of a Czech type beer is in a traditional labelling 12° pale larger. Its most significant characteristics are strong intensity of bitterness with a slow fading of a bitter taste. A Czech type beer should also have a weak odour intensity, strong palatefulness and sharpness, excellent foaming power and relatively lower content of alcohol (ethanol). A Czech type beer has won its high popularity all over the world mainly because it respects human physiology very well. The basic effective component of taste is the above-mentioned strong but for a longer time dying away bitterness stimulating the activity of main sialadens and also other parts of the digestive system, it evokes thirst and supports intake of solid food. When the beer is strongly bitter, it must be palateful too as strong bitterness in beer of empty flavour unpleasantly excels. Therefore a Czech type beer must be rather less fermented. Thus it also has a relatively lower content of alcohol and is less intoxicating. Therefore it can be consumed in a bigger quantity.

For the historical reasons, in a long period of the years 1938 – 1990, the Czech breweries could not buy new technologies, which is the main reason why Czech beer is produced in all Czech breweries by the classical decoction technology of mashing, long hop boiling and bottom fermentation at low temperatures. At the same time the Czech brewing industry also avoided drastic cost reduction dominating all advanced brewing countries since the 60s. Decrease of production costs has brought to these breweries an aggravated sensorial character with the consequence of lowered drinkability of beer.

In 1996 the RIBM began a project with the aim to preserve a sensorial character of a Czech type beer also under the new economic conditions. Today we are aware of the immense effect of the beer production technology on its sensorial character and drinkability. The sensorial beer character is influenced by the floor or pneumatic malting technology. We know how great effect the used barley variety has. In the scope of this research we succeeded in characterizing beer from the aspect of the production technology and also from the chemical aspect.

The current requirements on the quality of the malting barley prefer only the varieties with a strong enzymatic activity, high content of extract and high values of final attenuation. On the contrary, lower final attenuation bringing residual extract is characteristic for a Czech type beer (lager). For the above-mentioned reasons for preserving a Czech type beer, the basic features were characterized the malting barley variety suitable for a Czech type beer should have.

Based on the results of the above-mentioned research and based on the technological characteristics of older varieties of malting barley and current assortment of barley varieties, values of individual basic technological features of barley varieties suitable for the production of a Czech type beer were proposed.

Extract in malt d.m. (%)	min.	81.5	%
Relative extract at 45 °C	max.	38.0	%
Kolbach index		39.0 ± 1	%
Diastatic power	min.	220	u.WK
Final attenuation	max.	80.0	%
Friability	min.	75.0	%
β-glucans in wort	max.	250	mg/l

At the same time the brewing tests were conducted in some of the wide spread varieties of the current assortment of Czech spring barley malting varieties. The varieties Jersey and Prestige are typical modern varieties with high extract, low portion of non-fermentable extract, lower content of proteins and low colour which probably together results in lower foaming power, colour, weak trend to a lower palatefulness of beers and a weak trend to a higher diacetyl production. In comparison with the variety Kompakt, fresh beers from the varieties Jersey and Prestige had a worse evaluation of the total subjective impression after drinking. These varieties are more suitable for intensified beer production, especially of dispensed beers. The advantage of the variety Jersey is a high colloid beer stability. The advantage of the variety Prestige is a very good sensorial stability.

Kompakt is the most suitable variety of all tested varieties from the point of view of the beer quality (colour, foaming power, palatefulness and total subjective impression after drinking). Good colloid and sensorial beer stability belong to further advantages of the variety Kompakt. For newly bred domestic varieties the variety Kompakt can be recommended as a certain example. The variety of barley suitable for the production of a Czech type beer.

References

PSOTA V., JUREČKA D. (2003): Barley varieties registered in the Czech Republic in the year 2003. Kvasny Prum., **49**: 142–146.

ANONYMOUS (2003): Committee for quality evaluation of malting barley varieties at RIBM, PLC. Kvasny Prum., **49**: 73–74.

MIKYŠKA A. (2003): Hodnocení pivovarské kvality nových odrůd sladovnického ječmene [Evaluation of brewing quality of new malting barley varieties], Final Report RIBM, Praha (in Czech).

Are We Any Closer to Predicting How Well a Malt Will Behave in the Brewhouse?

E.D. Baxter, C.D. Booer and A. Faulkner

Brewing Research International, Lyttel Hall, Nutfield, Surrey, RH1 4HY, United Kingdom

Abstract
The ease and efficiency with which a malt can be processed in the brewery is an important factor in the choice of barley variety and of malt suppliers. Malt specifications cover many quality parameters but prediction of brewhouse behaviour remains elusive, in spite of the number of predictive tests which have been developed. In this study the brewhouse performance of six commercial malts of similar specification was determined and compared with the results obtained by the same malts in a range of predictive tests and quality analyses. Malts were scored for ease of lautering, fermentation behaviour and yield of ethanol, using the pilot brewery at Brewing Research International (BRi). Although the malts had similar specifications, they differed significantly in their brewing behaviour. The results suggested that laboratory tests of viscosity and wort filterability were the best predictors of processability.
Keywords: malt; brewing; processability; prediction

Introduction

Malt specifications are the primary trading tool for the purchase of malt. The most widely used of these specifications, extract and total nitrogen, give an indication of the potential yield of fermentable carbohydrate. Other parameters, such as malt colour and specific flavour-related compounds, relate to the style of beer being brewed. However, as vessels become larger, turn-around times shorter, and production margins slimmer, the efficiency of processing, that is, the ease with which the fermentable carbohydrate potentially available can actually be extracted from the malt and converted into alcohol, becomes ever more important. As a consequence, many "functional" tests for malt have been developed in recent years. These are designed to predict some aspect of malt processability. Such predictions are important not only for the brewer in choosing a malt supplier, but also for the maltster and barley breeder, in selecting barley varieties which yield malt which can be easily processed in the brewhouse.The separation of sweet wort from the mash is usually the main rate-limiting step in the brewhouse in terms of cycle and turn-around times, and so is one of the main targets for those tests which aim to predict processability. The extent of cell wall modification is generally considered to be a key factor affecting lautering. Consequently, many tests are designed to measure the extent of cell wall modification, for example the sanded slab technique, in which un-degraded β-glucan is stained with Calcofluor (MUNCK *et al.* 1983). Measurement of the viscosity and/or the β-glucan content of laboratory worts (LEE *et al.*1998) can give also some indication of cell wall degradation. A number of bench-scale filtration tests, using laboratory worts, have been developed (BROWN & FREEMAN 1991; MARTELL *et al.* 1993; STEWART *et al.* 2000), and these also are claimed to have some value in predicting brewhouse performance. Measurement of amylolytic enzymes, such as α-amylase or β-amylase, or more empirical methods such as DP, are widely used to predict fermentation performance.

Small-scale laboratory tests are also widely used by organisations which are responsible for the registration and / or evaluation of new barley varieties, in order to try and ensure that varieties grown in their area are suitable for the needs of both home and export markets. There is, however, less information available to compare these tests for their predictive value.

This paper describes a series of trials the aim of which was to compare a number of "functional" malt analyses for their ability to predict the actual processability of malt in a brewery. A set of commercial malts, sourced from around the world and all conforming to a typical specification for a premium lager malt, were brewed on the pilot scale. The brews were scored for key aspects of processability, and the overall score was compared with the results obtained in a range of standard and functional tests.

Methods

Commercial malts, all conforming to the same specifications for a number of standard parameters, were obtained from international brewing companies. Wherever possible at least two samples, from different malt suppliers, but considered suitable for use in brewing the same brand of beer, were obtained from each company. Details of the agreed specification are shown in Table 1.

Parameter	**Specification**
Extract (EBC)	80.5% minimum
β-glucan (Skalar)	< 230 mg/litre
Colour	3 – 4.5 °EBC
Total Nitrogen	1.65 – 1.85 %
Total soluble nitrogen	0.65 – 0.80 %
Kolbach Index	38 - 45
Apparent Attenuation	78% minimum
Friability	80% minimum

Table 1. Agreed standard malt specification

At BRi the malts were subjected to a range of standard and functional tests. Extract, colour, TSN, TN, Kolbach index, Free amino nitrogen, pH, and friability were carried out according to EBC methods (EUROPEAN BREWERY CONVENTION 1998). DP was measured according to the ASBC Recommended method (ASBC 1999). Wort viscosity was measured using a rotary viscometer. Modification and homogeneity were estimated using the Carlsberg sanded slab technique (MUNCK *et al.* 1983) and the Light Transmission meter (LTm) (SHARPE *et al.* 2000). Wort β-glucan was measured using the McCleary method (McCLEARY & CODD 1991). Mash filtration was measured by the Mash filtration cell (MULLER *et al.*). Each malt was then brewed by three standard protocols, each selected to test a different aspect of processability. In the first set of brews, the grist contained a high proportion (81% by weight) of the test malt, in order to emphasise the inherent properties of the malt. The second set used a high proportion (40%) of maize grits, since this would place demands on the amylolytic capabilities of the malt. The third set of brews contained a significant proportion (13%) of under-modified malt in order to test the cellulytic capabilities of the test malt. Each brew was scored for a number of key indicators of processability, both in the brewhouse and during fermentation.

Results

Malt Analyses

All six malts fell within the agreed specifications and very similar in terms of standard analyses (results not shown), except that Malt A was higher in nitrogen (1.84%) than the other malts, all of which contained between 1.63 and 1.69% total nitrogen. However, the nitrogen content of Malt A was commercially acceptable for a malt from that country.

The functional tests displayed more variation (Table 2). It was noticeable that Malt A gave relatively poor scores for Calcofluor, LTm, friability and wort β-glucan, but a very good score for DP. Malt E gave good scores with the LTm and Calcofluor (although homogeneity by Calcofluor was significantly poorer). With the mash filtration cell, results of commercial trials (MORRALL *et al.,* in press) suggest that malts above a certain threshold do not tend to give lautering problems. Malts below this threshold may give lautering problems, with an inverse correlation between Mmax and lautering rate (MULLER *et al.*). The actual threshold varies somewhat with the brewhouse involved, but is around 35g. Thus this test suggested that Malts C and E could have lautering problems. Malt G gave particularly good scores in all tests.

Parameter	**A**	**C**	**D**	**E**	**F**	**G**
DP (°WK)	372	253	285	274	298	326
Viscosity (mPa)	1.52	1.56	1.53	1.54	1.53	1.49
β-glucan of lab wort (mg/litre)	83	90	12	32	38	0
Mealiness -LTm (%)	69	83	80	87	85	84
Homogeneity – LTm (%)	39	54	46	84	65	97
Mash filtration (Mmax)	38	27	51	24	42	136
Modification by calcofluor (%)	91	Not done	99	97	98	100
Homogeneity by calcofluor (%)	73	Not done	82	78	83	100

Table 2. Functional analyses of malts used for brewing

Pilot Brewing: High Malt Grist

Brewhouse yields for pilot brews using a high malt grist are shown in Table 3. Malt E gave the highest yield of extract overall, followed by Malt F. Extract yields from Malts A and C were below average for the malt set.

Parameter	**A**	**C**	**D**	**E**	**F**	**G**
Extract yield (litre ° Plato at fermentation gravity)	1049	1044	1082	1125	1103	1086

Table 3. Brewhouse data for brews with a high malt grist

A quantitative measure of ease of lautering is the differential pressure across the lauter base plate during run-off (Figure 1). The sharp rises in pressure show where raking was required, resulting in a sharp drop in pressure. The time which elapses before raking is required is an indication of the ease with which the wort could be run off. These data confirm that malts A, C and G ran off very easily, while lautering problems were experienced with malts D and E.

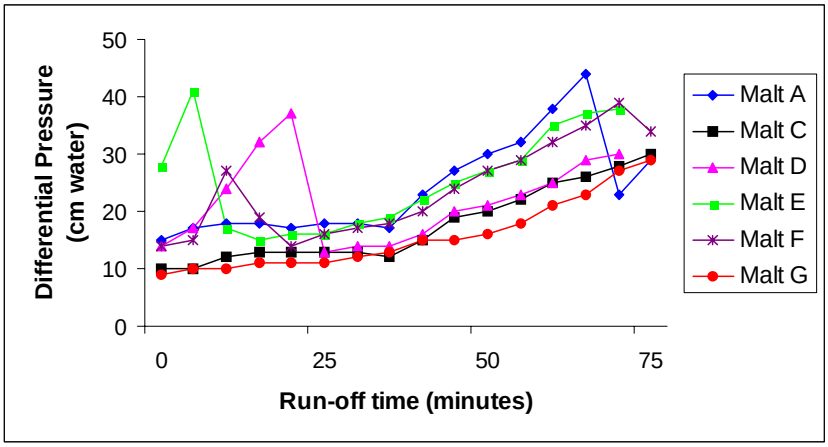


Figure 1. Lautering performance for brews with a high malt grist

The clarity of the sweet wort is another important quality parameter. Wort turbidity was therefore monitored during lautering. Wort C, which had run off very readily, and Wort E, which had not run off well, were both very turbid throughout. All the other malts, including Wort D, which had experienced lautering problems, were quite clear.

Fermentation Performance; High Malt Brews

The fermenting worts were monitored daily for gravity. All six worts fermented satisfactorily and that there were no major differences between them in their ability to support yeast growth and fermentation (results not shown).

Beer Quality

The final beers were analysed for standard quality parameters. As expected, these did not show any major differences between the malts, other than the slightly higher nitrogen content of beer from Malt A, as expected from the higher nitrogen content. Malts F and G gave above average levels of ethanol, while both Malt E and Malt C were below the average ethanol concentration for this set of malts. The total yield of ethanol from the grist was calculated from the total volume of wort obtained at fermentation gravity and the concentration of ethanol in the final beer. The highest yields of ethanol overall were obtained from Malts F and G. Malt E, although it gave the highest yield of extract in the brewhouse, gave slightly below the average yield of ethanol, suggesting that some of the extract was not fermentable. Conversely, the total yield of alcohol from Malt A was close to the mean, in spite of the low brewhouse yield.

Brewing with High Maize Adjunct and Under-Modified Malt

The results obtained with the other two brewing protocols (high maize adjunct and grist with under-modified malt) in general showed the same trends as the high malt brews. With the high

maize brews, lautering performance and wort clarity was good for all malts. During fermentation, Malt E took longer to reach final gravity than did the other brews, and specific alcohol yield was lower. However, the total yield of alcohol from Malt E was slightly higher than the mean because of its high brewhouse yield (Table 7).

Parameter	A	C	D	E	F	G
Extract yield in brewhouse (litre ° Plato at fermentation gravity)	1323	1371	1287	1454	1464	1342
Ethanol (%)	4.85	4.67	4.75	4.58	4.78	4.97
Total yield of ethanol (kg)	5.844	5.861	5.558	6.069	6.405	6.063

Table 7. Performance data for high maize brews

Lautering performance was satisfactory for all the malts in the series of brews containing under-modified malt, although wort clarity was again poor for Malt E. Extract yields in the brewhouse were lower than those obtained with the maize brews and with the high malt brews, as was to be expected. However, similar trends were observed, in that Malt E gave the highest brewhouse yield, but not all of it was fermentable, so that the percentage of ethanol in the final beer was lower than for the other malts. Again, the yield of ethanol (both specific and total) from Malt A was much better than expected from its brewhouse extract (Table 8).

Parameter	A	C	D	E	F	G
Extract yield in brewhouse (litre ° Plato at fermentation gravity)	1060	1055	1056	1117	1030	1069
Ethanol (%)	4.73	4.60	4.70	4.56	4.72	4.87
Total yield of ethanol (kg)	4.588	4.393	4.512	4.628	4.460	4.724

Table 8. Performance data for brews with under-modified malt

Discussion

Comparison of Actual Processability with Predictions

In order to compare the effectiveness of the laboratory tests at predicting actual processability in the brewery, overall processability was scored both qualitatively and quantitatively. Each of the three sets of brews, each malt was scored as poor, fair, good or very good for each of a number of key processability parameters. (It should be emphasised that these descriptors were relative only to this set of malts, which were all of good quality). The processability parameters scored were ease of lautering, wort clarity, total brewhouse extract, fermentation performance, specific ethanol yield and total yield of ethanol per brew. Using a scoring system of poor = 0, fair = 1, good = 2 and very good = 3, a score was derived for overall processability for each malt.

It is apparent (Table 9) from this exercise that the test malts fell into two groups, Malts C and E, (scores < 20), and Malts A, D, F and G (scores > 30).

These processability scores were then compared with the results obtained for several "functional" malt analyses (Table 10). Several of the tests, in particular those affected by cell wall

Malt	A	C	D	E	F	G
Over-all procesability score	33	16	30	19	37	39

Table 9. Overall processability scores for each malt

modification such as Calcofluor staining, LTm, wort β-glucan and friability, significantly under-rated the processability of Malt A. Equally, these tests all over-rated the processability of Malt E. Viscosity, DP and mash filtration were the three tests which gave the best predictions for these two malts. The relatively poor processability of Malt C, and the good performance of Malt G were predicted by most of the tests.

Malt	Prediction						Actual processability
	Calcofl. (Mod)	LTm (Homog)	Wort β-glucan	Viscosity	DP	Mash filtration	
A	Poor	Poor	Poor	Good	Good	Good	GOOD
C	ND	Fair	Poor	Poor	Poor	Poor	POOR
D	Good	Poor	Good	Fair	Fair	Good	GOOD
E	Good	Good	Good	Fair	Poor	Poor	POOR
F	Good	Fair	Good	Fair	Fair	Good	GOOD
G	Good	V. good	V.good	Good	Good	Good	GOOD

Table 10. Comparison of predictions with actual processability in the brewery

Parameter	Correlation coefficient (R^2)	Type
Viscosity	0.7162	linear
Mmax	0.6046	exponential
DP (WK)	0.4776	linear
KI	0.4156	linear
Homogeneity (Calcofluor)	0.3035	linear
β-glucan (SKALAR)	0.2649	linear
Total nitrogen	0.1359	linear
LTm mealiness	0.0566	linear
Modification (Calcofluor)	0.0207	linear
LTm homogeneity	0.0197	linear
β-glucan (McCleary)	0.0102	linear
Friability	0.0079	linear
HWE coarse	0.0067	linear
β-glucanase	0.0002	linear

Table 11. Correlations for a number of malt parameters with overall processability

The correlation coefficient (R^2) for the overall processability score was then calculated for a number of malt analytical parameters (Table 11). Viscosity and mash filtration, together with DP, gave the best predictions overall.

Conclusions

The trials described in this paper confirm that even when malts fall within a set specification they can still vary significantly in their processability in the brewery. Within this small trial set, viscosity and mash filtration tests, together with DP, gave the closest prediction of processability. Tests which rely on measures of cell wall breakdown could, if used alone, give misleading predictions for some malts, particularly those with high levels of hydrolytic enzymes.

Acknowledgements

This work was supported by funding from the Home-Grown Cereals Authority (HGCA) in the UK.

References

AMER. SOC. BREW. CHEMISTS (1999): Recommended Methods of Analysis.

BROWN, A.T., FREEMAN, P.L. (1991): The use of alternative laboratory mashing and filtration techniques in malt quality control. Proc. Conv. Inst. Brew. (Cent. & South African Sect.), Victoria Falls, **3**: 66-71.

EUROPEAN BREWERY CONVENTION Analytica-EBC. EBC Analysis Committee, Verlag Hans Carl Getranke-Fachverlag, Nurnberg.

LEE, R., ALLAN, G., GREIG, R., DUNN, C. & STUART, M., (1998): The effect of (1-3),(1-4) beta-glucan and arabinoxylan on malt quality and brewhouse performance. Proceedings of the Convention of the Institute of Brewing, (Asia Pacific Sect.), Perth, **25:** 33-37.

MARTEL, C., FLAYEUX, R. & CARNIELO, M., (1993): Advantages of the Tepral filtration apparatus in the barley/malt/beer industry: Part 1. *Bios,* April 1993, **24**(4): 35-39.

CLEARY, B.V., CODD, R. J. (1991): Measurement of (1-3),(1-4)-beta-D-glucan in barley and oats: a streamlined enzymic procedure. Sci. Food Agric., **55**(2): 303-312.

MORRALL, P., GREENSWORD, S., MACINTOSH, S. & BLOOMFIELD, C. (2003): A logical approach to determining the relationship and importance of factors in malt production and malt quality to their effect on brewhouse run off. Proceedings of the European Brewery Convention Congress, Dublin, 2003. in press.

MULLER, R.M., GRANT, J.P. & BOOER, C.B.: Measurement of mash filtration in the laboratory as a means of predicting the processability of malts in the brewhouse. Proceedings of the Institute and Guild of Brewing (Africa Sect.), Victoria Falls, in press.

MUNCK, L., GIBBONS, G.C., JENSEN, S.A., HELVETED, F. & AASTRUP, S. (1983): The use of fluorochromes as an analytical tool in the malting and brewing industry. Proceedings of the European Brewery Convention Congress, London: 635-642.

SHARPE, F.R., CHANDRA, G.S., DICKIE, K.H., MULLER, R.E. & WHEATON, L.K. (2001): Light transflectance- a new method for evaluating barley and malt endosperm quality. Proceedings of the Convention of the Institute and Guild of Brewing, (Africa Sect.), Sun City, **8:** 41-46.

STEWART, D., FREEMAN, G. & EVANS, E. (2000): Better prediction of the beer filtration efficiency of malt. Proceedings of the Convention of the Institute of Brewing (Asia Pacific Sect.), Singapore, **26:** 47-51.

Distilling Barley – Bringing Certainty to the Future

J.M. Brosnan[1], T.A. Bringhurst[1], K. Denyer[2], J.S. Swanston[3] and W.T.B. Thomas[3]

[1]Scotch Whisky Research Institute, Research Park North, Riccarton Edinburgh, EH14 4AP, UK; [2]John Innes Institute, Norwich Research Park, Norwich NR4 7UH, UK; [3]Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, UK

Abstract

The distilling industry is one of the most historic and economically important end users of barley. The Scotch Whisky industry alone earns over 3 billion euros in exports for the United Kingdom. The quality of malting barley for distilling in the United Kingdom has been greatly improved because of a rigorous varietal selection procedure endorsed by all those in the barley supply chain. From Golden Promise in the 1960's to Optic today distillers have seen a steady increase in the alcohol yield potential of barley varieties. However, the challenges which face distillers in the near future demand more than high alcohol yield from barley. Exciting opportunities do exist to significantly improve distilling barley by utilising non-GMO genetic techniques combined with an understanding of the phenotypic variation already available in the barley gene pool. Making the jump in technology transfer between knowledge of barley genes to innovation in a distillery requires partnership. In this paper the future needs of distillers will be described in the context of research work carried out by the distilling industry in Scotland in collaboration with other barley research groups.

Introduction

Scotch whisky is one of the most distinctive and popular alcoholic beverages in the world, accounting for approximately 9 percent of the global spirits market (GORDON 2003). According to the Scotch Whisky Association, Scotch whisky represents an annual export value of more than 3 billion Euros, and comprises 20 percent of United Kingdom food and drink exports, making it one of the top 5 UK exports. The industry is a major end user of both UK barley and wheat. Overall, distillers use about 0.5 million tonnes of barley, about 28 percent of all the malting barley produced in the UK.

Table 1. Scotch Whisky Industry agreed List of Desirable Attributes for Distilling Barley (Scotch Whisky Association Cereals Committee)

Maximum Alcohol Yield Potential
Ease of Processing
High Levels of Enzymes
Minimise Ethyl Carbamate precursor (epiheterodendrin (EPH))

In an industry where there is considerable (and continuing) pressure to reduce costs, distillers wish to obtain the maximum possible benefit from their raw materials. During 2002 the Scotch whisky industry decided to review its quality requirements for new distilling barley varieties. The review, which was endorsed by the Cereals Committee of the Scotch Whisky Association, was intended to give a clear message to barley breeders, about the attributes they would like to see incorporated into new malting barley varieties for distilling (Table 1).

In order to secure a continuing supply of barley which is suitable for distilling, the Scotch whisky industry is an active participant in the system for the selection of barley, which is

currently managed by the Maltsters Association of Great Britain (MAGB). The selection process, has resulted in a gradual progression from 'traditional' varieties, such as Golden Promise, producing alcohol yields of around 390 litres of alcohol per tonne (DOLAN 2003) to more modern, varieties such as Optic, Chalice and Decanter which give much higher spirit yields (>440 litres of alcohol per tonne). This process has been very effective at promoting the development of new barley varieties, but is very time consuming and expensive to run. The risks to plant breeders are significant, as only a small number of candidates progress to the final stages.

There is now a perception that the benefits of the normal selection process are beginning to diminish (BURRELL 2003), particularly in terms of agronomic yield and end user parameters such as hot water extract (THOMAS 2003). Distillers have also noted that the potential alcohol yields of current varieties are now approaching their theoretical maxima (BATHGATE 1998; STEELE 2001) and that we may have to look beyond these parameters in order to deliver the next major advance in the quality of distilling barley.

In recent years there has been a growing interest in applying the field of molecular biology to the selection process for new barley varieties and the Scotch Whisky Research Institute (SWRI) has been involved in work with the Scottish Crop Research Institute (SCRI) and Advanta Seeds, to extend our understanding of the barley genetics governing important economic traits (MEYER *et a.l* 2001). This has resulted in extensive mapping of the barley genome, and the identification of regions of the genome, known as quantitative trait loci (QTL) which can be used as markers for these traits (SWANSTON *et al.* 2000; THOMAS 2003). QTL have been identified for hot water extract, fermentability and predicted spirit yield (SWANSTON *et al.* 1999; SWANSTON *et al.* 2000; MEYER *et al.* 2000; POWELL *et al.* 2004) as well as for the main precursor of ethyl carbamate (MEYER *et al.* 2001).

The aim of this paper is to show how the Scotch whisky industry hope to promote the development of new distilling barley varieties, by conjoining in partnerships between the Scotch Whisky Research Institute and other research organisations such as the SCRI, the John Innes Centre (JIC), together with plant breeders and commercial malting companies. We would like to show how these collaborations have resulted in major advances in our understanding of barley, facilitating the technology transfer jump between knowledge of the barley genome to distillery innovation.

Discussion

There are two forms of Scotch whisky, malt whisky and grain whisky. While the production processes for these spirits are completely distinct, the same principles apply to both products. In both cases, cereal starch is solubilised and converted to fermentable sugars, which are the fermented by yeast, to give alcohol which is collected by distillation and then matured in oak casks (for at least 3 years) to give the final product. Malt and grain spirits form the basis for blended whisky, which accounts for 90 percent of whisky sold.

The production process is defined in UK and EU law (The Scotch Whisky Act 1988, The Scotch Whisky Order 1990 and EEC No 1576/89). This specifies that Scotch whisky must be produced in Scotland solely from cereals, the starch of which must be saccharified by endogenous malt enzymes. This means that the use of additives such as commercial enzymes, or other process aids is not permitted. This legal limitation puts severe constraints on changes to the process options available to the Scotch whisky industry.

The flow chart shown in Figure 1 identifies the areas where cereals have an impact on the Scotch whisky process and indicates where the potential benefits of new barley attributes might be realised.

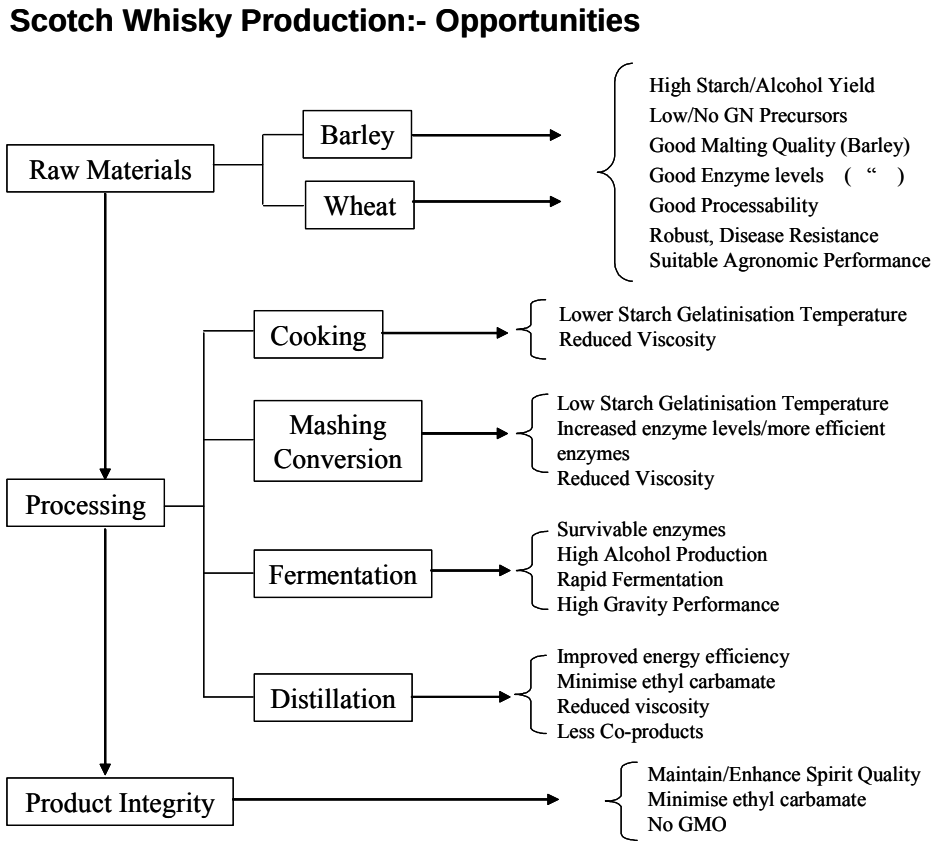


Figure 1. Diagram showing the main stages in the production of Scotch New Make Spirit, outlining the areas of research which could give potential benefits in production efficiency and enhance product integrity

Alcohol Yield

Selection of suitable distilling barley varieties is perhaps the most important part of the Scotch whisky production process, since the cost of cereals accounts for a large proportion of production costs (somewhere in the region of 70 – 80 percent) (NICOL 1990). Thus it is essential that the Scotch whisky industry is able to select good distilling barley varieties for maximum alcohol production.

One area of raw materials research, of increasing interest to malted barley end users, is that of malt deriving from hull-less or naked barley. Genetically these are similar to normal varieties, but have important physiological differences. While currently, very few naked varieties of distilling quality are available, some are beginning to enter the production chain, as barley breeders begin to select suitable parent varieties which are genetically similar to existing high quality cultivars.

Some work was recently carried out at SWRI, in conjunction with the Crisp Malting Group Ltd and a plant breeder, on a range of naked barley micro-malts. These were tested using a laboratory mashing and fermentation method, which closely simulates malt distillery

conditions (BRINGHURST *et al.* 1996). In this experiment 8 naked barley varieties were assessed for alcohol yield and compared with 2 modern malting varieties, Optic and Cellar.

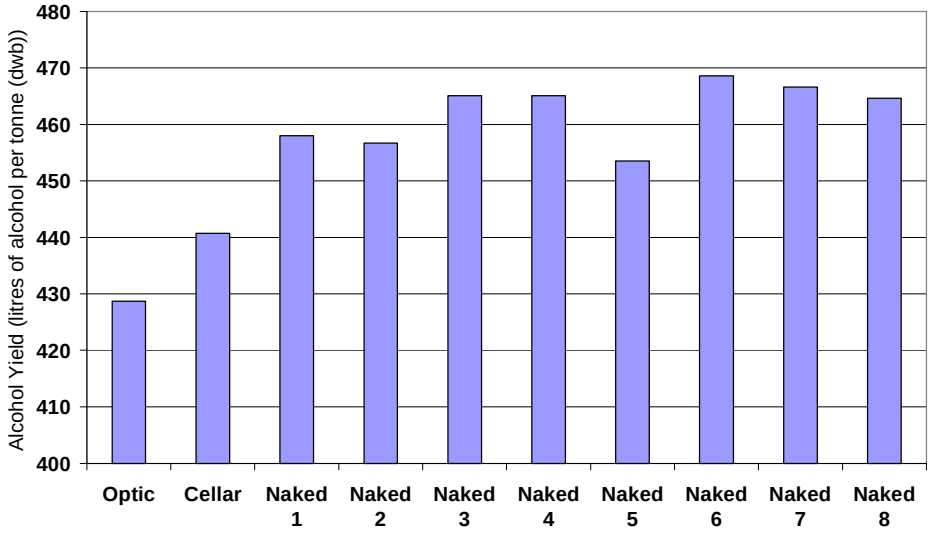


Figure 2. Comparison of the spirit yields of 8 Hull-less (Naked) barley varieties with 2 current conventional malting varieties using a mashing and fermentation procedure, simulating a malt distillery (BRINGHURST *et al.* 1996)

The results summarised in Figure 2 show that malt deriving from distilling quality naked barley can give a potential alcohol yield which is much higher than normal hulled varieties. On average, the improvement was around 25 litres of alcohol per tonne, which could potentially amount to £15 million per year (22 million Euros). Importantly, this potential was realised without the need to optimise growing or malting conditions, so there is further potential for improvements.

Ease of Processing

Different processing systems are used for the production of malt and grain whisky. In grain whisky production, the major raw materials are unmalted cereals, such as wheat or maize. These are generally cooked to gelatinise and release the starch. The solubilised starch is then mixed with malted barley so that the endogenous starch degrading enzymes can convert it into fermentable sugars.

In the production of malt whisky, barley malt is mashed over a range of temperatures (63-95°C), generally in a lauter type mash tun, and filtered worts extracted until as much as possible of the wort sugars are collected. This allows enzyme activity to survive into fermentation, where the enzymes are able to further degrade dextrins into fermentable sugars.

While both of these processes seem very different, they have many common features. The temperatures used must be sufficient to fully gelatinise the starch, and there must be enough enzyme activity to efficiently convert the starch and any remaining dextrins to fermentable sugars. This allows us to identify where developments in cereals might be focussed to deliver processing improvements. One benefit would be a reduction in energy costs, if the gelatinisation temperature of barley (and cereal) starch could be reduced sufficiently to allow low temperature processing. Another would be improved enzyme performance.

Lower Gelatinisation Temperature

The gelatinisation temperature of starch is largely dependent on its granular structure. In barley and wheat starch, the granules occur in 2 forms, large 'A' type granules and much smaller 'B' granules. In both cereals, the large 'A' granules are much fewer in number, but account for the major proportion of starch (about 85 – 90 percent for barley, 75 percent for wheat). The small 'B' granules can be present in very large numbers, but these are very tightly packed, require more energy to gelatinise and release a relatively small proportion of starch.

GREBER *et al.* (2000) identified low starch mutant barleys which lack iso-amylase activity. When such varieties were studied in more detail (BURTON *et al.* 2002) it was found that the starch granules did not fall into the normal 'A' and 'B' type categories, but appeared to be of a 'composite' character, falling between the two extremes. In these mutants, instead of starch (amylose and amylopectin) being laid down in the amyloplasts during development, substantial amounts of a water soluble fraction of phytoglycogen, together with high levels of soluble α-glucans, were produced.

Samples of two of these varieties (Notch 2 and Risø 17), together with parent varieties (Bomi, NP113), were obtained from the John Innes Centre, along with a standard distillery malt (Chariot), and micro-malted. The micro-malts were assessed using the Institute of Brewing fermentability method (IOB Method 2.16). The analysis was carried out at the standard mashing temperature (65°C), and compared with an identical procedure carried out at a lower temperature of 45°C. The results are summarised in Table 2.

Table 2. Comparison of the IOB Fermentability at 65° and 45°C of micromalts deriving from low starch mutant barley varieties (Notch 2, Risø), parent varieties (Bomi, NP113) and a conventional control variety (Chariot). The results were expressed as the percentage ratio of the fermentability at 45°C to that at 65°C.

Sample/Variety	% Fermentability (45°C)	% Fermentability (65°C)	Fermentability Ratio 45°/65° (%)
Chariot (Control)	73.7	86.9	85
Bomi (Parent)	72.6	84.5	86
Notch 2 (Mutant)	60.1	54.9	109
NP 113 (Parent)	68.0	82.1	83
Risø 17 (Mutant)	66.3	65.5	101

Although this was a preliminary experiment, the fermentability data produced were extremely interesting. While the fermentabilities of the mutant micro-malts were very low in comparison to the parent varieties and the control distilling malt, the results indicate that it is possible to achieve or even exceed the normal fermentability at a much lower temperature than would be possible for standard distilling/malting barley varieties. As fermentability is essentially a measure of how easily the malt extract can be converted to alcohol, this is an important result.

The barley varieties used for this work were low-starch mutants, designed to study starch synthesis, and would certainly not be suitable for industrial use in the production of Scotch whisky. However, some of these attributes could be incorporated into future generations of a proper malting/distilling barley breeding line. While in the short term it might be difficult to

produce barley varieties such as these commercially, there is potential in promoting future research into developing distilling barley varieties with these unusual properties. If successful, such varieties would allow distillery processing to proceed efficiently at lower temperatures, reducing energy usage, resulting in substantial improvements in distillery and malting efficiency.

Enzyme Performance

Unlike in brewing, in the Scotch whisky process the wort is not boiled prior to fermentation and some of the malt enzymes can survive in the fermenter, to give further conversion of wort dextrins into fermentable sugars. These enzymes (α-amylase, β-amylase, limit dextrinase and iso-amylase) normally work together to efficiently degrade amylose, amylopectin, and branched dextrins, into fermentable material, which can be converted to alcohol by yeast. There would be clear benefits in developing new barley varieties containing enzymes with enhanced thermostability, or improved pH stability.

Normally β-amylase, which is one of the most important of the starch degrading enzymes in barley malt, is heat labile under distillery conditions and significant levels of this do not generally survive mashing (MACGREGOR 1990). However, recent work reported by MA *et al.* (2000) has shown that it is now possible to develop new varieties with increased β-amylase thermostability. KANEKO *et al.* (2000) have identified several existing varieties possessing this trait in East Asia and Ethiopia. Thus we are now beginning to understand the genetic basis of high β-amylase thermostability, and it should be feasible to use this knowledge to facilitate the conventional breeding of distilling barley varieties with β-amylase thermostability.

Distillation and Co-Products

In a grain distillery, after distillation, the residues (spent wash) are collected and evaporated in special evaporators, to give a syrup concentrate which can be mixed with other spent grains, dried and sold as animal feed. One of the major problems is the residual levels of non-fermentable β-glucans and gum-like materials, such as arabinoxylans, which are associated with cereals such as barley and wheat. These survive the process and can substantially increase the viscosity of the spent wash, causing fouling and potential failure of the evaporators. These problems can often cause severe process interruptions and the resulting downtime can add substantially to production costs. It should now be possible to apply fundamental knowledge of the genetics and starch structure of cereals to provide practical solutions to these problems, by allowing us to select varieties which will give substantial improvements in distillery processing.

Product Profile

Ethyl carbamate can be present at trace levels in whisky (as well as in many other common foods and beverages), and as this is an undesirable component, much effort is spent in ensuring levels are minimised in final products. Ethyl carbamate derives from a precursor, epiheterodendrin (EPH), which is a cyanogenic glycoside that is normally present in many barley varieties. EPH is produced during the germination of barley, and is degraded to ethyl carbamate during processing (COOK *et al.* 1990). It has been well established that certain barley varieties, such as Derkado and Decanter do not contain this ethyl carbamate precursor, and THOMAS (2003) describes the development of Simple Sequence Repeat (SSR) markers, which could be used in marker assisted selection programmes to select cultivars which do not produce EPH (MEYER *et al.* 2001). While the link between these markers and EPH production is currently not strong enough to be reliable in all cases, work is currently in

progress to refine them. When this is fully developed, we should be able to reliably identify barley varieties with the potential for EPH formation, so that we can promote non-producers at an early stage of the breeding cycle.

Conclusions

With recent developments in our understanding of the barley genome, and the development of genetic markers for important traits, there are now exciting opportunities to use a combination of non-GMO genetics and an understanding of the phenotypic variation present in the barley gene pool, to give improvements in distilling barley. This will allow us to predict and select for characteristics providing benefits at virtually every stage of the distillery production process. These will potentially improve process efficiency or protect the integrity of the final products to a degree which was not possible in the past. These advances are now on the horizon and only await the final jump in the transfer of this knowledge to real innovation in the distillery.

It is clear that ways of identifying and defining (and communicating) what end users really want, at as early a stage as possible, would yield great benefits in reducing the risks to barley breeders, and improving the chances of success for new varieties. One of the prerequisites for this is the development of partnerships between the distilling industry and other research groups and organisations, which will allow distilling end users to indicate, to researchers, the areas of fundamental cereals research which they consider will bring long term benefits. This will facilitate the transfer of the knowledge of barley genetics to its practical application in the distillery, so that we can deliver innovations, which will be of value to the Scotch whisky industry, as well as the rural (and urban) economies which depend on it.

Acknowledgements

The Authors would like to thank the Directors of SWRI for their support and their permission to publish this paper, as well as all our collaborators in the UK cereals supply chain. We would also like to acknowledge the contribution of Stan Sole of the Crisp Malting Group Ltd.

References

BATHGATE G.N. (1998): The Recipe for Scotch Whisky. In BEACH R.H.B (ed): Brewing Room Book 1998 – 2000 (85th Edition), Pauls Malt, Bury St Edmunds, U.K. 81-84.

BRINGHURST T.A., BROSNAN, J.M., MCINNES B., STEELE G.M. (1996): Methods for Determining the Fermentability and Predicted Spirit Yield of Distilling Malts, Journal of the Institute of Brewing, **102:** 433-437.

BURRELL M.M. (2003): Starch the need for Improved Quality or Quantity, Journal of Experimental Botany, **54:** 451-456

BURTON R.A., JENNER H., CARRANGIS L., FAHY B., FINCHER G.B., HYLTON C., LAURIE D.A., PARKER M., WAITE D., VAN WEGEN S., VERHOEVEN S., DENYER K. (2002) Starch Granule Initiation and Growth are Altered in Barley Mutants that lack Isoamylsae Activity, The Plant Journal, **31:** 1-17

COOK R., MCCAIG N., MCMILLAN, J.M.B., LUMSDEN W.B. (1990): Ethyl Carbamate Formation in Grain-Based Spirits, Part III. The Primary Source, Journal of the Institute of Brewing, **96:** 233-244

DOLAN T.C.S. (2003): Malt Whiskies: Raw Materials and Processing. In RUSSELL I. (ed): Whisky: Technology, Production and Marketing. Academic Press, London, U.K.: 27-74.

GORDON G.E. (2003): Malt Whiskies: Raw Materials and Processing. In RUSSELL I. (ed): Whisky: Technology, Production and Marketing. Academic Press, London, U.K.: 311-351

GREBER B., WAITE D., FAHY B., HYLTON C., PARKER M., LAURIE D., DENYER K. (2000): Use of Barley Mutants to Understand Starch Synthesis. In LOGUE S. (ed.): Proceedings of the 8th International Genetics Sypmosium, Adelaide, South Australia, Volume I, Invited Papers.:196-198

ANALYSIS COMMITTEE OF THE INSTITUTE OF BREWING (1997); Institute of Brewing Methods of Analysis, Volume 1, Analytical. The Institute of Brewing, London, U.K.: 2.16

KANEKO T., KIHARA M., ZHANG W.S., ITO K., AIDA Y., TAKEDA K. (2000): Variation and Utilisation of β-Amylase Thermostability in Barley. In LOGUE S. (ed.): Proceedings of the 8th International Genetics Sypmosium, Adelaide, South Australia, Volume II, Contributed Papers.: 252-254

MA Y., EVANS D.E., LOGUE S.J., LANGRIDGE P. (2000): Improving Thermostability and Substrate Binding Affinity of Barley β-amylase by Protein Engineering. In LOGUE S. (ed.): Proceedings of the 8th International Genetics Sypmosium, Adelaide, South Australia, Volume III, Contributed Papers.: 111-113

MACGREGOR A.W. (1990): Current State of Research into Barley Carbohydrates. In CAMBELL I. (ed.): Proceedings of the Third Aviemore Conference on Malting, Brewing and Distilling. Institute of Brewing, London, U.K.: 10-33

MEYER R.C., SWANSTON J.S., YOUNG G.R., LAWRENCE P.E., BERTIE A., RITCHIE J., WILSON A., BROSNAN J. , PEARSON S., BRINGHURST T., STEELE G., POWELL W., THOMAS W.T.B (2000): Genetic Approaches to Improving Distilling Quality in Barley. In LOGUE S. (ed.): Proceedings of the 8th International Genetics Sypmosium, Adelaide, South Australia, Volume I, Invited Papers.: 107-114

MEYER R.C., SWANSTON J.S., YOUNG G.R., LAWRENCE P.E., BERTIE A., RITCHIE J., WILSON A., BROSNAN J. , PEARSON S., BRINGHURST T., STEELE G., ALDISS P.R., FIELD M., JOLLIFFE T., POWELL W., THOMAS W.T.B (2001): A Genome based Approach to Improving Barley for the Malting and Distilling Industries. Home Grown Cereals Authority Project Report no 264.

NICOL D.A. (1990): Developments in Distillery Practices in Response to External Pressures. In CAMBELL I. (ed.): Proceedings of the Third Aviemore Conference on Malting, Brewing and Distilling. Institute of Brewing, London, U.K.: 117-137.

POWELL W. MACHRAY G.C., SWANSTON J.S. (2004): New Technological Opportunities in Distilling Raw Materials. In BRYCE J.H. and STEWART G.G. Distilled Spirits, Tradition and Innovation, Nottingham University Press, Nottingham. U.K.: 59-67

STEELE G. (2001): The Whisky Distilling Industry in the 21st Century. In Pauls Brewing Room Book 2001 – 2003 (86th Edition), Pauls Malt, Bury St Edmunds, U.K.: 81-84.

SWANSTON J.S., THOMAS W.T.B., POWELL W., MEYER R., MACHRAY G.C., HEDLEY P.E. (1999): Breeding Barley for Malt Whisky Distilling - A Genome Based Approach. In: CAMBELL I. (ed.): Proceedings of the Fifth Aviemore Conference on Malting, Brewing and Distilling. Institute of Brewing, London, U.K.: 177-180

SWANSTON J.S., THOMAS W.T.B., POWELL W., MEYER R., BRINGHURST T.A., BROSNAN J.M., PEARSON S.Y., BROADHEAD A. (2000): Assessment of Spirit Yield in Barley Breeding Lines, Journal of the Institute of Brewing, **106:** 53–58.

THOMAS W.T.B. (2003): Prospects for Molecular Breeding of Barley, Annals of Applied Biology, **142** (1):1-12

S 6 – BARLEY PROTEIN, FEED AND FOOD

Functional Genomics in the Productivity and End-Use Quality of Barley

E. Newbigin[1], A. Bacic[1], P. Langridge[2] and G.B. Fincher[2]

[1]School of Botany, University of Melbourne, VIC 3010, Australia;
[2]Australian Centre for Plant Functional Genomics, University of Adelaide, Waite Campus, Glen Osmond, SA 5064, Australia

Abstract

Functional genomics is a technology through which large sets of genes that influence a particular biological process are identified. Functional genomics comprises several sub-disciplines, including genomics, proteomics and metabolomics. The total number of genes in plant genomes lies in the range of 25,000 to 40,000. Thus, functional genomics technologies must be geared to high throughput data collection and high throughput analyses of gene structure and function. Additional specialised resources, including barley mutant libraries, barley DNA microarrays and high-density genetic maps of barley, represent critical support technologies. The technologies are illustrated here by reference to specific applications relating to malting and brewing performance, including the biosynthesis of cell wall polysaccharides and early grain development, and to productivity, where tolerance to a range of abiotic stresses is a key determinant of yield. Finally, the potential impacts and benefits of functional genomics research for the barley industry are addressed.

Introduction

In this brief report, the potential applications of emerging functional genomics technologies to the enhancement of barley productivity and quality are described. In particular, the roles of early endosperm and embryo development, together with the role of cell wall biosynthesis, are discussed in relation to grain quality. From the productivity viewpoint, the potential of functional genomics in the identification of key genes that mediate in grain quality is addressed, and the subsequent use of these genes for the improvement of grain quality is addressed.

The early stages of seed development in barley play a key role in determining a range of quality characteristics of the resultant grain, and greatly influence grain characteristics and grain yield. The importance of these developmental stages has motivated large research efforts over several decades. Over the past few years there has been a heavy focus of genomics research onto trying to identify genes involved in the control of grain development with a view to identify gene systems for modifying grain characteristics. However, most groups have focused on gene identification from advanced developmental stages since these have been the only stages for which mRNA could be readily isolated. Here we have produced cDNA libraries and transcript databases from the very earliest stages of embryo and endosperm development starting from the egg and central cells and moving through the earliest cell divisions. The very early stages are of critical importance to grain development since it is during these stages that the cell number in the final embryo and endosperm are determined.

In contrast to animals, where specialized skeletal systems provide structural integrity and strength, the strength, flexibility, texture and overall shape of higher plants depend on the cumulative properties of cell walls, which surround individual cells (BACIC *et al.* 1998; CARPITA 1996). Cell wall components make a relatively minor contribution to the total weight of cereal grains, but they can have a disproportionately large impact on grain technology, utilization and nutrition (BHATTY 1993). Thus, in malting and brewing, wall polysaccharides can adversely affect the efficiency of malt extraction, filtration processes and the quality of the final beer. Wall polysaccharides can also have undesirable effects on the digestibility of barley-based stockfeeds by monogastric animals such as pigs and poultry. In contrast, they are important constituents of the 'dietary fibre' component of human foods, which is considered to be of salutary importance in several areas of human digestion and health (BHATTY 1993; FINCHER & STONE 2004). Despite the importance and contributions of different wall components to agro-industrial processes such as paper and pulping, food quality and texture, dietary fibre and ruminant digestibility grows, genetic manipulation of the major wall components, the polysaccharides, has been hampered by the paucity of our knowledge of the mechanism(s) and control of the biosynthetic steps and, until recently, the lack of information on the genes encoding plant polysaccharide synthases. The cloning of plant cellulose synthase genes (PEAR *et al*. 1996; DOBLIN *et al*. 2002) and the identification of mutants carrying lesions in cell wall components have paved the way for genetic tailoring of wall phenotypes for specific cereal-based processes, and functional genomics technologies provide a high throughput procedure for the identification of candidate genes that influence wall biosynthesis and hence grain quality.

From the barley productivity viewpoint, functional genomics can be used to identify genes that confer tolerance to abiotic stresses in adapted barley cultivars and related species, including genes that are important in tolerance to drought, salinity, cold and frost, and to deficiencies or toxicities in mineral nutrients. Given that yields of barley crops can be severely diminished following exposure to these abiotic stresses, the generation of varieties with increased abiotic stress tolerance remains a central objective in barley improvement programs. Perhaps the most striking feature of plant adaptation to abiotic stresses is that multiple responses, involving complex networks that are interconnected at many levels, are activated when abiotic stresses are encountered. Plants increase their tolerance to 'environmental insults' through both physical and interactive molecular and cellular changes that are triggered by the stress (KNIGHT & KNIGHT 2001). It is not always possible, therefore, to attribute a particular response to a specific abiotic stress. For instance, freezing temperatures, low water availability and high salt concentrations can all cause a lowering of cellular osmotic potential and thereby activate osmotic stress responses. These osmotic stress responses can operate *via* both an ABA-dependent and an ABA-independent signalling pathway. In addition to the induction of osmotic response pathways, salt stress simultaneously activates a second, ionic response, through which ion transporters shuttle ions between various cellular compartments in attempts to maintain ionic homeostasis (ZHU 2001). Drought and cold stresses will similarly activate additional, more specific response pathways. In another example, drought tolerance and tolerance to boron toxicity are closely related in barley, where boron-toxic soils restrict root development. Thus, stresses induced by soil drying might incorporate stress attributable to water shortage, osmotic stress and nutrient deficiency.

Plant responses to abiotic stress are effected at several levels, and these eventually result in slowing or cessation of growth. Following perception of the stress conditions, signal transduction pathways are activated and lead to alterations in gene expression, as measured by the abundance of mRNA species, in the protein profiles of cells, in the activities of key

enzymes, and in the relative flux through and between different metabolic pathways. In turn, the alterations in cellular activity result in molecular and cellular changes that constitute the network of abiotic stress responses invoked to protect the plant against the unfavourable environmental conditions.

New technologies in genomics, proteomics and metabolomics (FIEHN 2001), coupled with a strong bioinformatics capability, now enable a 'systems' approach to be taken in the study of plant responses to abiotic stresses. Thus, the entire system of networks of signalling pathways and key interconnecting processes that lead to the multiple defensive responses can be described in detail. This level of understanding of plant responses to abiotic stresses and the basis for diversity will be achievable for the first time, and will allow the manipulation of the responses, or their transfer from adapted species to important cereal crop species, for the benefit of barley producers and for the wider benefit of society and the environment.

Material and Methods

Plant Material

Barley has been the target plant system, although model systems such as rice and Arabidopsis are used where appropriate. However, it should be emphasised that grasses, and particularly the Triticeae, have evolved novel mechanisms, not found in model dicots, which enable the grasses to more efficiently deal with a range of abiotic stresses, notably micronutrient deficiencies. Similarly, genes for key quality parameters in barley might not be present in dicots or even other cereals. An additional reason for using barley for functional genomics studies is related to the extensive range of genetic resources and germplasm available, including high density genetic maps and BAC libraries (Table 1). Observations that cereal genomes exhibit a high level of synteny, that is the order of individual genes along a segment of the genomes of different cereals is conserved, have allowed cross-referencing of genome sequences and genetic maps between different cereal species. For example, if the rice genome-sequencing program reveals a particular order of *CesA* genes along a region of the rice genome, it can be confidently predicted that the gene order would be conserved, in large part, along the homologous region of the barley genome.

Table 1. Genomic resources for barley and other major cereals

Species	Genome Size (base pairs)	Genetic Maps	ESTs (2003)	BAC Libraries	Mutant libraries	Physical Maps
Barley	5×10^9 (diploid)	Yes	346,000	Yes	Limited	Limited regions only
Rice	4.3×10^8 (diploid)	Yes	202,000	Yes	Yes	Whole genome sequenced
Wheat	1.6×10^{10} (hexaploid)	Yes	420,000	Yes	No	D-genome map available
Maize	2.5×10^9 (diploid)	Yes	229,000	Yes	Yes	Maps available

Transcript Analysis

To identify candidate genes involved in the biological processes of interest here, expression patterns of genes, as indicated by the relative abundance of mRNA species, can be correlated with existing knowledge on the tissue and time frame in which the process is occurring. For example, large amounts of (1,3;1,4)-β-glucan are deposited in the cell wall of developing barley endosperm 20-25 days post anthesis, so candidate (1,3;1,4)-β-glucan synthase genes might be identified from abundant mRNAs that appear in developing grain at that time.

Transcript profiles can be obtained from mass sequencing of short segments of clones in cDNA libraries; the 'expressed sequence tags' or EST sequences so generated are indicative of gene expression in a particular tissue under defined conditions. There are over a million publicly available ESTs for the major cereal species (Tables 1). Microarraying is another method for transcript analysis. In this case, large numbers of gene fragments are immobilised in an ordered array on a solid support. Chips comprising more than 10,000 barley genes are now commercially available for the generation of transcript profiles. Through these chips, entire populations of mRNAs from a tissue can be hybridised, in a single experiment, with thousands of known DNA probes on the chip. Following hybridisation, the expression levels of each gene can be determined with a high-resolution laser scanner.

Candidate Genes for Grain Development

Transcript analyses have been used to reveal sequences that show differential expression during the targeted stages of early embryo and endosperm development. These sequences represent genes that are candidates for loci involved in the control of the early grain development. The genes have been further analysed to accumulate functional information on the genes and their promoters. The map positions of candidate genes relative to genetic loci controlling grain quality characteristics have been defined in barley and transgenic lines over-expressing or under-expression candidate genes are under preparation.

Candidate Genes for Cell Wall Biosynthesis

Available databases from *Arabidopsis*, barley and rice have been searched to identify sequences related to known plant polysaccharide synthases (e.g. *CesA* and *Csl* gene families) and glycosyl hydrolases. High density cDNA arrays, northern hybridisation analyses, EST libraries and quantitative PCR have been used to study differences in gene expression at various stages of grain development, and between adapted and non-adapted barley lines. This has enabled the identification of candidate genes, based on information on their expression patterns. Candidate genes that come out of the gene identification program have been mapped to the barley genome, where co-location with significant QTLs for cell wall components or structure, or other traits such as grain quality, provides evidence of possible function.

Candidate Genes for Abiotic Stress Tolerance

Abiotic stresses examined include drought stress (heat, light and water stress), cold stress, salt stress, and stresses imposed by mineral deficiency or toxicity. To fully realise the potential for real improvement of cereal tolerance to abiotic stress, the examination of multiple stresses is more appropriate than the selection of fewer, arbitrarily chosen stress responses. Initially, adapted varieties of barley and its relatives have been challenged with various abiotic stresses

and their responses monitored and compared with non-stressed plants at the transcriptional level, at the level of cellular protein complement, and at the metabolic level. Similarly, adapted and non-adapted varieties of barley will be compared. Species tolerant to extreme stress are considered to be prime candidates for comparative genomics and proteomics, and metabolomics profiling for the precise definition of adaptive strategies for cold and salt stresses.

Functional Analysis of Candidate Genes

Once key genes, enzymes, proteins and metabolites are identified, their putative roles in grain quality, stress perception, signal transduction or in defensive responses are confirmed through a range of functional analysis systems. These include loss-of-function systems, effected through double-stranded RNA interference techniques for gene silencing (FIRE *et al*. 1998), and gain-of-function through transient expression of candidate genes in heterologous plants or through stable transformation of barley. In addition, protein-protein interactions can be examined at an individual level through the yeast two-hybrid system, and wider protein-protein interaction networks can be defined through yeast mass mating techniques.

Bioinformatics

The development of robust bioinformatic databases, methods to continually update such databases, and their integration through automatic cross-referencing is one of the most important single factors for ensuring that the biological significance of functional genomics data is realised. An advanced database management system is required to store these diverse forms of data in a retrievable and accessible manner, and to allow the data to be queried in a biologically sensible manner. The bioinformatics component of the work therefore represents a centrally important core activity, and has three major parts: data integration and distribution, data analysis and decision trees.

Results and Discussion

Early Embryo and Endosperm Development

Close to 50 genes that are transcribed in early developing embryo and endosperm tissues have been identified and cloned. These include genes involved in tissue polarity and genes encoding transcription factors that could play important roles in the coordination of gene expression in the developing grain. Furthermore, the yeast two-hybrid system has been used to identify groups of interacting proteins that make up highly complex transcription complexes. Although similar genes have been implicated in endosperm and embryo development or in cellular differentiation in other plant species, their precise roles in developing barley grain have not yet been defined unequivocally.

Cell Wall Composition in Cereal Grains

Barley cellulose synthase genes (*CesA*s) and representatives of all the cellulose-synthase-like genes (*Csl*s) have been successfully cloned. The participation of barley *CesA* genes in cellulose biosynthesis has been demonstrated (BURTON *et al.* 2000) and it has been shown for the first time that the *CesA* genes operate in groups of three, probably for the synthesis of primary and secondary walls (BURTON *et al.* 2004). Sequence data from cDNAs, coupled with analyses of EST databases, indicated that the cellulose synthase (*CesA*) gene family from

barley has at least eight members, which are distributed across the genome. Quantitative PCR has been used to determine the relative abundance of mRNA transcripts for individual *HvCesA* genes in vegetative and floral tissues, at different stages of development (BURTON *et al.* 2004). The *HvCesA* genes fall into two general groups of three genes with respect to mRNA abundance, and the co-expression of the groups identifies their products as candidates for the rosettes that are involved in cellulose biosynthesis at the plasma membrane. In addition, *CesA* knockout mutants were generated in barley for the first time, and the mutants show greatly reduced vegetative growth and lower wall cellulose content.

The (1,3;1,4)-β-D-glucans and arabinoxylans are key components of the cell walls of cells in the starchy endosperm of the grain, where together they account for about 90% of the wall. For these reasons, the cloning of genes encoding the (1,3;1,4)-β-D-glucan synthases and the xylan synthases has become the major target for our functional genomics program. We now have close to 20 candidate genes for (1,3;1,4)-β-glucan and xylan synthase genes; the functions of these are being systematically analysed. In addition to the candidate genes identified amongst the barley *Csl* family, the possible participation of XTH and/or callose synthase genes in (1,3;1,4)-β-glucan and xylan synthesis has been investigated.

Analytical Procedures

A number of new functional analysis techniques have been developed, together with the rapid adoption of new techniques for transcript (gene expression) analysis. In particular, quantitative-PCR and microarray procedures have been valuable for the analysis of transcriptional activity of candidate genes, and double-stranded RNA interference has been used both transiently and in stably transformed barley to demonstrate the function of key genes. In addition, monoclonal antibodies specific for cell wall polysaccharides have been used for the immunolocalization of wall polysaccharides in specific tissues and cells during grain development.

Relationships of Cell Wall and Grain Development

Cellularization of the starchy endosperm early in grain development involves 'atypical' growth of cell walls around individual nuclei in a multinucleate syncytium. The fertilised endosperm mother cell divides for about 70 h to form up to 2000 free, individual nuclei in the cytoplasm of the central cell. Walls subsequently grow between the free nuclei. After a lag phase, further expansion of the cellular endosperm occurs through meristematic activity of peripheral cells that eventually differentiate to form the aleurone. There are a number of important implications in this developmental pattern for the quality of the mature grain. For example, the number of nuclei formed before cellularization begins is likely to affect the final number of cells in the starchy endosperm, as is the length of the meristemic division phase. Cell number could well represent a prime determinant of final grain size or "plumpness potential", which is an important quality parameter of commercially valuable cereals. Similarly, during the deposition of wall polysaccharides in the 13-40 dpa period, the final levels of (1,3;1,4)-β-glucans and arabinoxylans will be determined. These wall polysaccharides exert a major influence on grain quality for malting and brewing, and for human and animal nutrition.

Economic, Environmental and Social Benefits

Several commercially relevant outputs can accrue from cereal functional genomics programs. Firstly, novel genes will almost certainly be discovered, but the value of any novel gene will depend upon the significance of the altered phenotype and the breadth of application. For example, a gene that stabilised yield under drought conditions and provided an average 10% yield benefit over an extended period would be very valuable. Where the gene controlling an important quality or productivity trait has been isolated, 'perfect' diagnostic markers could be used in high throughput screening of germplasm in breeding programs.

Thus, the delivery to breeding programs of genes that significantly enhance the end-use quality of barley will carry a significant economic benefit. However, the generation through functional genomics programs of barley varieties that are more sustainable, require less fertiliser, and have improved water use efficiency and tolerance to salinity would have a far larger impact, which would extend well beyond economic outcomes, into social and environmental benefits. Internationally, abiotic stresses represent the major cause of yield losses in cereal crops. With the developing world accounting for almost half of the world's 550 to 600 million tonne annual wheat crop, any improvement in yield stability under stress will have major social, economic and environmental impacts internationally. In many cases, genes conferring abiotic stress tolerance in barley might find equally important applications in cereals more generally.

Acknowledgements

This work has been supported by grants from the Grains Research and Development Corporation and the Australian Research Council. We especially acknowledge the talented and enthusiastic postgraduate students and postdoctoral fellows working in our laboratories at the University of Melbourne and the University of Adelaide for their contributions to the work.

References

BACIC A, HARRIS PJ, STONE BA (1988): Structure and function of plant cell walls. In: Preiss J (ed.) *The biochemistry of plants*. pp 297-371. Academic Press, New York/London/San Francisco.

BHATTY RS (1993): Non-malting uses of barley. In: MacGregor AW and Bhatty RS (eds.) *Barley: Chemistry and Technology*. Chapter 8, pp 355-417. American Association of Cereal Chemists, St Paul, Minnesota, USA.

BURTON RA, GIBEAUT DM, BACIC A, FINDLAY K, ROBERTS K, HAMILTON A, BAULCOMBE DC, FINCHER GB (2000): Virus-induced silencing of a plant cellulose synthase gene. Plant Cell, **12:** 691-705.

BURTON RA, SHIRLEY NJ, KING BJ, HARVEY AJ, FINCHER GB (2004): The *CesA* Gene Family of Barley (*Hordeum vulgare*): Quantitative Analysis of Transcripts Reveals Two Groups of Co-Expressed Genes. Plant Physiol., **134**: 224-236.

CARPITA NC (1996): Structure and biogenesis of the cell walls of grasses. Annu Rev Plant Physiol Plant Mol Biol, **47**: 445-476.

DOBLIN MS, KUREK I, JACOB-WILK D, DELMER DP (2002): Cellulose biosynthesis in plants: from genes to rosettes. Plant Cell Physiol, **43:** 1407-1420.

FIEHN O. (2001): Combining genomics, metabolome analysis, and biochemical modelling to understand metabolic networks. Comp. Funct. Genomics, **2:** 155-168.

FINCHER GB, STONE BA (2004): Chemistry of non-starch polysaccharides from cereal grains. Encyclop. Grain Sci., in the press.

FIRE A, XU S, MONTGOMERY MK, KOSTAS SA, DRIVER SE, MELLO CC (1998): Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature, **391:** 806-811.

KNIGHT H, KNIGHT MR (2001): Abiotic stress signalling pathways: specificity and cross-talk. Trends Plant Sci., **6:** 262-267.

PEAR JR, KAWAGOE Y, SCHRECKENGOST WE, DELMER DP, STALKER DM (1996): Higher plants contain homologs of the bacterial celA genes encoding the catalytic subunit of cellulose synthase. Proc Natl Acad Sci USA, **93:** 12637-12642.

ZHU JK (2001): Plant salt tolerance. Trends Plant Sci., **6**(2): 66-71.

Barley Foods for Good Nutrition and Health

C. W. Newman and R. K. Newman

College of Agriculture, Montana State University, Bozeman MT 59717 USA

Abstract
Barley furnishes a multitude of dietary nutrients including amino acids, minerals, vitamins, insoluble and soluble dietary fiber and physiologically active phytochemicals. Many genotypes of barley are available for processing into foods and some are more concentrated in various nutrients than others and differ in processing requirements. Concentration of nutrients in barley can be altered by standard breeding practices. New breeding techniques are being used to reduce phytate, which is considered to be an antinutrient, although some reports indicate beneficial effects of phytate. Barley can be used in numerous ways to prepare delicious, nutritious and health promoting foods, depending upon the innovation of a food processor. Traditional use of barley as a food has been pearl barley in soups, stews and as a replacement or extender for white rice. Processing techniques, such as milling, flaking and extrusion increase the variety of barley food products. Research has shown that the consumption of barley food products is effective in lowering blood cholesterol thus reducing risk of heart disease. Barley also has a very low glycemic index which helps to prevent high peaks in blood sugar and insulin after meals. This effect is beneficial in the prevention of type 2 diabetes and controlling blood sugar in both type 1 and type 2 diabetes. Because of the nutritional and health benefits of barley, its consumption in human foods should be actively promoted.
Keywords: barley foods; health; nutrition; heart disease; diabetes

Introduction

Barley has been a nutrient resource for man and animals throughout history. In addition to providing the basic ingredient for brewing, barley contains a multitude of nutritional components including energy (calories), essential and non essential amino acids, triacylgylcerols (fats), vitamins, soluble and insoluble dietary fiber and several physiologically active phytochemicals. There are many genotypes of barley that can be processed into foods and food products; and just as with malt barley, certain cultivars are more desirable than others for use as food. Barley breeding programs have made vast strides in developing cultivars having greater yield and characteristics important for malt and more recently for feed. Currently greater emphasis is being placed on the development of food barleys, principally hulless genotypes. Hulless barley is an obvious choice for use as food; however pearled hulled barley is currently the most readily available barley food product.

Although major advances have been made in hulless barley production, it is generally accepted that hulless barley has lower yield than hulled barley which limits its choice as a commercial crop. Yield is an extremely important criterion for any cereal grain; however as with malt barley there are certain characteristics for food applications that preclude maximum yield per unit of area. The dilemma in utilizing hulled barley for food is that the hull must be removed by pearling, thereby producing a “hulless” barley product.

Composition

There are a number of references in the literature on barley composition including but not limited to these citations (NEWMAN & McGUIRE 1985; NEWMAN & NEWMAN 1992; BHATTY 1993; FASTNAUGHT 2001; NEWMAN & NEWMAN 2004). Evidence presented by these and other authors demonstrates that it is not sufficient to simply state the average composition of barley. Certain of the major constituents of barley have received considerable attention in recent years. High-lysine genes in barley and more recently starch and fiber types have been researched in depth. The beta-glucan content of barley varies considerably, being influenced by both genetics and environment. Cultivars have been developed in Australia, Canada and the USA that contain 10 to 16% beta-glucan. The high levels in some cultivars are due to reduced starch content (NEWMAN & NEWMAN 2004). Azhul, a waxy hulless barley released by the USDA having 55-60% starch has been shown to contain 10 to 11% beta-glucan (NEWMAN & NEWMAN 1989a). Reports such as XUE *et al.* (1997) present convincing evidence of increased beta-glucan content in the presence of the waxy gene. Major and even minor changes in barley composition have major effects on production as well as product development. Recent releases of cultivars that contain reduced amounts of starch (20-30 %), yet contain high dietary fiber (25-30%) have yields that are seriously below accepted norms.

Processing-Preparing Barley for Food

Pearling. Pearled barley is the most commonly used form of barley for food in North America and most of Asia. We recently reported the effects of pearling on composition (NEWMAN *et al.* 2002) and color scores before and after cooking (FASTNAUGHT *et al.* 2002) of three hulled and three hulless cultivars. Pearling reduced protein, fat, ash, insoluble and total dietary fiber, but increased starch, insoluble and total beta-glucans and extract viscosity. Growing location had no effect on soluble dietary fiber; and soluble beta-glucan levels in pearled barley were not different from that in whole barleys within growing location. Hulless barleys were higher in protein, fat, soluble fiber, total, soluble and insoluble beta-glucans and extract viscosity than were hulled barleys prior to and after pearling. The major effect of growing location was on protein and starch contents.

Blending pearled barley and rice is a simple and effective way to increase fiber in the diet, especially soluble fiber. However, consumer acceptance of blended products can be affected by appearance, i.e., color. We found that color components, whiteness, red/green and blue/yellow were affected by cultivar, growing environment and cooking. A genetic*environment interaction occurred in that five of the six cultivars had a darker color in the raw and cooked state in the more arid environment. The exception was a waxy hulled cultivar. Cooking reduced whiteness in all cultivars, but the cooked grain of the waxy starch, hulless cultivars had lower whiteness scores than the hulled cultivars. Pearled kernels of the waxy starch cultivars were whiter than that of nonwaxy cultivars. Whiteness of raw pearled barley was significantly correlated to soluble dietary fiber ($r = 0.6$), reflecting the lighter color and higher soluble fiber of the waxy genotypes. Significant negative correlations of whiteness with protein ($r = -0.74$) and insoluble dietary fiber ($r = -0.82$) are consistent with the reported high polyphenol levels and polyphenoloxidase activity in hulless barley (QUINDE & BALK 2001). No relationship was found between color and beta-glucan content.

Milling. The term milling refers to two types of processing, grinding and roller milling. In the

first instance the whole grain is ground to various particle size specifications producing whole grain flour. Because of the coarse hull, grinding for food is generally limited to pearled barley or hulless genotypes. Traditional roller milling of barley as with wheat is not a routine practice in the cereal industry in North America to produce refined flour, bran and shorts. Nevertheless, there have been numerous reports of laboratory experiments on roller milling barley, both hulled and hulless types indicating significant differences in flour yield, color and ease of processing (CHEIGH 1979; McGUIRE 1979; BHATTY 1997). Roller milling barleys that are currently available, using equipment designed for wheat and using methods applied to wheat milling, presents a challenge to millers. Hulless barleys should be more acceptable for milling than hulled types as sifting to remove hull fragments will be unnecessary. Essentially no selection pressure has been applied to barley populations to improve extraction rates of flour from barley grain as with wheat. For commercial flour production from hulless barley, milling would need to be optimized by selection of variety, mill flow, temper conditions and roll settings.

Flaking and Other Processes. The procedure used to produce barley flakes is not unlike the procedure used in producing oat flakes. The kernel or groat is generally cut in two or more pieces with steel knives or corrugated rollers to produce the desired sized and thickness of flakes. Although information is lacking in the literature, it is certain that genetic and environmental growing differences will affect the quality of barley flakes. Other commonly used processing methods, such as steel cutting, extrusion and puffing will certainly require innovation to develop desirable food products from barley.

Products

Bread, Cakes, Cookies, Muffins, Breakfast Cereals. Barley can be considered a competitor to other cereal grains, especially wheat and oats. A large segment of the baking industry has developed the concept that "white, light and volume" are highly desirable, almost necessary characteristics in bread and pastry products. Many products can be made with various portions of barley, ranging from 100% barley breakfast food flakes to very small amounts of malted barley added to a multitude of foods for flavor. Nutritious and tasty breads can be made with up to 40-50% barley flour replacing wheat flour without significantly changing the appearance and appeal of the products. A common and successful approach to adding barley fiber is to include barley flakes in the dough mixture for bread, muffins and other pastries. Baked products containing barley flour are darker and have less volume than all-wheat products; however the color and volume are not as objectionable by today's standards as was the case in the past.

Health Benefits of Barley

There is currently an intense focus on the relationship of diet and health. Certain chronic diseases, namely coronary heart disease (CHD) and diabetes are increasing in the USA and life style practices including diet are considered causative factors. The major cause of death in the USA and other industrialized countries is CHD and can often be prevented and controlled by diet. Diabetes, a condition where metabolic control and utilization of glucose is disrupted, is often associated with and may be a causative agent in CHD.

Blood Cholesterol. Elevated blood cholesterol, a major risk factor, is strongly related to lifestyle and food choices in particular. When consumed in significant amounts, the soluble fiber in barley and oats, principally beta-glucan, can play a major role in reducing blood cholesterol. It has been

suggested that barley soluble fiber reduces blood cholesterol by increasing digestive tract viscosity, removing bile acids, and increasing the production of short chain fatty acids which is thought by some researchers to provide a feedback mechanism that reduces cholesterol synthesis in the liver.

In one of our early animal studies, we found that barley beta-glucan was the responsible agent in reducing total and LDL cholesterol in hypercholesterolemic chicks fed waxy hulless barley. Since then numerous animal studies have documented the effectiveness of barley in lowering plasma cholesterol (FASTNAUGHT 2001). We conducted two human studies preparing foods from Azhul, a waxy hulless barley which contained 10% beta-glucan (NEWMAN *et al*. 1989a; NEWMAN *et al.* 1989b). In the first study subjects who ate wheat foods had significantly increased serum total and LDL cholesterol when compared to pretreatment levels. Subjects consuming barley who had average pre-treatment levels showed no significant effects on cholesterol; but for those subjects who had higher pre-treatment levels, total and LDL-cholesterol levels were significantly reduced after four weeks. In the second study, we showed that barley was equivalent to oats in lowering serum cholesterol in men and women with elevated cholesterol (>250 mg/dl). The results in later clinical studies with barley confirmed our findings that the consumption of a variety of barley foods will lower serum cholesterol, especially the LDL cholesterol (McINTOSH *et al*. 1991; IKEGAMI *et al*. 1996; BOURDON *et al.* 1999; LI *et al*. 2003; BEHALL *et al*. 2003; BEHALL *et al*. 2004). These are summarized in Table 1.

Table 1. Summary of clinical trials showing evidence of barley lowering blood cholesterol.

Reference	Subjects	Cholesterol Reduction, %
Newman et al. 1989a	Healthy men and women	3.3
Newman et al. 1989b	Hypercholesterolemic men and women	5.0
McIntosh et al. 1991	Hypercholesterolemic men	6.0
Ikegami et al. 1996	Hypercholesterolemic men and women	10.0
Li et al. 2003	Healthy women	14.5
Behall et al. 2003	Hypercholesterolemic men	14.0-20.0
Behall et al. 2004	Hypercholesterolemic men and women	5.0-10.0

In addition to the effects of beta-glucans, lipid associated components in barley may have a role in cholesterol control. Barley oil is a concentrated source of tocotrienols which have been shown to be effective in suppressing cholesterol synthesis in the liver (QURESHI 1987). Tocopherol (vitamin E), also found in generous amounts in barley oil, blocks the oxidation of low density lipoproteins, the first step in development of arterial plaque (PACKER *et al*. 2001).

Elevated blood pressure is a second risk factor in CHD. Whole grain consumption has been reported in several instances to lower blood pressure in individuals at risk. However, the results have been mixed, probably because of inconsistent experimental design and uncontrolled factors within the experiments. In a very controlled study, HALLFRISCH *et al*. (2003) found that barley

flakes, barley flour, and pearled barley were equally effective as a mixture of brown rice and whole wheat foods in reducing blood pressure in non-hypertensive men with elevated cholesterol levels.

Diabetes. When starch from a cereal grain is ingested by man or animal, blood glucose is quickly elevated because of rapid digestion of starch. In the normal individual this is followed by the release of insulin from pancreatic beta cells to effect its proper utilization by the body. When insulin is not produced in sufficient quantities or if there is insulin resistance, the absorbed glucose cannot be properly metabolized resulting in abnormally high blood glucose and/or insulin levels leading to severe life-threatening complications. The glycemic index (GI) is a method to classify foods to the degree of glucose response they elicit when eaten by normal individuals. Foods that cause a rapid and steep rise in blood glucose are considered to have a high GI number and vice versa. Factors that give foods a low GI number include the presence of resistant starch and/or soluble dietary fiber, heat processing and a large starch particle size. Barley is among the foods with very low GI numbers, particularly those varieties that contain the *amol 1* gene. The *amol 1* gene produces a starch that contains 40-45% amylose in comparison to the 25-27% amylose in other varieties. The CSIRO in Australia has developed a barley cultivar that is reported to contain .70% amylose which is under different genetic control (D. TOPPING, personal communication).

Swedish researchers at the University of Lund (BJORCK *et al.* 1990) reported increased levels of resistant starch in High-amylose Glacier barley and consequently low GI values. In a follow up study (GRANFELDT *et al.* 1994), products produced from Glacier and High-amylose Glacier barleys gave significantly lower GI values than white bread and intact boiled kernels gave lower values than barley porridge. Further investigations by this group (LILJEBERG *et al.* 1996) showed the lowest blood glucose and insulin responses after consumption of high-fiber barley products. These researchers suggested that the blunted glycemic and insulinemic responses were due not to the amylose content, but to the soluble fiber (beta-glucan) concentration in the barley tested, cv. Sustagrain. In a clinical study, RENDELL *et al.* (1998) reported a substantial reduction of the post-prandial glycemic peak following ingestion of Sustagrain barley flakes compared to oatmeal or a commercial liquid meal replacer in both normal and diabetic patients. LIFSCHITZ *et al.* (2002) concluded that Sustagrain barley is an appropriate food for obese and diabetic patients due to reduced absorption in the gastrointestinal tract. The Australian CISRO high-amylose barley tested in clinical trials produced glucose and insulin responses very similar to those reported for Sustagrain barley (D. TOPPING, personal communication). USDA researchers, YOKOYAMA *et al.* (1997), reported that wheat pasta supplemented with barley beta-glucan produced lower glycemic responses compared to standard wheat pasta. The long term effects of incorporating waxy hulless barley in bread fed to non-insulin dependent diabetic men were reported by PICK *et al.* (1998). Incorporation of barley bread products into the diet of these patients improved the post-prandial glycemic responses. Insulinemic responses were also improved and some subjects were able to reduce oral medication dose levels. These reports are summarized in Table 2.

Table 2. Summary of clinical trials showing evidence of barley moderating blood glucose and/or insulin responses.

Björck et al. 1990	Resistant starch in high-amylose Glacier barley produced low glycemic index values
Granfeldt et al. 1994	Low glycemic index values in boiled kernels and porridge made from Glacier barley
Liljeberg et al. 1996	Sustagrain barley reduced post-prandial blood glucose and insulin responses
Yokoyama et al. 1997	Beta-glucan supplemented wheat pasta lowered the glycemic index value
Pick et al. 1998	Positive response to long-term consumption of wheat bread containing waxy hulless barley flour by type 2 diabetics
Rendell et al. 1998	Sustagrain barley reduced post-prandial blood glucose and insulin responses in type 2 diabetics and nondiabetics
Lifschitz et al. 2002	Reduced absorption of glucose from Sustagrain barley due to high dietary fiber content
Topping 2003	Reduction in glucose and insulin peaks after consumption of high-amylose, high-fiber barley product

References

BEHALL K.M., SCHOLFIELD D.J., HALLFRISCH J. (2003): Barley consumption lowers cholesterol in men and overweight women. FASEB J., **17**: A1.

BEHALL K.M., SCHOLFIELD D.J., HALLFRISCH J. (2004): Lipids significantly reduced by barley in moderately hypercholesterolemic men. J. Am. Col. Nutr., **23**: 55-62.

BHATTY R.S. (1993): Nonmalting uses of barley. In: MacGregor, A.W. and Bhatty, R.S. (eds.): Barley Chemistry and Technology. Am. Assoc. Cereal Chem. St. Paul, MN USA.

BHATTY R.S. (1997): Milling of regular and waxy starch hull-less barleys for the production of bran and flour. Cereal Chem., **74**: 693-699.

BJORCK I., ELIASSON A.-C., DREWS A., GUDMUNDSSON M., KARLSSON R. (1990): Some nutritional properties of starch and dietary fiber in barley genotypes containing different levels of amylose. Cereal Chem., **67**: 327-333.

BOURDON I., YOKOYAMA W., DAVIS P., HUDSON C., BACKUS R., RICHTER D., KNUCKLES B., SCHNEEMAN B.O. (1999): Post-prandial lipid, glucose, insulin, and cholecystokinin responses in man fed barley pasta enriched with beta-glucan. Am. J. Clin. Nutr., **69**: 55-63.

CHEIGH H.S. (1979): Rheological baking and noodle making properties of barley-wheat flour blends. In: Proc. Joint Barley Utilization Seminar, Korean Sci. Eng. Foundation Suweon, Korea: 48-71.

FASTNAUGHT C.E. (2001): Barley fiber. In: CHO, S.S. and DREHER, M.L. (eds.): Handbook of Dietary Fiber. Marcel Dekker, Inc., NY, NY USA: 519-542.

FASTNAUGHT C.E., NEWMAN R.K., NEWMAN C.W., BERGLUND P.T. (2002): Variety and cooking effects on pearl barley color. Am. Assoc. Cereal Chem. Ann. Meet., Montrðal, Can. Abstr. #405.

GRANFELDT Y., LILJEBERG H., DREWS A., NEWMAN R., BJORCK I. (1994): Glucose and insulin responses to barley products: Influences of food structure and amylose-amylopectin ratio. Am. J. Clin. Nutr., **59**: 1075-1082.

HALLFRISCH J., SCHOLFIELD D.J., BEHALL K.M. (2003b). Blood pressure reduced by whole grain diet containing barley or whole wheat and brown rice in moderately hypercholesterolemic men. Nutr. Res., **23**: 1631-1642.

IKEGAMI S., TOMITA M., HONDA S., YAMAGUCHI M., MIZUKAWA R., SUZUKI Y., ISHII K., OHASAWA S., KIYOOKA N., HIGUCHI M., KOBAYASHI S. (1996): Effect of boiled barley-rice-feeding in hypercholesterolemic and normolipemic subjects. Plant Foods Human Nutr., **49**: 317-328.

LI J., KANEKO T., QIN L.-Q., WANG J., WANG Y. (2003): Effects of barley intake on glucose tolerance, lipid metabolism, and bowel function in women. Nutr., **19**: 926-929.

LIFSCHITZ C.A., GRUSAK M.A., BUTTE N.F. (2002): Carbohydrate digestion in humans from a beta-glucan-enriched barley is reduced. J. Nutr., **132**. 2593-2596.

LILJEBERG H.G.M., GRANDFELDT Y.E., BJORCK I. (1996): Products based on a high fiber barley genotype, but not on common barley or oats, lower postprandial glucose and insulin responses in healthy humans. J. Nutr., **126**: 458-466.

McGUIRE C.F. (1979): Roller milling and quality evaluation of barley flour. In: Proc. Joint Barley Utilization Seminar, Korean Sci. Eng. Foundation, Suweon, Korea: 89-93.

McINTOSH G.H., WHYTE J., McARTHUR R., NESTEL P.J. (1991): Barley and wheat foods: influence on plasma cholesterol concentrations in hypercholesterolemic men. Am. J. Clin. Nutr., **53**: 1205-1209.

NEWMAN C.W., McGUIRE C.F. (1985): Nutritional quality of barley. In: RASMUSSON D.C. (ed.): Barley. Am. Soc. Agron., Crop Sci. Soc. of Am., Soil Sci. Soc. of Am. Madison, WI USA: 403-456.

NEWMAN C.W., NEWMAN R.K. (1992): Nutritional aspects of barley seed structure and composition. In: SHEWRY, P.R. (ed.): Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology. C.A.B. Int., Wallingford UK: 351-368.

NEWMAN R.K., LEWIS S.E., NEWMAN CW., BOIK R.J., RAMAGE R.T. (1989a): Hypocholesterolemic effect of barley foods on healthy men. Nutr. Rep. Int., **39**: 749-760.

NEWMAN R.K., NEWMAN C.W., GRAHAM H. (1989b): The hypocholesterolemic function of barley beta-glucans. Cereal Foods World, **34**: 883-886.

NEWMAN C.W., NEWMAN R.K., FASTNAUGHT C.E. (2002): Comparative composition of whole and pearl barley. Am. Assoc. Cereal Chem. Ann. Meet., Montrðal, Can. Abstr. #321.

NEWMAN C.W., NEWMAN R.K. (2004): Hulless barley for food and feed. In: ABDELAAL and WOOD (eds.): Specialty Grains for Food and Feed. Am Assoc. Cereal Chem. St. Paul MN USA: (at press).

PACKER L., WEBER S.U., RIMBACH G. (2001): Molecular mechanisms of protective effects of vitamin E in atherosclerosis. J. Nutr., **131**: 369s-373s.

PICK M.E., HAWRYSH Z.J., GEE M.I., TOTH E. (1998): Barley bread products improved glycemic control of type 2 subjects. Int. J. Food Sci. Nutr., **49**: 71-81.

RENDELL M., VANDERHOOF J., VENN M., SHEHAN M.A., ARNDT E., RAO C.S., GEOFFREY G. (1998): A grain with reduced blood sugar response in diabetes. Ann. Meet. Am. Soc. Clin. Nutr., Albuquerque, NM USA, Oct 2,1998.

QUINDE Z., BALK B.-K. (2001): Discoloration potential of various types of barley in food products. Am. Assoc. Cereal Chem. 2001 Ann. Meet. Abstr. #13, Charlotte, NC USA.

QURESHI A.A., CRENSHAW T.D., ABUIRMEILEH N., PETERSON D.M., ELSON C.E. (1987): Influence of minor plant constituents on porcine hepatic lipid metabolism. Atherosclerosis, **64:** 109-115.

XUE, Q., WANG L., NEWMAN R.K., NEWMAN C.W., GRAHAM H. (1997): Influence of the hulless, waxy starch and short-awn genes on the composition of barleys. J. Cereal Sci., **26**: 251-257.

YOKOYAMA W.H., HUDSON C.A., KNUCKLES B.E., CHIU M.-C., SAYRE R.N., TURNLUND J.R., SCHNEEMAN B.O. (1997): Effect of barley beta-glucan in Durum wheat pasta on human glycemic response. Cereal Chem., **74**: 293-296.

Genetic Studies of Barley Seed Phosphate and Phytic Acid

V. Raboy[1], H. Saneoka[2], J.A. Dorsch[1,3], K. Peterson[1], D. Bowen[4], J. Fu[4], M. Guttieri[4] and E. Souza[4]

[1]USDA-ARS, Aberdeen, Idaho, U.S.A.; [2]Hiroshima University, Hiroshima, Japan; [3]Current Address, BASF Corporation, North Carolina, U.S.A.; [4]University of Idaho, Aberdeen, Idaho, U.S.A.

Abstract

Phytic acid represents about 65% of seed total P. More than 20 heritable barley *low phytic acid* (*lpa*) mutants have been isolated. These mutations result in reductions in seed phytic acid P ranging from 50% (*lpa*1-1) to >90% (M 955), but have little effect on seed total P. The pathway to phytic acid begins with the synthesis of *myo*-inositol (Ins), via the conversion of glucose 6-P to Ins(3)P_1, catalyzed by the enzyme D-*myo*-inositol 3-phosphate synthase (MIPS, E.C. 5.5.1.4), and then hydrolysis of Ins(3)P_1 to Ins, catalyzed by an Ins monophosphatase (IMP, EC 3.1.3.25). We isolated and mapped (to chromosome 4H) the barley genome's single copy MIPS gene, and are now working on IMP-encoding sequences. We plan to screen for mutations in these sequences using the "TILLING" reverse genetics approach. We are also studying the impact of *lpa* mutations on expression of genes outside those in the pathways of phytic acid synthesis, in an effort to characterize the metabolic effects of substantial blocks in phosphate and Ins phosphate seed metabolism. For example, preliminary results indicate that during seed development B1 Hordein and granule-bound starch synthase 1 expression are reduced by about 75%, or substantially delayed, in M 955 as compared with wild-type, whereas both MIPS and IMP expression are unchanged or increased in M 955 versus wild-type. This work will lead to a fuller understanding of both phosphate and Ins phosphate/phytic acid molecular biology in seeds, and will contribute to long-term efforts to metabolically engineer "optimized" Low Phytate crops.

Introduction

Approximately 65% to 75% of mature seed total phosphorus (P) is found as *myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate ($InsP_6$. mw 660), or "phytic acid" (Figure 1) (RABOY 2003). This compound is the most abundant representative of a class of compounds, the inositol phosphates, important in signal transduction, RNA and DNA metabolism, and other aspects of eukaryotic cell biology (SHEARS 2001). The biology of seed storage P, and some aspects of the biology of seed mineral cation distribution and storage, revolve around the metabolism of phytic acid (RABOY 2003). Applied interest in seed phytic acid primarilly concerns its role in human and animal nutrition and health (ADAMS *et al.* 2002; VEUM *et al.* 2002). Non-ruminant species such as poultry, swine and fish do not efficiently digest and utilize seed phytic acid P. Most of seed-derived P they consume is excreted, a management and environmental problem. In terms of human nutrition, dietary phytic acid might contribute to mineral deficiencies, such as iron and zinc deficiency, especially in populations that rely on grains and legumes as staple foods. However, dietary phytic acid might also function as an antioxidant and anti-cancer agent (GRAF *et al.* 1987). To develope materials useful for studying these applied and basic questions we have been purusing two types of research: isolation and study of *low phytic acid* mutants and mutations; isolation and study of specific genes of known or proposed importance to seed phytic acid metabolism. We isolated more than 20 heritable barley *low phytic acid* mutants. Initial chemical and genetic work focused on a subset of four mutations.

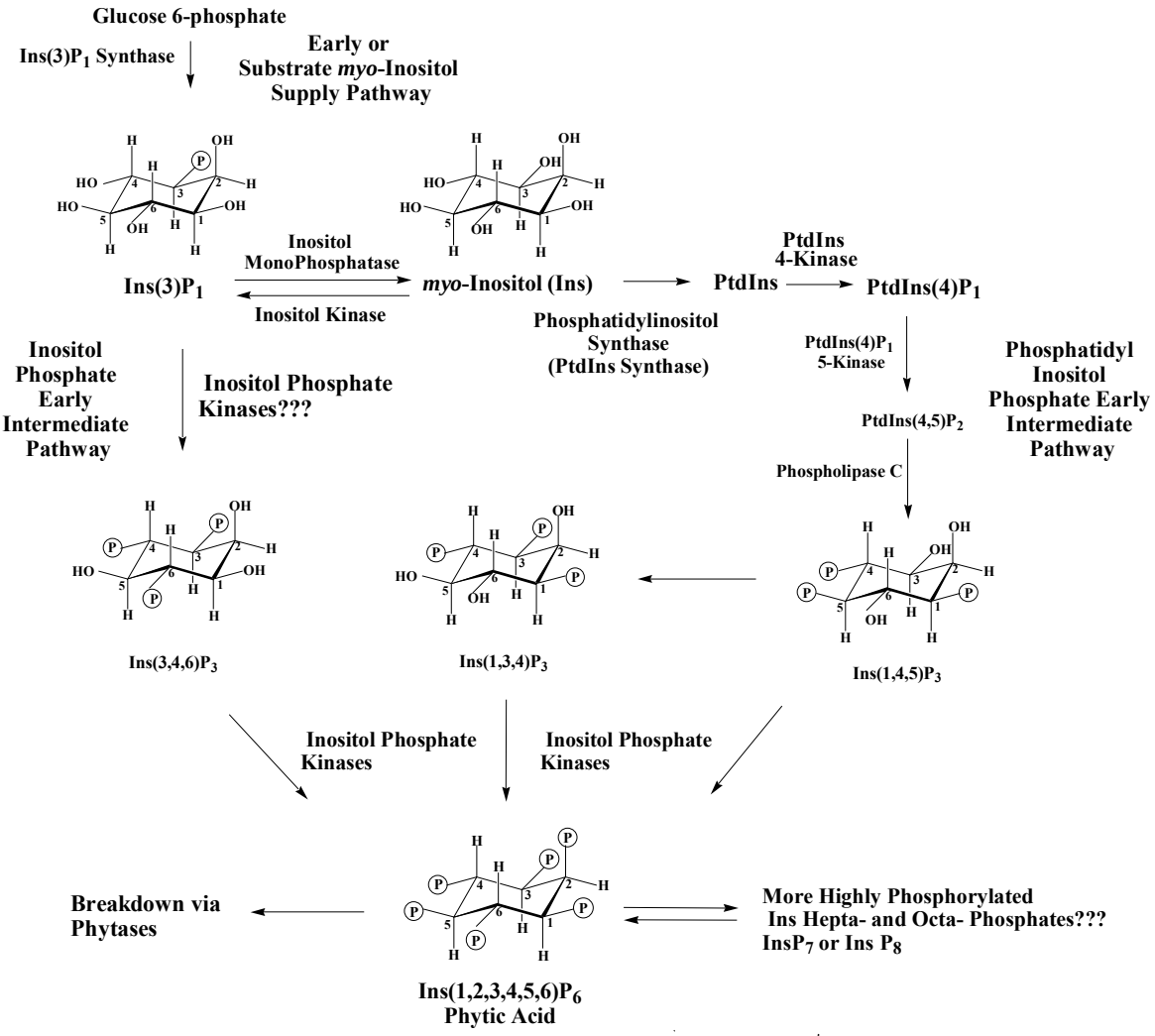


Figure 1. Biosynthetic pathways important to phytic acid synthesis in seeds

This subset conisisted of the *lpa* mutants M 422, M635, M 955 and M 1070, selected to represent the range of phenotypes observed in the larger collection. M 422 and M 1070 were shown to be recessive alleles at two loci, termed barley *low phytic acid* 1 (M422) and barley *low phytic acid* 2 (*lpa*2), mapping to chromosome 2H and 7H, respectively (LARSON *et al*. 1998). M 635 we next shown to be a recessive allele at a third locus, termed barley *low phytic acid* 3 (*lpa*3; Roslinsky, Ecksein, Rossnagel, Skoles, and Raboy, work presented separately at this Symposium). Here we present the initial phenotypic description, in terms of seed phosphorus fractions, of our complete set of barley *lpa* mutations.

The biochemical pathways to phytic acid begin with the synthesis of *myo*-inositol, a two-step process: the conversion of glucose 6-P to *myo*-inositol(3)P_1 (Ins(3)P_1, an Ins monophosphate with the single phosphate ester at "D-3" position) catalyzed by *myo*-inositol 1-phosphate synthase, or MIPS; the hydrolysis of Ins(3)P_1 to Ins and inorganic P, catalyzed by Ins monophosphatase, or IMP. Initial sequence and gene expression analyses (LARSON & RABOY 1999) focused on sequences encoding MIPS. Here we describe the first steps in studying sequences encoding barley and wheat IMPs. Finally, we are interested in the downstream effects of mutations that greatly perturb phosphate and Ins phosphate metabolism in seeds, as do *lpa* mutations. Here we will describe initial experiments looking at downstream gene expression effects of a selected barley *lpa* mutation.

Material and Methods

Survey of Seed Phosphorus Fractions in lpa Mutants. All *lpa* mutants described here were first isolated in the cv. Harrington genetic background following chemical mutagenesis of seed with sodium azide treatment (DORSCH *et al*. 2003). Lines are referred to as their original "M" number (M 281, M 955 etc.) pending determination of inheritance, mapping and allelic assignment. Lines homozygous for a given mutation, or homogeneous for a given mutant phenotype (in those cases where the genetics or inheritance of a given mutant phenotype are not yet known), were grown in 2000 in a field nursery located at the University of Idaho Aberdeen Research and Extension Center, Aberdeen Idaho, U.S.A.. Homozygosity or homogeneity were determined using the single-seed HIP assay. The *lpa* seed analyzed in Table 1 was from plants representing the M_5 to M_8 generations (obtained via self-pollination of the original M_2s), with the exception of M 593, which was in the F_5 generation. Rows of the cultivars Harrington, Steptoe and Morex were grown in the same nursery as controls. Plants were harvested by hand and threshed individually, and seed stored in a seed storage facility at 4°C until analysis. Samples of mature seeds were dried for 48 h at 60 °C, milled to pass through a 20-mm screen, and stored in a desiccator until analysis. All methods were as described in DORSCH *et al.* (2003).

Isolation of Barley and Wheat myo-Inositol Monophosphatase Sequences. Total RNA was isolated from developing seeds of cv. Harrington barley and cv. Idaho 563 wheat. mRNA was purified using a Poly(A)Purist kit (Ambion, Austin, TX), and used for the synthesis of double stranded cDNA. Expression libraries were constructed using the SMART cDNA Library Construction System (BD Biosciences Clontech, Palo Alto, CA) according the manufacturer's recommendation. Approximately 10^6 plaque-forming units (pfu) of the primary phage libraries were converted into plasmid libraries hosted in the E. coli strain BM25.8. The coding regions of the *myo*-inositol monophosphatases (IMPs) were PCR-amplified using primers based on barley and wheat Expressed Sequence Tags (ESTs) homologous to tomato IMP cDNA (accession #AY227666, www.ncbi.org), and then cloned into pCR4-TOPO vector. DNA sequencing was conducted using the BigDye method by Sequetech Corp. (California, USA). The sequence alignments given in Figure 2 were prepared using the "Multalin" program (CORPET 1988).

Quantitative Real-Time Reverse-Transcription (RT)-PCR. The near-isogenic sibling lines used in this study were either homozygous wild-type or homozygous for the single-gene M 955 mutation, and were from the BC_2 F_5 generation. These materials were grown in the 2003 summer nursery at Aberdeen, ID. Seed were harvested during development ("watery ripe" stage), and immediately frozen in liquid N_2. Total RNA was extracted using the Concert Plant RNA reagent (Invitrogen), and total RNA was quantified using Ribogreen RNA Quantitation kit (Molecular Probes Inc.). The Sybr-green One-step PCR protocol (Quiagen) was used for Real Time RT-PCR following the manufacturer's instructions. Total RNA (0.2 µg) was added to 50 µL reactions for all reactions except those for IMP, in which 2.0 µg total RNA was used. Assays were conducted using an ABI Prism 7000 Sequence Detection System.

Results and Discussion

Our current set of barley *lpa* mutants consists of 22 independently-isolated mutants (Table 1). In all cases where a clear reduction in phytic acid P is observed, these reductions are largely matched by increases in inorganic P or both inorganic P and inositol phosphate precursors to phytic acid, so that seed total P remains unchanged and similar to wild-type. In some cases such as M 499, M 1083, M AZ 397 and M AZ 423, large reductions in phytic acid

particular do a lot with it (LOEWUS & MURTHY 2000). For example, in addition to functioning as the head-group for the parent (PtdIns) of a major class of lipids; one of two alternative pathways to plant cell wall polysaccharides proceeds via Ins oxidation; Ins is the parent to all cyclitols; IAA metabolism utilizes Ins as does the pathway to raffinosaccharides.

Table 1. Mean (±SD) seed dry weight and phosphorus fractions of twenty two barley *low phytic acid* mutants. For those cultivars or genotypes were no Standard Deviation is given, only one plant was analyzed, in duplicate. For the rest, two plants representing each genotype were analyzed duplicate.

Cultivar or Genotype	Seed Dry Weight mg seed^{-1}	Total P mg g^{-1}	Phytic Acid P mg g^{-1}	Inorganic P mg g^{-1}
Harrington	40.1	5.53	3.03	0.80
Steptoe	41.4	4.58	2.32	0.44
Morex	32.9±0.86	4.69±0.330	2.97±0.349	0.56±0.018
M 281	29.0±0.41	3.71±0.028	1.24±0.113	1.35±0.029
*lpa*1-1 (M 422)	33.4±1.94	3.84±0.013	1.49±0.015	1.50±0.000
M 499	23.9±1.49	6.14±0.059	3.11±0.258	1.84±0.015
M 593	35.0±3.88	6.28±0.276	1.49±0.183	3.17±0.185
lpa 3-1 (M 635)	29.9±0.68	5.85±0.423	1.07±0.166	3.23±0.099
M 640	24.0	4.46	1.31	1.66
M 678	19.5	6.28	0.18	4.60
M 882	32.4±1.03	3.89±0.131	1.40±0.103	1.54±0.004
M 889	40.4±2.57	5.80±1.147	1.89±0.043	2.50±0.082
M 955	30.5±0.37	5.98±0.036	0.24±0.015	3.86±0.007
*lpa*2-1(M 1070)	31.2±0.10	5.66±0.662	0.87±0.097	3.39±0.200
M 1083	31.2±0.04	5.59±1.036	3.19±0.707	0.89±0.059
M 1154	33.4±0.34	3.80±0.132	1.320.162	1.35±0.004
M 1572	31.7±1.17	6.33±0.453	2.23±0.207	2.59±0.060
M 1580	33.4±2.85	4.30±0.055	1.56±0.131	1.55±0.002
M 1673	32.7±1,61	4.03±0.408	1.61±0.160	1.40±0.042
M 1954	31.1±1.34	5.97±0.637	2.54±0.281	1.83±0.085
M 2002	33.2±0.36	4.08±0.683	1.39±0.226	1.46±0.076
M 2080	31.5±4.12	6.01±0.266	2.31±0.004	2.53±0.116
M 2172	31.9±0.33	4.04±0.324	1.50±0.170	1.60±0.036
M AZ 397	29.3±8.58	5.84±0.362	3.14±0.016	1.75±0.197
M AZ 423	30.9±2.45	6.06±0.200	3.52±0.751	1.48±0.025

Finally, the regulation of cellular P, “P homeostasis”, is also critical to nominal cellular function (STROTHER 1980). Thus it is interesting to determine the distal or downstream impacts of mutations that greatly alter the metabolism of these compounds. This type of knowledge might also be of practical value in understanding how phytic acid mutations negatively impact plant and seed function, and thus important in the development of strategies of overcome these negative impacts.

One approach to determining this downstream impact is to identify changes in gene expression genome-wide in developing seeds homozygous for a given *lpa* mutation, as compared with developing seeds that are homozyous wild-type. An excellent experimental model for such studies are near-isogenic lines. The initial approaches we are taking use micro-array analysis and for selected genes, real-time reverse-transcription PCR (RT-PCR). While preliminary in nature, requiring replication and the use of additional controls, Figure 3 illustrates the interesting results of the first RT-PCR assays we conducted with a barley *lpa* mutation, M 955, and its corresponding wild-type isoline as control.

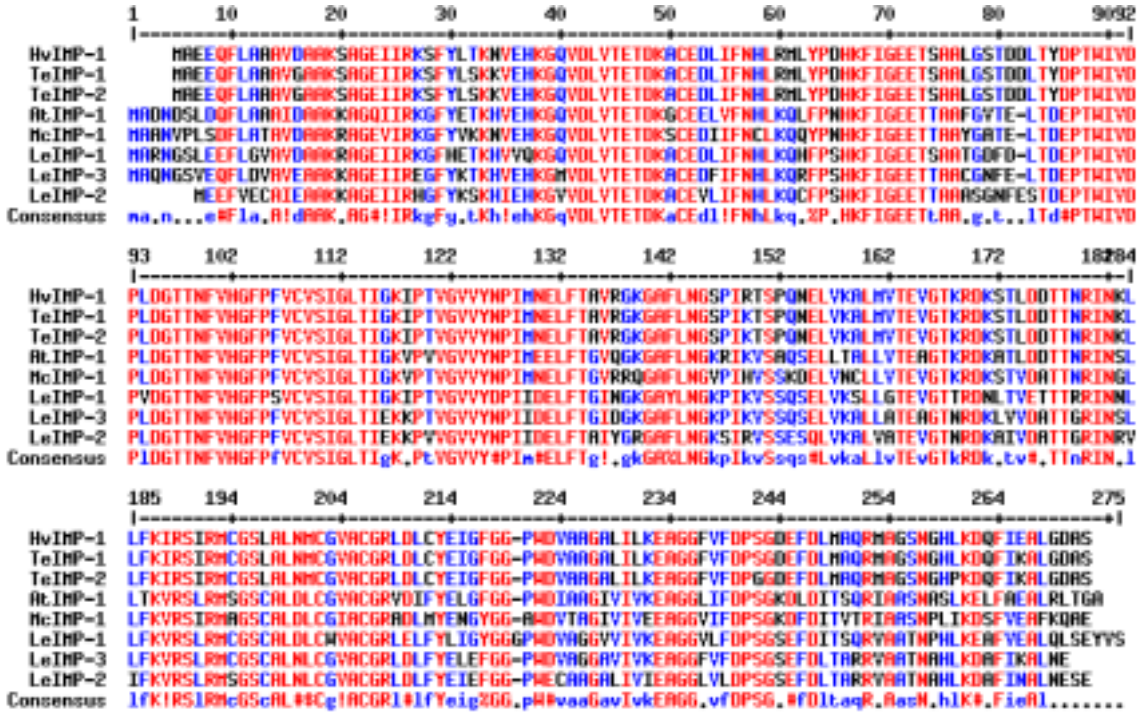


Figure 2. Sequence alignment of seed-expressed *myo*-inositol monophosphatases (IMPs) from barley (HeIMP-1)and wheat (TeIMP-1 and TeIMP-2). These are compared with IMPs from *Arabidopsis thaliana* (AtIMP-1, Accession No. AAM62772), ice plant (*Mesembryanthemum crystallinum*, McIMP-1, Accession No. T12205) and tomato (LeIMP-1, Accession No. AAP15454; LeIMIP-2, Accession No. AAP15455; LeIMP-3, P54928).

The data points (Ct values) in Figure 3 represent the PCR cycle number at which maximal exponential accumulation of PCR product for a given reaction was observed. Variation in this point in product accumulation reflects variation in substrate RNA amount. One estimate of the difference in locus RNA amount between M 955 and wild-type is $(1/2^n)$, where n=cycle number difference. If there is a 2-cycle difference in the assays of GBSS and Hor in M 955 versus wild-type, then there is about ¼ as much RNA for these genes expressed in the M 955 seed, as compared with wild-type, at that point in development. This may clearly translate into a significant difference in storage protein or starch in M 955 seeds as compared with wild-type, or into a substantial delay in developmental accumulation of these important

storage products. Reductions in expression of these genes might represent one component contributing to the reduced seed weight, a pleiotropic phenotype, observed in M 955 and many other *lpa* mutants (Table 1; RABOY *et al.* 2000; DORSCH *et al.* 2003). Differences in MIPS and IMP mRNA amounts in M 955 versus wild-type are not nearly as great. If anything, expression of these two genes appears to be increased in M 955 as compared with wild-type. This result may be similar to the observation (K.A. Young, H.-I. Rhee and V. Raboy, unpublished) that Ins kinase and Ins 1-phosphate kinase activities were increased in maize *lpa* 1-1 seed as compared with its wild-type isoline control. Perhaps in both these cases blocks at one point in the pathway to phytic acid, and the resulting increase in cellular inorganic P, in turn results in enhanced expression of genes at other points in the pathway, as part of cellular attempt at homeostatic compensation.

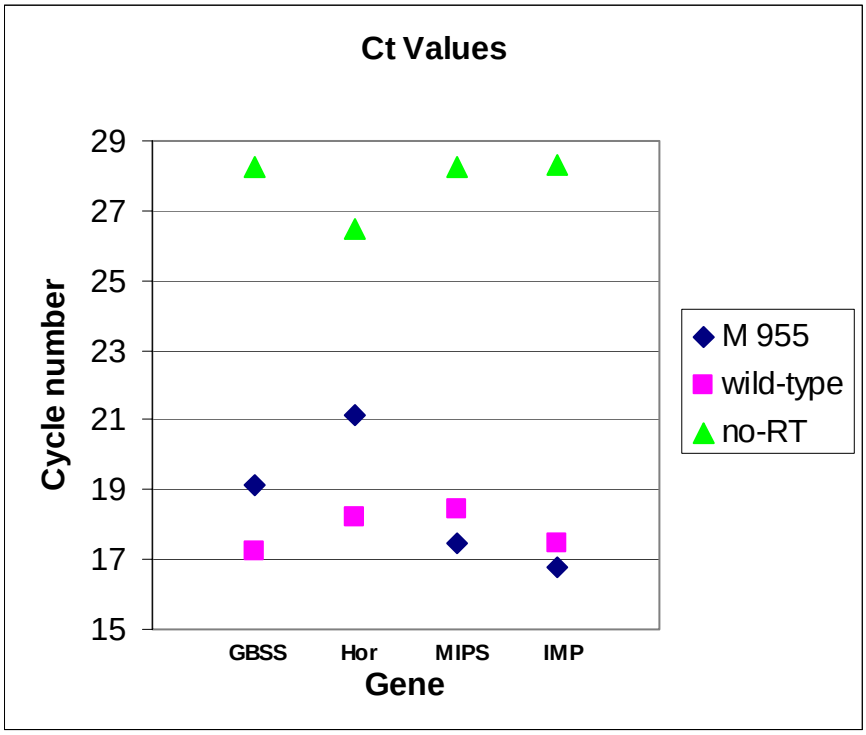


Figure 3. Real-time Reverse-Transcription PCR (RT-PCR) of gene expression in developing M 955 seeds as compared with wild-type. "Ct" values shown represent point of maximal exponential increase of a given product. "GBSS"=barley granule-bound starch synthase 1, Accession no. AF486519.1. "Hor"=barley B1 hordein, the Hor2-4 gene, Accession no. X87232. "IMP"=barley inositol monophosphase (see Figure 2.); "MIPS"=barley *myo*-inositol 1-phosphate synthase gene, Accession no. AF056325. Forward and Reverse primers were designed to amplify fragments of about 100 bp in length. The data points represents means of assays of five M 955 isolines and three wild-type isolines. The largest standard deviation of these means was 0.54 cycles (for M 955 Hor), so the difference between M 955 and wild-type in the results for GBSS and Hor are clearly statistically significant. "No-RT"=no reverse transcriptase, and this serves as a negative control.

This work provides genetics resources (mutants, mutations and genes) useful in research aimed at developing a fuller understanding of both phosphate and Ins phosphate/phytic acid molecular biology in seeds. These resources provide an experimental model useful for an wide array of basic and applied studies such, as those into the agronomics

of Low Phytate crops, or those that address their potential value of Low Phytate crops for human nutrition and health, or for animal production. These resources are also useful in basic studies into plant and seed metabolic pathways connected to P and Ins phosphate metabolism. Finally, these resources mightl be valuabe to long-term efforts to metabolically engineer "optimized" Low Phytate crops.

Acknowledgements

This work was supported in part by the USDA-CSREES Special Project, "The North American Barley Genome Mapping Project". It was also supported in part by the U.S. National Science Foundation Molecular Biosciences Program Grant No. 0110748, "Molecular Mechanisms of Inositol and Inositolhexakisphosphate Metabolism in Barley and Wheat Seed".

References

ADAMS C.L., HAMBIDGE M., RABOY V., DORSCH, J.A., SIAN L., WESTCOTT J.L., KREBS N.F. (2002): Zinc absorption from a low-phytic acid maize. Am. J. Clin. Nutr., **76**: 556-559.

CORPET F. (1988): Multiple sequence alignment with hierarchical clustering. Nucl. Acids Res., **16**: 10881-10890.

DORSCH J.A., COOK A., YOUNG K.A., ANDERSON J.M., BAUMAN A.T., VOLKMANN C.J., MURTHY P.P.N., RABOY V. (2003):Seed phosphorus and inositol phosphate phenotype of barley *low phytic acid* genotypes. Phytochemistry, **62**: 691-706.

GRAF E., EPSON K.L., EATON J.W. (1987): Phytic acid: a natural antioxidant. J. Biol. Chem., **262:** 11647-11650.

LARSON S.R., RABOY V. (1999): Linkage mapping of maize and barley *myo*-inositol 1-phosphate synthase DNA sequences: correspondence with a *low phytic acid* mutation. Theor. Appl. Genet., **99**: 27-36.

LARSON S.R., YOUNG K.A., COOK A., BLAKE T.K., RABOY V. (1998): Linkage mapping two mutations that reduce phytic acid content of barley grain. Theor. Appl. Genet., **97:**141-146.

LOEWUS F.A., MURTHY P.P.N. (2000): *myo*-Inositol metabolism in plants. Plant Sci., **150**: 1-19.

RABOY V., GERBASI P.F., YOUNG K.A., STONEBERG S.D., PICKETT S.G., BAUMAN A.T., MURTHY P.P.N., SHERIDAN W.F., ERTL D.S. (2000): Origin and seed phenotype of maize *low phytic acid 1-1* and *low phytic acid 2-1*. Plant Physiol., **124:** 355-368.

RABOY V. (2003): *Myo*-Inositol-1,2,3,4,5,6-hexa*kis*phosphate. Phytochemistry, **64**:1033-1043. SHEARS S.B. (2001): Assessing the omnipotence of inositol hexakisphosphate. Cellular Signaling, **13**: 151-158.

STROTHER S. (1980): Homeostasis in germinating seeds. Ann. Bot., **45**: 217-218.

VEUM T.L., LEDOUX D.R., BOLLINGER D.W., RABOY V., COOK A. (2002): Low-phytic acid barley improves calcium and phosphorus utilization and growth performance in growing pigs. J. Animal Science, **80**: 2663-2670.

Investigation of Barley Proteome

J. Chmelík and P. Řehulka

Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic,
Veveří 97, 611 42 Brno, Czech Republic

Abstract
Recent research advances at gene level are providing a lot of nucleotide sequences, genetic map and DNA markers for barley. However, knowledge of the genotype alone does not permit either to select better cultivars, or to determine the environmental effects on grain quality. To this aim, detailed elucidation of the protein composition is necessary in order to assess the quality barley. In addition, identification of individual proteins as markers of phenotype has great value at all stages of barley production. Detailed knowledge of the grain protein composition may produce further insight into the relationships between protein composition and the technological properties of barley. Identification of several proteins extracted with various solvents from barley grains by the proteomic strategy based on a combination of gel electrophoresis, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and bioinformatics was performed. The mass spectrometry-based strategy designed for protein identification exhibits good sensitivity and rapid protein identification in comparison to other methods. In order to simplify the protein mixture extracted from grains, we used sequential extractions of grains with various solvents (based on modified Osborne system). Several proteins (e.g. beta-amylase, B3 hordein) were identified. It seems that this approach can become an important tool for the investigation of proteome of cereals.

Introduction

Barley is one of major world crops. It contributes to the food supply in the world as human food, malt products, and livestock feed. As an important experimental and model plant, barley is useful for various studies in malting and brewing chemistry, plant breeding methodology, genetics, virology, physiology, biotechnology and other fields.

From the Middle East center of barley cultivation, barley has spread around the world. Cultivated barley is adapted over a wider range of various environmental conditions in comparison to any other cereal. Barley is relatively cold tolerant and is considered the most drought, alkali, and salt tolerant among the small-grain cereal crop species. However, acid and wet soil conditions are not well suited for barley production. The main role in barley adaptation to drought and temperature extremes plays its relatively early maturity and relatively low water use. Tolerance to cold weather is quite good for spring types, whereas the winter types are less tolerant to cold than wheat, rye, and triticale.

In order to understand barley properties, it is necessary to describe its chemical composition in any stage of barley life. Proteins are the major functional molecules of life. The ability to unambiguously identify the proteins is a prerequisite for their functional investigation. Global analysis of biological systems is becoming increasingly feasible as technologies that facilitate proteome analyses are developed. The modern technologies allow the global analysis of expressed proteins (including posttranslational modifications) and establishing the relationship between genome sequence, expressed proteins, protein-protein interactions, and cell and tissue phenotype. As proteins derived from the same gene can be largely identical, and might differ only in small but functionally relevant details, protein identification tools must not only identify a large number of proteins but also be able to differentiate between close relatives.

Proteomics represents nowadays an important tool for understanding of processes in living organisms. It can be defined as the systematic analysis of proteins for their identity, quantity and functions. Proteomic methodology consists of combination of a suitable separation technique, mass spectrometry (MS) measurements and data evaluation using bioinformatics tools (MANN *et al.* 2001). The most used separation technique for rapid identification of proteins in proteomics is two-dimensional gel electrophoresis (2D-GE) that is capable of separation of thousands proteins present in a protein extract from the analyzed cell material. Sensitive MS measurements enable identification of protein spots visualized by silver staining method (SHEVCHENKO *et al.* 1996). Because only a small part of extracted peptides is consumed by peptide mass fingerprinting (PMF) with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), the remaining part can be used for further analysis (primary structure determination) by on-line combination of HPLC separation and tandem MS.

Barley Seed Proteins

Proteins accounts for about 8-15 % of the dry weight of the mature barley grain. They are quantitatively the minor component in comparison to carbohydrates (starch forms about 70 % of the dry weight of the grain). However, they are very important for both plant develoment and the suitability and quality of the grain for its final end-uses.

The pioneering work on classification of cereal proteins into groups on the basis of their extraction and solubility in a series of solvents was done by T. B. Osborne (OSBORNE 1895; OSBORNE 1924) and this has formed the basis for the modern study of cereal proteins. Osborne recognized four groups of seed proteins in his work:

1. albumins – water soluble proteins;
2. globulins – proteins soluble in dilute salt solutions;
3. prolamins – proteins soluble in alcohol-water mixtures; they are called hordeins in barley;
4. glutelins – proteins that are not extracted using previous solvents; they are clasically extracted with dilute acid or alkali, however, more usual is to use a detergent (e.g. SDS) and/or chaotropic agent (e.g. urea) often in the presence of a reducing agent.

This classification is very advantageous, however, sometimes is suitable to consider barley proteins as two groups according to their function:

a) Storage proteins – this group includes only those proteins that functions solely for storage. These are members of two solubility groups: the major alcohol-soluble prolamins (hordeins) and the quantitatively minor globulins.

b) Nonstorage proteins – here are all the structural and metabolic proteins; some of them have (accumulating in sufficient quantities) a secondary storage role (e.g. beta-amylase).

All the endosperm proteins can be considered as proteins having also storage role, because they can be mobilized during germination to provide nutrients for the developing seedling.

The prolamins of barley (hordeins) are the major storage proteins and this fraction appears to exert a key influence on the technological characteristics of the harvested barley grain. Hordeins account for around 50 % of the total grain protein. Hordein consists of three major groups of polypeptides called B, C and D hordeins, which account for 75–90, 10–20 and 2–4 % respectively of the total fraction. They have a high content of glutamine and proline (50 % of the total amino acids) and this also relates to the name prolamins. The low content of the essential amino acids in barley grain is caused due to this fact and that is why the some farm animals cannot successively grow on a complete barley diet without any supplementation. The missing amino acids are lysine (only around 1 %), threonine, valine and isoleucine.

The low amount of lysine and other essential amino acids can be solved by supplementation, but the adverse effect of the presence of large amount of hordeins during the brewing process is hard to overcome. The higher protein content in the original barley grain, the less amount of

extract can be derived from malt. It seems that displacement of starch by protein is not the principal problem. It is thought that the constitution of the protein matrix surrounding the starch granules determines the rate of conversion of starch to soluble extract. This may be due to the aggregation of the small starch granules with hordein that improves the resistance to amylolysis. Endosperm degradation during malting may also depend on the packing of small starch granules and storage proteins. The loosely packed endosperms may be modified more easily than endosperms with compact matrix area of proteins and small starch granules. It follows that good malting barley should have a low amount of proteins with high capacity for the synthesis of starch-degrading enzymes during malting.

Nonstorage proteins, especially albumins and globulins are important not only due to their high amount of lysine (chymotrypsin inhibitors CI-1 and CI-2, beta-amylase, protein Z, protein synthesis inhibitor), but they include many enzymes that are important for degradation of starch during malting. These enzymes can be divided as follows:

1) Alpha-amylases – endohydrolases that cleave internal (1–4)-alpha-glucosyl linkages of amylose or amylopectin in an essentially random fashion. They do not hydrolyze the (1–6)-alpha-glucosyl linkages at branch points in amylopectin and may vary in the ability to hydrolyze (1–4)-alpha-glucosyl linkages close to the branch points. These enzymes are Ca^{2+} metalloproteins consisting of a single polypeptide chain with approximate molecular mass of 45000 Da. According to their pI values, they form two groups: low-pI alpha-amylases (AMY1 group) with approximate pI value 4.6 and high-pI alpha-amylases (AMY2 group) with pI value around 5.9.

2) Beta-amylases – exohydrolases that cleave the penultimate (1–4)-alpha-linkage from the nonreducing termini of (1–4)-alpha-glucans to release the disaccharide maltose. Theoreticaly they can completely depolymerize unbranched or unsubstituted amylose molecules, but they are unable to bypass (1–6)-alpha-glucosyl linkages at branch points in amylopectin. Beta-amylases are single polypeptide chains of molecular mass of about 54000 – 60000 Da and their pI values fall in the range 5.2–5.7. Beta-amylases may account for 1–2 % of total protein in starchy endosperm and it is mostly located on the outer surface of starch granules. Beta-amylases are synthetized exclusively in the starchy endosperm during grain maturation (in contrast to the majority of hydrolytic enzymes responsible for endosperm mobilization).

3) Limit dextrinase is an enzyme that hydrolyzes (1–6)-alpha-glucosyl linkages in amylopectin or derived limit dextrins and increases the abundance of linear (1–4)-alpha-glucan chains that can be extensively depolymerized by the action of alpha-amylases and beta-amylases. There are several isoforms of limit dextrinase with molecular mass in the range of 80000–104000 Da and pI values of 4.2–5.0.

4) Alpha-glucosidase releases glucose from a variety of alpha-glucosides and mainly functions in the final conversion of maltose and other small dextrins to glucose.

Further information about barley, its chemistry, genetics and technology can be found for example in (MacGREGOR & BHATTY 1993).

Proteins separated by gel electrophoresis can be digested using enzymes directly in cut gel spots and the digestion-derived peptides are extracted for further MS analysis. MALDI-TOF mass spectrum of a peptide extract after in-gel digestion of the gel spot often contains not only the expected peptide signals, but also peaks of different origin (autolysis peptides, post-translational modifications, contaminants in the sample, nonspecific cleavages etc.) (KARTY *et al.* 2002). Whereas enzyme autolysis peptides or contaminants in the sample make the mass spectrometry analysis more difficult (with the only exception of their use for internal calibration of the mass spectra), nonspecific peptides can be used for protein identification. They originate from unexpected enzyme activities or they are products of the chemical treatment during protein cleavage and/or peptide extraction. The use of MS/MS analysis of the nonspecific peptides is useful for protein identification because it helps to find

a protein sequence tag and it also improves the coverage of the primary structure of the identified protein.
The first characterization of barley proteome by using the combination of gel electrophoresis, mass spectrometry and bioinformatics was done by CHMELÍK *et al.* (2001). The following work continued on proteomics of barley and employed the modified Osborne solvent system for extraction of proteins from barley grain (CHMELÍK *et al.* 2002). This solvent system divides barley proteins into several groups that differ in their solubilities in various solutions. The protein extracts were separated by one-dimensional gel electrophoresis (1-D GE) and several proteins were identified based on peptide mass fingerprinting analysis by MALDI-TOF MS. Another approach utilizing extraction of proteins from barley grain with a single buffer and separation by 2-D GE in neutral pI range (pI 4–7) was published recently (ØSTERGAARD *et al.* 2002; FINNIE *et al.* 2002). The barley starch granule proteome was studied by BOREN *et al.* (2004). Our work at the present stage is focused on the identification of proteins from various extracts of barley grains in order to enhance studies on malt quality. Peptide mass fingerprinting together with post-source decay (PSD) analysis of both tryptic and nonspecific peptides was employed for this purpose.

Material and Methods

Sample

For this study, a barley variety Monaco was selected. 1 g of milled barley grains was extracted with 10 ml of deionized water for 20 minutes while shaking. The mixture was centrifuged at 7 000×g for 15 minutes. Supernatant was taken as the aqueous extract. This procedure was sequentially repeated with the other solvents (aqueous 5% (w/v) NaCl solution, aqueous 70% (v/v) ethanol and aqueous 0.2% (w/v) NaOH solution).

Gel Electrophoresis

These sample extracts were then individually mixed with common sample buffer for gel electrophoresis (i.e. 50 mM TRIS-HCl, pH 6.8, 4% SDS, 12% glycerol, 2% β-mercaptoethanol, 0.01% bromophenol blue) in the volume ratio 1:1. After short boiling (5 min) in a water bath, 20 µl of this mixture were applied onto the polyacrylamide gel (concentrating layer–6%, separating layer–20%, size 150 mm×150 mm×1 mm). Visualization was carried out with Coomassie Brilliant Blue R-250 by fixing in 45.4% methanol/4.6% acetic acid (1 hour), staining in 45.4% methanol/4.6% acetic acid/0.1% Coomassie Brilliant Blue R-250 (1 hour) and subsequent destaining in 5% methanol/7.5% acetic acid (24 hours).

Protein Digestion

After washing of the stained gel (water, 2 changes, 10 min each), the protein spots were excised using a scalpel. The gel pieces were washed with water and water/acetonitrile 1:1 (v/v) (one change, 15 min/change). All liquid was then removed and acetonitrile was added to cover the gel pieces. After the gel pieces have shrunk, acetonitrile was removed and the gel pieces were rehydrated in 0.1 M NH_4HCO_3 for 5 min. An equal volume of acetonitrile was added and the gel pieces were incubated for 15 min. The mixture was then removed and gel pieces were dried down in a vacuum centrifuge. Proteins contained in gel pieces were reduced in 10 mM DTT/0.1 M NH_4HCO_3 for 45 min at 56 °C. Then the excess of the liquid was removed and proteins were alkylated with 55 mM iodoacetamide/0.1 M NH_4HCO_3 for 30 min at room temperature in the dark. After the reduction and alkylation an additional 0.1 M NH_4HCO_3-acetonitrile cycle was performed in order to remove the residual Coomassie staining. Gel particles were dried in a vacuum centrifuge and rehydrated in the digestion buffer containing 50 mM NH_4HCO_3, 5 mM $CaCl_2$, 12.5 ng/µl of trypsin (Roche, Modified

Sequencing Grade) for 45 min at 4 °C on ice. The remaining enzyme supernatant was removed and the same buffer (but without enzyme) was added in order to keep the gel pieces wet during the overnight digestion process. The peptides were extracted by addition of sufficient volume of 25 mM NH_4HCO_3 for 15 min and by addition of the same volume acetonitrile for next 15 min. After the supernatant was recovered, the extraction continued with three changes of 5% formic acid/acetonitrile (1:1, v/v) solution for 30 min each. All extracts were pooled and dried down in a vacuum centrifuge.

Mass Spectrometry
Mass spectrometric measurements were performed with MALDI-SEQ and AXIMA-CFR (Kratos Analytical) TOF instrument. A saturated solution of α-cyano-4-hydroxycinnamic in acetone mixed together with nitrocellulose solution (10 mg/ml, acetone: isopropanol=1:1, v/v) in the volume ratio 4:1 was used as a matrix. The dried peptide extracts were redissolved in 20 µl of 1% trifluoroacetic acid, purified using ZipTip C_{18} technology and eluted directly on the matrix layer.

Bioinformatics
Internet accessible tools in Protein Prospector (http://prospector.ucsf.edu) and Mascot (http://www.matrixscience.com) were used for interpretation of measured data. MS-Fit program was used for evaluation of PMF experiments and MS-Tag together with MS-Pattern program were used for evaluation of the results from PSD experiments. MS-Tag is dedicated to protein identification from determined masses of peptide parent ion and its fragments, whereas MS-Pattern identifies proteins on the basis of submitted peptide sequence tag gained from the interpretation of PSD spectra. SwissProt (22.1.2003) and NCBInr (22.1.2003) were used as searching databases.

Results and Discussion
Both PMF and PSD analysis by MALDI-TOF MS were used for protein identification in this work. PMF is a rapid method for protein identification, however, when this approach fails, a MS/MS experiment for sequencing of extracted peptides is necessary to employ. Using a MALDI-TOF instrument equipped with an ion gate and a reflectron mirror, PSD experiments can be immediately carried out with the rest of the sample on the target (SPENGLER 1997). No further sample handling steps are required in comparison to standard sample preparation for PMF experiments. The method is sensitive and the use of a curved-field reflectron (CORNISH & COTTER 1994) focusing all fragment ions simultaneously makes the whole procedure also rapid.
Because no further sample-handling step is required in comparison to the standard sample preparation for PMF experiments, PSD experiments were immediately carried out with the rest of the sample on the target. While PMF is based on determination of exact masses of the extracted peptides, PSD experiments use the analysis of fragments occurring in a field free region of time-of-flight mass analyzer that arise from a parent ion isolated by an ion gate. Reflectron mirror then resolves the fragments that are detected by a reflectron detector. Although the whole process is sensitive and (due to the use of the curved-field reflectron) also rapid, certain disadvantage can be the formation of various internal fragments that makes the interpretation of spectra more difficult. However, a-, b- and y- ions are the most abundant peptide fragments, because PSD is a type of soft-fragmentation method. Another problem is the localization of the proton in the fixed position at basic residues (lysine, arginine) of tryptic peptides, which leads to a specific peptide chain fragmentation and it limits the gained sequence information from the fragmentation spectra (GEVAERT & VANDEKERCKHOVE

2000). In this case, PSD analysis of nonspecific peptides is advantageous, because of lower probability of the presence of arginine at the N-terminus.
1-D gel electropherogram of protein mixtures sequentially extracted from the barley grains with various solvents is shown in Fig. 1. After tryptic in-gel digestion of the separated proteins, the isolated and purified peptides were transferred on a MALDI target covered with a layer of the matrix. β-amylase as a water soluble protein was identified in aqueous protein extract with high sequence coverage based only on PMF. An example of the MALDI-TOF spectrum of peptides from alkaline extract is shown in Fig. 2. The labelled peaks are tryptic peptides of the protein identified as B3-hordein by using PMF. This finding was supported by partial PSD data of the peak 997.64, where C-terminus sequence was determined as TR and part of internal sequence was determined as PLAID. This information together leads to unambiguous identification of B3-hordein.
However, PMF failed in the case of the peptides extracted from the spot A (Fig. 3), where no relevant protein identification was obtained. For this peptide mixture, several PSD experiments were carried out. Interpretation of PSD spectra of peptide peaks 973.59 Da, resp. 1001.66 Da enabled obtaining short peptide sequence tags and subtilisin-chymotrypsin inhibitor CI-1A (8.9 kDa, P16062), resp. subtilisin-chymotrypsin inhibitor CI-1B (9.0 kDa, P16063) were identified after submitting corresponding regular expressions into MS-Pattern program.
PSD analysis of the peak 1163.54 Da revealed a nice fragmentation spectrum (Fig. 4) with a complete y-ion series, containing some b-ions, a-ions and many internal peptide fragments. Thus unambiguous determination of the peptide sequence (with the exception of leucine/isoleucine identification) was possible and this information was again submitted into MS-Pattern program with enzyme specificity set to "No enzyme" and nonspecific lipid-transfer protein 1 precursor (12.3 kDa, P07597) was unambiguously identified.
All identified proteins are summarized in Table 1.

Label	Identified Proteins
A	subtilisin-chymotrypsin inhibitor CI-1A subtilisin-chymotrypsin inhibitor CI-1B nonspecific lipid-transfer protein 1 precursor (LTP 1) (PAPI)
B	trypsin/amylase inhibitor pUP13
C	triosephosphate isomerase, cytosolic (TIM)
D	glyceraldehyde 3-phosphate dehydrogenase, cytosolic protein Z (Z4) (major endosperm albumin)
E	beta-glucosidase BGQ60 precursor beta-amylase
F	beta-amylase
G	protein synthesis inhibitor I (ribosome inactivating protein I) (RRNA N-glycosidase) protein synthesis inhibitor II (ribosome inactivating protein II) (RRNA N-glycosidase)
H	B3-hordein
I	B3-hordein

Table 1. The summary of the proteins so far identified from the extracts separated by 1-D GE

Acknowledgements
The authors express their thanks to Professor Günter Allmaier of the Institute of Chemical Technologies and Analytics, Vienna University of Technology, for the opportunity to carry

out some experiments with the AXIMA-CFR TOF instrument. This work was partially supported by the grant QD 1023 from NAZV of the Czech Republic.

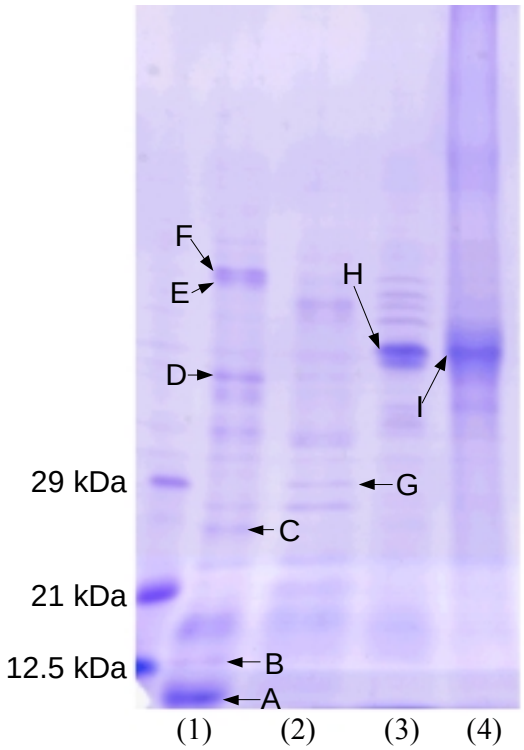


Figure 1. 1-D GE separation of subsequent protein extracts from barley variety Monaco. (1) – aqueous extract, (2) – 5% NaCl extract, (3) – 70% ethanol extract, (4) – 0.2% NaOH extract. Spots with identified proteins are labelled with capital letters (see Tab. 1).

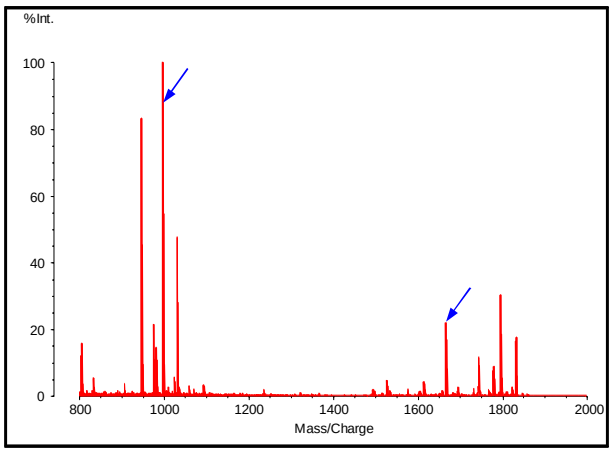


Figure 2. MALDI-TOF MS spectrum of an in-gel digest from spot I. Arrows denote peaks of B3 hordein identified with the support of PSD analysis of the peak 997.64.

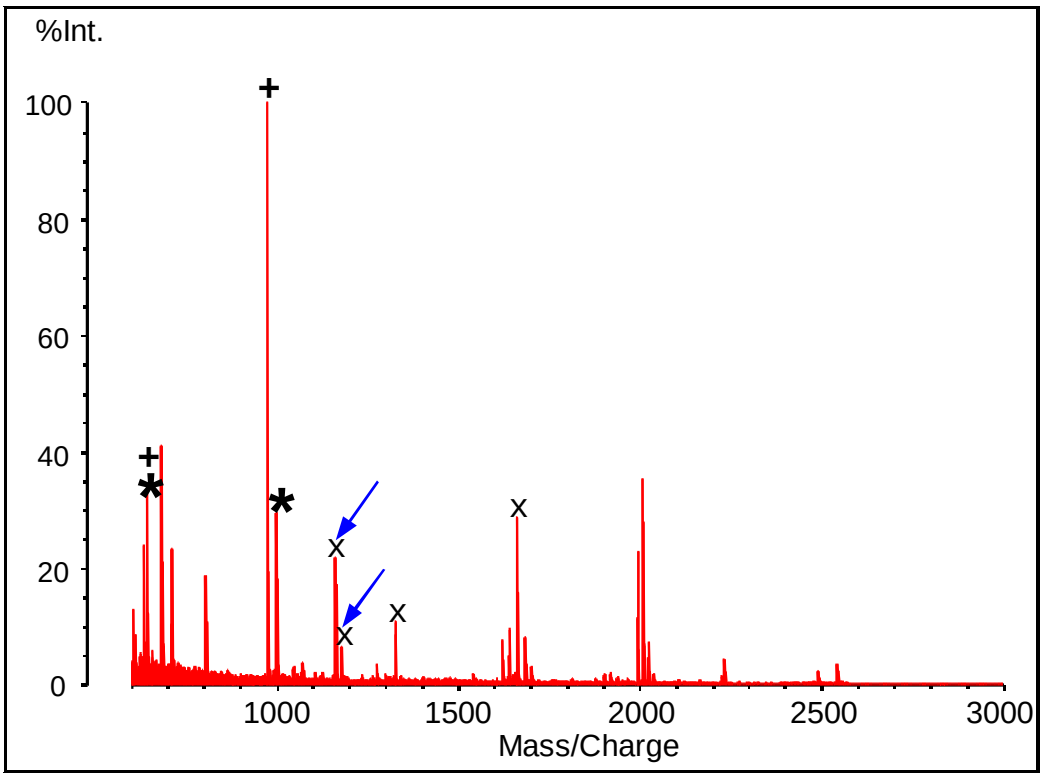


Figure 3. MALDI-TOF MS spectrum of an in-gel digest from spot A; **+**, resp. *****, resp. **x** denotes peaks of subtilisin-chymotrypsin inhibitor CI-1A, resp. subtilisin-chymotrypsin inhibitor CI-1B, resp. nonspecific lipid-transfer protein 1 precursor (LTP 1) (PAPI). Identification of these proteins was based on PSD analysis. The peptides labeled with an arrow are the nonspecific ones.

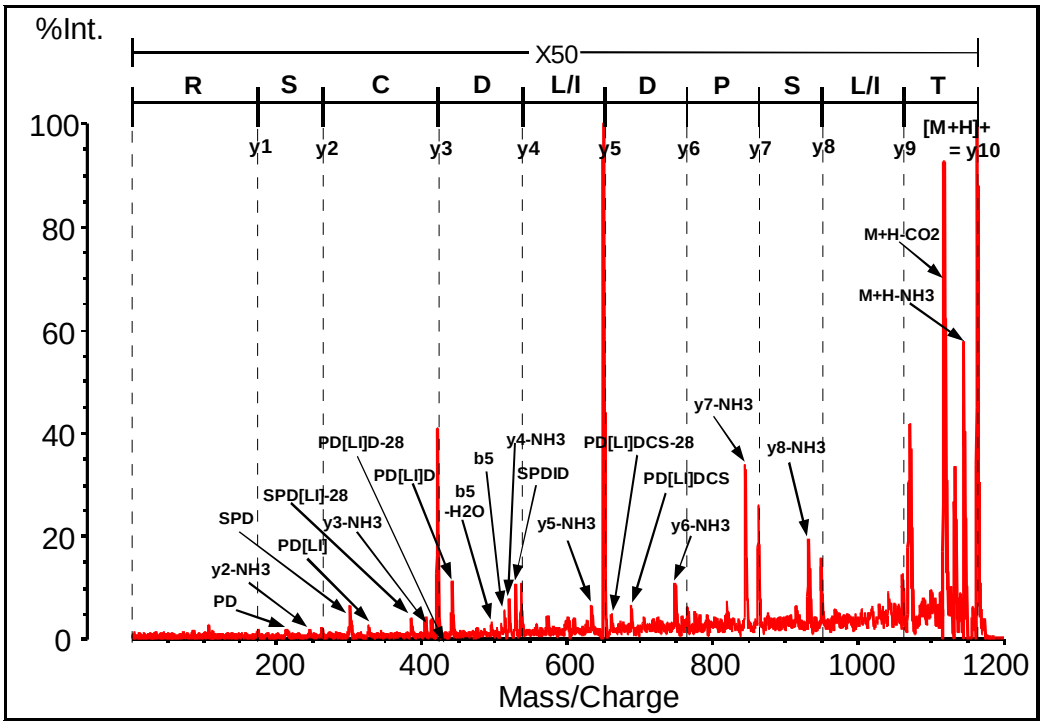


Figure 4. PSD spectrum of the peptide 1163.54 from nonspecific lipid-transfer protein 1 precursor (LTP 1) (PAPI). This peptide was a product of a nonspecific enzymatic cleavage. Various fragment ions of peptide backbone including internal peptide cleavages are denoted in the figure.

References

BOREN M., LARSSON H., FALK A., JANSSON C. (2004): The barley starch granule proteome – internalized granule polypeptides of the mature endosperm. Plant Sci., **166**: 617–626.

CHMELÍK J., ŘEHULKA P., MAYRHOFER C., ALLMAIER G. (2001): Proteomics – a new and fast tool for identification of barley varieties. Kvasný Prům., **47**: 159–163.

CHMELÍK J., ŘEHULKA P., STŘELCOVÁ M., KUBÁŇ V., MAYRHOFER C., ALLMAIER, G. (2002): Proteomic analysis of different extracts from barley grains. Rostl. Výroba, **48**: 261–264.

CORNISH T.J., COTTER R.J. (1994): A curved field reflectron time-of-flight mass-spectrometer for the simultaneous focusing of metastable product ions. Rapid Commun. Mass Spectrom., **8**: 781–785.

FINNIE C., MELCHIOR S., ROEPSTORFF P., SVENSSON B. (2002): Proteome analysis of grain filling and seed maturation in barley. Plant Physiol., **129**: 1308–1319.

GEVAERT K., VANDEKERCKHOVE J. (2000): Protein identification methods in proteomics. Electrophoresis, **21**: 1145-1154.

KARTY J.A., IRELAND M.M.E., BRUN Y.V., REILLY J.P. (2002): Artifacts and unassigned masses encountered in peptide mass mapping. J. Chromatogr. B, **782**: 363–383.

MacGREGOR A.W., BHATTY R.S. (1993): Barley: Chemistry and Technology. American Association of Cereal Chemists, Inc., St. Paul, Minnesota, U.S.A.

MANN M., HENDRICKSON R.C., PANDEY A. (2001): Analysis of proteins and proteomes by mass spectrometry. Annu. Rev. Biochem., **70**: 437–473.

OSBORNE T.B. (1895): The proteids of barley. J Am. Chem. Soc., **17**: 539–567.

OSBORNE T.B. (1924): The Vegetable Proteins. Longmans, Green & Co., London, U.K.

ØSTERGAARD O., MELCHIOR S., ROEPSTORFF P., SVENSSON B. (2002): Initial proteome analysis of mature barley seeds and malt. Proteomics, **2**: 733–739.

SHEVCHENKO A., WILM M., VORM O., MANN M. (1996): Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. Anal. Chem., **68**: 850–858.

SPENGLER B. (1997): Post-source decay analysis in matrix-assisted laser desorption/ionization mass spectrometry of biomolecules. J. Mass Spectrom., **32**: 1019–1036.

S 7 – DISEASE AND PEST RESISTANCE I – GENERALLY, LEAF DISEASES

Optimizing Breeding Strategies Based on the Evolutionary Potential of Barley Pathogens

B.A. McDonald and C.C. Linde

Institute of Plant Sciences, Swiss Federal Institute of Technology (ETH) Zurich, CH-8092 Switzerland

Abstract
Pathogen populations evolve in response to the deployment of resistance genes (R-genes) in agroecosystems. One consequence of this evolution is the familiar boom-and-bust cycle whereby major R-genes lose their effectiveness after being grown over a wide area. The evolutionary response of the pathogen population is affected by the mutation rate, mating/reproduction system, gene flow, effective population size, type of resistance gene, and R-gene deployment strategy. Population genetic structure may prove useful for predicting the evolutionary potential of pathogen populations. We will present a strategy that may prove useful for deciding whether to focus on major R-gene or quantitative R-gene resistance and for extending the life expectancy of R-genes in breeding programs. Examples of proposed breeding strategies will be presented for major barley pathogens.

Introduction
While this paper does present some relatively recent and unpublished analyses, most of the ideas and text have been borrowed from our previously published papers listed in the citations. We point the reader to these publications to obtain a more complete description of the theory and analytical methods. The primary novelty is reflected in Figure 1, where special consideration was given to rank the evolutionary potential of common barley pathogens.

Plant pathologists have seen many boom-and-bust cycles following the deployment of resistant varieties. These cycles result when pathogen populations adapt to the presence of a major resistance gene by evolving a new population that can overcome this resistance gene. In these cases, the breakdown of genetic resistance is due to the evolution of the local pathogen population because of selection for mutants, recombinants, or immigrants that are better adapted to the resistant cultivar. To understand the process that leads to breakdown of a resistance gene, we need to understand the processes that govern pathogen evolution. Population geneticists have identified five evolutionary forces that interact to affect the evolution of organisms. In Table 1, we summarize the risks associated with each of these forces. We ranked these risks and developed a quantitative framework to predict the risk that a pathogen will evolve to overcome major resistance genes (McDONALD & LINDE 2002ab). Our hypothesis is that much of the durability of resistance genes is due to the nature of the pathogen population rather than to the nature of the resistance gene. The framework we developed (McDONALD & LINDE 2002ab), which is described briefly here, can be used as a hypothesis to test against a large number of plant pathosystems. The underlying principles of the framework can be tested individually or in combination according to the available knowledge of the population genetics for any pathogen. We propose that this framework can be used to design breeding strategies to break the boom-and-bust cycle and lead to durable resistance (McDONALD & LINDE 2002ab).

Material and Methods

Figure 1 is a simplified diagram that we proposed as a model framework for assessing the evolutionary risk posed by most plant pathogens. The evaluation of evolutionary risk is relevant not only for breakdown of resistance genes, but also for development of resistance to fungicides or antibiotics. Figure 1 considers only the evolutionary risk due to differences in reproduction/mating system, gene/genotype flow, and effective population size. Mutation rate was not included in this diagram because we assumed that mutation rates would be low and relatively constant across pathogens. For pathogens known to have very high mutation rates (i.e. active transposable elements), or for bacteria and viruses where mutation is likely to play a more important role in evolution, these risk values can be increased accordingly. Selection was not included in this diagram under the assumption that selection is likely to be efficient in the genetically uniform monocultures that dominate modern agricultural ecosystems. Selection risk is increased by increasing the land area covered to the same resistance gene (or the area sprayed to fungicides), or decreased through resistance gene (fungicide) deployment strategies, such as gene (fungicide) rotations or mixtures. We expect that the population size for most pathogen populations is large, so it is likely that virulent (or fungicide-resistant) mutants will be present and the effects of genetic drift will be small. However, we recognize that some pathogen populations have smaller effective population sizes because of a founder effect, regular bottlenecks, or short-lived overseasoning propagules. Other pathogen populations have larger effective sizes due to year-round multiplication, short latent periods, and production of long-lived overseasoning propagules. This results in a range of values for each cell in the matrix (Figure 1). The proposed risk categories may need to be adjusted in many cases as a result of anthropogenic activities. For example, gene/genotype flow may be increased beyond the normal biological limits of spore dispersal by movement of inoculum or infected plant material through international commerce and travel.

The risk values presented in Figure 1 are on a 3--9 scale. This ranking system assumes that reproduction/mating system, gene/genotype flow, and effective population size affect evolutionary potential equally. A further assumption is that these effects are additive. The proposed scale offers many possibilities for developing testable hypotheses and assigning relative evolutionary risks. For example, pathogens that have exclusively asexual reproduction and little potential for gene flow are assigned to the lowest risk category. This category includes some bacterial and viral pathogens and the *Fusarium oxysporum* formae speciales causing wilt diseases of many crops. At the other extreme, pathogens that have mixed reproduction and asexual spores that are disseminated over long distances by wind are assigned to the highest risk category. This category includes pathogens such as the powdery mildew fungi. In the intermediate risk categories are pathogens that we expect to have more limited evolutionary potential as a result of lack of an asexual propagule that has high gene flow potential (i.e. splash-dispersed spores), or lack of regular outcrossing that produces new recombinants (Figure 1). Figure 1 hypothesizes that pathogens with regular sexual cycles will evolve faster than pathogens without recombination. It also hypothesizes that pathogens producing asexual propagules distributed over long distances will break down resistance genes faster than pathogens with short distance dispersal of asexual propagules.

Validation of the Risk Assessment Model

To test the model, our first analysis considered 34 plant pathosystems and used Spearman rank order correlation analysis to determine the correlations between the four "expected" risk factors and the "observed" risk values for all 34 pathosystems (McDONALD & LINDE 2002a). When the analysis was conducted using the sum of mutation risk values and

gene/genotype flow risk values, the correlation was -0.35 (P=0.044, all probabilities are based on a two-tailed t-test). When the largest outlier in the correlation matrix, the nematode *Meloidogyne incognita*, was removed from the analysis, the correlation rose to -0.46 (P=0.007). This preliminary analysis suggested that the contributions of the evolutionary forces may not be equal. It also indicated that gene/genotype flow and mutation may be the dominant forces driving pathogen evolution in the 34 plant pathosystems we considered. The correlations improved significantly when the analysis was conducted separately for different major categories of pathogens. The second analysis was conducted focussing exclusively on virus plant pathosystems (29 viruses, 35 pathosystems, GARCIA-ARENAL & McDONALD 2003) or fungal plant pathosystems (30 fungal pathosystems, C.C. Linde unpublished). For fungi the correlation with migration was significant (r_s = -0.72; P = 0.0001), but effective population size and reproduction system did not show significant correlations. Similarly, for viruses, the effective population size and reproduction system did not show significant correlations, but the correlation with migration was significant (r_s = -0.39, P = 0.0207). For nematodes, 24 plant pathosystem examples (10 nematodes, 24 pathosystems) could be found to test the model (P. Roberts, unpublished). In those, the correlations with migration (r_s = -0.62, P = 0.0013) and reproduction system (r_s = -0.43, P = 0.0293) were significant, whereas the correlation with effective population size was not significant. A fourth analysis was conducted using fungi and fungicide resistance (54 published cases evaluated) as the selective agent. The results of this analysis were compared to results based on a risk assessment model published by the Fungicide Resistance Action Committee (FRAC), an industry-sponsored think tank. In our risk model, the correlation with mating/reproduction system and with effective population size was non-significant, but the correlation with migration was -0.71 (P = 0.0001). FRAC's predicted risk assignments showed no correlation with the observed number of years before the appearance of fungicide resistance (r_s = -0.06, P = 0.6474). We conclude from these analyses that the population genetic model shows some promise as a predictor of pathogen evolution, and, in the special case of fungicide resistance, that pathogen biological properties are better predictors than chemical parameters.

Results and Discussion

A Decision Diagram to Aid Resistance Breeding

We proposed guidelines based on the evolutionary potential of the pathogen to choose appropriate types of resistance and to decide how to deploy major resistance genes in a breeding program (McDONALD & LINDE 2002a). The simple decision diagram is shown in Figure 2. This diagram offers some broad guidelines to consider before embarking on a resistance-breeding project, with the objective of choosing the appropriate type of genetic resistance and then applying a resistance gene management strategy that will match the pathogen's biology and minimize the likelihood that the pathogen population will evolve to overcome the resistance. The outcome of the decision diagram is a general recommendation for choosing the type of resistance to use and the optimum deployment method with the aim of maximizing the useful lifespan of the resistance genes. We point out that the same general recommendations can be applied to guide applications of fungicides.

At one end of the decision diagram are pathogens that have strictly asexual reproduction, a low potential for gene/genotype flow, and small effective population sizes. In our risk model, these are pathogens with the lowest evolutionary potential. For these pathogens, a breeding strategy that relies on single major resistance genes is likely to be durable because the mutation to virulence will occur in a limited number of genetic backgrounds and the virulent lineages that inevitably arise are unlikely to move quickly to new fields planted to the same major resistance gene. An example of a class of pathogens that follow this life history is the

Fusarium oxysporum wilts on many crops. A specific example for barley is the soil-borne wheat mosaic virus (SBWMV).

The next category in the decision diagram is asexual or inbreeding pathogens that exhibit a high potential for genotype flow. These pathogens also exhibit low genotypic diversity, but when the virulent lineage arises by mutation, it is moved efficiently to neighboring fields or adjacent agricultural regions. For these pathogens, a breeding strategy that pyramids major resistance genes is likely to be durable because it is unlikely that a sequence of multiple mutations to virulence (loss of several elicitors simultaneously) will occur in the same clonal lineage. Examples of pathogens that follow this life history are the asexual rusts and barley yellow dwarf virus (BYDV). Pathogens that have a sexual cycle, but appear to be mainly inbreeding, such as *Tilletia controversa* or other smuts and bunts, may also fall into this category.

Pathogens that exhibit mixed reproduction that includes regular recombination exhibit higher genotype diversity as a result of recombination and have greater potential for local adaptation to a changing environment. After a mutation to virulence occurs, it can be recombined into many different genetic backgrounds, and it can be recombined with other virulence mutations that occur at unlinked loci. Thus pyramids are not an optimum approach for these pathogens. Pathogens with a mixed reproduction system and a low potential for gene/genotype flow are placed in an intermediate risk category. For these pathogens, breeders should focus on quantitative resistance instead of major gene resistance. If quantitative resistance is not available, then major gene resistance can be deployed in rotations through time or space. The rationale for these choices are explained in McDONALD & LINDE (2002ab).

The highest risk pathogens have a mixed reproduction system and a high degree of gene/genotype flow. We believe that these pathogens will require the greatest effort to achieve durable resistance because the mutations to virulence can be recombined into many genetic backgrounds until a pathogen clone with high fitness appears, and then this adapted genotype can be dispersed across long distances and into new populations. For pathogens in this risk category (e.g. *Blumeria graminis* f. sp. *hordei*), we suggest the breeding effort should concentrate on quantitative resistance that will need to be renewed regularly to stay ahead of the pathogen. If quantitative resistance is not available, then major gene resistance should be managed aggressively, including development of cultivar mixtures and multilines that can be used in combination with regional and temporal deployment strategies.

Genetically Engineered Resistance and the Risk Assessment for Pathogen Evolution

Genetic engineering technologies offer great potential, but present a number of uncharacterized risks that require further investigation. One risk is that genetically engineered resistance genes will face the same boom-and-bust cycles as the major resistance genes incorporated by traditional breeding methods. Our present knowledge indicates that plants evolved leucine-rich repeat (LRR)-types of receptors to recognize a diverse array of pathogen elicitors, and it is likely that pathogens coevolved with these receptors for millions of generations before agriculture arose. With this long history of coevolution, it seems unlikely that we will be able to eliminate plant diseases by engineering new receptors or combinations of receptors and putting them into our crops. Pathogens will continue to evolve. However, genetic engineering offers new opportunities to stay a few steps ahead of the pathogen. Genetic engineering can be used to create novel pyramids of major resistance alleles that can be transferred into plants as a cassette of linked genes. It may become possible to create a pyramid more quickly through a single transformation step than through a

series of hybridizations and backcrosses. Of course, plants already have evolved cassettes of linked resistance genes over evolutionary time scales, and pathogens are still with us. Genetic engineering also could be used to synthesize multilines quickly and efficiently by inserting different resistance alleles into superior agronomic genotypes as they are developed. This approach may allow us to impose disruptive selection that slows pathogen evolution, but it is unlikely to eliminate the pathogen. It is most likely that pathogen populations will continue to evolve and respond to the new forms of genetic resistance that we deploy through genetic engineering. But with careful management of these new, engineered resistance genes, we may be able to create truly durable forms of genetic resistance. The best way to insure the durability of these new engineered resistance genes is to manage them wisely using knowledge of the evolutionary potential of the pathogen population.

References

GARCIA-ARENAL, F., MCDONALD B.A. (2003): An analysis of the durability of resistance to plant viruses. Phytopathology, **93**: 941-952.

MCDONALD B.A., LINDE, C. (2002a): Pathogen population genetics, evolutionary potential, and durable resistance. Annu. Rev. Phytopathol., **40**: 349-379.

MCDONALD B.A., LINDE, C. (2002b): Pathogen population genetics and the durability of disease resistance. Euphytica, **124**: 163-180.

Table 1. Extremes of evolutionary risk posed by plant pathogens and examples of factors that affect risk assessment

Highest risk of pathogen evolution	Lowest risk of pathogen evolution
High mutation rate	Low mutation rate
Transposable elements active	No transposons
Large effective population sizes	Small effective population sizes
Large overseasoning population	No overseasoning propagules
Extinction of local populations rare	Extinction of local populations common
No genetic drift, no loss of alleles	Significant genetic drift, alleles lost
High gene/genotype flow	Low gene/genotype flow
Asexual propagules dispersed by air over long distances	Asexual propagules soilborne
Human-mediated long-distance movement common	Quarantines effective
Mixed reproduction system	Asexual reproduction system
Annual sexual outcrossing and asexual propagules produced	Only asexual propagules produced
Efficient directional selection	Disruptive selection
R-gene deployed in genetically uniform monoculture	R-genes deployed in mixtures/multilines
R-gene deployed continuously over large area	R-genes deployed as rotations in time or space

Figure 1. A risk assessment model for quantifying the evolutionary risk posed by different barley pathogens. The scale of evolutionary risk is organized according to the risk factors reproduction/mating system, gene/genotype flow and effective population size and based on published knowledge at the present time. Assignment of total risk value assumes that all effects are additive. Placement of barley pathogens is according to principles explained in McDONALD & LINDE (2002ab).

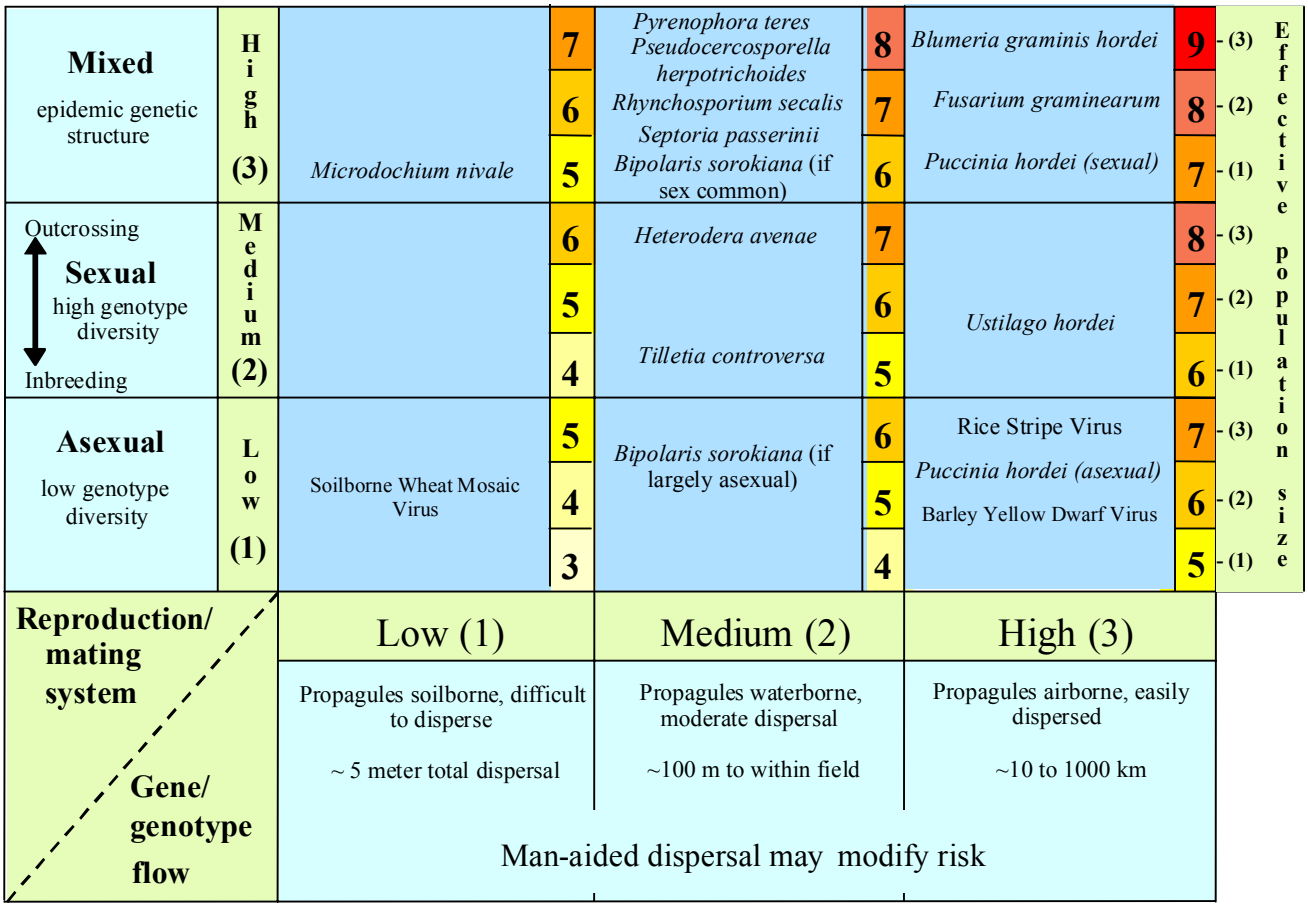
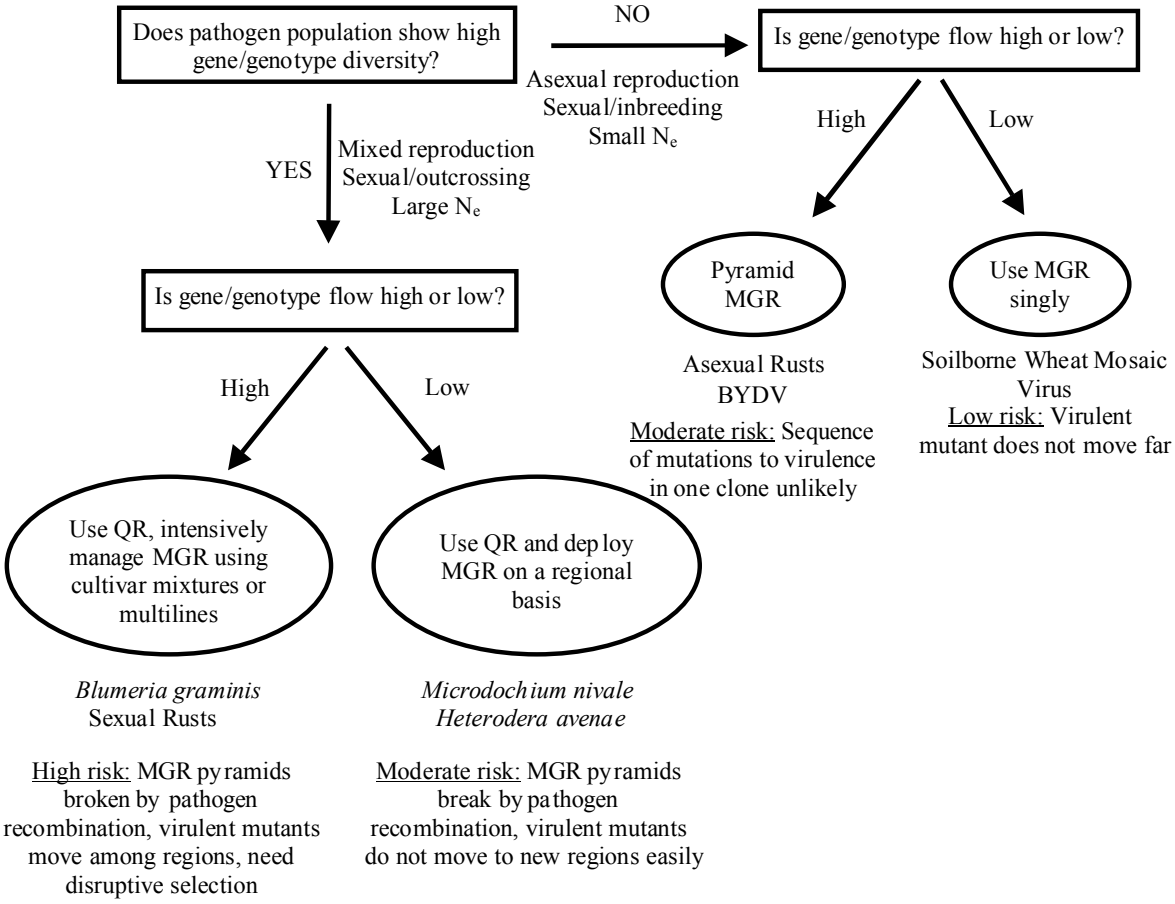


Figure 2. A simplified decision diagram to assist with developing resistance-breeding strategies to achieve durable disease resistance based on knowledge of pathogen population genetics. Major gene resistance (MGR): resistance that has large effects, is based on the hypersensitive response and follows the receptor-elicitor model of the gene-for-gene interaction. Quantitative resistance (QR): resistance that has, on average, small, nearly equal, and additive effects that are equally effective against all strains of the pathogen.



Importance of Secondary and Tertiary Genepools in Barley Genetics and Breeding. I. Cytogenetics and Molecular Analysis

R. Pickering[1], P.A. Johnston[1] and B. Ruge[2]

[1]New Zealand Institute for Crop & Food Research Limited, Private Bag 4704, Christchurch, New Zealand;
[2]Federal Centre for Breeding Research on Cultivated Plants, Institute of Agricultural Crops, Rudolf-Schick-Platz 3a, D-18190 Gross Lüsewitz, Germany

Abstract
There have been no plant breeding developments using species from the tertiary genepool of cultivated barley for breeding or genetics since the VIIIth International Barley Genetics Symposium in 2000. Hence, the first part of this review describes progress since 2000 in developing and characterising recombinant lines derived from hybridisations between the sole species in the secondary genepool, *Hordeum bulbosum* L., and cultivated barley, *Hordeum vulgare* L. The topics discussed in part I are cytogenetics and molecular analysis of recombinant lines.

Cytogenetics

Introduction

In situ hybridisation, which combines cytogenetic and molecular methods, can be used to identify and locate introgressions transferred from *Hordeum bulbosum* L. into the cultivated barley (*H. vulgare* L.) genome (PICKERING *et al.* 2000). The first step is performed on somatic chromosome preparations (e.g. root tip cells) using genomic in situ hybridisation (GISH) to identify the site of the introgression on one or more unknown chromosomes. This is followed sequentially on the same chromosome preparation by fluorescent in situ hybridisation (FISH) with the microsatellite sequence $(CTT)_{10}$ or $(GAA)_7$ to identify individual barley chromosomes (PEDERSEN & LINDE-LAURSEN 1994). Although the methods are laborious and require expensive equipment, the chromosome location of introgressions and approximate physical size can be established prior to more critical genetic mapping with molecular markers.

By these means we have identified and characterised more than 70 recombinant lines (RLs) containing introgressions of *H. bulbosum* chromatin on 13 out of the 14 chromosome arms of the *H. vulgare* genome (Table 1). One problem using these RLs in breeding programmes is linkage drag where the introgressed segment cannot be reduced in size by genetic recombination. To overcome this linkage drag it is necessary to reduce the size of the introgression and remove unwanted DNA linked to the locus of interest – a process that necessitates screening large segregating populations. Does this linkage drag result from reduced meiotic pairing between recombinant and non-recombinant chromosomes? To investigate this possibility we used FISH to determine the levels of meiotic chromosome pairing in heterozygous RLs and compared these with homozygous controls.

Material and Methods

Details of the seven heterozygous RLs and two homozygous RLs are presented in Table 2; full details of each RL, including the original code numbers, can be supplied on request. Each

RL contained one or more distal introgressions of *H. bulbosum* chromatin on particular *H. vulgare* chromosomes. Heterozygous RLs were obtained either by crossing together two homozygous RLs containing introgressions on different chromosomes or by backcrossing a homozygous RL to barley cultivars Emir or Golden Promise. The plants were grown in a heated glasshouse maintained at 21/15ºC ± 2ºC (16 h day/8 h night). Natural daylight was supplemented when necessary with 400 W mercury and sodium vapour lamps to extend the daylength to 16 h.

Table 1. Numbers of recombinant lines with introgressions on particular chromosomes obtained from *Hordeum vulgare* x *H. bulbosum* hybrids and the identifiable traits associated with the introgressions

Chromosome location of introgression	Number of recombinant progeny	Transferred trait
1HS	1	
1HL	3	resistance to leaf rust
2HS	12	resistance to leaf rust and powdery mildew; glossy spike and leaf sheath
2HL	13	resistance to leaf rust
3HS	1	
3HL	0	
4HS	1	resistance to scald
4HL	13	resistance to Septoria speckled leaf blotch; pubescent leaf and leaf sheath; black aleurone
5HS	1	response to DDT
5HL	7	resistance to leaf rust; very susceptible to powdery mildew; vernalisation requirement
6HS	7	resistance to BaYMV/BaMMV
6HL	5	
7HS	3	
7HL	5	resistance to powdery mildew; resistance to stem rust?

Table 2. Recombinant lines used for cytogenetic analyses. *denotes homozygous RLs; ** denotes RLs used for FISH. E (Emir) and G (Golden Promise) refer to the *H. vulgare* parent followed by the chromosome location of the *H. bulbosum* introgression(s).

Code	*H. bulbosum* parents
E-2HL-4HS	A17/1, HB2032
E-2HL-7HL	A17/1, HB2032
E-4HS-7HL	A17/1
EG-5HL-6HS**	Cb2920/4
E-6HS-7HS	Cb2920/4 x Cb2929/1
G-6HS-7HS**	Cb2920/4
E-2HL-6HS-7HS**	Cb2920/4 x Cb2929/1; HB2032
E-2HL*	HB2032
G-2HL*	A17/1

Spikes were fixed in 3 ethanol : 1 acetic acid and conventional meiotic pairing analyses were carried out by squashing a single anther in 1% aceto-carmine and examining pollen mother cells (PMCs) at metaphase I. To perform FISH, each anther containing PMCs at metaphase I was incubated on a microscope slide in one drop of 2% pectinase + 2% cellulase (Sigma) for 1 h at 37°C in a humid chamber. The enzyme was removed by carefully blotting around the anther with filter paper and replaced with a drop of 45% acetic-acid and left for 3-5 mins at room temperature. Anthers were macerated and squashed under 18 x 18 mm cover slips, which were removed with a scalpel blade after immersion in liquid nitrogen. The slides were stored at 4°C until use. To prepare the probe, BAC T15P10 from *Arabidopsis thaliana* containing 45S rDNA (LYSAK *et al*. 2003) was labelled with digoxigenin by nick translation according to the manufacturer's (Roche) instructions. The 45S rDNA probe hybridises to the short arms of barley chromosomes 5H and 6H. Subsequent FISH methods were based on those of TOUBIA-RAHME *et al*. (2003). Detection of hybridisation signals was performed as described by HOUBEN *et al*. (2001) using anti-digoxigenin rhodamine. Slides were mounted in Vectashield (Vector Laboratories) with 1.0 µg/ml 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) as a counterstain.

Results

Meiotic Pairing Using Aceto-Carmine Squashes

Apart from E-6HS-7HS, E-2HL-6HS-7HS and, to a smaller extent G-6HS-7HS, chromosome pairing in the RLs was similar or even higher than the *H. vulgare* controls (Table 3).

Table 3. Mean (range) of univalent (I) and bivalent (II) formation in PMCs of heterozygous and homozygous (*) recombinant lines derived from *Hordeum vulgare* x *H. bulbosum* crosses using aceto-carmine squash preparations

Code	No. of PMCs	I	II	
			rod	ring
E-2HL-4HS	212	0	0.34 (0-2)	6.66 (5-7)
E-2HL-7HL	365	0	0.34 (0-3)	6.66 (4-7)
E-4HS-7HL	206	0	0.38 (0-3)	6.62 (4-7)
EG-5HL-6HS	331	0	0.36 (0-2)	6.64 (5-7)
E-6HS-7HS	265	0.03 (0-2)	0.68 (0-4)	6.31 (3-7)
G-6HS-7HS	442	0	0.31 (0-2)	6.69 (5-7)
E-2HL-6HS-7HS	209	0	1.01 (0-3)	5.99 (4-7)
E-2HL*	236	0.02 (0-2)	0.27 (1-2)	6.72 (5-7)
G-2HL*	184	0	0.07 (0-1)	6.93 (6-7)
Emir	334	0	0.40 (0-3)	6.60 (4-7)
Golden Promise	241	0	0.18 (0-2)	6.82 (5-7)
H. bulbosum (Cb2920/4)	390	0	0.09 (0-2)	6.91 (5-7)

Highest pairing was observed for the homozygous RL, G-2HL and lowest mean ring bivalent formation was in E-2HL-6HS-7HS (5.99) and E-6HS-7HS (6.31). Both of these RLs contained 6HS and 7HS introgressions derived from a common *H. bulbosum* parent (Cb2920/4 x Cb2929/1) but E-2HL-6HS-7HS had an extra introgression on 2HL transferred from *H. bulbosum* genotype HB2032. Another RL (G-6HS-7HS) with a different pedigree also contained 6HS and 7HS introgressions but had higher pairing (6.69 ring bivalents) although somewhat lower than GP.

Meiotic Pairing Using FISH

In the three heterozygous RLs studied, most of the unpaired chromosomes involved the short arms of satellite chromosomes 5H or 6H (Table 4), but these chromosomes could not be distinguished from each other with the 45S rDNA probe. In E-2HL-6HS-7HS, 25 of the 91 PMCs analysed contained two or more rod bivalents. Despite the absence of an introgression on chromosome 5H in this RL, 36.4% of the rods in these 25 PMCs involved 5HL-5HL and 6HL-6HL pairing, which was much higher than expected by chance. The short arms of both pairs of chromosomes remained unpaired.

Table 4. Mean (range) of rod and ring bivalents in PMCs of three recombinant lines heterozygous for *H. bulbosum* introgressions and the % rod bivalents involving 5H-5H or 6H-6H pairing as determined by FISH analysis

Code	No of PMCs	II		% of rods occurring as 5HL-5HL or 6HL-6HL*
		rods	rings	
G-6HS-7HS	59	0.19 (0-1)	6.81 (6-7)	72.7
E-2HL-6HS-7HS	91	1.09 (0-4)	5.91 (3-7)	69.7
EG-5HL-6HS	66	0.24 (0-2)	6.76 (5-7)	81.3

* denotes pairing between the long arms with the short arms remaining unpaired

Discussion

Overall, chromosome pairing was normal in the homozygous RLs and in all but two out of the seven heterozygous RLs. The source of the 6HS and 7HS introgressions in these two RLs was identical and they also shared the same *H. vulgare* parent, Emir. Conversely, another RL with 6HS and 7HS introgressions but a different pedigree had higher pairing. Hence, chromosome pairing may be influenced by genotype, but introgression size may be another factor. Since chromosome pairing initiation occurs at the telomeres in barley (KASHA & BURNHAM 1965) large terminal introgressions may be more disruptive to chromosome pairing than small introgressions in heterozygous RLs and interstitial introgressions may allow nearly normal pairing and recombination. We lack precise physical and genetic map information on the introgression sizes to assess these effects.

FISH carried out on three heterozygous RLs indicated that most of the failures in pairing were because the short arms of one or both of the satellite chromosomes (5HS and 6HS) did not pair and resulted in rod bivalents. We could not distinguish between these two *H. vulgare* chromosomes using 45S rDNA as a probe. However, it was assumed that most of the pairing failure involved 6HS since there were no introgressions on 5HS. But in E-2HL-6HS-7HS, rod bivalents in which the short arms of 5H remained unpaired were commonly observed. Reduced pairing between homologous satellite chromosomes in *H. vulgare* has been described previously. STOINOVA (1994) reported that in eight cultivars most of the open ring bivalents at diakinesis involved the satellite chromosomes, probably the short arms. BURNHAM *et al*. (1954) also observed the frequent occurrence of one rod and six ring bivalents and that the rod was most likely the chromosome pair with the small satellite (i.e. 5H). In two trisomic series of *H. spontaneum* and *H. vulgare* greatest numbers of Y-type trivalents were observed for chromosome 5H (TSUCHIYA 1960, 1967). Y-type trivalents arise from chiasmata restricted to one of the two arms and we can speculate that pairing was reduced between the 5HS arms.

In conclusion, reduced chromosome pairing probably contributes to linkage drag, but will depend on the size and location of the introgressed segment. Other factors such as reduced recombination between paired chromosomes (ZHANG *et al.* 1999), parental genotype and

certation effects are also likely to be important. None of these impediments to exploiting RLs in breeding programmes will be easily overcome.

Molecular Analyses

Introduction

To complement GISH and FISH, molecular methods have been used successfully to characterise RLs derived from *H. vulgare* x *H. bulbosum* hybrids. An initial screen to identify RLs that contain *H. bulbosum* chromatin introgressed into *H. vulgare* has been developed (JOHNSTON & PICKERING 2002). These RLs can then be further characterised with GISH and FISH (PICKERING *et al*. 2000) and the genetic size of the introgression determined by molecular markers. Because of the variation between barley and *H. bulbosum* genomic DNA, PCR-based markers such as microsatellites may not amplify DNA sequences of both species. To develop our own markers we have used cDNA-based sequences, which are more likely to be conserved between *H. vulgare* and *H. bulbosum* but still show enough polymorphisms for reliably analysing RLs. We describe here two examples of these procedures; first the production of PCR markers derived from cDNA-based RFLP probes for identifying diagnostic expressed sequence tags (EST) and, second, cDNA-AFLP for developing PCR markers closely linked to loci conferring resistance to the soil-borne virus complex barley yellow mosaic virus (BaYMV) and barley mild mosaic virus (BaMMV).

Material and Methods

EST Marker Development

cDNA-derived barley RFLP distal markers were selected for developing into EST PCR markers. Sequences from these markers were used to identify matching barley ESTs by searching the BLASTn algorithm. From the EST database HarvEST (WANAMAKER & CLOSE 2003) contiguous consensus sequences that matched the original RFLP clone were identified. These consensus cDNA sequence contigs were searched against the non-redundant or high-throughput genomic sequencing databases using discontinuous megaBLAST to find genomic matches to the cDNA sequence. Genomic matches were most often recovered from the rice genome. The likely positions on introns in the barley genome could, therefore, be estimated. Primers to amplify across a putative intron were designed using Primer3 (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi). A second criterion was that primers should match sequences between the barley cDNA contig and matching wheat ESTs to increase the likelihood of the primers being conserved between barley and *H. bulbosum*. Amplified sequences were examined using single-stranded conformational polymorphism (SSCP) to identify alleles originating from the *H. bulbosum* parental genotype in putative RLs.

cDNA-AFLP

An RL, which was resistant to the soil-borne virus complex BaMMV, BaYMV-1 and –2, was derived from an *H. vulgare* x *H. bulbosum* cross. Inheritance of resistance was determined from segregating F5 and F6 families based on field trials and glasshouse tests using mechanical inoculation. The methods for carrying out the cDNA-AFLP analysis have been published (RUGE *et al*. 2003) but, briefly, RNA was isolated from homozygous resistant and susceptible pooled populations of F5 plants. cDNA was synthesised from the RNA, fragmented with *Taq1* and adaptors ligated. Sixty-four primer combinations were used to amplify the cDNA. PCR products were separated on polyacrylamide gels and diagnostic fragments cloned into a plasmid vector for later use as RFLP probes on a mapping population.

Results and Discussion

EST Marker Development

Recombination between *H. vulgare* and *H. bulbosum* chromosomes usually takes place distally and arises from a single crossover. EST markers were, therefore, developed for the distal regions of most chromosome arms where suitable cDNA-derived RFLP markers already exist (Figure 1). By carefully designing primers, almost all the EST markers in both species were amplified. Anchoring the PCR primers in conserved cDNA sequences but amplifying across less conserved introns maximised the production of codominant polymorphic markers. This strategy also allows many markers to be developed *in silico* from large public databases and reduces the cost of developing new markers.

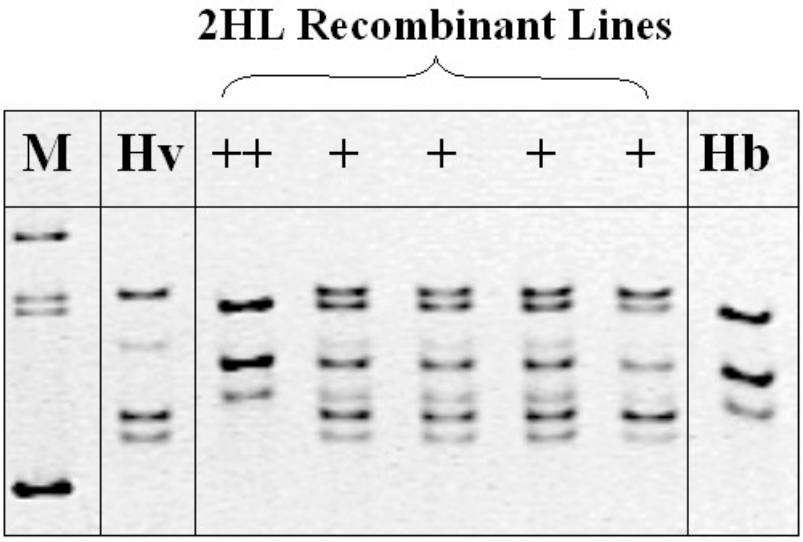


Figure 1. HarvEST contig 5825 (assembly #31) derived from RFLP probe cMWG720 (2HL). Single stranded conformational analysis of parental (*H. vulgare* (Hv) and *H. bulbosum* (Hb)), heterozygous (+) and homozygous (++) recombinant lines. M = dsDNA marker as control.

cDNA-AFLP

Resistance to the BaYMV-BaMMV complex in European cultivars is based on the recessive resistance genes *rym4* and *rym5* on barley chromosome 3 (WEYEN *et al.* 1996, GRANER *et al*. 1999). However, the inheritance of the resistance in the RL, designated *Rym14*^{Hb}, was dominant and positioned on chromosome 6HS using a set of barley anchor markers. Hence the locus is non-allelic with *rym4* and *rym5* and provides breeders with a novel source of resistance. To develop markers linked with *Rym14*^{Hb}, cDNA-AFLP analysis was carried out. One of 64 primer combinations amplified a differentially expressed 250 bp transcript, which was only observed in the resistant populations. Southern hybridisation with the cloned fragment revealed a single-copy hybridisation pattern that displayed a codominant polymorphism between resistant and susceptible genotypes. The marker cosegregated with *Rym14*^{Hb} and a BLAST search against the Genbank database (NCBI Blast Homepage) found no sequence similarities for the amplified 250 bp cDNA-AFLP fragment. The RFLP revealed by use of this fragment was converted to a PCR-based STS marker, which was also codominantly inherited, and is being used in a backcross breeding programme. The size of the introgression has been reduced following further recombination in a segregating population.

In conclusion, we have shown that useful PCR-based markers can be developed for marker assisted selection and breeding programmes without incurring a huge investment in time or equipment. Our aim is also to produce these markers for each chromosome arm so we can

locate introgressions of *H. bulbosum* chromatin prior to carrying out more precise molecular mapping.

Acknowledgements
We thank Ruth Butler (Crop & Food Research, New Zealand) for advice on statistical analysis, and Professor Ingo Schubert (Institute of Plant Genetics, Gatersleben, Germany) for allowing R Pickering to perform part of this research in his laboratory. The programme in New Zealand is supported by the Foundation for Research, Science and Technology.

References

BURNHAM C.R., WHITE F.H., LIVERS R. (1954): Chromosomal interchanges in barley. Cytologia, **19:** 191-202.

GRANER A., STRENG S., KELLERMANN A., SCHIEMANN A., BAUER E., WAUGH R., PELLIO B., ORDON F. (1999): Molecular mapping and genetic fine-structure of the *rym5* locus encoding resistance to different strains of Barley Yellow Mosaic Virus Complex. Theor. Appl. Genet., **98**: 285-290.

HOUBEN A., FIELD B.L., SAUNDERS V.A. (2001): Microdissection and chromosome painting of plant B chromosomes. Meth. Cell. Sci., **23:** 115-124.

JOHNSTON P.A., PICKERING R.A. (2002): PCR detection of *Hordeum bulbosum* introgressions in an *H. vulgare* background using a retrotransposon-like sequence. Theor. Appl. Genet., **104:** 720-726.

KASHA K.J., BURNHAM C.R. (1965): The location of interchange breakpoints in barley. II. Chromosome pairing and the intercross method. Can. J. Genet. Cytol., **7:** 620-632.

LYSAK M.A., PECINKA A., SCHUBERT I. (2003): Recent progress in chromosome painting of *Arabidopsis* and related species. Chromosome Research, **11:** 195-204.

PEDERSEN C., LINDE-LAURSEN I. (1994): Chromosomal locations of four minor rDNA loci and a marker microsatellite sequence in barley. Chromosome Research, **2:** 65-71.

PICKERING R.A., MALYSHEV S., KÜNZEL G., JOHNSTON P.A., KORZUN V., MENKE M., SCHUBERT I. (2000): Locating introgressions of *Hordeum bulbosum* chromatin within the *H. vulgare* genome. Theor. Appl. Genet., **100:** 27-31.

RUGE B., LINZ A., PICKERING R., PROESELER G., GREIF P., WEHLING P. (2003): Mapping of $Rym14^{Hb}$, a gene introgressed from *Hordeum bulbosum* and conferring resistance to BaMMV and BaYMV in barley. Theor. Appl. Genet., **107:** 965-971.

STOINOVA J. (1994): Characteristics of meiosis in some winter barley varieties. Cytologia, **59:** 423-426.

TOUBIA-RAHME H., JOHNSTON P.A., PICKERING R.A., STEFFENSON B.J. (2003): Inheritance and chromosomal location of *Septoria passerinii* resistance introgressed from *Hordeum bulbosum* into *Hordeum vulgare*. Plant Breeding, **122:** 405-409.

TSUCHIYA T. (1960): Cytogenetic studies of trisomics in barley. Jap. J. Bot., **17:** 177-213.

TSUCHIYA T. (1967): The establishment of a trisomic series in a two-rowed cultivated variety of barley. Can. J. Gen. Cytol., **9:** 667-682.

WANAMAKER S., CLOSE T. (2003): HarvEST. http://harvest.ucr.edu/

WEYEN, J., BAUER, E., GRANER, A., FRIEDT, W., ORDON, F. (1996): RAPD-mapping of the distal portion of chromosome 3 of barley, including the BaMMV/BaYMV resistance gene *ym4*. Plant Breeding, **115**: 285-287.

ZHANG L., PICKERING R., MURRAY B. (1999): Direct measurement of recombination frequency in interspecific hybrids between *Hordeum vulgare* and *H. bulbosum* using genomic in situ hybridization. Heredity, **83:** 304-309.

Importance of Secondary and Tertiary Genepools in Barley Genetics and Breeding. II. Disease Resistance, Agronomic Performance and Quality

R. Pickering[1], R.E. Niks[2], P.A. Johnston[1] and R.C. Butler[1]

[1]New Zealand Institute for Crop & Food Research Limited, Private Bag 4704, Christchurch, New Zealand;
[2]Laboratory of Plant Breeding, Wageningen University, PO Box 386, 6700 AJ Wageningen, The Netherlands

Abstract

In this second paper on the use of secondary and tertiary genepools in barley improvement, we describe the characterisation of leaf rust resistant recombinant lines (RLs) derived from *Hordeum vulgare* x *H. bulbosum* crosses. Twelve RLs were inoculated with leaf rust and the early stages of disease development were observed. Several RLs showed complete resistance to the pathogen, but others had a high level of partial resistance, which may be durable. Some of these RLs and others were tested in yield trials to determine the effects of introgressed chromatin from *H. bulbosum* on yield and quality. We conclude that there are no major adverse effects that cannot be overcome through normal breeding techniques.

Disease Resistance

Introduction

Resistance to several diseases has been transferred from *Hordeum vulgare* L. into *H. bulbosum* L. (PICKERING & JOHNSTON 2004). Since *H. bulbosum* is probably not a host to barley leaf rust (*Puccinia hordei* Otth; XU & SNAPE 1989; ANIKSTER 1989) it would be desirable to exploit the genes for nonhost resistance of that species in barley breeding. Such genes may confer a more durable type of resistance than the genes available in the cultivated host species. Little is known about the genetics of nonhost resistance but resistance to heterologous rust species may be the joint effect of many quantitative genes for non-hypersensitive resistance (HOOGKAMP *et al.* 1998). The resistance of wheat to the powdery mildew forma specialis of *Agropyron* has been reported to be based on a gene-for-gene response (TOSA 1989).

In New Zealand 33 lines carrying introgressed DNA from *H. bulbosum* (called here recombinant lines, RLs) showed complete or incomplete hypersensitivity resistance to *P. hordei* in the field and glasshouse, or had a reduced rate of infection compared to the recipient cultivars Emir and Golden Promise. Twelve of these RLs were screened to determine more precisely the level and mechanism of their resistance to leaf rust. The research was carried out in the Netherlands and Spain (Dr Diego Rubiales) using similar methods.

Material and Methods

The 12 RLs used in the experiments are shown in Table 1. The parental *H. vulgare* cultivars are designated as E (Emir) and G (Golden Promise) followed by the chromosomal location of the *H. bulbosum* introgression(s). Where two or more RLs have the same chromosomal introgression location, another letter is added (a, b, or c). The full identity including original code numbers of each RL can be supplied on request.

Eleven days after sowing, first leaves were fixed horizontally and inoculated with leaf rust isolates 1.2.1, Córdoba and Tunisia. The inoculum was mixed with 9x the volume of Lycopodium powder, and applied in a settling tower at about 200 spores per cm^2. After

inoculation the plants were incubated overnight (10 h) in a dark mist chamber, and then transferred to a greenhouse at 20 to 25°C. After 6 days uredia were counted daily in a marked area containing about 20 to 50 pale flecks before mature uredia appeared. The latency period (LP) was estimated as the time from the start of incubation to the time when 50% of the uredia had appeared. Infection type (IT) was assessed on a 0 – 9 scale (McNEAL *et al*. 1971).
Three RLs (E-1HL, E-2HL-a and G-2HL-b) with contrasting infection types to *P. hordei* were used to determine the infection frequency (IF) and resistance mechanism with isolate 1.2.1. On the fourth day after inoculation we collected central segments from three leaves per line and stained them for UV microscopy (HOOGKAMP *et al*. 1998). At least 60 infection units were evaluated per leaf segment and classified according to their stage of development (HOOGKAMP *et al.* 1998). Infection units that were arrested after primary infection hyphae had formed but before the formation of six mycelial branches per infection unit were classed as "early aborted". Infection units with six or more mycelial branches were classified as "established". Yellow autofluorescence of plant cells directly associated with infection units was recorded as "necrosis" and occurred after haustorium formation indicating post-haustorial resistance. The diameter of 20 established colonies per leaf segment was measured and the IF on any unsampled leaves or leaf stubs was determined by counting the number of pustules in 1 cm^2. On leaves with a low IF a larger segment of the leaf was cut, measured and the number of pustules counted to calculate the IF in uredia per cm^2.
In a third experiment the three RLs were inoculated with four different *P. hordei* isolates (1.2.1., 17, 26 and Uppsala) to check the race-specificity of the resistance by measuring LP and IF.

Results and Discussion
Table 1 shows the LPs and ITs of the 12 RLs with isolate 1.2.1. Results from the Córdoba and Tunisia isolates were generally similar (data not presented). On four RLs no pustules had developed (IT = 0 or 1), or a few pustules appeared but were associated with strong necrosis or chlorosis. In these RLs the *H. bulbosum* introgressed DNA must contain a gene(s) for hypersensitive resistance to *P. hordei.* L94 was the most susceptible accession since the leaf rust fungus had the shortest LP. The LP on Vada was 34% longer than on L94 due to its high level of partial resistance. The LPs on Emir and Golden Promise were generally intermediate to L94 and Vada, indicating a moderate level of partial resistance. On four lines (E-1HL, E-5HL, G-2HL-b and G-5HL-6HS) the LPs were longer than on Emir or Golden Promise, and equal to or longer than on Vada. The level of partial resistance of G-2HL-b was outstanding since the LP was even longer than on Vada and 46% longer than on L94.
The second experiment (Table 2) confirmed the resistance rankings. No uredia were formed on E-2HL-a and its resistance was complete and the response very rapid. Almost all infection units were arrested within 24 hours before they formed at least six mycelial branches. Most (90%) of such aborted colonies were associated with autofluorescent plant cells indicating a hypersensitive reaction. The high degree of partial resistance in G-2HL-b was apparent too, since the number of pustules was much lower than on Vada and not associated with chlorosis or necrosis (infection type 9), which would have indicated hypersensitivity. High partial resistance is also associated with high frequencies of early aborted infection units. Although the percentage of early abortion was unexpectedly low for Vada, RLs E-1HL and G-2HL-b had even higher percentages of early abortion and smaller established colonies than Vada and Emir, indicating a higher level of partial resistance. Since most of the early aborted colonies were unassociated with autofluorescent plant cells, the resistance mechanism is probably not based on hypersensitivity but on blocking haustorium formation at most of the plant cell wall penetration sites.

Table 1. Latency period (LP, hours in parenthesis for L94; and as a % of L94), and infection type (IT) of isolate 1.2.1 of *Puccinia hordei* on 12 barley recombinant lines with introgressed chromatin from *Hordeum bulbosum*

Barley line[§]	*H. bulbosum* parent	Relative LP	IT[#]
L94		100 (136 h)	9
Vada		134	9
Emir		111	9
E-4HL-a	Cb2920/4	113	9
E-4HL-b	Cb2920/4	114	9
E-5HL	Cb2920/4	124	9
E-1HL	A17	129	9
E-4HL-c	A17/1	-[*]	0
E-2HL-a	HB2032	-	0
E-2HL-b	HB2032	-	0
E-2HS-a	Cb2920/4	-	4
E-6HS-7HS-7HL	Cb2920/4 x Cb2929/1	-	5
Golden Promise		113	9
G-5HL-6HS	Cb2920/4	129	9
G-2HL-b	A17/1	146	9
G-2HL-a	Cb2920/4	-	1

§ In bold the lines used in further leaf rust experiments.
# Infection type (IT): 0 = no symptoms; 1 = minute necrotic flecks; 4, 5 = necrotic flecks and pustules surrounded by necrotic or chlorotic tissue; 9 = fully compatible pustules that may be surrounded by pale green halos.
* LP could not be determined because of low numbers of uredia, due to the low IT.

Table 2. Infection frequency (IF in uredia/cm^2), percentage of early aborted (EA) infection units (cessation of growth before at least 6 mycelial branches), and average longitudinal diameter of established colonies of *Puccinia hordei* isolate 1.2.1. in seedling leaves of three recombinant lines and two barley cultivars

Barley line	IF	% EA	% EA without autofluorescent plant cells	Length of established colonies (μm)
Emir	115	10	93	322
Vada	76	12	94	314
E-1HL	65	26	72	228
G-2HL-b	27	51	86	212
E-2HL-a	0	96	10	156

The resistances of E-1HL, E-2HL-a and G-2HL-b were confirmed in the third experiment (data not presented). Compared with Emir, the introgression in E-1HL caused a longer LP and lower IF to all four isolates. The complete resistance of E-2HL-a was also effective against the four isolates and the level of partial resistance in G-2HL-b was higher than in Vada since the LP was longer and the IF lower. Both these RLs contain introgressions on 2HL although

the sizes differ and they have different *H. vulgare* and *H. bulbosum* parents. We will establish whether the gene conferring the hypersensitive resistance in E-2HL-a is allelic to the gene for non-hypersensitive resistance in G-2HL-b.
In conclusion, the resistances in these RLs must be due to the introgressed segments of DNA from *H. bulbosum*. Although the complete resistance in some of the RLs may not be durable, the partial resistances in E-1HL and G-2HL-b, which are manifested by long LPs, low IFs and early abortion of infection units, may be long-lived.

Agronomic Performance and Quality

Introduction

In this part of the programme we investigated whether introgressions of DNA from *H. bulbosum* into cultivated barley may affect yield and quality in the presence or absence of disease. Some of the RLs tested are resistant to one or more diseases whereas others are as susceptible as their barley parents. Yield trials were carried out in two years in New Zealand. Half the plots were treated with fungicide to control powdery mildew and leaf rust (caused by *Blumeria graminis* f. sp. *hordei* L. and *Puccinia hordei* Otth, respectively), the prevalent diseases on spring-sown barley in Canterbury (New Zealand). Malting quality was assessed on samples from harvest year 1 only. Five of the lines had also been tested in the leaf rust experiments described above.

Material and Methods

Trials were laid out in a randomised block design with five replicates of 30 plots per replicate; plot area was 4 m^2. Each replicate consisted of 15 entries x 2 plots, one of which was treated with fungicide. The 15 entries comprised 13 recombinant lines (RLs) derived from *H. vulgare* x *H. bulbosum* crosses and their respective barley parents, Emir (feed barley) and Golden Promise (GP; malting barley) (Table 3). Ten of the 13 RLs were trialled again in year 2 with extra replicates for GP and E-4HL-a. Code numbering follows the same format as previously. The RLs were all different selections apart from G-4HL-b, which was derived from G-4HL-a after reducing the size of the 4HL introgression through backcrossing to GP. In year 1, the fungicides applied to control powdery mildew and leaf rust were Opus® (epoxiconazole) or Twist® (trifloxystrobin) + Folicur® (terbuconazole), each applied twice with a final application of Merit® (propiconazole + fenpropimorph). In year 2 three applications of Opus® + Fortress® (quinoxyfen) were made during the growing season. Disease in year 1 was scored on four dates for powdery mildew and once for leaf rust, which only appeared later in the season. Area under the disease progress curve (AUDPC) was calculated for the mildew data using standard methods. Other agronomic characters such as earliness, height and lodging were recorded.
Eight months after harvest year 1, thousand grain weights (tgw) and germinations were carried out at Lincoln (New Zealand) and 120 g of grain taken from each plot were kindly micromalted by Coors Brewing Company (Golden, Colorado, USA). Samples from two complete replicates were then analysed for malting quality at University of Adelaide (Australia) using standard methods (LOGUE *et al.* 2002). Owing to financial constraints only grain protein, diastatic power, malt protein, hot water extract (IOB), viscosity, soluble protein and the Kolbach Index were determined.
Data were analysed with analysis of variance; a few plots were excluded because of sowing errors. There were some spatial trends, particularly for the harvest data, but adjusting for these trends produced similar results to those from the simple analysis of variance presented here. Comparisons among the RLs and their respective parents were made as contrasts within the analysis.

Results and Discussion

Yield Trial

Agronomic data (year 1) are presented in Table 3. Two RLs, E-2HS-b and G-5HL, were highly susceptible to leaf rust and powdery mildew, respectively. Fungicide applications did not have a significant effect ($p>0.2$) on ear emergence or straw height, but lodging was increased slightly (by about 3%; $p=0.09$) with fungicide, perhaps because of greater spike weight.

Table 3. Means of fungicide treated and untreated plots for ear emergence (days ± Emir), straw height and lodging (maximum of two scores) on 13 recombinant lines and their respective barley parents. Disease incidence is for untreated plots only. Leaf rust incidence: mean % leaf area infected for one scoring date. AUDPC: area under the disease progress curve for powdery mildew incidence recorded as % leaf area infected on four dates.

Code	*H. bulbosum* parent	Ear emergence	Straw height (cm)	Lodging (%)	Leaf rust incidence	Powdery mildew AUDPC
E-2HS-a	Cb2920/4	+2.3	84	12	3	1942
E-2HS-b	A17/1	+6.4	77	0	62	1624
E-2HL-a	HB2032	-0.3	93	17	0	1505
E-4HL-a	Cb2920/4	+6.9	78	21	10	342
E-6HS	Cb2920/4 x Cb2929/1	+4.0	84	18	33	1744
E-7HS	Cb2920/4	+10.4	76	0	44	2308
E-7HL	A17/1	+1.0	90	6	27	120
Emir		**0**	**92**	**23**	**42**	**1634**
G-2HL-a	Cb2920/4	+3.2	71	3	1	1712
G-2HL-b	A17/1	+3.5	75	22	3	528
G-4HL-a	Cb2920/4	+9.5	70	30	7	455
G-4HL-b	Cb2920/4	+3.4	71	14	70	1633
G-5HL	Cb2920/4	+6.3	73	0	38	3212
G-6HS	Cb2920/4	+3.5	74	10	54	2054
Golden Promise		**+1.2**	**75**	**17**	**74**	**1698**
Lsd 5%*		**0.6**	**3**	**10**	**10**	**291**

*Lsd 5%: Least significant difference between the means at the 5% level. Associated degrees of freedom are 111, 111, 111, 53, 49 for the measurements, respectively.

Ear emergence for all RLs was significantly ($p<0.05$) later than their parents for all RLs except E-2HL-a. GP was significantly later than Emir but by just over one day. All RLs were shorter strawed than their respective parents except for E-2HL-a.

Yield data (year 1) are presented in Figure 1. GP and its RLs were generally higher yielding than Emir and the Emir RLs. Of the 13 RLs tested, seven equalled or exceeded the yield (4-22% increases) of the barley parent when leaf rust and powdery mildew were present (i.e. without fungicide application). Conversely, when disease was controlled most of the RLs were similar to or lower ($p<0.05$) in yield than their parents and a yield penalty of up to 36% was associated with the *H. bulbosum* introgression. Yields of all the RLs and the recurrent parents increased when disease was controlled by fungicides ($p<0.001$ for the overall effect of the fungicide), and this effect varied among genotypes ($p=0.041$ for the genotype x fungicide interaction).

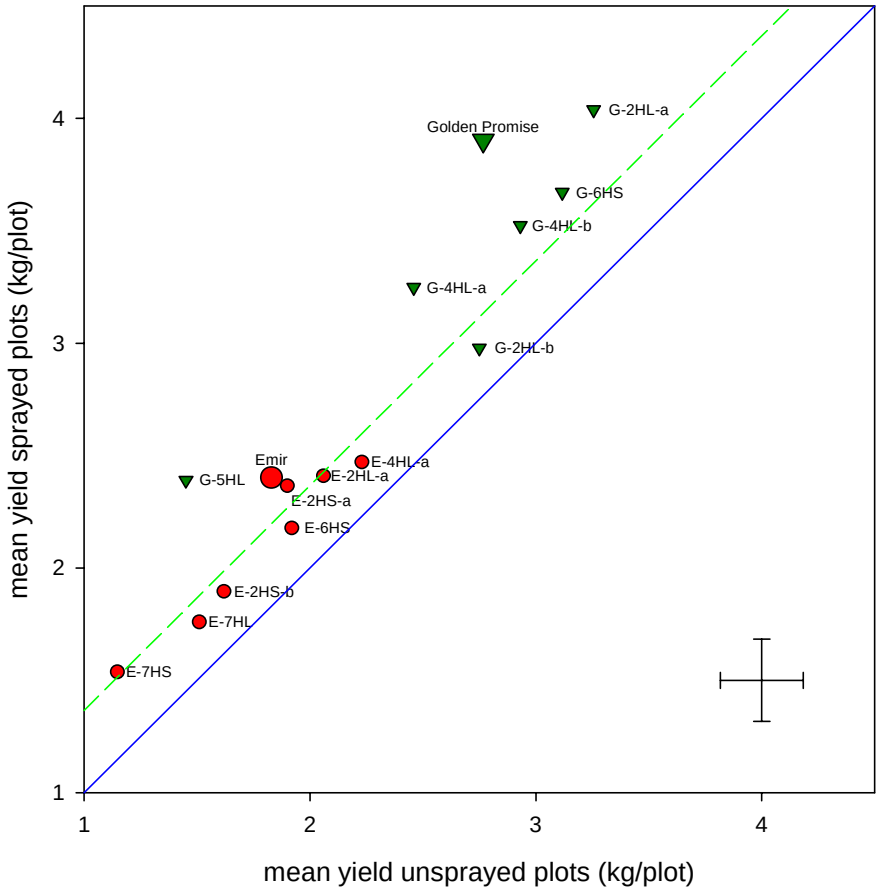


Figure 1. Grain yields (after screening over a 2.4 mm sieve) of recombinant lines derived from Golden Promise (▼) and Emir (●) and their *H. vulgare* parents. Error bars (bottom right corner) are Lsd 5% (df=111) to compare between means. The solid line indicates where sprayed mean yield equals that for untreated plots and the dotted line is the solid line plus the Lsd 5%. Values outside and to the left of this line were significantly higher yielding in the fungicide treated plots.

There were some inconsistencies in the fungicide effect. Two RLs with low disease incidence (G-2HL-a and G-4HL-a) responded positively to fungicide applications whereas these had only slight effect on the disease susceptible RLs E-2HS-b and E-7HS. Thus, other factors, such as the use of strobilurin fungicides, must explain these anomalies. This was confirmed statistically, since including disease data in the analysis (leaf rust incidence, mildew incidence or AUDPC) did not fully account for the fungicide effect or the significant interaction.
In year 2, E-2HL-a, E-7HS and E-7HL were omitted. There was little lodging or disease and negligible fungicide effect on yield (no strobilurin fungicides were applied). The RL rankings for ear emergence and straw height were similar to year 1. Mean yields were higher than year 1 by 57 and 29% for fungicide treated and untreated plots, respectively. G-5HL again yielded poorly, whereas all the other GP RLs gave similar or lower yields (G-2HL-b and G-4HL-a) than GP. E-2HS-b was the lowest yielding Emir RL, but E-4HL-a significantly outyielded Emir before taking into account screening losses. In both years screening losses (2.4mm sieve size) were most pronounced for RLs with low tgw's (E-4HL-a, G-2HL-b, G-4HL-a and G-5HL).

Malting Analysis (Table 4)

Fungicide did not have a significant effect on % germination, diastatic power, grain protein or soluble protein levels ($p>0.1$). For other traits, the effect of fungicide was similar for all genotypes ($p>0.2$ for the genotype x fungicide interaction). Germinations were all above 89% but there were some differences in tgw, which ranged from 31.7 g (G-4HL-a) to 41.3 g (E-2HL-a). Tgw from fungicide-treated plots were on average 1.2 g higher than those for untreated plots.

Table 4. Year 1 means for fungicide-treated plots (2 replicates only) for grain and malting parameters. Tgw (1000 grain weight: grams); GP (grain protein, %); MP (malt protein, %); SP (soluble protein, %); DP (diastatic power, µmoles maltose equivalent/minute/g dry weight); HWE (hot water extract, % dry weight (IOB)); V (viscosity, centipoise); KI (Kolbach Index).

Line	Tgw	GP	MP	SP	DP	HWE	V	KI
E-2HS-a	38.4	16.3	15.9	3.91	605	67.4	1.58	25.6
E-2HS-b	40.5	18.0	18.0	4.85	670	65.9	1.50	27.7
E-2HL-a	41.3	16.7	16.0	3.95	550	66.9	1.60	25.8
E-4HL-a	34.8	16.9	15.6	3.92	630	67.7	1.59	26.2
E-6HS	38.4	17.6	17.3	4.05	669	65.0	1.45	24.1
E-7HS	40.7	15.0	17.4	4.05	671	65.6	1.50	24.0
E-7HL	39.7	16.7	16.0	4.07	592	66.1	1.53	26.4
Emir	**40.2**	**17.9**	**16.2**	**3.51**	**644**	**64.7**	**1.86**	**22.5**
G-2HL-a	39.2	16.2	14.5	3.82	614	68.4	1.78	27.9
G-2HL-b	35.6	14.4	14.3	3.74	587	68.0	1.69	27.8
G-4HL-a	31.7	15.6	14.5	4.06	658	67.7	1.71	29.7
G-4HL-b	38.1	16.0	14.5	4.20	707	66.3	1.86	30.6
G-5HL	32.3	16.7	16.4	4.28	741	63.8	1.56	27.0
G-6HS	38.3	16.0	14.3	4.70	696	66.2	1.61	35.1
Golden Promise	**39.4**	**14.0**	**12.8**	**3.89**	**637**	**69.5**	**1.93**	**32.9**
Lsd 5%* (df= 27)	2.0	2.8	1.2	0.43	93	2.0	0.15	3.8
Mean change with no fungicide	1.2	0.5	0.3	-0.06	-4	-0.9	0.04	-1.1
Lsd change (df=27)	0.5	0.7	0.3	0.11	24.0	0.5	0.04	1.0

*Lsd 5%: Least significant difference between the means at the 5% level.

Grain protein contents were generally lower for the RLs derived from the malting cultivar GP than those derived from the feeding variety Emir. Grain and malt protein contents of all genotypes were high, and consequently diastatic power (DP) was also high and extracts low with rather poor modification. The RLs had higher (Emir) or lower (GP) HWEs than their respective parents with some significant differences among the RLs. Unfortunately, it is hard to draw conclusions about the effects of particular introgressions on quality. This is not surprising since the traits contributing to malting quality have complex inheritance (HAYES & JONES 2000). However, tgw's were significantly lower for two of the three 4HL RLs, whereas G-4HL-b, which was derived from G-4HL-a, has a smaller introgression following further recombination and its tgw was similar to GP. The differences in tgw and protein

between E-2HS-a and E-2HS-b, and for tgw between G-2HL-a and G-2HL-b, might be due to differences in introgression size as well as the genotype of the *H. bulbosum* parent.
In conclusion, the introduction of new genetic material can be accommodated in the barley genome without too many detrimental effects on agronomic performance and quality. Hence, useful disease resistant lines can be developed. No firm conclusions can be drawn about the effects of particular introgressions on yield and quality or the introgression size in all RLs.

Acknowledgements
We thank Coors Brewing Company, Golden, Colorado, USA, for micromalting the grain; BASF (NZ) Ltd and Bayer (NZ) Ltd for supplying fungicides; Dr Diego Rubiales, Institute for Sustainable Agriculture, Córdoba, Spain, for supplementary inoculations, and Andy Hay (Crop & Food Research) for help with field work. The programme in New Zealand is supported by the Foundation for Research, Science and Technology.

References

ANIKSTER Y. (1989): Host specificity versus plurivority in barley leaf rusts and their microcyclic relatives. Mycol. Res., **93:** 175-181.

HAYES P.M., JONES B.L. (2000): Malting quality from a QTL perspective. In: 8th International Barley Genetics Symposium, Adelaide, Australia: **Vol. 1:** 99-105.

HOOGKAMP T.J.H., CHEN W.-Q., NIKS R.E. (1998): Specificity of prehaustorial resistance to *Puccinia hordei* and to two inappropriate rust fungi in barley. Phytopathology, **88:** 856-861.

LOGUE S., TANSING P., ROUMELIOTIS S. (2002): 2000 Season Barley Quality Report, University of Adelaide, Waite Campus, Glen Osmond, South Australia: 20-22.

McNEAL F.H., KONZAK C.F., SMITH E.P., TATE W.S., RUSSELL T.S. (1971). In: A uniform system for recording and processing cereal research data. USDA Agricultural Research Service, Washington, D.C., U.S.A.: 34-121.

PICKERING R., JOHNSTON P. (2004): Recent progress in barley improvement using wild species of *Hordeum*. Cytogenetic and Genome Research (in press).

TOSA Y. (1989): Evidence on wheat for gene-for-gene relationship between formae speciales of *Erysiphe graminis* and genera of gramineous plants. Genome, **32:** 918-924.

XU J., SNAPE J.W. (1989): The resistance of *Hordeum bulbosum* and its hybrids with *H. vulgare* to common fungal pathogens. Euphytica, **41:** 273-276.

Adaptation of Biotrophic Barley Pathogens to Genetic Resistance in Central Europe

A. Dreiseitl

Agricultural Research Institute Kroměříž Ltd., Havlíčkova 2787,
CZ-767 01 Kroměříž, Czech Republic, dreiseitl@vukrom.cz

Abstract
The contribution is based on earlier studies aiming at postulation of resistance genes to powdery mildew and leaf rust in barley varieties, multiyear data on resistance evaluations of these varieties in the official variety trials in the Czech Republic, and investigations of respective pathogen populations (*Blumeria graminis* f.sp. *hordei* and *Puccinia hordei*). The examples of some barley varieties demonstrate the role of individual evolutionary forces (in particular, direct selection, indirect selection, migration, and recombination) for the population adaptation of the causal agents of these diseases to individual resistance genes and increasing population virulence complexity. Considering some aspects of breeding and evolutionary potential of the pathogens, it seems that a combination of at least two original and fully effective resistance genes in a variety could be a good way to prolong the durability of resistance to both powdery mildew and leaf rust. However, the progress in barley breeding for disease resistance will depend not only on the resistance sources and extending the diversity of this character in commercial varieties, but also on the critical selection of varieties that combine different effective resistance genes. The use of molecular markers seems to be essential to achieve these aims.
Keywords: *Hordeum vulgare*; *Blumeria graminis* f.sp. *hordei*; *Puccinia hordei*; powdery mildew; leaf rust; evolutionary forces

Introduction
Barley (*Hordeum vulgare* L.) is the second widely grown crop in the Czech Republic. Powdery mildew is the most common disease and leaf rust is the second one of spring barley (DREISEITL & JUREČKA 1996). To limit the harmful effects of the biotrophic fungi like *Blumeria graminis* f.sp. *hordei* (*Bgh*) and *Puccinia hordei* (*Ph*), genetic resistance is an effective, economically sound and safe alternative to fungicides. Growing resistant varieties is the most rational way of controlling these barley diseases. The trend in breeding European spring barley varieties for powdery mildew resistance was from growing susceptible varieties, to developing varieties carrying a specific resistance gene (*Mlg*), then to varieties possessing genes at the *Mla* locus, which were later combined with other genes (*Mlg*, *Mlk1*, *MlLa*, *Mlat*, and some others). The current predominating strategy is to utilize the gene *mlo* and newly described resistance genes (JAHOOR & FISCHBECK 1987, 1993).

Material and Methods
The material and methods used are described in contributions referred to in section "Results".

Results
Results of postulated powdery mildew and leaf rust resistance of spring barley varieties (DREISEITL & JØRGENSEN 2000; DREISEITL & STEFFENSON 2000a; DREISEITL & SVAČINA 2001) as well as their scoring in variety trials conducted by the Central Institute for Supervising and Testing in Agriculture in 1971-2000 (DREISEITL 1990; DREISEITL 2003a; DREISEITL & JUREČKA 2003; DREISEITL & PAŘÍZEK 2003) were used.

Discussion

The durability of disease resistance depends on the evolutionary potential of the pathogen population (McDONALD & LINDE 2002). The occurrence (qualitative aspect) and frequency (quantitative aspect) of matching virulence in the pathogen population define the effectiveness of major resistance genes. The expression of variety resistance in the field is affected by the environment and especially by actual inoculation potential of the pathogen (DREISEITL & PAŘÍZEK 2003). If the pathogen is absent, all varieties appear to be resistant, and under such conditions, a potential change in resistance cannot be determined. Therefore, only data on resistance of varieties from trials with high disease severity were used (DREISEITL & JUREČKA 2003).

The varieties can be divided into the two groups: firstly, those that could not induce changes in the pathogen population (the varieties without a resistance gene, the varieties with resistance genes originating from old domestic varieties like *Mla8* to powdery mildew and *Rph2* and *Rph4* to leaf rust, and the varieties possessing the resistance gene *mlo* to powdery mildew), and secondly, the varieties carrying other specific resistance genes to which *Bgh* and *Ph* populations could adapt (DREISEITL & JØRGENSEN 2000; DREISEITL & STEFFENSON 2000a).

A qualitative change, it means occurrence of the first individuals virulent on a certain resistance gene, is a result of mutation in the pathogen population in the territory studied or virulence immigration from other territory. Increasing the frequency of matching virulence in the pathogen population is a quantitative change. It is followed by decreasing effectiveness of the corresponding specific resistance. Increasing frequency of a matching virulence takes place most often due to direct selection of virulent pathotypes on varieties with corresponding specific resistance genes.

A typical example of the **direct selection** is breakdown of the resistance conditioned by the gene *Mla13* (Table 1). Denmark (since 1972) and former Czechoslovakia (since 1978) have priority in growing varieties possessing the gene *Mla13*. In contrast to a low proportion of the planting area of varieties possessing the *Mla13* in Denmark (MUNK *et al*. 1991), the proportion of such varieties exceeded 55% of the spring barley area (470,000 ha) in the Czech Republic and Slovakia already in 1983 (DREISEITL 1993). However, these varieties resistant till that time, in spite of their percentage of the barley harvesting area, were grown under conditions of high inoculation potential (**large pathogen population**) that was produced by winter varieties that were not resistant to powdery mildew (DREISEITL & JUREČKA 2003). A large size of the pathogen population, together with a high percentage of the varieties possessing the gene *Mla13*, induced a strong selection pressure in the pathogen population. A rapid direct selection of virulent pathotypes accompanied by a rapid decrease in the resistance of the corresponding varieties was only a logical consequent of this state. Considering the variety resistance, adaptation of the pathogen population to the gene *Mla13* has been the most important change within the entire host-pathogen system. Owing to subsequent emigration of virulent pathotypes (DREISEITL 2002a), it became an event of European importance (WOLFE *et al*. 1992).

The mentioned process from development of virulent *Va13* individuals due to mutation (BRÜCKNER 1982) through an intensive direct selection of virulent pathotypes up to total breakdown of the resistance conditioned by the gene *Mla13* (DREISEITL & PAŘÍZEK 2003) took place in the domestic territory. The direct selection undoubtedly played a primary role also in breakdown of resistances controlled by other genes *Mlg*, *Mla1*, *Mla6*, *Mla7*, *Mla9*,

Mlk1 and *MlLa* to powdery mildew (DREISEITL 2003a) and the gene *Rph3* to leaf rust (DREISEITL 1990).

Table 1. Varieties of spring barley, their resistance genes and powdery mildew resistance in field trials with high powdery mildew severity (Official Trials of the Czech Republic)

Variety	*Ml* genes[1]	Years										
		1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990
Spartan	*a9,k1*	7.75[2]	4.53	5.67	**3.38**							
Zefir	*a12,g*	6.00	**3.24**	**5.00**	3.92	**2.80**	**3.00**	**2.50**				
Koral/Krystal	*a13,g*	8.50	**8.71**	**9.00**	8.54	8.80	7.13	5.50	6.00	4.81	**3.95**	**3.67**
Jarek	*(Kr),La*					8.80	7.25	6.50	-	-	5.57	4.70
Resistant[I]		8.63	8.71	9.00	8.62	9.00	8.25	8.25	6.69	6.86	6.40	7.10
Susceptible[II]		4.25	3.24	5.00	3.38	2.80	3.00	2.50	3.31	2.48	3.95	3.67
Mean[III]		7.21	6.71	8.11	6.58	7.16	6.13	5.24	5.32	5.09	5.38	5.79

[1]DREISEITL and JØRGENSEN 2000; [2]According to the 1–9 scale, 9 = variety fully resistant, plants are without visible symptoms of infection; -Testing the variety was interrupted; [I]The most resistant variety; [II]The most susceptible variety; [III]A mean of all varieties included in the trials in the respective year.

Important changes in the pathogen population are also induced by **indirect selection**. It is obviously the case of breakdown of the resistance controlled by the gene *Mla12*. In the Czech Republic, the gene *Mla12* was present for the first time in the variety Zefir (DREISEITL & JØRGENSEN 2000). This variety was registered in 1981, i.e. in the same year when its resistance dramatically decreased. Its planting area was negligible in 1981 (DREISEITL 1993). The resistance of the variety Zefir decreased in the same year when the resistance of Spartan drastically declined. The common breakdown of these different resistances cannot be explained by other way than the (above-described) direct selection of pathotypes virulent on the resistance genes of the variety Spartan (*Mla9*,*Mlk1*) at the simultaneous indirect selection (hitch-hiking) of (obviously associated) virulence *Va12*. A similar situation was also recorded in breakdown of the resistance controlled by the gene *Ml(Kr)* (DREISEITL 2003a). The decrease in the resistance controlled by the originally fully effective gene *Ml(Kr)* copied the decline of the resistance conditioned by the above-mentioned gene *Mla13*. The variety Kredit was registered in 1984 (the original resistance was designated according to this variety) and already the year after, its field resistance markedly decreased (Table 1).

The increase in the virulence frequency *Va3* in the Czech population of *Bgh* from 7% in 1992 (DREISEITL & SCHWARZBACH 1994) to 13% in 1995 (DREISEITL and STEFFENSON 2000b) and to 24% in 2000 (DREISEITL unpublished) was caused by **migration**. The variety Mars is the only barley possessing the resistance gene *Mla3* grown in the Czech Republic until now. It was registered in 1983 and two years later, Mars achieved its largest planting area (nearly 24,000 ha=3.4% of barley area) (DREISEITL 1993). During the following period, the area planted with Mars decreased very quickly and since 1990 Mars has been registered in the Czech Republic for seed production for export only. Despite that no barley carrying the resistance gene *Mla3* is grown in the territory of the Czech Republic, the frequency of corresponding virulence *Va3* significantly increases. Seed of Mars was exported mostly to Hungary where this variety was grown. That probably caused high frequency of *Va3* (38%) found in 1996 in the south of Slovakia (KRIŽANOVÁ 1997). Besides Hungary, the varieties possessing the resistance gene *Mla3* (Sewa, Dorina, Korinna, and Baronesse) were grown in Germany and variety Rodos in Poland (DREISEITL 1996). So, there is no direct selection of *Va3* in the Czech Republic, but in surrounding countries. Due to wind

dispersal (migration) of conidia, virulence frequency increases not only in the population created by direct selection but as well as in its surroundings.

Fast adaptation of the pathogen to genetic resistance is also enabled by a large **recombination** ability of the *Bgh* population. Among 295 isolates obtained from the air in the territory of the Czech Republic in 2001, 41 isolates exhibited virulence on the original gene *Ml(N81)* (BRÜCKNER 1986) firstly possessed by the variety Maridol registered in the Czech Republic in 1999 (DREISEITL & JØRGENSEN 2000). These 41 isolates belonged to 33 different pathotypes. This is evidence for a large recombination ability of the pathogen and importance of the sexual stage on creating high diversity of the population (DREISEITL 2003b).

The above mentioned **evolutionary forces** act in the direction of increasing the virulence frequency on individual major resistance genes. It leads towards increasing complexity (accumulation of virulence genes) of individuals in the pathogen population. It was found that the population complexity to 12 barley powdery mildew resistance genes increased in the Czech territory from the estimated value of 0.90 in 1971 to the assessed value of 8.73 in 2001 (DREISEITL 2002b). Such an increase in virulence complexity brings about the decrease in diversity of the pathogen population (DREISEITL 2001), but only when considering the virulences examined for a long time (LIMPERT & DREISEITL 1996). At present, new grown barley varieties possess some new resistance genes to which the pathogen population successfully adapts again (DREISEITL unpublished). Due to increasing the virulence frequency to these new resistance genes, both complexity and diversity of the pathogen population continue to increase.

It can be concluded that practical durability of each resistance is limited by adaptability of the pathogen population. Considering the importance of the pathogens, particularly in central and northwest Europe, much effort is necessary to develop programmes aimed at breeding resistant barley varieties. The diversity of barley powdery mildew and leaf rust resistances in commercial varieties should be extended and combinations of new and effective resistance genes (JAHOOR & FISCHBECK 1993; DREISEITL & BOCKELMAN 2003; DREISEITL & DINOOR 2004) should be used.

Effectiveness and sufficient durability (at least for a natural lifetime of the variety) are basic demands on the genetic resistance. It seems that a combination of at least two original and fully effective resistance genes in a variety could be a good way to prolong the durability of resistances to both powdery mildew and leaf rust (JIN *et al*. 1995; DREISEITL & BOCKELMAN 2003; DREISEITL & DINOOR 2004). However, the progress in barley breeding for disease resistance will depend not only on the resistance sources and extending the diversity of this character in commercial varieties, but also on the critical selection of varieties that combine different effective resistance genes. The use of molecular markers seems to be essential to achieve these aims (DREISEITL *et al*. 2003).

Acknowledgements
Financial support of our research within project no. 522/03/0112 by the Grant Agency of the Czech Republic is acknowledged.

References

BRÜCKNER F. (1982): The finding of powdery mildew (*Erysiphe graminis* DC. var. *hordei* Marchal) race on barley: a race virulent to resistance genes *Mla9* and *Mla14*. Ochrana Rostlin, **18**: 101−105.

BRÜCKNER F. (1986): Possibilities of the use of the 'Nepal 81' cultivar to spring barley breeding for resistance to powdery mildew. Genetika a Šlechtění, **22**: 97−102.

DREISEITL A. (1990): Occurrence of leaf rust of barley (*Puccinia hordei*) in a set of selected genotypes of spring barley in 1979-1989. Genetika a Šlechtění, **26**: 219−224.

DREISEITL A. (1993): Analysis of growing of Czechoslovak spring barley varieties resistant to powdery mildew. Rostlinná Výroba, **39**: 337−344.

DREISEITL A. (1996): Powdery mildew resistance of barley cultivars grown in the Visegrad-group countries. In: LIMPERT E., FINCKH M.R. and WOLFE M.S. (eds.): Integrated control of cereal mildews and rusts: Towards coordination of research across Europe, Brussels, 1996, pp. 141−148.

DREISEITL A. (2000): Direct selection in the *Blumeria graminis* f.sp. *hordei* population in the Czech Republic. Acta Phytopathologica et Entomologica Hungarica, **35**: 317−322.

DREISEITL A. (2001): Diversity in the *Hordeum vulgare-Blumeria graminis* f.sp. *hordei* pathosystem. Proceedings of the 5th Congress of the European Foundation for Plant Pathology. Biodiversity in Plant Pathology, Taormina-Giardini Naxos, 18−22 September 2000, Societá Italiana di Patologia Vegetale, Catania, Italy, 2001, pp. 407−409.

DREISEITL A. (2002a): Migration and Czech population of *Blumeria graminis* f.sp. *hordei.* Petria, **12**: 209−212.

DREISEITL A. (2002b): Virulence complexity and its increasing in the Czech population of *Blumeria graminis* f.sp. *hordei.* Plant Protection Science, **38** (Special Issue 2): 531−532.

DREISEITL A. (2003a): Adaptation of *Blumeria graminis* f.sp. *hordei* to barley genetic resistance in the Czech Republic in 1971-2000. Plant, Soil and Environment, **49**: 241−248.

DREISEITL A. (2003b): Recombination in the Czech population of *Blumeria graminis* f.sp. *hordei*. 8th International Congress of Plant Pathology, Proceedings of Abstracts, Vol. 2-Offered Papers, 2−8 February 2003, Christchurch, New Zealand, p. 257.

DREISEITL A., BOCKELMAN H.E. (2003): Sources of powdery mildew resistance in a wild barley collection. Genetic Resources and Crop Evolution, **50**: 345−350.

DREISEITL A., DINOOR A. (2004): Phenotypic diversity of barley powdery mildew resistance sources. Genetic Resources and Crop Evolution, **51**: 251-258.

DREISEITL A., JØRGENSEN J.H. (2000): Powdery mildew resistance in Czech and Slovak barley cultivars. Plant Breeding, **119**: 203−209.

DREISEITL A., JUREČKA D. (1996): Disease occurrence on spring barley in the Czech Republic in 1989-1995. Ochrana Rostlin, **32**: 221−229.

DREISEITL A., JUREČKA D. (2003): Severity of powdery mildew on spring barley in the Czech Republic in 1971-2000. Plant Protection Science, **39**: 39−51.

DREISEITL A., PAŘÍZEK P. (2003): Resistance of spring barley varieties to powdery mildew in the Czech Republic in 1971-2000. Czech Journal of Genetics and Plant Breeding, **39**: 31−44.

DREISEITL A., PICKERING R.A. (1999): Pathogenicity of *Blumeria graminis* f.sp. *hordei* in New Zealand in 1997. New Zealand J. of Crop and Horticultural Science, **27**: 273−280.

DREISEITL A., RASHAL I. (2004): Powdery mildew resistance genes in Latvian barley varieties. Euphytica 135: 325-332.

DREISEITL A., REPKOVA J., KYJOVSKA Z., LIZAL P., JAHOOR A. (2003): First steps towards development of DNA markers for detection of new barley powdery mildew resistance genes. Book of Abstracts, Arnel R. Hallauer International Symposium on Plant Breeding, Mexico City, Mexico, 17−22 August 2003, 86−87.

DREISEITL A., SCHWARZBACH E. (1994): Composition of the powdery mildew population on barley in Central Moravia (Czech Republic) in 1992, (in Czech, English abstract). Rostlinná Výroba, **40**: 545-554.

DREISEITL A., STEFFENSON B.J. (2000a): Postulation of leaf rust resistance genes in Czech and Slovak barley cultivars and breeding lines. Plant Breeding, **119**: 211−214.

DREISEITL A., STEFFENSON B.J. (2000b): Structures of barley mildew population in the Czech Republic and North Dakota in 1995. Cereal Rusts and Powdery Mildews Bulletin http://www.crpmb.org/2000/0608dreiseitl/.

DREISEITL A., SVAČINA P. (2001): Registration of 'Heris' Barley. Crop Science, **41**: 1992−1993.

JAHOOR A., FISCHBECK G. (1987): Sources of resistance to powdery mildew in barley lines derived from *Hordeum spontaneum* collected in Israel. Plant Breeding, **99**: 274−281.

JAHOOR A., FISCHBECK G. (1993): Identification of new genes for mildew resistance of barley at the Mla locus in lines derived from *Hordeum spontaneum*. Plant Breeding, **110**: 116−122.

JIN Y., STEFFENSON B.J., BOCKELMAN H.E. (1995): Evaluation of cultivated and wild barley for resistance to pathotypes of *Puccinia hordei* with wide virulence. Genetic Resources and Crop Evolution, **42**: 1−6.

KRIŽANOVÁ K. (1997): Virulence of local population of *Erysiphe graminis* f.sp. *hordei* in Sládkovičovo in 1996. Proceedings of the XIVth Slovak and Czech Plant Protection Conference in Nitra, September 3−4, 1997, pp. 153−154.

LIMPERT E., DREISEITL A. (1996): Barley mildew in Europe: Towards standardisation of pathotype nomenclature. In: LIMPERT E., FINCKH M.R., WOLFE M.S. (eds.): Integrated control of cereal mildews and rusts: Towards coordination of research across Europe, Brussels, 1996, pp. 51−53.

McDONALD B., LINDE C. (2002): Pathogen population genetics, evolutionary potential, and durable resistance. Annual Revue of Phytopathology, **40**: 349−379.

MUNK L., JENSEN H.P., JØRGENSEN J.H. (1991): Virulence and severity of barley powdery mildew in Denmark 1974−1989. In: J. H. JØRGENSEN (ed.): Integrated Control of Cereal Mildews: Virulence and Their Change. Risø National Laboratory, Roskilde, pp. 55−65.

WOLFE M.S., BRÄNDLE U., KOLLER B., LIMPERT E., McDERMOTT J.M., MÜLLER K., SCHAFFNER D. (1992): Barley mildew in Europe: population biology and host resistance. Euphytica, **63**: 125−139.

Linkage Disequilibrium Mapping for Yield and Leaf Rust Resistance in Barley

R.E. Niks, A.T.W. Kraakman, P. Stam and F.A. Van Eeuwijk

Wageningen University and Research Center, Laboratory of Plant Breeding, PO Box 386, 6700 AJ, Wageningen, The Netherlands

Abstract

The recent development of linkage disequilibrium (LD) mapping methodology has widened the scope for the search of quantitative trait loci (QTL). Whereas the applicability of classical QTL studies was restricted to populations of offspring from biparental crosses, LD approaches allow QTL detection in collections of genotypes. We used LD methodology to detect marker-trait associations in a collection of 146 modern European two-row spring barley cultivars. The traits were yield, yield adaptability, yield stability, and resistance to barley leaf rust (*Puccinia hordei*). The marker system consisted of 236 AFLP-markers. Linkage disequilibrium existed up to more than 10 cM distance. A number of markers were found associated with the traits. These markers were often located in regions where earlier QTLs had been found in standard QTL experiments. Thus, we were able to verify existing QTLs and detect new ones. LD mapping appears a promising technology for studying the genetical basis of qualitative and quantitative traits in barley.

Introduction

Many of the economically most relevant traits in crops have a complex, polygenic inheritance. The genetic dissection of such traits still presents a challenge. Yield is the classical example of a complex trait. It is under polygenic control, and determined by various yield components. Even more complex traits are yield adaptability and yield stability across a range of environments.

Another example of an economically desirable but complex trait is polygenically inherited quantitative resistance against pathogens. It is generally assumed that pathogens cannot easily adapt to such resistance, and hence polygenic resistance would provide a durable protection of the crop.

Chromosome positions on which genes for quantitative and complex traits are located are called quantitative trait loci (QTLs). Such QTLs can be mapped on genome-wide marker maps. Marker genes flanking a QTL can be used in breeding programmes. Selection for these marker alleles may be much more convenient than selection for the complex trait itself. This approach is known as marker assisted selection.

Typically, QTLs are mapped in a population derived from a cross between two inbred lines. The co-segregation of alleles of mapped marker loci and alleles for phenotypic traits allows the identification of markers that are linked to loci that contribute to the trait values. For complex traits, especially those with considerable genotype x environment interaction, this approach is very cumbersome, since it requires large scale testing of mapping populations across a range of environments.

In this paper we explore the possibilities of mapping traits in a collection of modern cultivars of barley (*Hordeum vulgare*), instead of a segregating population derived from a biparental cross. We followed a methodology that has become popular in human genetics under names as

association mapping and linkage disequilibrium (LD) mapping (CARDON & BELL 2001). Quantitative geneticists working in crop plants have started to adapt this approach to their situation (*e.g.* JANNINK & WALSH 2002; see GAUT & LONG, 2003 for a review of LD in crop plants).

LD mapping is based on the following assumptions and conditions. Alleles on QTLs with a strong favourable effect on the trait of interest have been introduced by breeders into their germplasm. As a result, these alleles occur in a substantial proportion of the released cultivars. During the selection procedures leading to those cultivars, there is limited opportunity for recombination between the favourable alleles and the flanking DNA regions carrying marker alleles. The greater the effect of the favourable allele on the trait of interest and the closer the linkage between this allele and the flanking markers, the more significant the association will be between those markers and the trait. LD mapping is facilitated by dense marker maps, although a dense map still does not guarantee high resolution for QTL positions as LD may extent over substantial distance, especially in self fertilizing crops. The method will most likely uncover QTLs that have been derived from rather frequently used ancestors of the cultivar set under consideration.

Preliminary studies on rather small populations indicate that LD mapping in barley offers interesting perspectives. IGARTUA *et al.* (1999) concluded that marker-trait associations for heading date, found in mapping populations, were, to some extent, maintained in 32 cultivars. IVANDIC *et al.* (2003) found in 52 wild barley lines association between markers and the traits water-stress tolerance (chromosome 4H) and powdery mildew resistance.

The main objective of this paper was the detection of associations between marker alleles and the quantitative traits mean yield, yield adaptability, yield stability and resistance to barley leaf rust (caused by *Puccinia hordei*) in a set of 146 modern European two-row spring barley cultivars.

Material and Methods

Plant Material: The cultivar set consisted of 146 modern European two-row spring barley cultivars, that were representative for a large part of the European germplasm. They all featured in the official Danish National and Recommended List of Cultivars in the period 1993 to 2000 (see www.planteinfo.dk). Seed was kindly provided by the testing authorities and by the original breeders.

Yield, Yield Adaptation and Yield Stability Data: We used yield data from the official Danish barley variety trials performed between 1993 and 2000. Here we present the results from the trials that had been treated with pesticides to control leaf diseases. For more extensive data, see KRAAKMAN *et al.* (in press). Yield adaptability was defined as the slope of the regression of yield for an individual cultivar on the mean yield (over all cultivars) across environments (FINLAY & WILKINSON 1963). Yield stability was defined as the mean square of deviations from the Finlay-Wilkinson line (EBERHART & RUSSELL 1966). Both statistics were based on the regressions of yields of individual genotypes in a trial on an environmental index. This index was taken to represent the general growing conditions in the trial. We estimated the environmental index by the environmental effects obtained from the fit of an additive model (phenotype = genotype + environment). Values of s_i^2 were log-transformed for subsequent analyses.

Disease Data: For all cultivars we determined the level of susceptibility to barley leaf rust (*Puccinia hordei*) isolate 'IVP2000'. This isolate is virulent to the hypersensitivity genes *Rph*9 and *Rph12*, which are present in many European cultivars (NIKS *et al.* 2000), but avirulent to the

also commonly applied *Rph3*. The cultivars were tested in the seedling stage in a monocyclic test in a greenhouse compartment, and in a polycyclic field test in the adult plant stage.

- *Seedling Test:* Seedlings were raised in plant boxes. Per cultivar two to five seedlings were available. Each box contained seedlings of L94 and the cultivar 'Vada' as references. About 200 spores per cm^2 were applied on the first leaves in a settling tower. After incubation at a relative humidity of 100% overnight, the seedlings were transferred to a greenhouse. The seedlings were evaluated for the infection type on a scale ranging from 0-9. This scale rates the degree of sporulation and hypersensitivity reaction associated with infection sites (see NIKS & RUBIALES 1994). On the seedlings that showed a compatible infection type to the pathogen, the latency period of the rust was measured (PARLEVLIET 1975). The latency period was defined as the time between inoculation and the moment that 50% of the ultimate number of pustules had become visible. The relative latency period (RLP) was calculated relative to the LP of L94 seedlings, where L94 = 100. The seedling test was carried out twice, and averaged data were used as parameter describing the degree of resistance of the cultivars in the seedling stage.

- *Adult Plant Test:* The cultivars were planted in a randomised block design with two replications, where the second replication contained fewer cultivars because of seed limitations. The plot size was 0.75 x 1.25 m^2. Inoculation was done by placing a pot with sporulating L94 plants in front of each plot (QI *et al.* 1998). The amount of infection in the field was determined three times during the season. Pustules were counted on the upper three leaves of three tillers per plot. The leaf rust pustule counts were transformed according to the scale proposed by PARLEVLIET and VAN OMMEREN (1984). The Area Under the Disease Progress Curve (AUDPC) was calculated and used as parameter for resistance of the cultivars in the field.

AFLP markers: Collection of DNA from leaf tissue and AFLP analysis were done as described by QI and LINDHOUT (1997). DNA was digested with restriction enzymes *Eco*RI and *Mse*I. Fourteen primer combinations were employed. Markers were scored for presence (1) or absence (0) of a band. In total 286 polymorphic markers were scored within this germplasm. For analyses, 236 markers with band frequencies in between 5% and 95% were used.

Map Position Based on an Integrated Map: Map positions of markers were determined by integrating three marker maps: 1) L94 × Vada, 568 markers (QI & LINDHOUT 1997); 2) Apex × Prisma, 252 markers (YIN *et al.* 1999); 3) GEI119 × Gunhild, 137 markers (KOOREVAAR, unpublished data). The number of markers segregating and mapped in two or three populations was 89, amounting to at least eight markers per chromosome. The resulting map was aligned to the RFLP-map of the Proctor × Nudinka population (BECKER *et al.* 1995).

Linkage Disequilibrium: We quantified the degree of LD by calculating the squared Pearson correlation coefficient, r^2 between all pairs of loci, and plotted these r^2 values against the genetic distance in cM.

Population Structure: We performed cluster analysis on band incidence (UPGMA on distances obtained from Jaccard similarities , GORDON, 1981), to verify whether the cultivar set consisted of distinct subsets that could lead to associations that were not due to linkage. Also a correspondence analysis was applied (GREENACRE 1984) on the cultivar by marker matrix of band incidences. Both analyses indicated that the germplasm consisted of two subpopulations (KRAAKMAN *et al.* in press). This split could not be explained by geographic arguments, nor by a separation of fodder and malting barleys.

Marker – Trait Associations: Pearson correlation coefficients were calculated between the quantitative trait values on one hand, and band incidences for markers on the other hand. This is effectively equivalent to t-tests using marker incidence as grouping variable. The test statistic for

Pearson correlations, $t^* = r \cdot (n-2)^{1/2} / (1-r^2)^{1/2}$, with r the correlation and n the number of observations, follows a $t_{(n-2)}$ distribution under the null hypothesis.

Results

Integrated Map and Map Position: The final integrated map had a genetic genome length of 1052 cM, and consisted of 811 AFLP-markers. Out of the 236 markers that were found to be polymorphic across the cultivars, 123 appeared also on the integrated map of the crossing populations. The position of the other 113 markers was unknown, since they had not been mapped in any of the three mapping populations.

Linkage Disequilibrium: Figure 1 gives LD as a function of genetic distance. LD was very common for distances below 10 cM. Occasionally, LD occurred between loci further apart. The r^2 between unlinked loci on different chromosomes was always below 0.28, except for two markers on chromosomes 3 and 5, which had an r^2 of 0.40. These two markers were typical for the two subgroups in the cultivar set. There were in total 53 marker pairs with distance < 1 cM, of which 32 had a significant correlation ($p<0.01$), while, surprisingly, 19 pairs were not significantly correlated ($p \geq 0.01$), and thus in linkage equilibrium (LE). The three locus pairs in LE with the shortest distance between them (<0.06 cM) were all mapped in the L94 × Vada population. This shows that the possible inaccuracy in marker order and distance after map integration could not be the only explanation for apparent LE on short distances.

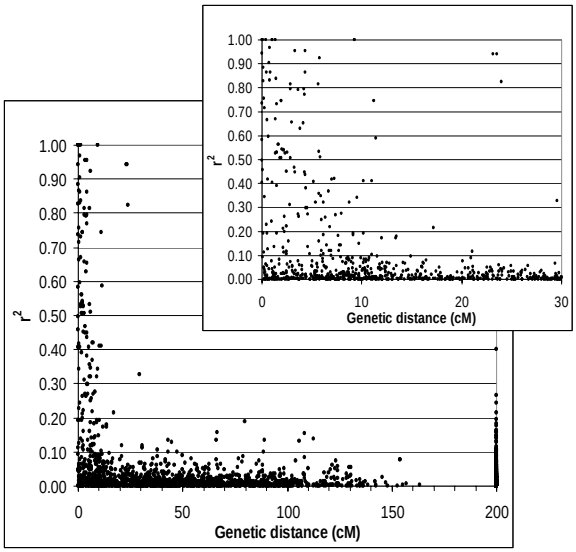


FIGURE 1. - Linkage disequilibrium (r^2) in a population of 146 modern barley cultivars as a function of genetic distance for 123 AFLP loci on the barley genome. The data points at 200 centimorgan represent unlinked loci on different chromosomes. The inset provides an enhanced view of LD decay.

Association: Table 1 gives an overview of markers with their genome positions and correlations with traits. The table presents markers that are correlated with yield, yield adaptability, yield stability and resistance to *P. hordei*, only for markers with known chromosome position. Several of the polymorphic markers with unknown position were also correlated with these traits (KRAAKMAN *et al.* in press).

The highest correlation was between marker E39M61-255 and infection type with isolate IVP2000 (r = 0.74). The positive association between this marker and the infection type score implies that the marker is linked to the allele for susceptibility. The position of this marker on the distal part of the long arm of chromosome 1 (7H) strongly suggests that the responsible gene is *Rph3* (CHELKOWSKI *et al.* 2003). The amplified DNA fragment occurred in 101 of the 110 cultivars with a susceptible reaction, and was absent in 27 of 31 cultivars with a hypersensitive reaction.

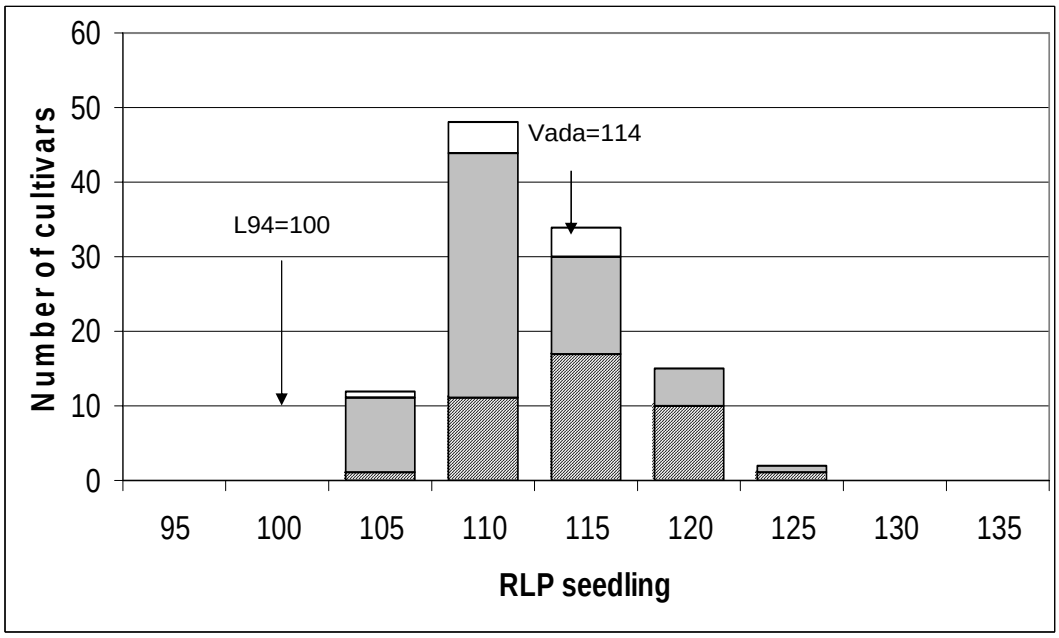
Another marker, E38M55-251, was associated with prolonged latency period. This is a marker that is close to the LP-prolonging allele of *Rphq2*, a QTL reported by QI *et al.* (1998), and located near the tip of the long arm of chromosome 2 (2H). On seedlings of cultivars carrying this marker, the rust fungus indeed had a longer latency period than on cultivars lacking this marker (Fig. 2). Since this QTL is only marginally effective in the adult plant stage and in field tests (QI *et al.* 1998), it is not surprising that the marker was not associated with AUDPC (Table 1).

TABLE 1. Correlation of AFLP markers with yield, yield adaptability, yield stability and resistance against *Puccinia hordei* in a set of 146 barley cultivars, and references to genes or QTLs for similar traits reported to be located in the same chromosome area as the AFLP marker.

Present study					Gene or QTL Reported in Vicinity	
AFLP marker	Chromosome / Position (cM)	*r*	*P*	Character	Reference	Character
E35M48-250	3 / 19.5	-0.29	***	Yield	BEZANT *et al.* 1997	Yield
E45M55-212	4 / 86.1	-0.22	*	Yield	TINKER *et al.* 1996	Yield
E38M55-114	7 / 0.0	0.27	**	Yield	BEZANT *et al.* 1997	Yield
E38M54-247	7 / 7.4	0.30	***			
E38M50-119	1 / 105.8	0.24	**	Adaptability	-	-
E45M55-086	2 / 36.2	-0.26	**	Stability	HAYES *et al.* 1993 VOLTAS *et al.* 2001	Yield GxE
E45M55-142	4 / 45.8	-0.26	**	Stability	MARQUEZ-CEDILLO *et al.* 2000	Yield
E35M55-262	4 / 105.0	0.30	***	Stability	HAYES *et al.* 1993 VOLTAS *et al.* 2001 FORSTER *et al.* 2000	Yield GxE Stress-response
E42M48-103	6 / 35.1	-0.32	***	Stability *Resistance to* Puccinia hordei	-	-
E39M61-255	1 / 151.5	0.74	***	Infection type	CHELKOWSKI *et al.* 2003	*Rph3*-resistance
E38M55-129	7 / 70.0	0.37	***	AUDPC	-	-
E35M54-078	2 / 89.2	0.36	***	LP	-	-
E38M55-251	2 / 137.7	0.39	***	LP	QI *et al.* 1998	PR to *P. hordei*
E45M55-172	3 / 56.3	0.35	***		-	-
E35M54-309	3 / 59.6	0.34	***	LP	-	-
E35M54-310	3 / 60.6	-0.34	***		-	-

Significance *, **, ***, $P < 0.01$, 0.005, 0.001, respectively.

FIGURE 2. Frequency distribution of the Relative Latency Period (RLP, on L94 = 100%) of *Puccinia hordei* isolate IVP2000 on seedlings of a set of 111 barley cultivars to which this isolate was virulent. The cultivars carrying the AFLP marker E38M55-251 (hatched) tended to cause a longer LP than the cultivars lacking this Vada-derived marker (solid grey). White bar sections represent cultivars with unknown presence of the marker.



The most significantly correlated markers for yield were located at the top of chromosome 7 (0 to 7.4 cM, two markers) and chromosome 3 (19.5 cM) (Table 1). There was a significant correlation for a marker on chromosome 1 for yield adaptability. The most significant correlations for yield stability were for a marker with unknown position ((KRAAKMAN *et al.* in press), and for markers on chromosomes 4 and 6. Nearly all markers that were associated with yield or yield stability were in regions where at least once before a yield-QTL had been reported. Besides, two of the three yield stability-associated markers also coincided with a region known to harbour QTL by environment interaction (VOLTAS *et al*. 2001; MALOSETTI *et al.* 2004). In general, markers were correlated with only one of the traits. None of the markers found to be associated to a trait differed in allele frequency between two subgroups of cultivars that were identified. Therefore we concluded that the associations were not caused by substructure in the germplasm, but were very likely the result of linkage.

Discussion

The present study demonstrates that in the set of North-West European barley cultivars LD-mapping is an attractive option to detect markers that are associated with high agronomic performance. We found that LD extended to as far as 10 cM distance. One of the advantages of LD-mapping over classical mapping on the basis of segregating mapping populations is that LD-mapping can be based on a representative genetic sample of agronomically relevant germplasm. Simultaneously markers linked to alleles for the same trait, but originating from different progenitors may be identified. This is illustrated by the identification of QTLs for one component of partial resistance to barley leaf rust, viz. latency period in the seedling stage. One QTL near the tip of chromosome 2 (2H), that had been reported before to be contributed by cv Vada (QI *et al.* 1998), was significantly associated with long latency period in the set of cultivars

(Table 1, Fig. 2). In addition, the LD-mapping indicated another QTL, located on chromosome 3 (3H), at approximate position of 60 cM (Table 1). The three most indicative markers E45M55-172, E35M54-309 and E35M54-310 had been mapped in L94 x Vada, suggesting that this QTL had been contributed by Vada. However, in the L94 x Vada mapping population this QTL had not appeared from the mapping by QI *et al.* (1998), so the linkage between these marker loci and the trait is absent in Vada/L94.

LD-mapping was very powerful to pinpoint the hypersensitivity resistance to the locus *Rph3*, which is indeed a frequently applied major gene for resistance in European barley (NIKS *et al.* 2000) and isolate IVP2000 is avirulent to this gene. The identified marker was linked to the susceptibility allele of this resistance locus. The marker had been mapped in Vada × L94 and in Prisma × Apex, none of which carries the dominant *Rph3* allele.

The present study demonstrates that LD mapping offers perspectives in locating QTLs for complex traits like yield, and even yield adaptability and yield stability. Most of the QTLs revealed in our LD-mapping approach had been reported in earlier linkage analyses that were based on biparental mapping populations (Table 1). This lends credibility to the relevance of the markers that were detected in the present study to be associated with yield aspects and resistance.

We conclude that LD-mapping has clear potential for improving barley, especially for complex traits, like yield and yield stability, for which measurements are costly and time-consuming. Combining existing phenotypic variety trial data and genotypic marker characterizations within an LD-approach may prove to be highly profitable.

Acknowledgements

Lars Kjær of the Danish Agricultural Advisory Center is gratefully acknowledged for the barley field data and seed of recently tested cultivars. More than thirty breeding companies from all over Europe have sent us seed of their cultivar(s), which we highly appreciate. We thank Pim Lindhout and Fien Meyer-Dekens for performing AFLP analyses, and Fernando Martínez and Bakhyt Mussiraliev for carrying out the disease tests. This research is supported by the Technology Foundation STW, applied science division of NWO and the technology programme of the Ministry of Economic Affairs of The Netherlands.

References

BECKER J., VOS P., KUIPER M., SALAMINI F., HEUN M. (1995): Combined mapping of AFLP and RFLP markers in barley. Mol. Gen. Genetics, **249**: 65-73.

BEZANT J.H., LAURIE A.D., PRATCHETT N., CHOJECKI J., KEARSEY M.J. (1997): Mapping of QTL controlling NIR predicted hot water extract and grain nitrogen content in a spring barley cross using marker-regression. Plant Breeding, **116**: 141-145.

CARDON L.R., BELL J.I. (2001): Association study designs for complex diseases. Nature Reviews Genetics, **2**: 91-99.

CHELKOWSKI, J., TYRKA M., SOBKIEWICZ A. (2003): Resistance genes in barley (*Hordeum vulgare* L.) and thier identification with molecular markers. J. appl. Genet., **44**: 291-309.

EBERHART S.A., RUSSELL W.A. (1966): Stability parameters for comparing varieties. Crop Sci., **6**: 36-40.

FORSTER B.P. , ELLIS R.P., THOMAS W.T.B., NEWTON A.C., TUBEROSA R. *et al.* (2000): The development and application of molecular markers for abiotic stress. J.Exp.Botany, **51**: 19-27.

GAUT B.S., LONG A.D. (2003): The lowdown on linkage disequilibrium. The Plant Cell, **15**: 1502-1506.

GORDON A.D. (1981): Classification. Monographs on applied probability and statistics. Chapman & Hall.

GREENACRE M.J. (1984): Theory and application of correspondence analysis. Academic Press, London.

HAYES P.M., LIU B.H., KNAPP S.J., CHEN F., JONES B. *et al.* (1993): Quantitative trait locus effects and environmental interaction in a sample of North American barley germplasm. Theor. Appl. Genet., **87**: 392-401.

IGARTUA E., CASAS A.M., CIUDAD F., MONTOYA J.L., ROMAGOSA I. (1999): RFLP markers associated with major genes controlling heading date evaluated in a barley germ plasm pool. Heredity, **83**: 551-559.

IVANDIC V., THOMAS W.T.B., NEVO E., ZHANG Z., FORSTER B.P. (2003): Association of SSRs with quantitative trait variation including biotic and abiotic stress tolerance in Hordeum spontaneum. Plant Breeding, **122**: 300-304.

JANNINK J.L., WALSH J.B., (2002): Association mapping in plant populations. In: M.S. KANG (ed.): Quantitative Genetics, genomics and plant breeding, edited by. CABI, Wallingford, UK.: 59-68.

KRAAKMAN A.T.W., NIKS R.E., VAN DEN BERG P.M.M.M., STAM P., VAN EEUWIJK F.A. (in press) Linkage disequilibrium mapping of yield and yield stability in modern spring barley cultivars. Genetics.

MALOSETTI M., VOLTAS J., ROMAGOSA I., ULLRICH S.E., VAN EEUWIJK F.A.. (2004): Mixed models including environmental variables for studying QTL by environment interaction. Euphtyica, in press.

MARQUEZ-CEDILLO L.A., HAYES P.M., JONES B.L., KLEINHOFS A., LEGGE W.G. *et al.*, (2000): QTL analysis of malting quality in barley based on the doubled-haploid progeny of two North American varieties representing different germplasm groups. Theor. Appl. Genet., **101:** 173-184.

NIKS R.E., RUBIALES D. (1994): Avirulence factors corresponding to barley genes *Pa3* and *Pa7* which confer resistance against *Puccinia hordei* in rust fungi other than *P. hordei*. Physiol. Mol. Plant Pathol., **45**: 321-331.

NIKS R.E., U. WALTHER, H. JAISER, F. MARTÍNEZ, D. RUBIALES, *et a.l* (2000): Resistance against barley leaf rust (*Puccinia hordei*) in West-European spring barley germplasm. Agronomie, **20**: 769-782.

PARLEVLIET J.E. (1975): Partial resistance of barley to leaf rust, *Puccinia hordei*. I. Effect of cultivar and development stage on latent period. Euphytica, **24**: 21-27.

PARLEVLIET J.E., VAN OMMEREN A. (1984): Interplot interference and the assessment of barley cultivars for partial resistance to leaf rust, *Puccinia hordei*. Euphytica, **33**:685-697.

QI X., LINDHOUT P. (1997): Development of AFLP markers in barley. Mol. Gen. Genet., **254**: 330-336.

QI X., NIKS R.E., STAM P., LINDHOUT P. (1998): Identification of QTLs for partial resistance to leaf rust (*Puccinia hordei*) in barley. Theor. Appl. Genet., **96:** 1205 - 1215.

TINKER, N.A., MATHER D.E., BLAKE T.K., BRIGGS K.G., CHOO T.M. *et al*. (1996): Loci that affect agronomic performance in two-row barley. Crop Sci., **36:** 1053-1062.

VOLTAS J., ROMAGOSA I., ULLRICH S.E., VAN EEUWIJK F.A. (2001): Identification of adaptive patterns in the 'Steptoe x Morex' barley mapping population integrating genetic, phenotypic and environmental information. 7th Quantitative Trait Locus mapping and marker-assisted selection workshop, Valencia.

YIN X., STAM P., DOURLEIJN C.J., KROPFF M.J. (1999): AFLP mapping of quantitative trait loci for yield-determining physiological traits in spring barley. Theor. Appl. Genet., **99:** 244-253.

Genetic Mapping of a Novel Scald Resistance Gene *Rrs15*$_{CI8288}$ in Barley

G. Schweizer[1], M. Herz[1], S. Mikolajewski[1], M. Brenner[1], L. Hartl[2] and M. Baumer[2]

Bavarian State Research Centre for Agriculture,
Institute for Crop Production and Plant Breeding
[1]Genome Analysis, Am Gereuth 2; [2]Barley Breeding, Am Gereuth 6
85354 Freising-Weihenstephan; Germany
Corresponding author: G. Schweizer; E-mail: guenther.schweizer@LfL.bayern.de

Abstract
Rhynchosporium secalis (Oudem) J.J. Davis, the causal agent of leaf scald is one of the major leaf diseases of barley (*Hordeum vulgare* L.). A number of resistance genes against the fungus were published up to now, most of them map either to the complex locus *Rrs1* (*Rh-Rh3-Rh4*) on chromosome 3H close to the centromere, or to the locus *Rrs2* (*Rh2*) on the short arm of chromosome 7H.
The objective of the present study was to map the major scald resistance gene in accession ´CIho8288´. A mapping population of 129 doubled haploid (DH) lines was based on a cross between the 6-rowed spring barley ´CIho8288´, carrying the unknown resistance gene *Rrs*$_{CI8288}$ and the 2-rowed malting barley cv. ´Steffi´, susceptible for *R. secalis*. The phenotypic evaluation of the population was carried out with a one-spore inoculum of *R. secalis* isolate 147/1 in a greenhouse camber. The resistance gene *Rrs*$_{CI8288}$ has been mapped on the short arm of chromosome 2H using bulked segregant analysis and SSR-markers. So far no scald resistance gene has been mapped to that chromosome arm and would be proposed as resistance locus *Rrs15*$_{CI8288}$.
Keywords: barley; *Hordeum vulgare*; scald; *Rhynchosporium secalis*; marker assisted selection

Introduction
Leaf scald, caused by the imperfect fungus *Rhynchosporium secalis* (Oudem) J. J. Davis, is one of the important foliar diseases in many barley growing regions of the world, as a result of intensive barley production and an interaction of cool and wet environmental conditions (XI *et al.* 2003). Yield losses due to scald can reach 20 to 36% (BUCHANNON & WALLACE 1962; XI *et al.* 2000) and the reduction of malting quality due to reduced kernel size and plumpness (KHAN & CROSBIE 1988) have been reported.
The pathogen is characterized by an extensive genetic variability and frequent changes of the population. XI *et al.* (2003) reported that shifts in the virulence of *R. secalis* and the potential for high variability in the virulence were responsible for the observed break down of barley cultivar resistance e.g. in Alberta. A better comprehension about variation in the virulence of the pathotypes of *R. secalis* and a clear understanding of the interaction between host and pathogen is important for new breeding programmes. So planting resistant cultivars is one of the major methods controlling scald in barley.
R. secalis is a pertotrophe fungus, unlike to other foliar growing pathogens, it does not degrade plant cell walls nor form haustoria to penetrate host cells for nutrient uptake (LEHNACKER & KNOGGE 1990). The infection cycle starts by the fungus with the spore attachment to the leave surface and penetration of the leave cuticula and stays confined to the subcuticular region throughout most of its life cycle. The early stages of pathogenesis show the collapse of a few epidermal cells. Subsequently, mesophyll cells underlying the affected epidermal cell collapse. In a later stage, the subcuticular mycelium forms a dense stroma and causes the formation of necrotic lesions and finally sporulates (KNOGGE & MARIE 1997).

The interaction of this pathogen and its host constitutes a model system for plant-fungus gene-for-gene relationship (ROHE *et al.* 1995). This process is controlled by a combination of diverse fungal factors and the interaction of the respective resistance factors/genes in the plant.
So molecular markers for the different scald resistance genes will be of strategic importance for all barley breeders, particularly if these genes are to be combined and the markers will be of diagnostic value.
Within the last decade four main resistance loci for scald resistance were mapped: BARUA *et al.* (1993), GRANER & TEKAUZ (1996), PENNER *et al.* (1996) and PATIL *et al.* (2003) mapped the *Rrs1* locus on chromosome 3H and a total of 11 alleles have been listed (BJÖRNSTAD *et al.* 2002). ABBOTT *et al.* (1992) and GARVIN *et al.* (1997) assigned a scald resistance gene to chromosome 4H. Another gene, *Rrs13*, was localised by ABBOTT *et al.* (1992) on chromosome 6H, SCHWEIZER *et al.* (1995) mapped the $Rh2_{\text{Atlas}}$ gene on chromosome 7H.
According to PATIL (2001) and BJÖRNSTAD *et al.* (2002), so far 14 major resistance genes or alleles against the fungus *R. secalis* have been described in barley, but not all of them have been mapped to specific loci (e.g. HABGOOD & HAYES 1971; SOGAARD & WETTSTEIN KNOWLES 1987; GOODWIN *et al.* 1990; BEER 1991; JÖRGENSEN 1993; ABBOTT *et al.* 1992, 1995; SCHWEIZER *et al.* 1995, 2000; GARVIN *et al*. 1997, 2000; BJÖRNSTAD *et al.* 2002; PATIL *et al.* 2003). Further resistance genes have been detected in genotypes of wild barley, *H. vulgare ssp. spontaneum*, collected in Iran, Israel and Turkey. (GENGER *et al.* 2000; BJÖRNSTAD *et al.* 2002) and designated as *Rrs12*, *Rrs13 and Rrs14*. As to our knowledge, no resistance gene against scald was mapped up to now on the short arm of chromosome 2H.
The nomenclature of the *R. secalis* resistance genes has to be transposed to the more correct Rrs/rrs system and to respective differential genotypes (for example: $Rrs2_{\text{Atlas}}$ for *Rh2*) in accordance to BJÖRNSTAD *et al.* (2002). At the moment there is still disorder in nomenclature because of missing test isolates and differential reference genotypes.
The objective of the present study was to map the major scald resistance gene in the accession ´CI8288´ ($Rrs15_{\text{CI8288}}$) and to identify molecular markers closely linked to the resistance locus for the application of marker based breeding programmes.

Material and Methods

Plant Material

A doubled haploid population (DH) of 129 lines, derived from anther-culture was developed from the F_1 hybrids of the cross between the *R. secalis* resistant *Hordeum vulgare* accession ´CI8288´ and the *R. secalis* susceptible barley cultivar ´Steffi´. The resistant parent ´CI8288´ (Gembloux 456), a six-rowed spring barley, was provided by the world barley collection (USDA) and possesses an unknown major resistance gene against scald. The susceptible cultivar ´Steffi´ (Saatzucht Ackermann, Irlbach, Germany) is a two-rowed spring barley with a good malting quality. The in vitro regeneration of anthers was conducted as described by DANIEL & BAUMANN (1990).

Resistance Assessment

The DH mapping population was tested for reaction to R. secalis in several independent experiments 2002-2003, according to SCHWEIZER et al. 1995 with some modifications. Disease severity of the 129 DH lines was assessed at seedling stage in a greenhouse camber. Therefore barley plants were grown at a temperature of 16-18°C in 9 x 9 cm plastic pots, each line being represented by four seedlings in a pot. The plants were inoculated at three leaves

stage, approximately 20 days after sowing. Parents and some differential genotypes were integrated in the experiment as internal controls.
The one-spore isolates 147/1 and 271 (Straßmoos, Bavaria) of *R. secalis*, provided by Dr. Sachs/BBA Kleinmachnow, were grown for approximately 20 days on 2.3% (w/v) Lima bean agar (Difco, Detroit, USA) in Petri-dishes at 16°C in the dark. The spores were harvested after addition of 5 ml of H_2O by gently rubbing of the mycel with a glass rod, the advanced spore suspension was decanted, filtrated and adjusted to 2-300.000 spores/ml. One inoculum preparation was used for all seedlings in a given experiment. High humidity and darkness were maintained by covering the inoculated plants in the greenhouse with black plastic hoods for 48 hours. Plants were assessed visually for scald symptoms on the lamina of the second leaf (the third leaf was used as further control) approximately 10-14 days after infection based on the scale described by JACKSON & WEBSTER (1976).

DNA Analysis
DNA were extracted from plant material based on SCHWEIZER *et al.* (1995) and BEHN *et al.* (2004). The AFLP-analyses were carried out according to VOS *et al.* (1995), HARTL *et al.* (1999) and BEHN *et al.* (2004) using the PstI/MseI enzyme system, combined with Fluorescein labelled PstI (SseI)-primers. The detection of the AFLP pattern was performed after a fragment separation on a 5% denatured PAA-gel by an ´Typhoon 9200´ laser scanner (Amersham Biosciences). For the bulked segregant analysis 256 primer combinations were used. The SSR assays in barley for the Bmag-, Bmac- and HVM-microsatellites were carried out according to LIU *et al.* (1996) and RAMSAY *et al.* (2000) and the GBM-microsatellites according to THIEL *et al.* (2003).

Linkage Analysis
Map construction and linkage analysis were conducted with MAPMAKER 3.0 computer software (LANDER *et al.* 1987), using a logarithm of odds (LOD) threshold of 3.0 and 30 cM as the maximal distance between two linked markers.

Results and Discussion
Inoculation Test
The reaction of the DH population from the cross between the *R. secalis* resistant accession ´CI8288´ and the susceptible barley cultivar ´Steffi´ on the fungus one-spore-isolates 147/1 and 271 was assessed by a seedling test in the greenhouse. No other fungus was observed during the test phase. The scald disease evaluation was started when the first symptoms appeared on the lamina of the second leaf and scored in two day intervals. The mean value of all DH lines per pot was formed and in table 1 the results of 4 independent inoculations, with a limited number of entries in test 3 and 4 are summarized.
The number of tested DH lines varied because of incomplete germination of some DH lines. The variation between tests 1 and test 2 is evident. The inoculation test 1 was assessed from 15. to 22.02.2002 with a constant increase of infection and a confidential scoring of the leaf necrosis, whereas the disease progression in inoculation test 2 was too fast (scoring data: 28. to 29.05.2002). Due to a defect in the dosing pump in test 2 a significant higher spore concentration than deliberated was applied. In fact, the data fit into the resistance ranking of the respective DH lines, equal to test 1, but the values already at the beginning of the score were to high and so test 2 was not used in the further mapping process.
To compare the resistance ranking in a set of random selected DH lines, the third and fourth inoculation test was performed with two different R. secalis isolates (271 and 147/1). The scored resistance data were in good accordance to the first inoculation test and confirms

earlier results (data not shown), that *R. secalis* isolate 271 is more aggressive than isolate 147/1 as we can see in mean disease score in table 1.

Table 1. Summary of results from four independent resistance tests with the ´CI8288´ x ´Steffi´ DH population after inoculation of *R. secalis* isolate 147/1 and 271

Item	1. Inoculation	2. Inoculation	3. Inoculation	4. Inoculation
R. secalis isolate	147/1	147/1	147/1	271
Number of lines tested	122	127	22	27
Min	0.00	0.00	0.33	1.33
Max	3.88	4.00	4.00	4.00
Mean disease score	1.98	2.91	2,12	2.84
Segregation ratio Res.: susc.	60:62	38:89	10:12	10:17
Mean score ´CI8288´	1.44	2.06	0.33	1.33
Mean score ´Steffi´	3.25	4.00	3.33	4.0
Correlation to Inoculation test 1	1.00	0.65	0,80	0.75

As resume, the mean score of the resistant parent ´CI8288´, and that of the susceptible parent ´Steffi´ differed significantly with respect to scald and ´CI8288´ is the donor of an efficient resistance gene against *R. secalis.* Within the DH population a high phenotypic variability and reproducible disease pattern for scald was observed and the segregation ratio of nearly 1:1 indicated the inheritance of single major resistance gene.

Test of Molecular Markers Linked to Scald Resistance

In a first step the two major scald resistance genes, the *Rrs1* locus at the centromere of chromosome 3H and the $Rrs2_{\text{Atlas}}$ on the short arm of chromosome 7H were analyzed for linkage with the scald resistance gene found in accession ´CI8288´.

Rrs1: The scald resistance gene *Rrs1* on chromosome 3H was mapped by GRANER & TEKAUZ (1996), PENNER *et al.* (1996) and PATIL *et al.* (2003) and the known STS- and SSR-markers (tab. 2) surround the *Rrs1* locus were tested for polymorphism between parents and were thereon applied in the segregating DH population. For the polymorphic markers a molecular map was constructed with MAPMAKER as shown in Fig. 1, the order of the markers is in accordance with published consensus maps. The segregating ratio of all markers at this chromosome interval was biased towards the ´Steffi´ allele, but the Rrs_{CI8288} resistance gene showed no linkage to this linkage group.

Table 2. Marker and their segregation ratio surround the *Rrs1* resistance gene locus of chromosome 3H

Marker	Segregation ratio ´CI8288´ : ´Steffi´	$\chi 2$*	Number of analyzed DH-lines
HVM27	47 : 76	6,83	123
YLM (PALTRIDGE *et al.* 1998)	48 : 80	8,00	128
HVM33	52 : 72	3,22	124
HVM60	53 : 74	3,47	127
Bmag0225	55 : 73	2,53	128
Falcon (PENNER *et al.* 1996)	No polymorphism	-	-

*$\chi 2_{\text{(Tab.)}}$: (p = 5%) = 3,84; (p = 1%) = 6,63

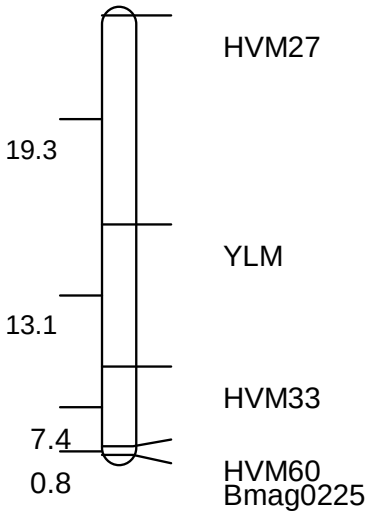


Figure 1. Genetic map of barley chromosome 3H. Linkage analysis was performed on 129 DH lines from a cross between ´CI8288´ x ´Steffi´. Distances (in cM) between loci are listed on the left side of the diagram.

Rrs2: For the scald resistance gene $Rrs2_{Atlas,}$ the STS marker ´Atlas14´ was developed and mapped at a distance of 0.07 cM to the $Rrs2_{Atlas}$ gene (SCHWEIZER et al. 1995, 2002). The STS marker was polymorphic between the parents ´CI8288´ and ´Steffi´ and could be mapped to the ´CI8288´x´Steffi´ population. A segregation ratio of 58:71 (´CI8288´:´Steffi´, χ^2=1,3) has been observed for this DNA locus, again the marker showed no linkage to the resistance gene in ´CI8288´.

Bulked Segregant Analysis

Because no correlation to the well known loci for scald resistance was found, we started a bulked segregant analysis (BSA) with the AFLP technology. Therefore the resistant DNA pool consists of 8 resistant scored DH lines with a mean score of 0.09 (inoculation test 1), the susceptible DNA pool consists of 9 DH lines and a mean disease score of 3.52. The bulks comprised of equal amounts of pooled DNAs from the respective lines and in addition the parents ´CI8288´ and ´Steffi´ were used as continuous control. The pool screening was carried out with 256 primer combinations. Thereof in 17 primer combinations the expected/respective AFLP-pattern with an identical and specific band in the resistant pool and the resistant parent ´CI8288´ was observed. For validation of the specific bands, each out of the 17 primer combinations was tested at 48 DH lines inclusive parents and three further controls. The fragment pattern from 11 out of 17 primer combinations showed a sufficient correlation to the scald disease scoring and marker analysis was expanded to the whole DH population of 129 plants.

After map construction (Fig. 2) the $Rrs15_{CI8288}$ resistance gene was integrated in a group of 4 AFLP markers, with an over all fragment length of 43,7 cM, whereas 2 AFLP markers (S13M52, S15M47) segregated at a distance of 3 cM. Based on comparative mapping with the ´Alexis´ x ´Steina´ population (HARTL *et al.* 2000) the linkage group could be assigned to the short arm of chromosome 2H (data not shown).

The SSR-anchor-markers for the short arm of chromosome 2H, Bmac0134 and HVM36, were polymorph and mapped into the present linkage group (Fig. 2). The resistance gene $Rrs15_{CI8288}$ was integrated in between the two SSR-markers. This result is a clear evidence, that the major resistance gene in ´CI8288´ was localized on short arm of chromosome 2H and will be named $Rrs15_{CI8288}$ according to the Rrs/rrs system and to the respective differentia l genotype in accordance to

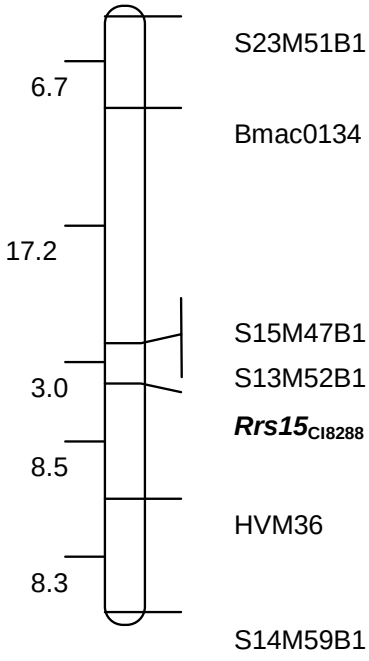


Figure 2. Genetic map of barley chromosome 2HS around the $Rrs15_{CI8288}$ resistance gene locus. Linkage analysis was performed on 129 DH lines from a cross between ´CI8288´ x ´Steffi´. Distances (in cM) between loci are listed on the left side of the diagram.

BJÖRNSTAD *et al.* (2002).
The mapping of the major resistance gene *Rrs15*$_{CI8288}$ against scald on a new linkage group on chromosome 2H will be the basis for further straight forward breeding strategies, particularly pyramiding of different scald resistant genes, because most of the known scald resistant donor plants carry their resistant gene/allel on chromosome 3H or 7H.
At the moment, a STS marker with a distance of 3.0 cM to *Rrs15*$_{CI8288}$ will be in development and tested for diagnostic value in European spring- and winter barley cultivars. For an efficient marker based selection, a SNP marker will be established by Pyrosequencing.

Acknowledgements
The authors thank Mr. A. Barth and Mrs. A. Jestadt for their excellent technical assistance and the Bavarian Ministry of Agriculture and Forestry for financial support.

References
ABBOTT D.C., BROWN A.H.D., BURDON J.J. (1992): Genes for scald resistance from wild barley (*Hordeum vulgare ssp. spontaneum*) and their linkage to isozyme markers. Euphytica, **61:** 225-231.
ABBOTT D.C., LAGUDAH E.S., BROWN A.H.D. (1995): Identification of RFLPs flanking a scald resistance gene on barley chromosme 6. J. Hered., **86:** 152-154.
BARUA U.M., CHALMERS K.J., HACKETT C.A., THOMAS W.T.B., POWELL W., WAUGH R. (1993): Identification of RAPD markers linked to a *Rhynchosporium secalis* locus in barley using near-isogenic lines and bulked segregant analysis. Heredity, **71**: 177-184.
BEER W.W. (1991): Leaf blotch of barley. Zentralb. Microbiol. **146:** 339-358.
BEHN A., HARTL L., SCHWEIZER G., WENZEL G., BAUMER M. (2004): QTL mapping for resistance against non-parasitic leaf spots in a spring barley doubled haploid population Theor. Appl. Genet. online DOI: 10.1007/s00122-003-1559-7.
BJÖRNSTAD A., PATIL V., TEKAUZ A., MAROY A.G., SKINNES H., JENSEN A., MAGNUS H., MACKEY J. (2002): Resistance to scald (*Rhynchosporium secalis*) in barley (*Hordeum vulgare*) studied by near-isogenic lines: I. Markers and differential isolates. Phytopathology, **92:** 710-720.
BUCHANNON K.W., WALLACE H.A.H. (1962): Note on the effect of leaf disease on yield, bushel weight and thousand-kernel weight of Parkland barley. Can. J. Plant Sci., **42:** 534-536.
DANIEL G., BAUMANN A. (1990): Einfluß verschiedener Faktoren auf die Planzenregeneration in der Antherenkutur bei Sommer- und Wintergerste. Bayer. Landw. Jahrbuch (ISSN 0375-8621), **64:** 609-617.
GENGER R.K., BROWN A:H:D., BURDON J.J. (2000): Molecular markers for wild barley derived scald resistance genes. Proceedings of the 8th International Barley Genetic Symposium 22.-27.10.2000 in Adelaide/Australia, Volume II, 117-119.
GENGER R.K., BROWN A.H.D., KNOGGE W., NESBITT K., BURDON J.J. (2003): Development of SCAR markers linked to a scald resistance gene derived from wild barley. Euphytica, **134:** 149-159.
GARVIN D. F., BROWN A.H.D., BURDON J.J. (1997): Inheritance and chromosome location of scald-resistance genes derived from Iranian and Turkish wild barleys. Theor. Appl. Genet., **94:** 1086-1091.
GARVIN D.F., BROWN A.H.D., RAMAN H., READ B.J. (2000): Genetic mapping of the barley *Rrs14* scald resistance gene with RFLP, isocyme and seed storage protein makers. Plant Breed., **119:** 193-196.

GOODWIN S.B., ALLARD R.W., WEBSTER R.K. (1990): A nomenclature for *Rhynchosporium secalis* pathotypes. Phytopathology, **80**: 1330-1336.

GRANER A., TEKAUZ A. (1996): RFLP mapping in barley of a dominant gene conferring resistance to scald (*Rhynchosporium secalis*). Theor. Appl. Genet., **93**:421-423.

HABGOOD R.M., HAYES J.D. (1971): The inheritance of resistance to *Rhynchosporium secalis* in barley. Heredity, **27:** 25-37.

HARTL L., MOHLER V., ZELLER F.J., HSAM S., SCHWEIZER G. (1999): Identification of AFLP markers closely linked to the powdery mildew resistance gene *Pm1c* and *Pm4a* in common wheat (*Triticum aestivum* L.). Genome, **42**: 322-329.

HARTL L., SCHWEIZER G., HERZ M., BAUMER M. (2000): Molekulargenetische Lokalisierung von QTL für die Malzqualität der Gerste. Bericht über die Arbeitstagung 2000 der Vereinigung österreichischer Pflanzenzüchter, 21. bis 23. November 2000, S. 117-S. 122.

JACKSON L.F., WEBSTER R.K. (1976): Race differentiation, distribution and frequency of *Rhynchosporium secalis* in California. Phytopath., **66:** 719-725.

JÖRGENSEN J.H.(1993): Coordinator's report: Disease and pest resistance genes. Barley Genet Newsletter **22:** 110-134 (http://wheat.pw.usda.gov/ggpages/bgn/22/cover.html.

KHAN T.N., CROSBIE G.B. (1988): Australian J. of Experimental. Agriculture.

Kilian A, Steffenson BJ, Saghai-Maroof MA, Kleinhofs A (1994) RFLP markers linked to the durable stem rust resistance gene *Rpg1* in barley. Mol Plant Microbe Interact , **7**: 298-301

KNOGGE W., MARIE C. (1997): Molecular characterization of fungal avirulence. In: CRUTE E.B., HOLUB E.B., BURDON J.J. (ed): The gene for gene relationship in plant parasite interactions. CAB international: 329-346.

LANDER E., GREEN P., ABRAHAMSON J., BARLOW A., DALY M., LINCOLN S., NEWBURG L. (1987): MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics, **1:** 174–181.

LEHNACKER H., KNOGGE W. (1990): Cytological studies on the infection of barley cultivars with known resistance genotypes by *Rhynchosporium secalis*. Can. J. Bot., **68:** 1953-1961.

LIU Z.W., BIYASHEV R.M., SHAGHAI-MAROOF M.A. (1996): Development of simple sequence repeat DNA markers and their integration into barley linkeage map. Theor. Appl. Genet., **93:** 869-876

PATIL V. (2001): Genetics of *Rhynchosporium secalis* (Oud.) J.J. Davis resistance in barley. In: PATIL A. (ed): Doctor scientiarum Theses 2001:21 Agricultural University of Norway ISSN 0802-3220, ISBN 82-575-0471-8.

PATIL V., BJÖRNSTAD A., MACKEY J.(2003): Molecular mapping o a new gene *Rrs4*$_{CI11549}$ for resistance to barley scald (*Rhynchosporium secalis)* Molecular breeding, **12:** 169-183.

PENNER G.A., TEKAUZ A., REIMER E., SCOLES G.J., ROSSNAGEL B.G., ECKSTEIN P.E., LEGGE W.G., BURNETT P.A., FERGUSON T., HELM J.F. (1996): The genetic basis of scald resistance in western Canadian barley cultivars. Euphytica, **92:** 367-374.

RAMSAY L., MACAULAY M., IVANISSEVICH S., MACLEAN K., CARDLE L., FULLER J., EDWARDS KJ., TUVESSON S., MORGANTE M., MASSARI A., MAESTRI E., MARMIROLI N., SJAKSTE T., GANAL M., POWELL W., WAUGH R. (2000): A simple sequence repeat-based linkage map of barley. Genetics, **156:** 1997–2005.

ROHE M., GIERLICH A., HERMANN H., HAHN M., SCHMIDT B., ROSAHL S., KNOGGE W. (1995): The race-specific elicitor NIP1 from the barley pathogen *Rhynchosporium secalis* determines avirulence on host plants on the *Rrs1* resistance genotype. EMBO J., **14:** 4168-4177.

SCHWEIZER G.F., BAUMER M., DANIEL G., RUGEL H., ROEDER M.S. (1995): RFLP markers linked to scald (*Rhynchosporium secalis*) resistance gene *Rh2* in barley. Theor. Appl. Genet., **90:** 920-924.

SCHWEIZER G., HARTL L., SCHÖNFELD M., ROEDER M., BAUMER M. (2000): Mapping of *Rhynchosporium secalis* resistance gens in barley. In: LOGUE S. (ed.) Barley Genetics VIII, Proceedings of the 8th International Barley Genetic Symposium 22.-27.10.2000 in Adelaide /Australia, Volume II, 172-174.

SCHWEIZER G., RÖDER M., HARTL L., BAUMER M. (2002): Entwicklung und Anwendung molekularer Selektionsmarker für *Rhynchosporium secalis* Resistenz bei Gerste. Vorträge Pflanzenzüchtung 54: 259-262. In: Vom Genom zur Sorte, Saatgut-Treuhandverwaltungs-GmbH, ISSN 0723-7812.

SOGAARD B., WETTSTEIN-KNOWLES P. (1987): Barley: Genes and chromosomes. Carlsberg Research Communications, **52:** 123-196.

THIEL T., MICHALEK W., VARSHNEY R.K., GRANER A. (2003): Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). Theor. Appl. Genet., **106:** 411–422.

VOS P., HOGERS R., BLEEKR M., REIJANS M., VAN DE LEE T., HORNES M., FRITJERS A., POT J., PELEMAN J., KUIPER M., ZABEAU, M. (1995): AFLP: A new technique for DNA fingerprinting. Nucl. Acid. Res., **23:** 4407-4414.

XI K., BURNETT A.G., HELM J.K., TURKINGTON T.K. (2000): Qantitative resistance of barley cultivars to *Rhynchosporium secalis*. Can. J. Plant Pathol., **22:** 221-227.

XI K., TURKINGTON T.K., HELM J.K., BRIGGS J.P., TEWARI J.P., FERGUSON T., KHARBANDA P.D. (2003): Distribution of pathotypes of *Rhynchosporium secalis* and cultivar reaction on barley in Alberta. Plant Disease, **87:** 391-396.

Suitability of a Selected Barley Differential Set for *Pyrenophora teres* f. *teres* Virulence Screening

M.J. Jalli

Boreal Plant Breeding Ltd, Myllytie 10, FIN-31600 Jokioinen, Finland
marja.jalli@boreal.fi

Abstract
A barley (*Hordeum vulgare* L.) differential set for *Pyrenophora teres* Drechsler f. *teres* (anamorph *Drechslera teres* [Sacc.] Shoemaker) virulence screening has been assessed in co-operation with barley breeders and researchers. Seedlings of the set (19 genotypes) were grown in the greenhouse to assess the virulence among 81 single-spore isolates collected from nine countries. A large variation for net blotch resistance was found among the 19 barley genotypes. None of the genotypes was either resistant or susceptible to all isolates tested. Genotypes CI 5791, CI 9214, CI 9819 and Beecher showed the highest resistance. The differential set used in this study was effective in screening for differences in the virulence of *P. teres* isolates and could therefore be useful for further development and use in disease resistance breeding programmes.
Keywords: barley; net blotch; *Pyrenophora teres*; resistance; virulence; *Hordeum vulgare*

Introduction

Pyrenophora teres Drechsler (anamorph *Drechslera teres* [Sacc.] Shoemaker), the cause of net blotch of barley (*Hordeum vulgare* L. emend. Bowden), is an economically important pathogen of barley throughout most barley growing regions. Breeding barley for improved resistance to net blotch is a priority in many plant breeding programmes around the world.

For breeding strategies to be successful, it is essential to have reliable information both on the pathogen population and on possible resistance sources. The virulence structure of *P. teres* populations have been studied at several sites around the world (STEFFENSON & WEBSTER 1992; SATO & TAKEDA 1993; ROBINSON & JALLI 1996; GUPTA 2001). However, the results are not entirely comparable because of the absence of a commonly used differential set.

In response to this fact, barley breeders and researchers have constructed a new barley differential set which includes several *P. teres* resistance genes available separately or in combination in different genetic backgrounds. The purpose of this study was to assess the suitability of this differential set in practical disease resistance breeding programmes.

Material and Methods

P. teres isolates were received from barley breeders and researchers from Australia, Canada, Czech Republic, Denmark, Finland, Lithuania, Norway, Slovakia and Sweden. Single spore cultures were grown on 2.3% lima-bean agar (LBA, 23 g Bacto-Lima Bean Agar, 1 L water) in petri dishes. The petri dishes were kept under near UV light and a 12 h photoperiod at 18ºC for 14 days. After two weeks, conidia were collected by flooding the petri dishes with distilled water. Density of conidia was adjusted to 2 x 10^4 conidia per ml.

The bases for the selection of differential genotypes were the sets of barley differentials determined by Steffenson & Webster (1992) and Afanasenko *et al*. (1995). The following differential genotypes selected by barley breeders and researchers were used in virulence test: 1) CI 2750, 2) Harbin, 3) K 8755, 4) K20019, 5) Manchurian, 6) Tifang, 7) CI 9819, 8) CI 9825, 9) CI 5791, 10) CI 2330, 11) Beecher, 12) CI 9214, 13) Skiff, 14) CI 11458, 15) Prior, 16) Corvette, 17) Pirkka (susceptible check), 18) Harrington (susceptible check), 19) Haruna Nijo (susceptible check).

Three viable barley seeds were sown per pot containing nutrient-supplement peat, and were placed in a greenhouse at 18ºC, 12 h photoperiod. The pots were arranged in a split plot design with two replicate blocks. Isolates were assigned to main plots and barley genotypes to sub plots.

Two weeks after planting, at GS12/GS13, humidity in the greenhouse was raised to 100%, and mail plots were inoculated with conidial suspension at the rate of 0.3 ml per pot. High humidity was maintained for 24 hours. Infection response on the second leaf was recorded using the 10 point scale of Tekauz (1985) 10 days after the infection. Isolates with scores ≤ 4 were categorized as avirulent and isolates with scores > 4 as virulent isolates.

Results

Net blotch symptoms developed on all barley genotypes examined. The results of ANOVA indicate significant differences between isolates, genotypes and their interactions. There was a high correlation between the mean infection response of the genotype and the percentage of virulent isolates on the genotype (Figure 1). *P. teres* isolates with different country origin showed similarities but also differences in their virulence spectrum (Table 1).

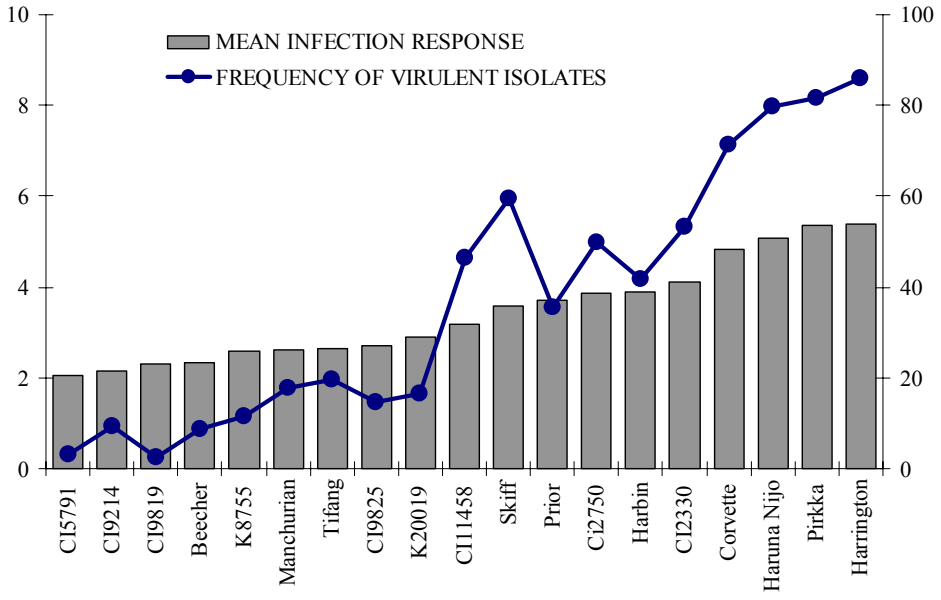


Figure 1. Mean net blotch infection response (scale 0-10) and the percentage of virulent *P. teres* f. *teres* isolates (scale 0-100) on the barley genotypes

Table 1. The percentage of virulent *P. teres* isolates with different origin on the barley differential set

	THE COUNTRY OF ORIGIN OF THE *P. TERES* ISOLATE		
GENOTYPE	Finland	Lithuania	Sweden
CI5791	2	0	4
CI9819	2	0	0
Beecher	8	6	7
CI9825	16	20	14
K8755	16	10	4
CI9214	17	5	0
K20019	19	15	14
Manchurian	21	15	14
Tifang	31	10	4
Prior	39	30	29
CI11458	46	80	64
Harbin	59	20	43
Skiff	61	100	79
CI2750	64	45	54
CI2330	70	55	50
Corvette	83	90	65
Pirkka	83	100	86
Haruna Nijo	87	100	75
Harrington	89	100	93

The base in disease resistance breeding, is the reliable knowledge on the history and on the present of a pathogen populations virulence spectrum. For understanding the philosophy *of P. teres* fungus, it is essential that the research done at different parts of the world is comparable. The studied barley differential set showed its usefulness in virulence studies of *Pyrenophora teres* f. *teres* isolates, and is recommended to be used as a common tool in further studies.

Acknowledgements
The author would like to thank colleagues for providing *P. teres* isolates and barley genotypes from different parts of the world. Colleagues working with net blotch are also thanked for the positive, open and encouraging atmosphere inside this interesting and fascinating net blotch world.

References

AFANASENKO O.S., HARTLEB H., GUSEVA N.N., MINARIKOVA V., JANOSHEVA M. (1995): A set of differentials to characterize populations of *Pyrenophora teres* Drechs. for international use. J. Phytopathology, **143:** 501-507.

GUPTA S. (2001): Current virulence of *Pyrenophroa teres* in Western Australia. Plant disease, **85:** 960-966.

ROBINSON J., JALLI M. (1996): Diversity among Finnish net blotch isolates and resistance in barley. Euphytica, **92:** 81-87.

SATO K., TAKEDA K. (1993): Pathogenic variation of *Pyrenophora teres* isolates collected from Japanese and Canadan spring barley. Bull Res Inst Bioresour Okoyama Univ, **1:** 147-158.

STEFFENSON B.J., WEBSTER R.K. (1992): Pathotype diversity of *Pyrenophora teres* f *teres* on barley. Phytopathology, **82:** 170-177.

TEKAUZ A. (1985): A numerical scale to classify reactions of barley to *Pyrenophora teres*. Can J Plant Pathol, **7:** 181-183.

Ramularia collo-cygni, Worldwide Evaluation

E. Sachs

Federal Biological Research Centre for Agriculture and Forestry, Institute of Plant Protection in Field Crops and Grassland. Bergholz-Rehbrücke, Germany

Abstract
Details of the history, disease symptoms and morphology of *Ramularia* leaf spot disease are presented to give an overview of this relatively unknown pathogen. Particular attention is paid to confusion of the disease with other diseases and for this reason to its diagnosis. In addition, the role of the disease is discussed, and distribution, epidemiology and first results on the formation of resistance and disease control are presented.
Keywords: barley; *Ramularia collo-cygni*; physiological leaf spots (PLS); symptoms; epidemiology; diagnosis; resistance; control

Introduction

The contribution reports about *Ramularia* leaf spot disease, which was described in literature already 100 years ago. The disease, however, is still relatively unknown. This might be explained by first the small size of fungal structures and numerous possibilities to confuse the disease with leaf spots of another origin.

History

- 1893 – The disease is first described in Northern Italy by CAVARA (1893), who used the name *Ophiocladium hordei*.
- 1985 – first findings in Austria (HUSS *et al.*1987), later on in other European states (HUSS and NEUHOLD 1995; SACHS 2002).
- 1988 – the disease was found on triticale in Mexico. It was re-named in *Ramularia collo-cygni* (SUTTON & WALLER 1988).
- 1992 – the disease was reported in New Zealand (SHERIDAN 1992).
- 2000 – disease was found in Argentina and Uruguay (SACHS 2002)

The Federal Biological Research Centre for Agriculture and Forestry in Germany (Biologische Bundesanstalt für Land- und Forstwirtschaft - BBA) has studied the spread of the disease since the end of the 1990ies. It monitored the appearance of the disease in Germany in 2000, started work about epidemiology and developed a method to isolate and culture the fungus. This was the prerequisite to fulfil *Koch's* postulates and develop methods for resistance testing. In addition studies served to correctly diagnose the disease and avoid confusion with other leaf spot diseases. An international conference on *Ramularia* in Aleppo, Syria in 2002 was the first big meeting of scientists making research into that disease throughout the world. As a result a homepage SACHS and HUSS established under the address http:www.bba.de.inst/a/*Ramularia*. Meanwhile, a number of countries are working on the *Ramularia* issue, including Scotland, Switzerland, Denmark, Norway, Argentina, and also the Czech Republic, in particular the Kromeriz institute. So far about the history of research into the disease.

Symptoms of the Disease and Morphology of the Fungus

Ramularia attack starts with pale punctual spots which turn into dark brown or brown-to-black necroses. Incubation takes about 6 days at 20 °C. *Ramularia* leaf spots have a

characteristic appearance: they are mostly 1-2 mm long and 0.5 mm wide, and clearly bounded by leaf veins. Chlorotic halos, which are attributed to the toxin formed by *Ramularia*, are formed along the leaf veins. The halos are darkest in the centre.
If infection is very strong, spots may remain tiny, with a size of between 0.2 and 0.4 mm.
These small spots may melt to form larger fields of dark colour, and leaves may appear nearly black in extreme cases. Infected flag leaves are dotted relatively evenly, while lower leaves, which grow upwards and are bent down at the tip, are dotted mostly only around the place where they are bent. This phenomenon may be explained by the distribution of dew on leaves. Dew also settles by preference on the more exposed parts of leaves. After first spots appear, leaves start to yellow from the edge inwards and die off. With strong infection, spots are not only on leaves but also on leaf sheaths, culms, awns and glumes. Late in the autumn and early in the spring, *Ramularia* spots on leaves of winter barley are round to oval. They are not as clearly bounded by leaf veins as spots formed in the summer, and sometimes they are a bit larger. The spots are all of about the same size at all varieties and clearly recognisable also after harvest.
100-fold enlargement brings out the unmistakable shape of conidiophores, which is like a swan's neck and unique in the genus (HUSS *et al.* 1992). It has given the fungus its name (*collo-cygni* means 'neck of a swan'). Conidia are on average 8 µm long and 4 µm wide. The surface is bristle, which is no longer recognisable under a simple light microscope. Conidiophores are arranged in clusters protruding from the stomata. Probably, the fungus prefers leaf undersides for sporulation because of the micro-climatic conditions there. With stereo-microscopic inspection, conidiophore clusters show small white dots in or around the leaf spots. Conidiophores are normally only formed after the tissue has died off, while leaf spot formation is completed before. With oblique light, reflection brings out conidiophores and conidia more clearly under the microscope. Heavy *Ramularia* attack with massive formation of hyaline conidiophores and conidia makes the leaf epidermis shimmer somewhat whitish. BRAUN (1998) has studied morphology and taxonomy of the fungus.

Confusion with Other Symptoms
Symptoms may be mistaken for a number of other leaf spot diseases if not looked at closely enough. In the first line, they may be confused with physiological leaf spots (PLS). The following survey shows the differences between the two kinds of leaf spots.

Feature	Ramularia leaf spots	Non-parasitic leaf spots
Cause of spots:	fungus	physiological
Infested organs:	leaves, stems, sheaths, awns	leaves mainly
Appearance:	whole year on winter barley	from beginning of April (?)
Wet chamber:	formation of conidia	no parasitic structures
Spot environment:	at first chlorotic rings around spots	no chlorotic rings
Spots bordered by:	leaf veins	no clear borders
Spots surrounded on agar	reddish	not reddish

Figure 1.

The size of *Ramularia* leaf spots does not depend on the barley variety, while the size of PLS does. For instance, variety 'Carola' may be recognised by physiological leaf spots which are up to 1 cm long, while the winter barley variety 'Marinka' always shows small PLS. *Ramularia* spots, in contrast, are the same size on both varieties. The possibility of confusion is given in particular when PLS are of the size shown in the photo. As both PLS and *Ramularia* spots often occur on leaves at the same time, *Ramularia* may be clearly diagnosed only under the microscope or by the method described below.
Ramularia spots may also be mistaken for emerging net blotch as caused by *Drechslera teres*, because both kinds of spots are bounded by leaf veins (*Ramularia* spots a bit more than net blotch), and net blotch spots are also very small in the beginning. Brown spots caused by *Bipolaris sorokiniana* also look quite similar to each other. Usually, they are darker, larger, and not as clearly bounded by leaf veins. MLO spots of some spring barley varieties may also be confused with *Ramularia*, in particular when MLO spots are not as typically developed as those found in plants of the variety 'Krona', such as those on 'Chariot'.
Mildew-hypersensitivity necroses formed by some mildew-resistant varieties infected with *Blumeria graminis* can also be confused with *Ramularia* spots, but they always show some residual mycelium on the necrotic tissue. The mycelium can only be seen using a looking glass.
The following survey illustrates possible confusion of *Ramularia* leaf spots with leaf spots of other origins and the differences.

Disease	**Differences to *Ramularia***
Net blotch (spot-type)	**Spots mostly larger (with exceptions), not limited by leaf veins, on agar sometimes red-brown colour**
Brown spot disease (spot blotch)	**Spots mostly larger, not clearly limited by leaf veins, on agar without red colour**
Mildew resistance-spots	**Spots differently large, not limited by leaf veins, on agar without red colour, residual mycelium on spots**
Mlo spots	**Occur only on spring barley, no chloroses, not limited by leaf veins, on agar without red colour**

Figure 2.

Diagnosis
A simple diagnostic method will allow to avoid such mistakes. The principle is as follows: Infected leaves are placed upside down on acid agar. If leaves are infected with *Ramularia*, the fungus forms the phytotoxin rubellin, which is dyed red. To confirm the outcome of the test, infected spots are scrutinised under the microscope for the typical conidiophores.

Diagnostic method for leaf spots on barley:

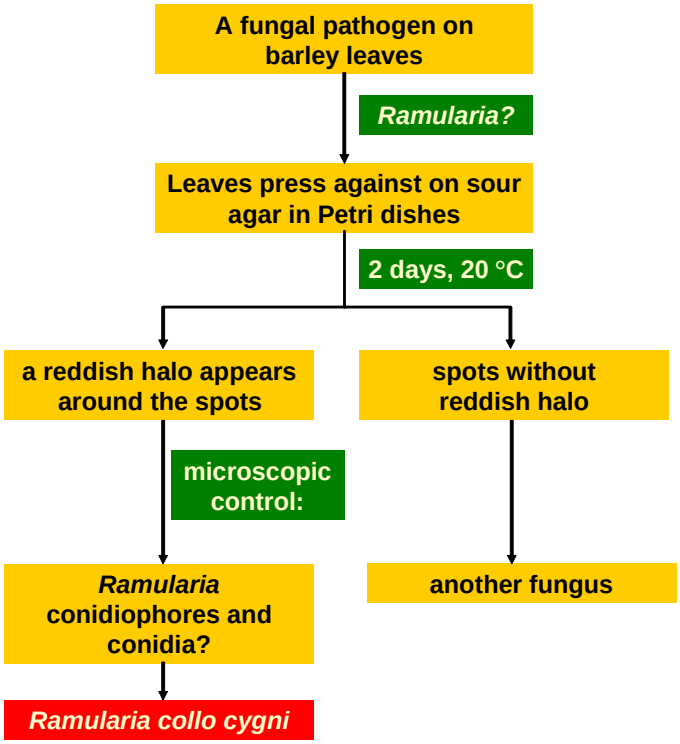


Figure 3.

Importance

In some regions where the disease is spread, it is actually the dominating post-maturity disease of barley. It is important because of the rapid advancement of attack and massive action of toxins. Leaves may completely turn brown and die off within 12 days. Trials in Upper Austria have shown that nightly dew plays a particular role in the development of infection. At times of dew fall, average spore flight was enough to cause heavy *Ramularia* infection. Grain quality and thousand grain weight deteriorated in consequence of infection and toxin action, which meant loss of yield. Further, accelerated maturity will probably affect deposition of kernel substances in the grain. The disease was found to cause yield losses between 16 and 26% (HUSS 2000; GREIF 2002). Whether the disease also affects brewing fitness has not yet been checked.

Spread and Preconditions for Attack

The European map marks those countries where the disease has been found, and the year when it was first detected.

- This map shows countries with disease foci in Western, Central and Northern Europe. Apart from Europe, *Ramularia* leaf spot disease has been found in
- New Zealand (in the South)
- South America (Argentina, Uruguay)
- Central America (Mexico).

It has to be assumed that the disease has also spread in neighbouring countries, because their small size and massive number allow *Ramularia* spores to be extremely easily spread by wind. But the way the disease actually spreads has not yet been studied more closely. The fact that *Ramularia* DNA (96% correspondence) was found on oak leaves in the Alps (HEUSER & ZIMMER 2001) shows they have been spread by wind.

Occurrence and importance of the disease strongly depend on the weather. In general, the pathogen prefers moderate, moist climate. The year 2003 was too dry for the fungus. The disease occurred only in regions where rainfall was above average or where there was enough dew. These were Bavaria, Baden-Wuerttemberg and some regions in Lower Saxony. In general, the climate in East Germany is too dry for the pathogen, but the disease may still appear in years with more rainfall. In Berlin's surroundings, the author found *Ramularia* in two out of five years. Regions with much dew formation are particularly at risk.

Epidemiology

To know why infection is coming on massively after emergence of ears, we investigated at which time symptoms become visible on winter barley. For that purpose, whole plants of barley were examined for *Ramularia* after the beginning of vegetation. The oldest leaves showed *Ramularia* spots as early as in the second decade of April (growth stage BBCH 31-33). By that time, the oldest leaves had already died off. So, leaves and spots obviously stemmed from the preceding autumn. We now observed how and when mass sporulation comes about, and how the fungus attacks winter barley seedlings in autumn. The following path of infection was found: Infection moves upwards across the leaf layers in the course of spring. Leaf levels F-1 and F are infected by the middle to the end of May (growth stages 71-77). Around that time, weather conditions are usually favourable to *R. collo-cygni* so that conidia are formed massively.

The fungus proceeds from winter barley to colonise summer barley plants. First symptoms show at growth stage BBCH 31. In principle, infection advances in the same way as with winter barley – the pathogen moves upwards through leaf layers so that awns, leaf sheaths and stems are infected by July.

Ramularia also attacks all other cereals and some grasses shortly before maturity. Volunteer cereals and maize are also attacked, sometimes heavily. Both species show symptoms from September on, and both, as well as the grasses, are still present in the field as a source of infection when freshly drilled winter barley is emerging. In Ireland, 80-90% of volunteer barley was found infected at the beginning of September 2001 (HACKETT, pers. comm., 2001). Attack on winter barley seedlings must also be expected in Central Europe on warm autumn days with nightly dewfall. Winter barley plants from Baden-Württemberg showed *Ramularia* spots on their oldest leaves in the middle of November. Checks in severe cold in January showed massive sporulation from these leaves after thawing. The fungus obviously survives frost periods well without suffering any damage. So, the development cycle of *Ramularia* leaf spot disease has been cleared up for the regions studied in Europe. From the development cycle the pathogen is assumed to spread by wind. Seed-borne and soil-borne paths of spread have not yet been thoroughly studied. However, both infected grains and straw have been found. But it is not clear how long the fungus can survive on these plant parts and whether they play a role in the infection cycle.

Resistance

The fungus seems to attack all barley varieties, but to different degrees. This is a starting point for breeding. For example: all barley varieties in the fungicide-free treatment of a variety trial in the Wehnen trial site of the Federal Office of Plant Varieties showed *Ramularia* leaf spots in 2001. The disease index was between 5 and 7 on a 1-to-9 assessment scale, with 1 meaning 'no symptoms'. Attack was noticeably less severe in the fungicide-treated plot, being mostly 3 to 4 degrees below that of the fungicide-free treatment.

Barley breeders in Germany and in Scotland have already found some strains which are less susceptible than others, but there are no accurate data yet. Relevant studies are made by BALZ at Göttingen University, Germany. . In general, early-maturing varieties are also attacked

earlier, what gives the impression that late-maturing varieties are more resistant. Winter barley variety 'Verticale' was authorised in 2003 among others for its lower susceptibility to *Ramularia* (Newsletter 8, May 2003, page 1 and 2).
In Upper Austria and in New Zealand, plant pathologists found a certain antagonism between appearance of *Ramularia* leaf spots and leaf rust (*Puccinia graminis*). Extreme attack of leaf rust in Upper Austria in 1989 with hardly any *R. collo-cygni* was followed by dominating *Ramularia* attacks and no leaf rust in the following year. It is also characteristic that *Ramularia* leaf spots have only been found on a leaf rust-resistant variety of winter barley ('Carola') in the Austrian Marchfeld region, which is usually dominated by leaf rust. Samples from New Zealand, which were heavily infected with *R. collo-cygni*, always showed only little infection with leaf rust, and vice versa. SALAMATI found a significant negative correlation of *Rhynchosporium* and *Ramularia* leaf spots in infected barley fields in Norway (SALAMATI 2002; mnorsk@plantevern.no).
Two-rowed varieties have been found to be more susceptible than six-rowed varieties, in general. The simplest method to test breeding material and varieties for susceptibility to *Ramularia* is to grow them in the field in areas where environmental conditions practically guarantee infection with *Ramularia collo-cygni*. A trial of that kind including a wide range of varieties is running in Upper Austria. It would be more ideal to test varieties under controlled conditions and with artificial infection, such as in air-conditioned greenhouse or in climatic chamber. This requires a suspension of conidia with defined density and to induce definite disease symptoms under the mentioned conditions.
The conditions for cultivating the fungus and for sporulation have been cleared up. Göttingen University has also completed first experiments on inoculating plants in the greenhouse (BALZ, pers. commun., 2003). The BBA has carried out inoculation trials with leaf segments. This method is a bit similar to that with leaf segments inoculated with mildew. In principle it is possible to artificially cause *Ramularia* infection, but the method should be improved.
Further, the phytotoxin produced by the fungus was employed in a resistance test. The phytotoxin was extracted and its structure cleared up. It is rubellin D, a substance dying intensively red and known as a phytotoxin from *Mycophaerella*. It is the red dye which is used in diagnosing the fungus. If rubellin D is placed on an injured spot of a barley leaf, the same brown necroses are formed as if *Ramularia* sporocysts penetrated the leaf. Unfortunately, the size of necroses did not correlate with varietal_resistance (HEISER *et al.* 2003). The following three possibilities remain, so:

- Field tests with varieties in areas which are sure to be attacked (natural infection).
- Greenhouse trial with inoculation by spraying. Method has still to be optimised.
- Leaf segments inoculated by spraying. Method has still to be optimised.

Disease Control
In Upper Austria, HUSS (2000) has carried out fungicide trials on disease control since 1986. In the years 1994, 1997, and 1999, which were clearly dominated by *Ramularia* attacks, winter barley crops were treated with Folicur (azole) at growth stage BBCH-39. Leaves remained green for a much longer time, but the disease was not completely eliminated. Yield levels ranged between 0.6 and 0.7 t, and yield losses were 18, 17 and 16% without treatment, so that application of the fungicide was judged to be profitable. Thousand grain weight and hectolitre weight increased with fungicide use. In New Zealand, disease control measures at growth stage 39 had efficiency degrees of between 26% (carbendazim) and 86% (two strobilurins) (CROMEY *et al.* 2002). In the region of Middle Franconia in Germany (GREIF 2002), yield losses of about 25% were attributed to the disease. Combined preparations including strobilurins and, in Scotland, epiconazole turned out most effective (OXLEY, pers. comm. 2001). The fungicide OPERA (strobilurin and epiconazole) and a newly developed

strobilurin provided effective *Ramularia* control in joint trials with BASF and Bayer CropScience. This shows that it is obviously not too difficult to control the disease by modern fungicides, though none has yet explicitly been authorised for the disease. Efficacy testing of treatment for different products would also require firm diagnosis of *Ramularia* leaf spots, which is a laboratory process.

References and further figures can be found on web site http://www.bba.de/inst/a/*Ramularia*.

Winter Barley Cultivar Mixtures: Why Isn't Everybody Growing Them?

A.C. Newton and J.S. Swanston

Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, Scotland, UK,
fax: +44 1382 568578; e-mail: a.newton@scri.sari.ac.uk

Abstract
There are many advantages to be gained from growing cultivars in mixtures from reduced disease and improved yield and quality, through to yield and quality stability. Disadvantages lie in the physical requirement to mix components and the perceived quality constraints. For spring barley grown for malting, quality concerns can be not only overcome, but turned to benefits. For winter barley, where yield benefits are already larger, there may be opportunity for spring barley quality performance. Furthermore, specific weights and lodging resistance are significant factors that can be substantially improved in mixtures, while differences in maturity and height are less problematic than expected due to convergence. The benefits to winter barley are probably greater due to a longer growth period exposed to biotic and abiotic stress when the competition and compensation interaction between components is maximised. We demonstrate that greater reduction of most diseases and increase in yield is correlated with mixture complexity, and that the contribution of components to quality and yield traits can be quantified by regression analysis. Yield sensitivity also affects performance in different mixtures through effects on competition behaviour.

Introduction

Against the cost requirement to mix grain for sowing, mixtures offer the potential for greater yield, less disease, improved quality, better fertiliser utilisation and reduced pesticides usage (NEWTON *et al*. 2004b), i.e. advantages for the environment in addition to those for grower and end-user which result in increased margins. However the magnitude of these responses is variable (MUNDT 2002; NEWTON 1997) and, in practice, the reason why cultivar mixtures are grown is often yield stability (FINCKH *et al*. 2002), since mixtures can frequently out-yield the mean of their component cultivars, but rarely exceed that of the best component. The presumed mechanism for this is that one or more of the components will be favoured in any environment, so these will out-compete the others, leading to a change in relative mixture composition at harvest. This cannot, however, explain responses of large magnitude observed for certain mixtures both for yield and for some quality traits (NEWTON *et al*. 1998; SWANSTON *et al*. 2000). It thus seems possible that some varieties may behave differently within a mixture than when grown in monoculture and may also influence mixture performance more strongly that other components. Data from our laboratory also suggests that mixtures may be less variable between sites, for quality components, relative to some cultivars and better able to deliver the 'acceptable range of heterogeneity' required by end-users. The variation within fields (AGU & PALMER 2003) in addition to that between sites precludes anything approaching uniformity.

For spring barley both the yield increase and disease reduction advantages of mixtures can be small, but for winter barley the heterogeneous interaction benefits are compounded over the longer growing season. This is probably substantially attributable to an increased variability resource available for response to biotic (e.g. pathogen challenge) and particularly, abiotic stress (e.g. frost damage) so competition and compensation interaction between components is maximised. The mixtures plant 'community' is able to better utilise the resources available to it

through niche exploitation, e.g. better light capture, and better nutrient and water uptake through complementary component root growth patterns and differing uptake efficiency mechanisms. Furthermore, in winter barley in the UK specific weights and lodging resistance are important factors that can be substantially improved in mixtures, while differences in maturity and height may be less problematic than expected due to convergence (SWANSTON & NEWTON 2004 (this volume)).

The greater reduction of most diseases and increase in yield is correlated with mixture complexity, i.e. the number of component genotypes, but components contribute differentially to both these characters (NEWTON *et al*. 1997), and therefore probably to quality components also. In this paper we make use of winter barley mixtures of varying complexity. We also address two areas. In the quality trial, the extent to which particular varieties consistently occur in successful or unsuccessful mixtures is assessed, while in the sensitivity trial we seek to determine whether variation in the yield sensitivity of individual components influences the sensitivity of mixtures across a range of input levels.

Material and Methods

Sensitivity Trial

The winter barley cultivars used represented a range of yield sensitivities (Table 1). A sensitivity greater than 1 indicates that the cultivar is more responsive than average to higher site yield potential, yielding relatively better under high-yielding conditions. Low sensitivity (<1) therefore indicates suitability to yielding relatively well under low-yielding conditions (NIAB 2001). Three of the cultivars, which were all high sensitivity types, were six-row types selected in order to distinguish them in the field. The twelve cultivars were grown in monoculture, while mixtures were derived from four groups of three cultivars (one being the 6-row types), using all 2- and 3-component within group combinations (total 16). In addition the three cultivars from groups A and C were grown in all two-component combinations with the three 6-row cultivars (total 18), while the six 6-, four 9-, and one 12- component combinations were derived from mixtures of the four groups of three cultivars (total 11) (Table 1). These were trialled under low and high fertiliser, low and high density and zero and full fungicide treatments to represent a wide range of competition environment scenarios.

Quality Trial

We selected seven winter malting cultivars comprising Melanie (W5907 x Br301a), Maris Otter (Proctor x Pioneer), and five cultivars with Maris Otter in their pedigrees: Halcyon (Maris Otter x Warboys), Pipkin (Maris Otter x Sergeant), Puffin ((Maris Otter x Athos) x Igri), Rifle (Puffin x NRPB87/5381), and Gleam (Puffin x Torrent). These were grown as monocultures and all possible equal proportion 2-, 3-, 4-, 5-, 6- and 7-component mixtures (total 127) in a trial at the Scottish Crop Research Institute, Dundee.

The trials comprised plots measuring 6.0 m (sensitivity trial) of 8.0 m (quality trial) x 1.55 m (excluding gaps) in a three replicate split plot design using with and without disease control as the main plot. Standard seed treatment was applied and seed were sown to a target of 360 seed / m^2 high density or 90 seed / m^2 low density. Fertiliser was 320kg/ha of 8:24:24 at sowing and 266kg/ha at first top dressing and 400kg/ha at the second of 30:0:0 N:P:K for the high treatment and half this for the low treatment. The fungicide treatment consisted BSPB recommended protocols at the time of the trials. Whole plots were harvested with a plot combine and yield recorded after drying to a constant 9% moisture level. Disease levels were assessed visually on a 1 to 9 scale (NEWTON & HACKETT 1994) on multiple occasions.

Following harvest, grain was sieved and the portion passing through a 2.5mm sieve was

retained Milling energy (ME) was determined as the mechanical energy required to mill a 5.0 g sample of grain using a Comparamill (ALLISON *et al*. 1979). A 30g sample, weighed to within 30mg, was malted, using the SCRI purpose-built malting apparatus (SWANSTON 1997) and the malted dry weight determined (Dwt). Malt moisture was measured by oven-drying for 3h as described in the Institute of Brewing (IOB) recommended method (1977) and malting loss was calculated from differences in dry weight before and after malting. The hot water extract (HWE) and fermentability, the latter determined 48h after addition of 0.5g distillers' yeast to 100ml of wort, were measured as described by SWANSTON and THOMAS (1996). Soluble nitrogen content was measured using a spectrophotometric method (HASELMORE & GILL 1995). Cell wall modification was determined by use of the Carlsberg malt modification analyser (AALSTRUP *et al*. 1981), with the degree of cell wall modification (CWM) and its homogeneity across the sample (HOM) calculated using the formulae of JENSEN and AASTRUP (1985). All data were analysed by ANOVA using 'Genstat' release 5.3.1.

Table 1. Cultivars and mixtures combinations used in mixtures convergence trial

Cultivar	**Sensitivity**	**Group**	**Head**	**Combinations**			
Halcyon	1.06	Mixed A	2-row	All 2- and	]		}
Pipkin	1.02	Mixed A	2-row	3-component		] All A group	}
Fanfare	0.84	Mixed A	2-row	mixtures	]	|	}
Regina	0.97	Low B	2-row	All 2- and		|	} Groups A,
Pearl	0.97	Low B	2-row	3-component		|	} b,
c, and D							
Pastoral	0.97	Low B	2-row	mixtures		|	} in all 6-, 9-
Opal	1.09	Med C	2-row	All 2- and	]	|	} and 12-
Leoni	1.03	Med C	2-row	3-component		] and C group	}
component							
Sumo	1.06	Med C	2-row	mixtures	]	cultivars with	} mixtures
Siberia	1.17	High D	6-row	All 2- and	]	|	}
Muscat	1.14	High D	6-row	3-component		] each D group	}
Manitou	1.12	High D	6-row	mixtures	]	cultivar	}
Total			**12**	**16**		**18**	**11**

Results and Discussion

Stability Trial

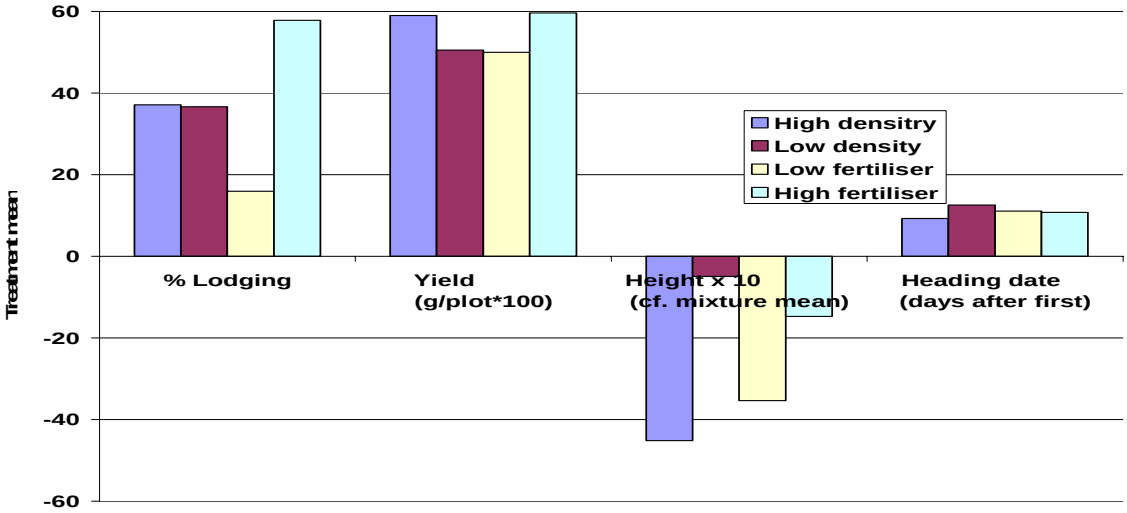
In the stability trial there was very little disease or interaction effects, so the fungicide treatments are combined. The effects of the treatments for all monocultures and mixtures are shown in figure 1: lodging showed no response to density but was substantially worse under high fertiliser; yield was higher under high density and high fertiliser; mixture height was most reduced compared with the mean of the components under high density and low fertiliser; heading date was later under low density.

Mean yield of individual mixtures ranged from -4.3% to 15.4% compared with the mean of the respective monoculture components (the expected), with the means being 4.4% and 5.4% greater in the high and low densities respectively, or 4.3% and 5.2% respectively for low and high fertiliser. Mean mixture heights measured on just the six-row + two-row combinations ranged from -5% to 15% with the mean being around - 2% for high fertiliser but no response at

low fertiliser. Overall the 6-row types were around 2.3% shorter in mixtures, and the 2-row types were 0.7% shorter. However, these means mask many interactions, for example under high density the mixtures were 7% taller than the mean of their components. There was evidence of convergence in some mixtures, but this was density- and fertiliser-dependent, i.e. environmentally-dependent competition effects. Mean heading date was just over two days later in mixtures compared with monocultures, slightly more in high than low density but not affected by fertiliser. Mean lodging was reduced 9% more in the mixtures than expected at high density and 13% at low density, particularly in the high fertiliser treatments.

These mixtures data were means of the diverse mixtures and treatments used, so represent overall trends. Analysis of the subsets of mixtures of different performance characteristics revealed many interactions still to be dissected. For example, the high sensitivity six-row + two-row combinations yielded notably less well under low density and low fertiliser conditions than most other combinations, many of which yielded better under these conditions. The contrast was greatest with the complex nine-component mixtures and different behaviour of the mixtures in the sensitivity groups was seen in the heading date and lodging data too.

Figure 1. Mean treatment effects on winter barley trial



Quality Trial

In the quality trial the disease levels were low but overall there was significantly less disease in the mixtures than the monocultures. There were only weak correlations between reduction in mildew (r^2 = 0.3219) or *R. secalis* (r^2 = 0.2416) and component number in the mixture. Yield response was correlated with component number, r^2 = 0.898 without fungicide and r^2 = 0.730 with fungicide treatment. The best yield response was about 12% without fungicide and 15% with fungicide in the 7- and 6-component mixtures respectively. Greater mixture complexity also had an effect on some malting quality parameters. In particular the correlation with HOM was both negative and highly significant (r^2 = 0.7568). Despite this, there was a highly significant positive effect on HWE (r^2 = 0.7879) and Predicted Spirit Yield (PSY) (HWE x fermentable extract). The monoculture mean for HWE was, however, lower than expected, given that all the cultivars were classed as malting quality and this reflected a relatively poorer performance from Puffin and its derivatives Gleam and Rifle. SN% showed some positive correlation (r^2 = 0.4315) whilst wort viscocity showed no correlation.

The principle components for ten quality or yield characteristics of the mixtures were visualised as biplots (not shown) for each of the mixture component-number groups. These

showed similar overall characteristics but a number of notable trends. Groupings became more apparent particularly in the more complex mixtures and four main groupings were identified. Group 1 had high PSY, and high cell wall modification and all four mixtures had Halcyon and Pipkin in them but Gleam was absent. Group 2 was characterised by high fermentability and all four mixtures had Gleam and also Puffin, but this time Pipkin was absent. Group 3 had high milling energy and all five mixtures had Melanie (no Maris Otter in its pedigree) but only two had Puffin and three had Pipkin or Gleam (cultivars which dominated group 2 and 4 mixtures). Group 4 had high cell wall modification but lower fermentable extract than group 1 and all seven mixtures had Pipkin and Gleam but only three had Melanie.

Which components of mixtures cause them to differ from their mean with respect to specific malting and yield parameters or combinations of parameters, in mixtures of different complexity, was deduced by calculating the percentage variance accounted for by each component in each component-number group of mixtures (Table 2) using a stepwise regression process. By light shading the positive and dark shading the negative contributions, the contrasting cultivar contributions are highlighted. Rifle, Halcyon and Puffin contribute in small but significant amounts in just a few mixtures for a few traits, whereas Melanie, Pipkin and Gleam have strong contributions to multiple traits, but often in opposite directions. The contributions are often significant in all component-number mixtures, but the magnitude of the contribution is frequently diminished in the more complex mixtures.

In many mixtures, particularly the less complex ones, Melanie contributes positively to yield, TGW, ME, HWE, FermExt, WortVisc and SN%. Gleam makes generally contrasting contributions apart from to yield where it too contributes positively. However, to TGW, HWE, FermExt, SN% and CWM it contributes negatively as well as small positive contributions to a few mixtures for Homog and Ferment. Pipkin contributes differentially again but has no effect on yield but a strong negative effect on TGW due to its small grain size. It also contributes the consistently strongest positive contributions to HWE, FermExt and SN%. Only Gleam and Melanie contribute substantially and consistently to yield, and this is positive across the 2-, 3- and 4-component mixtures. In the case of Melanie this may be through TGW which is positive in these as well as the 5-component mixtures. HWE, FermExt and SN% all follow similar patterns of contribution from the cultivars Pipkin, Gleam and Melanie. Pipkin contributes positively to these characters in all component-number mixtures, whereas Gleam shows consistently negative contributions. The contribution from Melanie is lesser and only in the lower component-number mixtures.

Clearly Melanie can make a substantial positive contribution to yield in a mixture although it has now become outclassed as a variety in its own right. This may be true of many other cultivars which are outclassed as monocultures as their strengths can still be exploited in mixtures, allowing other components to compensate for their weaknesses. In the case of Melanie it also makes a positive contribution to spirit yield, through high extract levels, even though it does not appear to affect fermentability. The positive contribution of Gleam to yield is offset by its malting performance under the conditions used, which favoured Melanie, as it may under-modify as shown by the low SN% results. While this slightly increases fermentability which peaks at an earlier stage in modification than extract (BATHGATE *et al*. 1978), it depresses extract and reduces spirit yield. Gleam and Melanie would not therefore be a suitable combination for a distilling quality mixture. In contrast, Pipkin contributes positively to spirit yield, through it high extract, and has no adverse effect on plot yield. It does, however, have small grain which appear to modify fairly quickly during malting giving high SN% levels. In some mixtures there appeared to be over-modification that slightly depressed fermentability. While previous results indicated that Pipkin performed well in combination with Maris Otter

and Halcyon (NEWTON *et al*. 1998), it may not complement a larger grained variety such as Melanie particularly well.

Designing Mixtures

This regression analysis allows such contributions to mixtures of different components to be objectively assessed and quantified. To apply this widely, such analyses need carrying out over multiple sites, seasons so assess environmental interactions, and using more cultivars, particularly some now outclassed cultivars and ones which failed to be recommended but nevertheless had particular strengths. However, to contain the extent of such trials, representative mixtures such as only all the 3-component combinations would be adequate. The effect of disease would also need to be assessed to determine the cost-effectiveness of fungicide treatment. In this case we might expect the opposite trend from quality components where the more complex mixtures reduced the component contributions as reduced disease tends to be correlated with increased mixture complexity (NEWTON *et al*. 1998).

The yield response to treatments in the sensitivity trial was as predicted, responding positively to increase in density and fertiliser. Lodging also became worse as fertiliser increased as expected, although there was no density effect. The competition effects of high density expressed as greater height and later heading date, although perhaps less intuitively the height was further enhanced by low fertiliser.

Table 2. Percentage variance accounted for by component cultivars for malting quality characteristics and yield in mixtures of winter barley with different component numbers

		B	C	D	E	F	G
		Halcyon	**Puffin**	**Pipkin**	**Rifle**	**Gleam**	**Melanie**
Yield	2-comp	-	-	-	-	15.65 +	9.21 +
	3-comp	4.29 -	-	-	-	9.76 +	7.12 +
	4-comp	-	-	-	-	4.41 +	2.48 +
	5-comp	-	-	-	-	-	-
TGW	2-comp	-	-	31.99 -	-	4.6 -	27.13 +
	3-comp	-	-	19.56 -	3.1 +	3.18 -	19.18 +
	4-comp	-	-	20.02 -	-	2.28 -	7.71 +
	5-comp	-	-	31.75 -	-	-	17.1 +
ME	2-comp	-	10.71 -	-	-	-	11.6 +
	3-comp	-	6.25 -	-	-	-	14.52 +
	4-comp	-	-	-	-	-	4.9 +
	5-comp	-	-	-	-	-	8.35 +
Homog	2-comp	-	-	-	-	-	6.25 -
	3-comp	2.77 -	-	-	-	2.45 +	-
	4-comp	-	-	-	-	-	-
	5-comp	-	-	-	-	-	-
HWE	2-comp	-	-	14.88 +	-	38.55 -	6.94 +
	3-comp	-	-	15.07 +	-	40.23 -	3.02 +
	4-comp	-	-	11.41 +	-	26.09 -	-
	5-comp	3.5 +	-	15.1 +	-	26.4 -	-
Ferment	2-comp	-	6.16 +	27.49 -	-	-	-
	3-comp	-	-	7.72 -	-	6.25 +	-
	4-comp	-	-	-	-	6.67 +	-
	5-comp	-	-	-	-	-	-
FermExt	2-comp	-	-	5.44 +	-	37.24 -	10.79 +

	3-comp	-	-	10.17 +	-	37.82 -	4.2 +
	4-comp	-	-	9.3 +	-	18.37 -	3.45 +
	5-comp	-	-	8.71 +	-	21.37 -	-
WortVisc	2-comp	-	-	4.23 -	-	-	4.41 +
	3-comp	-	-	-	-	-	-
	4-comp	-	-	-	-	-	-
	5-comp	-	-	-	-	-	-
SN%	2-comp	-	-	6.69 +	-	33.17 -	6.19 +
	3-comp	-	-	10.03 +	-	19.43 -	5.17 +
	4-comp	-	-	3.4 +	-	14.52 -	-
	5-comp	-	-	3.54 +	-	7.88 -	-
CWM	2-comp	-	-	-	-	4.14 -	-
	3-comp	-	-	-	-	-	-
	4-comp	-	-	-	-	-	-
	5-comp	-	-	-	-	6.31 -	-

The treatments had little effect overall on yield of mixtures compared with their monoculture means, but the mean increased around 5%, the best responses being low density and high fertiliser. However, different yield sensitivity types behaved differently, some high sensitivity types, for example, yielding relatively poorly under low density. A lack of contrasting performance characteristics in some mixtures was probably the reason for difference between mixtures performance, indicating that yield sensitivity measurements may be good indicators of interaction characteristics and therefore their utility as mixtures components and should be a factor in mixtures design for particular climatic or agronomic situations.

Height showed a small response to mixtures overall, a small reduction under high fertiliser but an increase under the competition of high density which also delayed heading date most, the mean delay for mixtures being two days. The heterogeneity of mixtures resulted in the stiffer strawed types reducing lodging overall, particularly at low density, and these later traits were also affected by the level of heterogeneity available, reflected in not only component numbers, but also yield sensitivity.

In the quality trial some strong interactions when deployed in complex mixtures were detected despite all except Melanie having Maris Otter in their pedigrees. Again disease levels were low so there was no more than an indication that they were controlling disease as well as expected. However, the yield response of the mixtures was considerable, up to 15% more than the component monoculture mean in the case of the fungicide treated six component mixture.

Homogeneity of cell wall modification was predicted by maltsters to be lower in mixtures and we confirmed this observation. Furthermore, it was directly correlated with complexity. Homogeneity was also lower in previous work where malting and feed varieties were used (NEWTON *et al.* 1998), due to differences in malting performance between the two types. Here differences between varieties occurred, as several did not achieve their malting potential. In a laboratory situation, where a whole trial is being malted, a single regime is used, so the procedure is not optimised for each variety. It was also important to avoid over-modification, as this will reduce fermentability (BATHGATE *et al.* 1978) and could obscure varietal differences in that character. Although cv. Pipkin gave a high extract in the previous experiment (NEWTON *et al*. 1998), neither it, nor its derivatives Gleam and Rifle malted as well here as cultivars such as Halcyon and Melanie. This is likely to be an effect of growing in a different season, or an interaction between the genotype and the environment and illustrates the variation that can occur in phenotypic expression of quality in a monoculture. The poorer levels of modification within some of the components thus led to a lower level of homogeneity in

certain mixtures. On a laboratory scale this did not appear to adversely affect other quality parameters, so the only likely problem would be if this caused practical processing difficulties on an industrial scale. There was, however, no obvious effect on wort viscosity and it should be remembered that both grain distilling and some forms of brewing use substantial quantities of unmalted adjunct. Differing levels of modification could have produced a combination of enzymes and substrates that gave more optimal starch modification in some mixtures. We have trialled a spring barley 3-component mixture on a farm scale (275 tonnes) and it malted successfully at a commercial maltsters and proved no problem to a distiller in milling where it gave a very high spirit yield (unpublished data) so we don't expect major problems with scale-up.

From an exploitation point of view, mixing six or more components in a mixture may not be practical as sourcing and mixing all the desired components adds cost. Ideally only those components of the mixture which contribute most across a combination of desirable traits need be selected and mixed. Desirable yield and quality were often not well correlated, so compromises are necessary. The percentage variance accounted for values provide a tool to make quantitative decisions about mixtures composition for different purposes. There is clearly much heterogeneity in winter barleys which can be exploited in mixtures. Benefits are easily achieved, but these data demonstrate the further considerable optimisation potential for exploitation. Thus there are few disadvantages to using mixtures in winter barley and many advantages, so it is surprising that they are not more widely exploited.

Acknowledgements

We thank the Scottish Executive Environment and Rural Affairs Department for funding.

References

AASTRUP S., GIBBONS G.C., MUNCK L. (1981): A rapid method for estimating the degree of modification in barley by measurement of cell wall breakdown. Carlsberg Res. Commun., **46**: 77-86.

ALLISON M.J., COWE I.M., BORZUCKI R., BRUCE F.M., McHALE R. (1979): Milling energy of barley. Jour. Inst. Brew., **85**: 262-264.

BATHGATE G.N., MARTINEZ-FRIAS J., STARK J.R. (1978): Factors controlling the fermentable extract in distillers malt. Jour. Inst. Brew., **84**: 22-29.

FINCKH M.R., GACEK E.S., GOYEAU H., LANNOU C., MERZ U., MUNDT C.C., MUNK L., NADZIAK J., NEWTON A.C., DE VALLAVIEILLE-POPE C., WOLFE M.S. (2000): Cereal cultivar and species mixtures in practice. Agronomie: Pl. Genet., Br. **20**: 813-837.

HASLEMORE R.M., GILL A.A. (1995). Rapid spectrophotometric measurement of soluble nitrogen in micromalts from a barley breeding programme. Jour. Inst. Brew., **101**: 469-472.

JENSEN S.A., AASTRUP S. (1985). Determination of malt modification. Cerevisia, **3**: 113-123.

MUNDT C.C. (2002): Use of multiline cultivars and cultivar mixtures for disease management. Annu. Rev. Phytopathol., **40**: 381-410.

NEWTON A.C., HACKETT C.A. (1994): Subjective components of mildew assessment on spring barley. European Jour. Pl. Pathol., **100**: 395-412.

NEWTON A.C. (1997): Cultivar mixtures in intensive agriculture. In: Gene-for-gene relationship in plant parasite interactions. Eds: I R Crute, J Burdon and E Holub, pp. 65-80. CAB International, Wallingford, Oxford.

NEWTON A.C., ELLIS R.P., HACKETT C.A., GUY D.C. (1997): The effect of component number on *Rhynchosporium secalis* infection and yield in mixtures of winter barley cultivars. Pl. Pathol., **46**: 930-938.

NEWTON A.C., SWANSTON J.S., GUY D.C., ELLIS R.P. (1998): The effect of cultivar mixtures on malting quality in winter barley. Jour. Inst. Brew., **104**: 41-45.

NEWTON A.C., GUY D.C. (2004): Genotype and phenotype deployment strategies to enhance durability and efficacy of cultivar mixtures. Proc. Crop Prot. Northern Britain 2004: 165-170.

NEWTON A.C., SWANSTON J.S., GUY D. (2004a): Enhanced durability and utility of genes for resistance by deployment in cultivar mixtures. Proc. Molec. Plant-Microbe Interactions XI, St Petersburg, 18-26 July 2003 (in press).

NEWTON A.C., BENGOUGH G., BEGG G., SWANSTON J.S., HAWES C. (2004b): The role and importance of canopy heterogeneity. Scottish Crop Research Institute Annual Report for 2002/3: 125-127.

NIAB (2001): UK recommended lists of cereals: 2001 cereals variety handbook. NIAB, Cambridge, UK: 62.

SWANSTON J.S., THOMAS W.T.B. (1996): Breeding barley for malt whisky distilling. In: SLINKARD A., SCOLES G., ROSSNAGEL B. (eds.) Proc. Fifth Int. Oat Conf. And Seventh Int. Barley Genetics Symp., Poster Sessions Vol. 1., University Extension Press, Univ. of Saskatoon, Canada: 38-40.

SWANSTON J.S. (1997): Assessment of malt. Ferment, **10**: 29-34.

SWANSTON J.S., GACEK E., GUY D.C., NEWTON A.C. (2000): Malting performance of barley cultivar mixtures from the UK and Poland. Jour. Inst. Brew., **106**: 239-243.

Trends in Biological and Molecular Variation of Barley Driven by Long-Term Breeding

J. Kraic and M. Benková

Division of Applied Genetics and Breeding, Research Institute of Plant Production, Bratislavská cesta 122, 92168 Piešťany, Slovakia

Abstract

Trends in diversity of morphological, agronomical, and molecular traits and characteristics have been studied in spring barleys released and cultivated in territory of Slovakia within the years 1900-2002. Temporal changes were detected in all studied characteristics. Average value of plant height in cultivars decreased within one century by 226 mm, resistance to lodging improved from 5.7 to 9, thousand kernel weight increased from 46.63 g to 49.69 g. Principal benefit in yield is related to cultivars after releasing of Diamant (after 1965), those average yield improved by 0.93 $t.ha^{-1}$, in comparison with cultivars of previous period, resistance against powdery milder increased from average value 6.2 to 7.3. The total number of microsatellite alelles was more or less equal in cultivars of periods up to 1985. Their significant rising has begun after this year. Nevertheless average number of microsatellite alelles per one cultivar is reversal and indicates progress in uniformity of newer cultivars.

Introduction

Barley is one of the most important crops of the Slovakian agriculture. Breeding of spring barley in Slovakia has long history initiated in late decade of the 19th century (JAMÁRIK, 1970, FISCHBECK, 2003). The first bulk and individual selections from landrace populations have been initiated in that time. Breeding progressed to qualitative higher level after the year 1912 but has been suspended by both World Wars. Moreover, in 1938 Slovakia lost south territory, where the greatest and most productive breeding stations were located (Diosek, Slovenský Meder, Palárikovo, Komjatice, and others). There remained only three other breeding stations at the rest territory, therefore ten new ones have been established. The spring barleys Lontovský 66, Lontovský 172, Lontovský 91, Perbetský hladkoostnatý, Radošinský najvýnosnejší, Radošinský skorý, Radošinský veleranný, Diosecký 62, Diosecký 236, Diosecký 496, Diosecký 738, Šuriansky 129 have been created and released to that time. Barley breeding continued also during the World War II. The end of war rejoined south territory to Slovakia but only station in Diosek (Sládkovičovo after 1948) was functional and possessed new cultivars - Slovenský jemný, Slovenský kvalitný, Šprinter, Slovenský Dunajský trh, Slovenský 802. Other stations finalized during a war additional cultivars - Terrasol Kneifel, Terrasol pivovarský, Radošinský sladár, Radošinský plnozrnný, Hruškov Slovák Export, Nitriansky export. Within the years 1947-1948 a power of breeding stations has been reconstructed and for next five years (1949-1953) have been defined breeding objectives (higher yield and quality, better resistance against diseases and drought, feasibility for machine harvest). The plant breeding in the first half of fifties was under high impression of the so-called Lysenko's-Mičurin's genetics. Return to Mendelian genetics and to modern breeding methods became at the end of fifties. Other breeding stations were established and two new cultivars - Dvoran and Sladár were registered in the sixties. Other new cultivars have been released after thirteen years. There were bred 22 spring barley cultivars in Slovakia since 1980 to this time. The latest is cultivar Adran released in 2004.

The overview of barley breeding and development in Slovakia disclosed that determining donors in barley breeding programs and cultivation were original landrace-derived cultivars

of „Haná pedigree“. Creation of cultivars by pedigree-based method finished generally by Kneifel's barley Opavský. Simple crossing between two cultivars opened other phase of barley breeding. Significant development in spring barley breeding started after the year 1946 (LEKEŠ, 1997). According to LEKEŠ (1997) spring malting barley breeding can be classified following basic donors: Haná landrace populations, Proskowetz's Haná pedigrée (1882), Kneifel's barley Opavský (1926), Valtický (1930-1939), Diamant (1965), series of cultivars of so-called „Diamant's set“ (1972-1985), and short-stemmed and high yielded cultivars (after 1986). Morphological, agronomical, phytopathological, and others traits and characteristics of barleys have been changed within single decades of the 20th century undoubtedly. Also changes in protein composition and quality, based on changes in DNA, have been occurred for sure, i.e. effective tools for evaluation of diversity and differences between genotypes are also protein and enzymes studies, and DNA markers.
The aim of this work is to study temporal flux of diversity of spring barley in morphological, agronomical, and molecular traits in our territory within last century. This study presents only preliminary results from the first year of planed three-year experiment.

Material and Methods
There are not conserved seeds of all original barleys in our collections. Some of them are lost irrecoverable some are maintained in different seed collections. An ultimate part of them, mainly originated from the first half of 20th century, we gathered also with serious aid of the ARI Kroměříž, Ltd. Due to small seed lots all cultivars have been sown in the first year of testing (2003) in equal plots, without replications in Piešťany. Evaluated barley set represents 101 spring cultivars originated and released in Slovakia, Czech Republic, and Czechoslovakia, respectively (Tab. 1). Two cultivars - Dobrovický staročeský and Lutskij were inserted only into molecular analyses, other two - Vígľašský polojemný and Semčický pivovar were evaluated only by morphological and agronomical traits. There were evaluated basic agronomical and morphological traits - vegetation period, plant height, thousand kernel weight-TKW, and yield) according to the IPGRI classificator (1994), phytopathological characteristics - level of field resistance to powdery mildew (*Blumeria graminis* f. sp. *hordei*) and to net blotch (*Pyrenophora teres* (Drechs.) by 9-point scale (9-resistant, 1-sensitive), and variation in microsatellite loci.
Molecular variation was evaluated in 12 microsatellite loci. Primers for amplifications of microsatellites located outside of coding sequences - SSR's (HVM3, HVM4, HVM40, HVM43, HVM44, HVM60, BMS32) and PCR reactions were performed according to LIU *et al*. (1996) and RUSSELL *et al*. (1997). Primers for microsatellites located in coding sequences, i.e. EST-SSR's (Hv13GIII, HvBAMY, HvBTAI3, TaCMD, HVWAXY) were designed by Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) and PCR followed PILLEN *et al*. (2000).
Statistical calculation and trends in individual traits have been done by Microsoft® Excel 2002.

Results and Discussion
Even though the main goal of the first year was to multiply seeds, the first informative evaluations of selected agronomical, morphological, and phytopathological traits and characteristics have been performed. After one-year study of cultivars segregated into groups, based on their genesis, we obtained preliminary data (Tab. 2). Plant height essentially and statistically significantly ($P<0.001$) decreased within the years 1900-2002. The tallest cultivar was Stupický plnozrný (1070 mm), the shortest Viktor (640 mm). The highest mean plant height (964 mm) had cultivars of period Valtický (1930-1939), the lowest (738 mm) the newest cultivars released between the years 1986-2002. Mean plant height between cultivars

of these periods decreased by 226 mm, i.e. the mean genetic gain in plant height was about 3.1 mm.year^{-1}. The substantial turning point in plant height was development of cultivar Diamant. Just cultivars of period of Diamant' set, i.e. in cultivars into which Diamant was intensively incorporated as donor of short stem, fundamentally depressed plant height over next decades. Significantly lower mean annual decreasing of 2-rowed spring barleys (2.0 mm.year^{-1}), during more or less comparable period documented ORTIZ *et al*. (2002) in Nordic barleys. An essential depression of plant height since the year 1900, after study of 24 spring barleys observed also GRAUSGRUBER *et al*. (2002). The reduction of plant height in our barleys, after more then centennial breeding is apparent (Fig. 1) and logical. The height decreasing was one the most important factor of progress in barley. Analyzed height of cultivars and trends within last century proposed to assess trend to the near future. Our rough account without regard of aspects of genetics and environment, requirements of agriculture and others, predicts subsequent decreasing of plant height to the year 2020 to 720 mm.

Plant height relates to resistance against lodging which is evidently lower in short-stemmed cultivars released in last forty years. The lowest resistance to lodging had cultivar Olešenský from the period of Valtický. Cultivars improved significantly ($P<0.001$), in the frame of used scale (9-1), from mean value 5.7 (cultivars of the period of Valtický, 1930-1939) to maximal value 9 (1986-2002). Serious improvement of lodging resistance denoted also ORTIZ *et al*. (2002) in Nordic barleys. Barley breeding quasi achieved its ultimate in this trait. Based on our study we estimate further improvement of this trait within the next 16 years related to decreasing of plant height.

Very important economical indicator of barley cultivation is thousand kernel weight (TKW), one of the yield components. Also TKW significantly ($P<0.001$) improved in time (Fig. 2) from mean value 46.63 g (period of Haná landrace populations, 1900-1926) to 49.69 g (1986-2002). Minimal TKW (around 42 g) had several cultivars, maximal value (52.90 g) cultivar Atribut registered in 1996. The average increasing of TKW in cultivars of the end of 20th century, in comparison to the oldest ones, was 3.06 g. It corresponds to mean genetic gain in TKW 0.03 g.year^{-1}. An anomaly in increasing of TKW occurred in barleys of Diamant's set (1972-1985) when the mean TKW reversed into the period of Valtický, i.e. forty years back. Trend announced by us for next 16 years indicates stagnation or smooth regress of mean values to 49.38 g.

The lowest yield revealed cultivar Kvasický (period of Valtický, 1930-1939), the highest Čelechovický hanácký (1946-1964). The lowest mean yield (3.90 t.ha^{-1}) had cultivars from period of Valtický, the highest cultivars from period of Diamant (1965-1971). The mean increasing of yield within 102 years was statistically significant ($P<0.001$) (Fig. 3). Yield of cultivars originated from 1900 to 1964 was similar. Principal benefit in yield is related to cultivars of period of Diamant (after 1965). Their mean yield improved by 0.93 t.ha^{-1}, i.e. mean increasing was approximately 14.5 kg.year^{-1} in that time. There became negative change in yield trend, similar to TKW, in cultivars of period of Diamant' set (1972-1985). The mean yield of cultivars of followed two periods, till 2002 were lower. The trend in yield till 2020, based on our study, predicts increasing to 5.02 t.ha^{-1}.

Yield of cultivars reflects morphological and agronomical traits and characteristics and individual yield parameters. Statistically significant negative correlation was detected between yield and plant height ($P<0.05$) and positive correlation between yield and resistance to lodging ($P<0.001$). TKW not correlated with yield what indicates that yield increasing based on morphological and physiological changes in plant habit, mainly with number of spikes per plant and number of kernels per spike. These additional parameters were not evaluated in the first year of this study.

Field resistance against powdery mildew expressed in the year 2003 was the lowest in Haná landrace populations (1900-1926) and the highest in the newest cultivars. Improvement of powdery mildew resistance within one century of breeding was significant ($P<0.001$) (Fig. 4). Resistance against *Pyrenophora teres* was not reliable detected. There was any prevalence of this pathogen in experimental field and differences between cultivars was nearly none.

Genetic improvement of barley in morphological and agronomical traits and characteristics in our territory within one hundred years is evident. Our breeding programs created new and advances spring barley cultivars as observed others (ORTIZ *et al*., 2002, GRAUSGRUBER *et al*., 2002).

Analysis of molecular variation reposed on evaluation of microsatellite alleles, including null ones. Altogether 50 alelles, 31 (62%) located outside and 19 (38%) inside of coding DNA sequences. The highest number of SSR, EST-SSR alelles and unique alelles was detected in the latest cultivars (Fig. 5). Analysis of total number of microsatellite alelles revealed trend similar to morphological and agronomical traits. Within the 102 years gradually increased number of microsatellite alelles but increasing was not steady. The total number of microsatellite alleles was more or less equal in cultivars from all periods up to 1985. As late as the year 1985 have begun their significant ($P<0.001$) rising caused by increasing of alelles in coding and also noncoding sequences. The highest proportion of all identified alelles (81.4%) possessed cultivars from period 1986-2002, the lowest (59.6%) cultivars from period Diamant (1965-1971). Variation in microsatellites outside of coding sequences was higher then in coding ones. The highest number of alelles possessed locus HVM3 (9 alelles).

There were identifies also unique alelles, i.e. alelles occured only in cultivars of single period. Such alelles have been revealed in cultivars of all evaluated periods except two (1946-1964 and 1972-1985). Some of alelles existed within the whole century. On the other hand some of them after emerging in one period persist in at least two followed periods, i.e. specific periods created new alelle. Such “enriching” periods were 1900-1926 and 1946-1965 which established by four microsatellite alelles.

Favourable trend in molecular variation of barley cultivars is advisable to confront also with number of cultivars in individual periods, which was not equal. If we respect different number of cultivars in individual periods than trends in average number of alelles per one cultivar is reversal (Fig. 6) even if not statistically significant. It indicates that uniformity of newer cultivars is in progress.

Retrospective analyses of barley cultivar development, discovered changes in diversity, and demonstrate genetic gains in consequence of intensive breeding within the last century. They were performed in genotypes originated from different regions of the Europe. Some observed general reduction of diversity caused by domestication (TANKSLEY, MCCOUCH, 1997) or breeding ALLARD (1996). RUSSELL *et al*. (2000) concluded reduction of microsatellite variation in Northern European spring barleys during last hundred years, mainly in cultivars registered after 1985. High proportion of microsatellite alelles (up to 72%) they detected only in nineteen so-called „foundation genotypes“. But reduction not affected all microsatellite loci. Other authors declare reversal results. BACKES *et al*. (2003) declared nearly identical molecular diversity possessed by old landraces and modern cultivars of spring barley by RFLP markers. KOEBNER *et al*. (2003) used AFLP markers for study of recommended cultivars in the UK within seventy years and they express that systematic breeding does not need to tend to reduction of genetic diversity. Based on our preliminary results of molecular diversity we can conclude that long-term breeding did not brought its reduction. On the contrary diversity has been increased and hypothesis of *de novo* diversity (RASMUSSON, PHILLIPS, 1997) should be supported.

Acknowledgements
This study was supported by the Department of Agriculture of the Slovak Republic (project no. 2003 SP27/0280D01/0280D01) and by the Agency for Science and Technology (project no. 20-017002).

References

ALLARD R.W. (1996): Genetic basis of the evolution and adaptadness in plants. Euphytica, **92**: 1-11.

BACKES G., HATZ B., JAHOOR A., FISCHBECK G. (2003): RFLP diversity within and between major groups of barley in Europe. Plant Breed., **122**: 291-299.

FISCHBECK G. (2003): Diversification through breeding. In: VON BOTHMER R., VAN HINTUM T., KNÜPFFER H., SATO K. (eds.): Diversity in Barley (*Hordeum vulgare*). Elsevier, Amsterdam: 29-52.

GRAUSGRUBER H., BOINTNER H., TUMPOLD R., RÜCKENBAUER P. (2002): Genetic improvement of agronomic and qualitative traits of spring barley. Plant Breed., **121**: 411-416.

IPGRI (1994): Descriptors for Barley (*Hordeum vulgare* L.). International Plant Genetic Resources Institute, Rome, pp. 45.

JAMÁRIK J. (1970): Vývoj šľachtenia rastlín od roku 1870 do roku 1948. In: KLINOVSKÝ M. (ed.): 100 rokov šľachtenia na Slovensku. Slovosivo-Príroda, Bratislava: 9-47.

KOEBNER R.M.D., DONINI P., REEVES J.C., COOKE R.J., LAW J.R. (2003): Temporal flux in the morphological and molecular diversity of UK barley. Theor. Appl. Genet., **106**: 550-558.

LEKEŠ J. (1997): Šlechtění obilovin na území Československa. Plant Select - Nakladatelství Brázda, Praha, pp. 280.

LIU W., BIYASHEV R.M., SAGHAI MAROOF M.A. (1996): Development of simple sequence repeat DNA markers and their integration into a barley linkage map. Theor. Appl. Genet., **93**: 869-876.

ORTIZ R., NURMINIEMI M., MADSEN S., ROGNLI O.A., BJØRNSTAD Å. (2002): Genetic gains in Nordic spring barley breeding over sixty years. Euphytica, **126**: 283-289.

PILLEN K., BINDER A., KREUZKAM B., RAMSAY L., WAUGH R., FÖRSTER J., LÉON J. (2000): Mapping of new EMBL-derived barley microsatellites and their use in differentiation of German barley cultivars. Theor. Appl. Genet., **101**: 652-660.

RASMUSSON D.C., PHILLIPS R.L. (1997): Plant breeding progress and gene diversity from de novo variation and elevated epistasis. Crop Sci., **37:** 303-310.

RUSSELL J.R., ELLIS R.P., THOMAS T.B., WAUGH R., PROVAN J., BOOTH A., FULLER J., LAWRENCE P., YOUNG G., POWELL W. (2000): A retrospective analysis of spring barley germplasm development from „foundation genotypes“ to currently successful cultivars. Molec. Breed., **6**: 553-568.

RUSSELL J., FULLER J., YOUNG G., THOMAS B., TARAMINO G., MACAULAY M., WAUGH R., POWELL W. (1997): Discrimination between barley genotypes using microsatellite markers. Genome, **40**: 442-450.

TANKSLEY S.D., MCCOUCH S.R. (1997): Seed banks and molecular maps: unlocking genetic potential from the wild. Science, **277**: 1063-1066.

Table 1. The set of analyzed spring barley genotypes

Period of creation of Haná landrace populations „Proskowetz's Haná pedigree" (1900-1926)	Dregerův, Jarohněvický, Hanácký Kargyn, Proskovcův, Zborovický Kargyn, Nolč Dregerův veleraný, Export Ratborský, Stupický Hanácky, Šumavský
Period of „Valtický" (1930-1939)	Janovický, Novodvorský Hanácky, Kvasický, Stupický plnozrnný, Nitriansky Exportný, Valtický, Olešenský
Period 1946-1964	Diosecký Sprinter, Bohatýr, Detěnický Kargyn, Diosecký Kneifl, Dobrovický Staročeský, Dregerův Imperiál, Hanácky jubilejný, Hanácky Exportný, Hanácky Moravan, Hanácky Staroveský, Hořický, Jindřichovický, Pisárecký, Pudmerický pivovar, Terrasol pivovarský, Židlochovický, Bučiansky Kneifl, Čelechovický hanácky, Semčický hospodársky, Semčický pivovar, Branišovický výnosný, Ekonom, Karát, Merkur
Period of „Diamant" (1965-1971)	Diamant, Dvoran, Jantar, Sladár, Denár, Dukát, Topás, Lutskij, Vígľašský polojemný
Period of „Diamant' set" (1972-1985)	Ametist, Favorit, Hana, Atlas, Diabas, Spartan, Koral, Rapid, Safir, Fatran, Opál, Krystal, Zefir, Horal, Rubín, Bonus, Kredit 21, Zenit
Period of short-stemmed, high productive cultivars (1986-2002)	Jaspis, Orbit, Jarek, Perun, Novum, Profit, Malvaz, Galan, Jubilant, Terno, Akcent, Heran, Ladík, Sladko, Svit, Donum, Forum, Stabil, Garant, Kosan, Viktor, Zlatan, Amos, Amulet, Granat, Kompakt, Atribut, Vladan, Olbram, Tolar, Progres, Expres, Cyril, Ludan

Table 2. Average values of morphological and agronomical traits and characteristics

Period of development	**Vegetation period [days]**	**Plant height [mm]**	**Resistance against lodging [9-1]**	**Resistance against *P. teres* [9-1]**	**Resistance against *B. graminis* [9-1]**	**TKW [g]**	**Yield [t.ha^{-1}]**
1900-1926	113	950	6.7	8.9	6.2	46.63	4.05
1930-1939	113	964	5.7	9	6.5	46.76	3.90
1946-1964	113	912	8.4	9	6.3	46.92	4.02
1965-1971	113	895	8.8	9	7.0	48.56	4.93
1972-1985	113	794	8.7	8.9	6.8	47.23	4.71
1986-2002	113	738	9.0	8.5	7.3	49.69	4.88

Figure 1. Trend in plant height

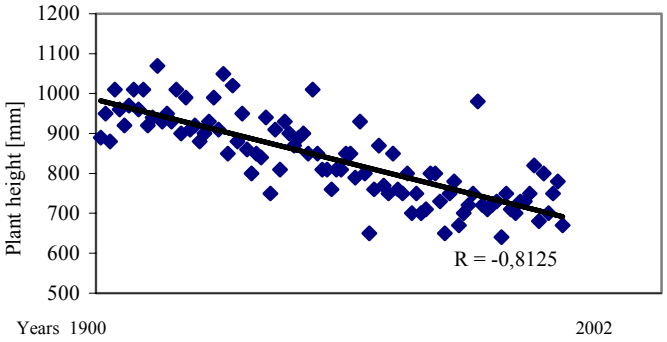


Figure 2. Trend in thousand kernel weight

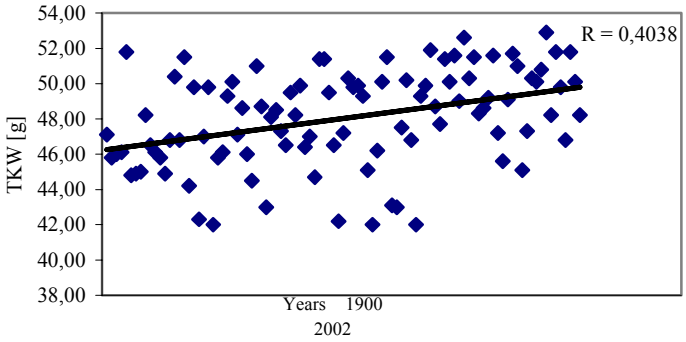


Figure 3. Trend in yield

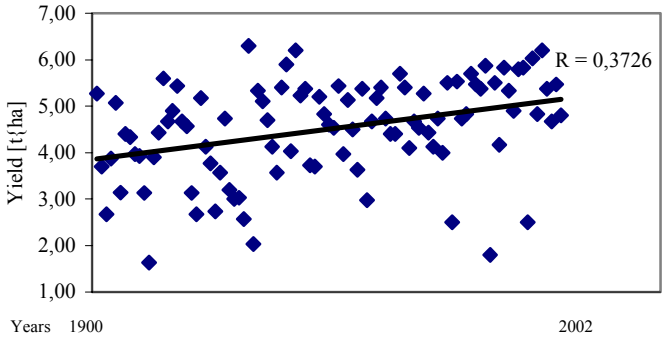


Figure 4. Trend in resistance against powdery mildew

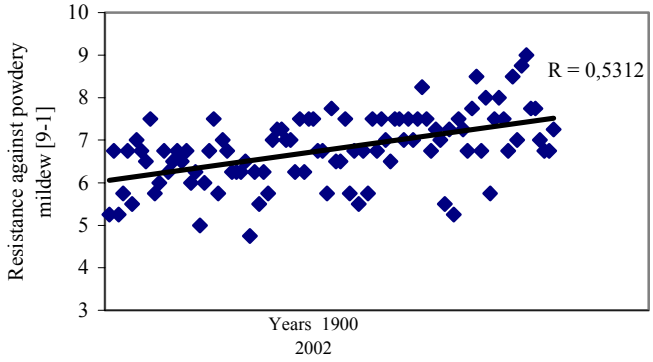


Figure 5. Total number of microsatellite alleles in coding and noncoding regions (SSR, EST-SSR) and number of unique SSR and EST-SSR alleles in cultivars of relevant periods

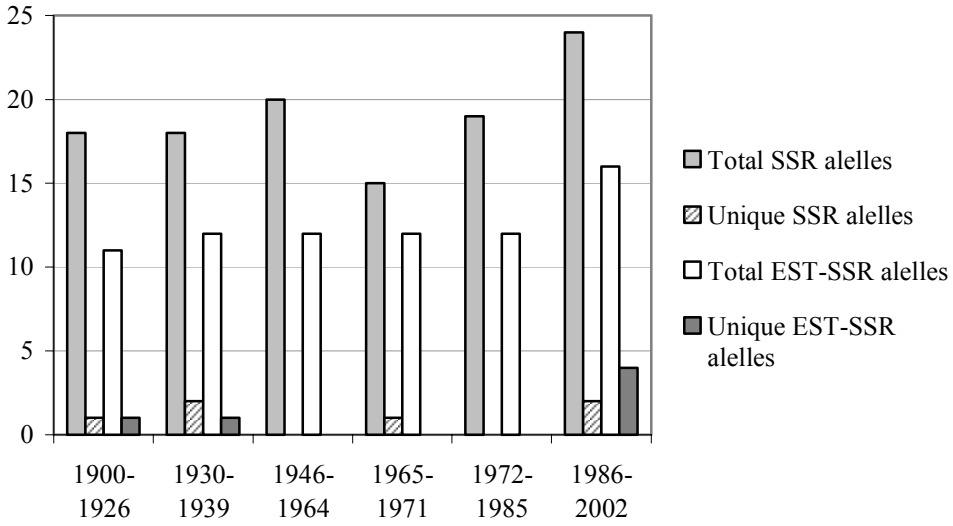
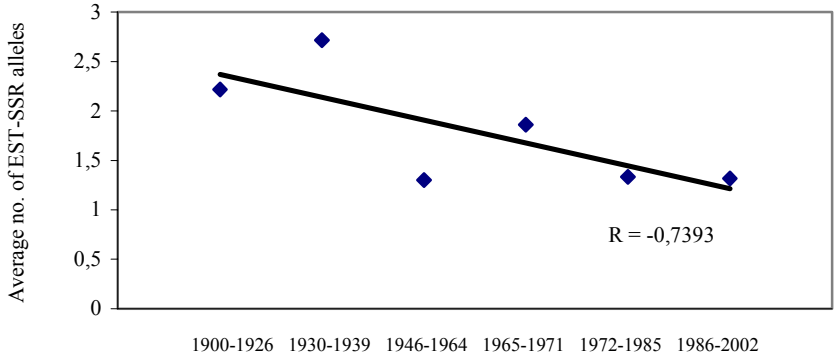


Figure 6. Trend in average number of EST-SSR alelles per cultivar



S 8 – DISEASE AND PEST RESISTANCE II – EAR DISEASES, FUSARIUM HEAD BLIGHT, VIROSES

Breeding Barley for Multiple Disease Resistance in the Upper Midwest Region of the USA

B.J. Steffenson[1] and K.P. Smith[2]

[1]Department of Plant Pathology and [2]Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN 55108 USA

Abstract
The Upper Midwest is one of the largest barley production areas in the USA. In this region, diseases can markedly reduce both the yield and quality of the crop. Molecular and classical breeding techniques are being employed to develop cultivars with resistance to five different diseases in the Minnesota barley improvement program. Stem rust and spot blotch have been successfully controlled for many years through the deployment of the major gene *Rpg1* and a major effect QTL, respectively. A sequence characterized amplified region (SCAR) marker developed from the sequence of *Rpg1* has made marker-assisted selection (MAS) for stem rust resistance highly effective. The major QTL controlling durable adult plant spot blotch resistance was first identified in the Steptoe/Morex population. This QTL was completely suppressed in the Harrington/Morex and Dicktoo/Morex populations, highlighting the importance of genetic background for the expression of resistance. The onset of Fusarium head blight (FHB) in 1993 led to dramatic changes in the focus of the breeding program. Significant resources have been expended to develop populations for mapping resistance QTL and identify closely linked markers for MAS. This is a difficult challenge because FHB resistance is controlled by many QTL with small effects. Sources of resistance to net blotch and Septoria speckled leaf blotch (SSLB) have been identified in a number of barley accessions. These resistances are simply inherited and are being introgressed into elite lines via phenotypic and MAS. Continued progress toward multiple disease resistance will require efficient phenotypic screening, MAS, and utilization of discoveries in barley genomics to manage numerous resistance genes and desirable gene complexes assembled over decades of breeding.
Keywords: barley; *Hordeum vulgare*; disease resistance; marker assisted selection

Introduction
The Upper Midwest region of the USA is one of the most productive cereal-growing regions in the northern Great Plains and the major source of barley for the malting and brewing industries. This region includes the states of Minnesota, North Dakota, and South Dakota and produces over 2,000 Mt of barley annually, most of which is intended for malting. In recent years, economic factors and disease pressure have pushed six-rowed malting barley production from an area centered in northwestern Minnesota and eastern North Dakota to north central North Dakota and southern Canada. Diseases that commonly impact barley production in this region include stem rust (caused by *Puccinia graminis* f. sp. *tritici*), spot blotch (caused by *Cochliobolus sativus* [anamorph: *Bipolaris sorokiniana*]), FHB (caused primarily by *Fusarium graminearum* [teleomorph: *Gibberella zeae*]), net blotch (caused by

Pyrenophora teres f. *teres* [anamorph: *Drechslera teres* f. *teres*]), and Septoria speckled leaf blotch (SSLB) (caused by both *Septoria passerinii* and *Phaeosphaeria avenaria* f. sp. *triticea* (anamorph: *Stagonospora avenae* f. sp. *triticea* (FETCH *et al*. 2003; STEFFENSON 2003). The deployment of host resistance is often the preferred method of control for these diseases because it is an effective, economical, and environmentally sound strategy.

One of the great challenges in breeding malting barley is to incorporate multiple disease resistance while maintaining favorable gene complexes responsible for regional adaptation and acceptable malting and brewing characteristics. Approval of a barley cultivar for use in malting and brewing is based on about 25 different quality traits (WYCH & RASMUSSON 1983). Additionally, an approved cultivar must also pass taste tests after it is malted and made into beer. These specific requirements have forced breeders to cross closely related parents that already possess superior malting and brewing characteristics. As a result, the Minnesota barley germplasm base has been drastically narrowed to the extent in which 50% of the parentage traces back to only five ancestors (MARTIN *et al*. 1991). Introgression of genes from exotic sources, such as in the case of disease resistance, requires a process of parent building or cyclical breeding. In this process, the most desirable progenies from crosses in early cycles of breeding are used as parents in subsequent breeding cycles. After several breeding cycles, progenies will be suitable for crosses that will potentially lead to new cultivar candidates. The number of breeding cycles necessary will depend on the ease and reliability of the screening methods and whether the trait exhibits simple or complex inheritance. For more challenging diseases under complex genetic control (e.g. FHB), it is likely that at least 4-5 breeding cycles will be necessary to generate breeding lines that can be used as parents to ultimately produce a new cultivar. Parent building is generally used to improve a single trait. Therefore, breeding for multiple disease resistance can be viewed as a multiple parent building enterprise that will ultimately lead to the combination of desired resistances in a single cultivar. The objective of this paper is to review current and past efforts in breeding six-rowed malting barley cultivars for multiple disease resistance in the Upper Midwest region of the USA. Successes and continuing challenges in this endeavor are discussed as well as prospects for the future.

Results and Discussion

Stem Rust. Stem rust has historically been one of the most devastating diseases of barley in the Upper Midwest region. Since 1942, losses to stem rust in barley have been minimal due to the planting of cultivars with the durable resistance gene *Rpg1* (STEFFENSON 1992). Pathotypes with virulence for *Rpg1* have been reported periodically in the Upper Midwest region since 1942 (STEFFENSON 1992). In 1989, a pathotype (QCCJ) with virulence for *Rpg1* became widespread in the Upper Midwest and damaged some barley fields (ROELFS *et al*. 1991). Pathotype QCCJ is still a threat to barley production in the region. To obtain stable stem rust control in the future, breeders may have to combine into cultivars *Rpg1* and gene(s) for resistance to pathotype QCCJ. The retention of *Rpg1* in new cultivars is essential because this gene has proven durable to many pathotypes of *P. g.* f. sp. *tritici* in the region for over 60 years. Resistance to pathotype QCCJ was identified in barley accession Q21861 (PI 584766) and is conferred by a single recessive gene *rpg4* (JIN *et al*. 1994). Prior to the appearance of pathotype QCCJ, breeding for stem rust resistance was easy because it only required the introgression of *Rpg1*. Since all of the elite parents carried *Rpg1*, stem rust resistance was maintained in the program without any phenotypic selection. The transfer of an additional gene (i.e. *rpg4*) for resistance to pathotype QCCJ will complicate the breeding effort. A significant advance for the high-throughput detection of *Rpg1* in the breeding program would be the development of a molecular marker in the gene itself. *Rpg1* was recently isolated by a

map-based approach (BRUEGGEMAN *et al*. 2002). By exploiting sequence variation in the gene, ECKSTEIN *et al*. (2003) developed a robust, allele specific SCAR marker that can differentiate between lines with the functional resistance gene and those that lack the gene or contain one of several susceptibility alleles. This *Rpg1* marker was 92% accurate in detecting stem rust resistance in a historical set of 100 Minnesota breeding lines and Midwestern cultivars (CONDON *et al*. 2004). Development of a molecular marker within the *rpg4* gene is in progress (A. KLEINHOFS *et al*., unpublished) and when completed will allow multiplexing molecular markers for the two stem rust resistance genes on parents and in early generation (F_2) segregating populations, thereby increasing the efficiency and throughput of stem rust resistance breeding (Table 1). Still, stem rust phenotyping (JIN *et al*. 1994) must be done to verify the presence of the genes and their expression, since the *Rpg1* marker has not proven infallible.

Table 1. Breeding scheme and timeline for disease screening in the Minnesota barley improvement program

Year	Breeding Generation	Time/Location	Disease Screening[1]
1	Parent Selection	Fall Greenhouse	SB(G), SR(M)
	F_1	Winter Greenhouse	
	F_2	Summer Field	FHB(M), SR(M), SSLB(M)
2	F_3	Fall Greenhouse	
	F_4	Winter Nursery (NZ)	SSLB(G), NB(G)
	$F_{4:5}$	Summer Field	FHB(F), SSLB(F), NB(F)
3	$F_{5:6}$	Winter Nursery (NZ)	
	$F_{5:7}$ Preliminary Yield	Summer Field	FHB(F), SSLB(F), NB(F)
4	$F_{5:8}$ Intermediate Yield	Summer Field	FHB(F), SSLB(F), NB(F)
5	$F_{5:8}$ Advanced Yield	Summer Field	FHB(F), SSLB(F), NB(F)

[1]SB=spot blotch; SR=stem rust; FHB=Fusarium head blight; SSLB=Septoria speckled leaf blotch; NB=net blotch; (G)=greenhouse disease screen; (M)=DNA marker screen; (F)=field disease screen; (NZ)=New Zealand.

Spot Blotch. Spot blotch was one of the most devastating foliar diseases of barley in the Upper Midwest region. The disease has been successfully controlled for over 40 years through the use of host resistance and is one of the great success stories in breeding barley for resistance. This durable spot blotch resistance was derived from the breeding line NDB112 and has been incorporated into all of the major six-rowed malting cultivars grown in the region (STEFFENSON *et al*. 1996). To elucidate the genetic basis of durable spot blotch resistance in six-rowed malting cultivars, we studied the Steptoe/Morex (S/M) population. Morex is a resistant six-rowed malting cultivar derived from NDB112, and Steptoe is a susceptible six-rowed feed cultivar. A single gene (designated *Rcs5*) located at the telomeric region of chromosome 1(7H) was found to confer spot blotch resistance at the seedling stage (STEFFENSON *et al*. 1996). Two quantitative trait loci (QTL) conferred adult plant resistance in the S/M population: one of major effect on chromosome 5(1H) explaining 62% of the variance and a second of minor effect on chromosome 1(7H) explaining 9% of the variance (Table 2). The QTL on chromosome 1(7H) mapped to the same region as *Rcs5*. Thus, durable spot blotch resistance in six-rowed malting barley cultivars is conferred mostly by a single QTL of major effect on chromosome 5(1H). To corroborate these findings, the same analysis was conducted on the two- × six-rowed cross of Harrington/Morex (H/M). Harrington is a susceptible two-rowed malting cultivar. As in the S/M population, a single gene (presumably *Rcs5*) on chromosome 1(7H) conferred spot blotch resistance at the

seedling stage. However, a different and quite unexpected result was obtained for adult plant resistance in the H/M population: no chromosome 5(1H) effect was detected. Instead, a single gene mapping at or near *Rcs5* on chromosome 1(7H) conferred resistance. When the disease severity data were subjected to quantitative analysis, a single major effect QTL explaining 75% of the variance was identified, again at or near *Rcs5* (Table 2). (STEFFENSON 2000; H. BILGIC AND B. STEFFENSON, unpublished). One additional population involving Morex (Dicktoo/Morex [D/M]) was tested for its reaction to spot blotch. In this case, the susceptible parent was the six-rowed feed cultivar Dicktoo; thus, the D/M population was used to test whether the Morex-derived chromosome 5(1H) adult plant resistance QTL first identified in the S/M population would again be expressed in a different six- × six-rowed cross. Three QTLs were detected at the adult plant stage in the D/M population: one on the short arm of chromosome 3(3H) explaining 36%, the second on the long arm of chromosome 3(3H) explaining 11%, and a third near *Rcs5* on the short arm of chromosome 1(7H) explaining 20% of the phenotypic variation (H. BILGIC AND B. STEFFENSON, unpublished). No effect whatsoever was detected in the chromosome 5(1H) region where the adult plant resistance QTL was first discovered in the S/M population (Table 2). Over the past 40 years, breeders have been very successful in retaining the chromosome 5(1H) resistance QTL in their six-rowed malting germplasm, presumably by fixing the resistance allele in elite parents and practicing occasional phenotypic selection. It appears that this resistance is highly expressed in the six-rowed genetic backgrounds of the major malting barley breeding programs in the Midwest. This resistance QTL may, however, be completely suppressed when introgressed into more diverse two- or six-rowed genetic backgrounds (e.g. H/M and D/M populations). Molecular markers for the chromosome 5(1H) spot blotch resistance QTL are being developed. Their utility in MAS for the chromosome 5(1H) QTL may be limited given the suppression that occurs in crosses with both two- and six-rowed susceptible parents. In the future, we will employ MAS to verify that parents used in the breeding program carry the resistance allele at the 5(1H) QTL (Table 1) and continue to screen advanced breeding lines in the field to ensure that the resistance is expressed in the current breeding background.

Table 2. Summary of major QTL (chromosomal location and % phenotypic variance explained) contributing to adult plant spot blotch resistance in three mapping populations derived from resistant parent Morex

Pop.	Chrom 1(7HS) *iEst5-ABC158*	Chrom 3(3HS) *Saflp119-saflp54*	Chrom 3(3HL) *saflp35-saflp53*	Chrom 5(1HL) *ABG500A-ABG452*
S/M	9%	--[1]	--	62%
D/M	20%	36%	11%	--
H/M	75%	--	--	--

[1]No significant QTL detected in this region.

Septoria Speckled Leaf Blotch. Septoria speckled leaf blotch (SSLB) is a disease complex caused by two different pathogens. In the Upper Midwest region, *S. passerinii* is the most common SSLB pathogen, although *P. a.* f. sp. *triticea* is also frequently isolated from symptomatic barley tissue (KRUPINSKY & STEFFENSON 1999). In recent years, SSLB has reemerged as one of the most important diseases of barley in the Upper Midwest region due to the increased use of minimum tillage and high rainfall during the growing season. Yield losses of 23-38% were reported on barley due to *S. passerinii* infection (TOUBIA-RAHME *et al*. 2003). All of the major malting and feed barley cultivars in the Upper Midwest region are highly susceptible to SSLB (TOUBIA-RAHME *et al*. 2003). Fortunately, many sources of resistance to *S. passerinii* have been identified in both cultivated (LEGGE *et*

al. 1996; RASMUSSON & ROGERS 1963) and wild barley (*H. vulgare* subsp. *spontaneum* and *H. bulbosum*) (FETCH *et al*. 2003; TOUBIA-RAHME *et al*. 2003). In the Minnesota barley improvement program, two sources of resistance are being used: CIho 4780 (an accession from northern China) and PC84 (a breeding line from the ICARDA/CIMMYT program in Mexico). Both accessions exhibit high levels of resistance in the field. Resistance in CIho 4780 is conferred by a single dominant gene *Rsp2* (RASMUSSON & ROGERS 1963), which was recently mapped to the short arm of chromosome 5(1H) (S. ZHONG and B. STEFFENSON, unpublished). A SCAR marker co-segregating with *Rsp2* was developed and evaluated for MAS of SSLB resistance. Selection of F_2 plants homozygous for the resistance allele of the SCAR marker in two segregating populations was 96-100% effective in identifying SSLB resistant F_5 lines. Resistance in PC84 is thought to be under the control of a single dominant gene that is different from the one present in CIho 4780 (B. STEFFENSON and K. SMITH, unpublished). Our goal is to increase the diversity of SSLB resistance by incorporating both genes into new cultivars.

Net Blotch. Net blotch is perhaps the most important foliar pathogen of barley in the Upper Midwest on an annual basis given the sporadic nature of SSLB epidemics and the success attained in controlling stem rust and spot blotch by host resistance. The disease is widely distributed and is often found in high severities in commercial fields (B. STEFFENSON, unpublished). Many sources of net blotch resistance have been described in cultivated and wild barley (FETCH *et al*. 2003; SHIPTON et al. 1973). The Canadian cultivar Heartland is currently being used as a source of net blotch resistance in the Minnesota program. Preliminary studies indicate that this resistance is simply inherited. We have initiated work to identify markers that will be useful in MAS for net blotch resistance. Currently, we screen for net blotch resistance in segregating populations during single seed descent using remnant F_4 seed in a greenhouse seedling assay (Table 1). Resistant lines ($F_{4:5}$) are advanced to a field screen on adult plants where selection is based on disease resistance as well as other traits (i.e. lodging, stem strength, height, maturity etc.).

Fusarium Head Blight. FHB is one of the most devastating and insidious diseases of barley. In addition to causing yield loss, the primary pathogen, *F. graminearum*, produces various mycotoxins (most notably deoxynivalenol or DON) that are hazardous to humans and animals (STEFFENSON 2003). FHB has been a relatively minor and sporadic disease problem of barley in the United States for many years. Over the past decade, however, it has re-emerged as the most important factor reducing the yield and quality of the crop in the Upper Midwest. The head blight epidemics of the 1990's were particularly devastating and caused severe economic losses, grain processing problems for producers and end-users alike, food/feed safety concerns, and human hardship (STEFFENSON 2003). These epidemics also forced breeders to make drastic changes in their programs. Today, a significant portion of the breeding effort is focused on breeding for resistance to FHB and the accumulation of DON. A number of conventional and molecular mapping studies have been made on the genetics of FHB resistance in barley (reviewed in STEFFENSON 2003). All have reported complex inheritance for the trait. The molecular mapping studies indicate that FHB resistance is a complex quantitative trait controlled, in most cases, by a number of loci with relatively small effects that are scattered across the barley genome. From these genetic studies, it is evident that FHB resistance in barley is under polygenic control and its heritability can vary greatly. Given the great importance of this disease, the numerous challenges in quantifying FHB severity, and the complex genetics of resistance, we have developed a modified FHB breeding strategy in the Minnesota program. The large experimental error and environmental effects on FHB severity have dictated that our early generation screening efforts employ multiple

locations and replications. For other diseases such as net blotch, it is possible to do greenhouse screening on seedlings using remnant seed from early generations (F_3, F_4) during single seed descent, followed by a single F_5 head row evaluation in the field for a number of traits. For FHB, we cannot effectively conduct greenhouse screening in early generations. In year two (F_5 generation), we evaluate FHB reaction in misted and inoculated field nurseries (Table 1). Each new breeding line is replicated twice at two locations and evaluated for FHB severity. We harvest grain from resistant lines and checks for quantification of DON. In addition, we grow a fifth row in a non-inoculated nursery and harvest the grain for malting quality evaluation. Because FHB resistance is linked to maturity and plant morphology traits, we have emphasized selection for resistance prior to selection for other traits in the early cycles of breeding.

The need for replication in early generations and the desire to work with more homozygous material (F_4-derived) have forced us to make changes in our single seed descent program. The initial protocols, however, are the same. We make most crosses in the fall, grow F_1's in the winter greenhouse, and F_2's in a summer field trial (Table 1). We then plant the F_3 generation immediately after harvest in early August to allow for an off-season F_4 generation in New Zealand. The F_4 generation is planted as spaced single plants to allow the harvest of sufficient $F_{4:5}$ seed for growing five 1.8 m rows in the disease and quality nurseries described above. This laborious screening effort has forced us to reduce the number of crosses and new lines that we can evaluate each year, but has given us much more confidence in our early generation selection. In year three, we evaluate lines selected from year two in five disease nurseries with three replications per nursery. These same lines are evaluated in preliminary yield trials at two locations. Lines that continue on in year four are evaluated in three location trials in Minnesota. The best lines from the advanced yield trials (year five) are evaluated in a collaborative regional FHB nursery with eight locations in Minnesota, North Dakota, and Canada.

Recently, we have begun to evaluate MAS for FHB resistance. We evaluated markers linked to two major QTLs for FHB resistance discovered (DE LA PENA *et al*. 1999) and validated (CANCI *et al*. 2003) from the Chevron source of resistance. The Chevron alleles at the QTL on chromosome 2(2H) reduced FHB by 43% and increased HD by two days as was predicted by the mapping studies (GUSTUS & SMITH 2001). Selection for the Chevron alleles at the chromosome 6(6H) region reduced FHB by 22%, but also increased grain protein by 14g kg^{-1}. We are continuing to evaluate these and other markers to increase the efficiency of FHB selection. MAS is generally used to select lines homozygous for the resistance marker allele in the F_2 generation prior to single seed descent (Table 1).

Conclusions and Future Directions

The successful development of malting barley cultivars with multiple disease resistance requires the introgression of resistance alleles that function in the target genetic background and are free of linkage to undesirable traits. Past progress has relied on parent building after fixing genes for resistance or by exploiting individual segregating populations using phenotypic selection. For several diseases, markers now allow breeders to track resistance alleles in the broad arrays of breeding lines within the program, thereby reducing the need for expensive and sometimes variable phenotypic screening. In the future, it may be possible to exploit phenotypic variation in the complex pedigree structure of breeding germplasm to identify new QTL through the use of association genetics (JANNINK *et al*. 2001). This approach exploits the tremendous amount of phenotypic data generated by breeding programs and the relatively inexpensive DNA genotyping technologies currently available to study

important traits. By routinely genotyping breeding lines with a strategic set of DNA markers, it will be possible to validate QTL in the relevant germplasm, identify new QTL for important breeding traits, and determine if alleles introgressed into breeding lines perform as predicted by genetic studies. The rapidly advancing field of genomics is providing information on the location, expression profile, and function of genes that will be important for continued progress in breeding as well as new tools for manipulating them in breeding programs. All of this new technology and information will facilitate the management of multiple disease resistance in barley.

References

BRUEGGEMAN, R., ROSTOCKS, N., KUDRNA, D., KILIAN, A., HAN, F., CHEN, J., DRUKA, A., STEFFENSON, B., KLEINHOFS, A. (2002): The barley stem rust-resistance gene *Rpg1* is a novel disease-resistance gene with homology to receptor kinases. Proc. Natl. Acad. Sci. (USA), **99**: 9328-9333.

CANCI, P. C., L.M. NDUULU, R. DILL-MACKY, G.J. MUEHLBAUER, D.C. RASMUSSON, AND K.P. SMITH. (2003): Genetic relationship between kernel discoloration and grain protein concentration in barley. Crop Sci., **43**: 1671-1679.

CONDON, F., SMITH, K.P., STEFFENSON, B.J. (2004): Detection of marker-trait associations within barley breeding germplasm. In: *Plant and Animal Genome XII Abstracts*, San Diego, CA. Jan 10–14, 2004, p. 187.

ECKSTEIN, P., ROSSNAGEL, B., SCOLES, G. (2003): Allele-specific markers within the barley stem rust resistance gene (*Rpg1*). Barley Genet. Newsl., **33:** 7-11.

DE LA PENA, R. C., SMITH, K. P., CAPETTINI, F., MUEHLBAUER, G. J, GALLO-MEAGHER, M., DILL-MACKY, R., SOMERS, D. A., AND RASMUSSON D. C. (1999): Quantitative trait loci associated with resistance to Fusarium head blight and kernel discoloration in barley. Theor. App. Genet., **99**: 561-569.

FETCH T.G., JR., STEFFENSON, B.J., NEVO, E. (2003): Diversity and sources of multiple disease resistance in *Hordeum spontaneum*. Plant Dis., **87**: 1439-1448.

GUSTUS, C., SMITH, K.P. (2001): Evaluating Phenotypic and Marker Assisted Selection in the F_2 Generation for Chevron-derived FHB Resistance in Barley. In: *Proceedings of the 2001 National Fusarium Head Blight Forum*. Erlanger, KY 12/8/01 - 12/10/01.

JANNINK, J.-L., BINK, M.C.A.M., and JANSEN, R. C. (2001): Using complex plant pedigrees to map valuable genes. Trends Plant Sci., **6**: 337-342.

JIN, Y., STEFFENSON, B.J., MILLER, J.D. (1994): Inheritance of resistance to pathotypes QCC and MCC of *Puccinia graminis* f. sp. *tritici* in barley line Q21861 and temperature effects on the expression of resistance. Phytopathology, **84**: 452-455.

KRUPINSKY, J.M., STEFFENSON, B.J. (1999): Septoria/Stagonospora leaf spot diseases on barley grown in North Dakota, USA. In: VAN GINKEL M., MCNAB A., KRUPINSKY J. (eds.): Proceedings of the 5th International Workshop on Septoria and Stagonospora Diseases of Cereals. CIMMYT, Mexico DF.: 37-38.

LEGGE, W.G., METCALFE, D.R., CHIKO, A.W., MARTENS, J.W., TEKAUZ, A. (1996): Reaction of Turkish barley accessions to Canadian barley pathogens. Can J. Plant Sci., **76**: 927-931.

MARTIN, J.M., BLAKE, T.K. and HOCKETT, E.A. (1991): Diversity among North American spring barley cultivars based on coefficients of parentage. Crop Sci., **31**: 1131-1137.

RASMUSSON, D.C., ROGERS, W.E. (1963): Inheritance of resistance to *Septoria* in barley. Crop Sci., **3**: 161-162.

ROELFS, A.P., CASPER, D.H., LONG, D.L, ROBERTS, J.J. (1991): Races of *Puccinia graminis* in the United States in 1989. Plant Dis., **75**: 1127-1130.

SHIPTON, W.A., KHAN, T.N., BOYD, W.J.R. (1973): Net blotch of barley. Rev. Plant Pathol., **52:** 269-290.

STEFFENSON, B.J. (1992): Analysis of durable resistance to stem rust in barley. Euphytica, **63**: 153-167.

STEFFENSON, B. J. (2000): Durable resistance to spot blotch and stem rust in barley. In: LOGUE, S. (ed.): Barley Genetics VIII. Volume I. Adelaide University, Glen Osmond. 39-44.

STEFFENSON, B. J. (2003): Fusarium head blight of barley: Impact, epidemics, management, and strategies for identifying and utilizing genetic resistance. In: LEONARD K.J. AND BUSHNELL, W.R. (eds.): Fusarium Head Blight of Wheat and Barley. APS Press. St. Paul. 241-295.

STEFFENSON, B.J., HAYES, P.M., KLEINHOFS, A. (1996): Genetics of seedling and adult plant resistance to net blotch (*Pyrenophora teres* f. *teres*) and spot blotch (*Cochliobolus sativus*) in barley. Theor. Appl. Genet., **92**: 552-558.

TOUBIA-RAHME, H., PICKERING, R.A., STEFFENSON, B.J. (2003): Inheritance and chromosomal location of *Septoria passerinii* resistance introgressed from *Hordeum bulbosum* into *Hordeum vulgare*. Plant Breed., **122**: 405-409.

WYCH, R.D., RASMUSSON, D. C. (1983): Genetic improvement in malting barley cultivars since 1920. Crop Sci., **23**: 1037-1040.

Inheritance of the Fusariun Head Blight Resistance in Barley

K. Takeda

Research Institute for Bioresources, Okayama University, Kurashiki, 710-0046, Japan

Abstract

Barley and wheat are sensitive to Fusarium head blight (FHB) around the time of flowering, and water is necessary for scattering and germination of the ascospores that are the first infectious source. Therefore, the 'cut-spike' inoculation method by which a large number of plants can be inoculated exactly at the flowering time was developed.

A total of 4,881 barley varieties were tested for their resistance to FHB. The two-rowed varieties showed higher resistance than the six-rowed ones. Some highly resistant varieties were found in the varieties collected from East Asia and Europe, but few from those collected from West Asia and Africa. After more than ten trials, 23 varieties that showed stable resistance were selected. All of them were two-rowed varieties and both dense-and lax-spike varieties were included.

For investigating host-pathogen interaction, 20 Fusarium strains were inoculated onto 20 barley varieties. Strain x Variety interaction was not statistically significant indicating the race differentiation is negligible in FHB.

The inheritance of the resistance to FHB was analyzed using F_1 plant from a set of 8×8 diallel cross. The heritability of the resistance to FHB was also estimated from selection response using F_2 generations from the five cross combinations. The resistance to FHB in barley was contro1led by quantitative genes fitting the additive dominance model, and the degree and direction of dominance varied with the cross combination. The maternal effect was not observed. The heritability of the resistance to FHB was about 0.3. QTL analysis detected two significant factors on the chromosome 2H and one on 5H.

Introduction

Many of the studies on the mechanism of infection and the resistance to FHB have been done using wheat. PUGH *et al.* (1933) found that the plants are infected during a short period around the time of flowering, the remains of anthers in the glume being infected first, and then growing hyphae invading into the grains. According to many studies conducted thereafter, it is generally believed that the infection occurs at the time of flowering or shortly before or after flowering.

The optimal temperature for the growth of the causal fungus of FHB is about 25ºC, and water is required for the scattering and germination of spores. Thus, the weather condition around the time of flowering is a critical factor for the outbreak of FHB. In fact, in humid areas such as the southwestern warm district of Japan and downstream basin of the Yangtze River in China, this disease is a major problem for the cultivation of barley and wheat. In the field of barley and wheat in North America, zero-tillage is spreading to prevent soil erosion and to save energy. However, the promotion of zero-tillage results in the increase of damages by FHB in barley and wheat, because plant remains are accumulated on the field surface, and FHB spreads on it.

It is not easy to evaluate accurately the resistance to FHB in the field, because the degree of infection varies with the flowering time and with the timing of rainfall and the flowering time. Therefore, we developed a highly accurate testing method of FHB, considering the evaluation of a large number of genetic resources and the screening of individuals in the breeding process. Then, highly resistant varieties were selected from the varieties collected from all over the world, by evaluating the resistance to FHB using that method. To clarify the presence or absence of race differentiation in the FHB, the host-pathogen relationship was analyzed. The inheritance of the resistance to FHB was analyzed using diallel crosses and segregating populations. And also quantitative trait loci (QTL) were detected in recombinant inbred (RI) lines. The present study includes following five parts:

A. Establishing the Testing Method and Search for the Resistant Varieties
Since Fusarium infects the spikes at around the time of flowering, we have to inoculate the plants at the flowering. However, it is not easy to adjust the time of flowering in many varieties. Here, the 'cut-spike' inoculation method that enables definite inoculation at the time of flowering has been developed.

B. Host-Pathogen Relationship
Because the resistance to the disease in plants is expressed as the interaction between the host and pathogen, the disease reaction varies with the combination of resistant gene of the host and the non-virulent gene of the pathogen (FLOR 1956). However, since Fusarium fungus is not considered to have striking host-specificity. Therefore, the presence or absence of the differentiation of parasitic behavior in the pathogen of FHB was examined.

C. Diallel Analysis of the Resistance
The resistance to FHB in barley is modified by many factors. Therefore, the resistance to this disease is considered to show quantitative inheritance to which many genes are involved. Therefore, in this study, the resistance to FHB of F_1 hybrids obtained by the diallel cross was analyzed to determine the inheritance of the resistance to FHB.

D. Selection Response of the Resistance
Diallel analysis revealed that the FHB resistance is under the control of additive-dominance gene system. Therefore segregation mode and selection response of FHB resistance were examined using several F_2 populations.

E. QTL Analysis of FHB Resistance
As mentioned above, FHB resistance seemed to be controlled by quantitative genes. Therefore the QTL analysis was conducted to detect the causal genes on the barley chromosome.

Material and Methods
A-1. Varietal Variation
Generally, FHB breaks out after the infection within a short period around the time of flowering, and high humidity near saturation is necessary for the germination of spores and infection. Therefore, the following method, by which the pathogen causes infection exactly at the time of flowering in a large number of plants with a different flowering time, has been developed.
A total of 4,881 barley varieties were divided into three groups and grown in the field in three years. The plants were grown by the ordinary method. At the flowering time, spikes were detached from the plants at the second internode from the top, and arranged in pans with

overflowing water. The use of a test-tube stand is recommended. The ascospores or conidia are suspended at a rate of about 15 spores per 200× microscope view, and sprayed onto the spikes.

The spikes were kept at 100% humidity for two days after the inoculation in a vinyl tent set in a phytotron controlled at 25ºC, and then placed in a phytotron at 18ºC and 95% humidity for six days. They were irradiated at 5,000~10,000 lux for 14 hours a day. Eight days after the inoculation, the percentage of infected spikelets was examined to determine the resistance to FHB.

A-2. Screening of Resistant Varieties

The resistant varieties were selected by the three-step screening method as shown in the result.

B. Host-Pathogen Relationship

From about 250 Fusarium strains preserved at the Barley Germplasm Center, Okayama University, 20 strains were used in this study. Twenty barley varieties from different sources and with different FHB resistance were selected from about 5,000 varieties preserved at our Institute, and used as the host barley.

C. Diallel Analysis of the Resistance

F_1 hybrids obtained by the reciprocal diallel cross of eight (four two-rowed and four six-rowed) and their parents were grown with two replication and were tested for FHB resistance by the 'cut-spike' inoculation method. The percentage of infected florets was transformed in to the resistance score: 0; 0%, 2; 1-5%, 4; 6-20%, 6; 21-40%, 8; 41-100%. The variance of resistance score was analyzed by HAYMAN (1954).

D. Selection Response of the Resistance

F_1 hybrids were obtained by the cross between CI3725 (two-rowed, susceptible) and four varieties of six-rowed moderately resistant varieties. 240–270 plants of F_2 population from each cross combination were tested for the resistance to FHB by the 'cut-spike' inoculation method. A total of 409 plants of F_2 population crossed between Russia 6 (two rowed, highly resistant) and HES 4 (six-rowed, highly susceptible) were also tested for the resistance to FHB. From each F_2 population, 5~20 plants with the highest resistance and 13~31 plants with the highest susceptibility were selected, and in the next year, about 15 plants from each F_3 line were grown and tested for their resistance to FHB to examine the selection efficiency.

E. QTL Analysis of FHB Resistance

A total of 93 recombinant inbred (RI) lines derived from the cross between Russia 6 (two-rowed, highly resistant) and H.E.S. 4 (six-rowed, highly susceptible) were scored for FHB resistance by 'cut-spike' inoculation method. A high density map with 1,172 loci（1,595.7cM） was constructed in this population (HORI *et al.* 2003). The simple interval mapping (SIM) and the composite interval mapping (CIM) were conducted to locate the QTL on the barley chromosomes.

Results and Discussion

A-1. Varietal Variation

FHB resistance score showed a continuous variation: many of the accessions expressed moderately to high sensitivity to FHB. On average two-rowed varieties were more resistant than six-rowed ones. Some varieties from East Asia and Europe showed low score (resistance) to FHB (TAKEDA & HETA 1989).

A-2. Screening of Resistant Varieties

First: The top 259 resistant varieties were selected from 4,881 barley varieties collected from all over the world and preserved at the Research Institute for Bioresources, Okayama University, and other 18 elite varieties selected from 1,515 varieties by co-workers were included (TAKEDA & HETA 1989).

Second: The top 277 varieties selected by the first screening were again evaluated by the 'cut-spike' inoculation method three or four times. The top 85 resistant varieties with stable resistance were selected.

Third: The top 85 varieties selected by the second screening were cultivated under eight environmental conditions with different sowing seasons, fertilizer levels and soil characters (upland or paddy field), and evaluated by the 'cut-spike' inoculation method. In addition, the same varieties were cultivated by the ordinary method in the field, and evaluated twice by the 'mist-field' inoculation method in which the plants were sprayed with mist sprinkler after heading (TAKEDA & HETA 1989). Finally, the top 23 varieties were selected as the FHB resistant varieties (Table 1).

Table 1. List of barley varieties resistant to FHB

Ear type	Varieties	
Two-row	A 008 Santana	J 211 Asahi 21
covered	J 216 Seijo 11	J 217 Aichi Wase Golden
Dense	J220 Fuji Nijo	J 812 Kyoto Nakate
	J 819 Kanto Nijo 2	U 121 Sirius 0-525
	U 634 Germany 11	U 689 Kombainiesis
	CI4196	Asahi 5
	Frederickson	Imperial
	Primus	Russia 6
Two-row	C 649 Harbin 2-row	U 158 Horni Peseky 2
covered	U 389 Maja	U 434 Niedzica 1
Lax	CI8826	CI187 Svanhals

B. Host-Pathogen Relationship

Table 2 shows the analysis of variance for the infection rate. The differences among varieties and Fusarium strains were significant at high levels, respectively, but the interaction between the barley varieties and Fusarium strains was not statistically significant. Such absence of a significant interaction between barley varieties and Fusarium strains shows that resistance is not reversed by the combination of barley variety and Fusarium strains.

C. Diallel Analysis of the Resistance

The score of the parents varied from highly resistant to highly susceptible. Table 3 shows the results of the analysis of variance by the method of HAYMAN (1954). The variance of item a, which shows the additive effect of genes, was the largest, and was significant at the 0.1%

Table 2. Analysis of variance for the persentage of infected florets in 20 barley varieties inoculated with 20 Fusarium strains

Source of Variarion	df	MS
Replication	1	443.5*
Barley varieties (V)	19	10,145.0***
Fusarium strains (S)	19	4,627.7***
V×S	361	107.1
Error	399	102.7

* , ***: Significant at the 5% and 0.1% levels, respectively.

Table 3. Analysis of variance for the diallel table of the score of resistance to FHB

Item	df	MS
a	7	19.138***
b	28	2.414**
b_1	1	0.844
b_2	7	2.271*
b_3	20	2.543**
c	7	1.124
d	21	0.875
Block	1	5.483*
Error	63	0.986

*,** and ***: Significant at the 5%, 1% and 0.1% levels, respectively.

level. Item b, which shows a dominance effect, was significant. Since this effect mainly depends on items b_2 and b_3, the dominance effect may be attributed to the specific parents or specific combination. The maternal effect (c and d) was not significant. The heritability of the resistance to FHB estimated from the variance components was 0.60 in the broad sense and 0.45 in the narrow sense. The average dominance was about 0.8, showing an incomplete dominance.

D-1. Selection Response of the Resistance in F_2 Population

The variation of the scores of resistance in five F_2 populations was continuous in all five cross combinations showing the resistance to FHB is under the control of quantitative genes. Since each F_2 population was segregated for ear types, the mean score of the resistance for each ear type was compared to examine whether the resistance to FHB is related to the ear type. In all cross combinations, the two-rowed group were significantly more resistance than the six-rowed group, indicating that the ear-type gene may secondarily affect the resistance to FHB.

D-2. Selection Experiment

The selection differential of the resistance score was 5.2~6.1, and the genetic gain was 1.1~1.7, and the heritability estimated from the ratio of the genetic gain to the selection differential was 0.21~ 0.32 (0.253 on the average) indicating the heritability of the trait is low in the early segregating generation.

E. QTL Analysis of FHB Resistance

As reported in detail in this symposium (HORI *et al.* 2004), three QTLs for FHB resistance were detected: two on the chromosome 2H and one on the 5H. Former two corresponded to the gene for row type (*vrs1*) and the gene for flowering type (cleistgamy), respectively, suggesting the row type and flowering type are important factor determining FHB resistance. The QTL on the 5H was independent of the genes reported so far. These three QTLs were responsible for about 40% of the total variation of FHB resistance again indicating the heritability of the trait is not very high.

References

FLOR H. H. (1956): The complementary genetic systems in flax and flax rust. Adv. Genet., **8**: 29-54. Acad. Press. New York.

HAYMAN B. (1954): The analysis of variance of diallel tables. Biometrics, **10**: 235-244.

HORI K., KOBAYASHI T., SIMIZU A., SATO K., TAKEDA K., KAWASAKI S. (2003): Efficient construction of high density linkage map and its application to QTL analysis in barley. Theor. Appl. Genet., **107**: 806-813.

HORI K., KOBAYASFHI T., SATO K., TAHKEDA K. (2004): QTL analysis of resistance to Fusarium head blight in barley RI Populations. Proc. 9th IBGS (in press)

PUGH C. W., JOHAN H., DICSON J. (1933): Factors affecting infection of wheat heads by *Gibberella saubinetii* J. Agn. Res., **46**: 771-797.

SELBY A. D. (1898): Some disease of wheat and oats. Ohio Agr. Expt. Sta. Bull., **97:** 40-42.

TAKEDA K., HETA H. (1989): Establishing the testing method and a search for the resistant varieties to Fusarium head blight in barley. Japan. J. Breed., **39**: 203-216.

Variation for Resistance to *Fusarium* Head Blight in Spring Barley

H. Buerstmayr[1], L. Legzdina[2], B. Steiner[1] and M. Lemmens[1]

[1]University of Natural Resources and Applied Life Sciences Vienna, Department for Agrobiotechnology Tulln, Division of Biotechnology in Plant Production. Konrad Lorenz Str. 20, A-3430 Tulln. www.ifa-tulln.ac.at, Email: hermann.buerstmayr@boku.ac.at;
[2]Priekuli Plant Breeding Station, Zinatnes Str. 1a, Priekuli LV-4126, Latvia

Abstract
Fusarium head blight (FHB) is a fungal disease of barley and other cereals, causing substantial yield and quality losses, mainly due to the contamination of the harvest with mycotoxins. We evaluated genetic variation for resistance to FHB and its association with other plant characters in diverse barley germplasm in order to identify useful lines for resistance breeding. The 143 barley lines consisted of 88 current European spring barley lines and cultivars, 33 accessions from the genebank at IPK Gatersleben, and 22 lines obtained from North American institutions. We conducted artificially inoculated field experiments with *Fusarium graminearum* Schwabe during two seasons. FHB severity was evaluated by repeated assessment of visual symptoms. On a set of 49 lines the content of the mycotoxin deoxynivalenol (DON) was analyzed. Variation for FHB severity was quantitative. The lines with lowest FHB severity were 'CIho 4196' and 'PI 566203'. Also within the European spring barley collection variation for FHB severity was highly significant. There was a significant negative correlation between plant height and FHB severity (r=-0.55). FHB severity assessed in the field and the amount of deoxynivalenol in the harvested grains were positively correlated (r=0.87). Several lines with a useful level of FHB resistance were found or confirmed and are recommended as crossing partners.
Keywords: *Fusarium graminearum*; deoxynivalenol; head blight; resistance

Introduction

Fusarium head blight (FHB, scab) is a widespread disease of cereals including wheat (*Triticum* spp.) and barley (*Hordeum vulgare* L.). The risk of FHB infection is high when warm and humid weather occurs around flowering. Apart from direct yield losses, the most serious concern is the contamination of the crop with mycotoxins. Barley lots contaminated with deoxynivalenol (DON) may be rejected by the malting and brewing industry (URREA *et al*. 2002). Agronomic and chemical measures are of only limited efficacy to control the disease. Genetic resistance offers the greatest potential for reducing FHB. Whereas in North America large collections of barley have been evaluated for potential sources of resistance to FHB (PROM *et al.* 1996; SCHOLZ *et al.* 1999; STEFFENSON & SCHOLZ 2001; SKOGLUND & MENERT 2002), Europe is lagging behind in this respect. This study comprises the first systematic survey of FHB resistance in current European spring barley germplasm in comparison to landraces and old cultivars obtained from the genebank at IPK Gatersleben and to lines obtained from overseas breeding programs. Additional results and a more extensive discussion on the same experiment will be published elsewhere (BUERSTMAYR *et al*. 2004).

Material and Methods

Plant Material

The 143 spring barley lines consisted of (1) 88 two-row lines or cultivars originating from breeding programs in Europe. These lines can be considered a random sample of lines adapted to European growing conditions and varying in malting quality. (2) 16 two-row and 17 six-row accessions from the genebank at IPK Gatersleben representing mostly landraces and old cultivars from a wide range of collection sites. (3) 22 lines obtained from North Dakota State University (NDSU) or the University of Minnesota (UMN), including breeding lines with US origin and germplasm introductions from other regions. For simplicity we will refer to these lines as the material obtained from North America in the following text.

Field Experiments for Evaluation of Fusarium Head Blight Resistance

The barley lines were evaluated during two seasons (2001 and 2002) at the experimental field of IFA-Tulln, Austria. The average temperature and annual precipitation were 9.4°C and 632 mm (2001) and 10.2°C and 672 mm (2002). The experimental layout was a randomized complete block design with three blocks. Sowing time was the first half of March in both years. The blocks were purposely sown several days apart, resulting in 1 to 3 days difference in anthesis between replications. Plots consisted of double rows with 17-cm row spacing and 1-m length. Sowing and crop management were done according to local procedures.

Macroconidia of the *F. graminearum* single-spore isolate 'IFA-65' were prepared as described by BUERSTMAYR *et al.* (2002). Inoculations were performed individually on each plot when > 50% of the plants had reached anthesis, and repeated 2 days later by spraying 100 ml inoculum [$5x10^4$ spores ml^{-1}] on each plot. Inoculations were carried out in the evenings on alternate days. An automated mist irrigation system was used to maintain humidity and keep the plants wet for 20 h after inoculation. In each plot the percentage of visibly infected spikelets was scored according to a linear 0 to 100% scale on a whole plot basis on days 10, 14, 18, 22, and 26 after inoculation and used to calculate the area under the disease progress curve (AUDPC) as an integrated measure of FHB severity (BUERSTMAYR *et al.* 2000). In 2002, 49 entries representing a wide range in FHB severity were chosen for harvesting using a Wintersteiger plot combine. The threshed samples were cleaned manually and grain from the three blocks was pooled in equal amounts and milled. Sample preparation and analysis of the trichothecene mycotoxin deoxynivalenol (DON) was performed as described by KRSKA *et al*. (2001).

Other Traits

The number of days from 1 May to anthesis was recorded. At the beginning of grain ripening, plant height was measured from the soil surface to the top of the heads, excluding awns. Every genotype was classified for ear type (two-row or six-row).

Statistical Analysis

The field data were analyzed as randomized complete block design over two years using the GLM procedure of SAS/STAT (SAS Institute 1989). Broad-sense heritabilities were estimated from variance components according to NYQUIST (1991). Phenotypic correlation coefficients were calculated with the CORR procedure of SAS/STAT (SAS Institute 1989).

Results

Fusarium Head Blight Severity

The barley lines showed continuous variation for percentage of diseased spikelets at all observation dates and for AUDPC. To illustrate the variation in disease severity, Figure 1 shows a frequency distribution of the AUDPC line mean values combined for the three groups of genotypes. Table 1 displays the line mean values of AUDPC for all 143 genotypes sorted in ascending order. As expected, many of the barley lines obtained from North America fell into the more resistant classes. The most resistant lines were 'CIho 4196' with an average of 26 AUDPC units (3% diseased spikelets 26 days after inoculation) and 'PI 566203' (30 AUDPC units) whereas the most susceptible was the six-row genebank accession 'HOR 214'. The European two-row spring barley germplasm showed a broad range in variation from as low as 82 AUDPC units (5.3% infected spikelets 26 days after inoculation) for the cultivar 'Hellana' up to 476 AUDPC units (42.5% diseased spikelets) for the cultivar 'Fontana'. The variation among the genebank accessions was also large, ranging from 72 AUDPC units (7.4% diseased spikelets) for 'Lubicki' to 704 AUDPC units (77% diseased spikelets) for 'HOR 214'.

Analysis of variance revealed highly significant *F*-values for the factor lines for all measured traits ($p<0.0001$) and significant line-by-year interactions for AUDPC ($p=0.05$), plant height and flowering date ($p<0.001$). Broad sense heritability was high for AUDPC ($H=0.81$) and very high for plant height ($H=0.96$) and flowering date ($H=0.96$).

Relation between FHB Severity and Other Traits

There was a significant difference between the mean performance of the two-row and that of the six-row lines. At 26 days after inoculation, the mean disease severity of the 120 two-row lines was 19.6% but that of the 23 six-row lines was 38.6%. Several of the six-row barley lines were amongst the most diseased ones (Table 1). The correlation between FHB severity (AUDPC) and plant height was significant and negative ($r=-0.55$, $p<0.0001$). There was no significant correlation between flowering date and AUDPC across all lines ($r = -0.14$, $p=0.08$).

Relation between FHB Severity and Trichothecene Content

The average level of DON in the samples was 14.5 mg kg^{-1} ranging from 1.5 mg kg^{-1} to 35.2 mg kg^{-1}. The line with the lowest DON content was the six-row hulless genebank accession 'HOR 1867' (='Boehmische Nackte'). The line with the highest DON content was the two-row cultivar 'Bartok'. Based on this set of 49 lines, sampled in one year but representing a wide range of FHB severity values, the correlation between DON content and FHB severity assessed in the field (AUDPC) was high ($r=0.87$, $p<0.001$) (Figure 2).

Discussion

The high heritability values indicate that (1) the genetic variation among the lines was large and (2) that the FHB resistance evaluation was highly reproducible across two years. Variation for FHB severity was clearly quantitative among the evaluated material. As expected, most of the lines obtained form the North American breeding programs were among the more resistant ones. The apparently most resistant line in this study was 'CIho 4196', which was described as highly resistant already by TAKEDA (1992), PROM *et al.* (1996) and URREA *et al.* (2002). Further lines with low FHB severity means were 'PI 566203', 'Fredrickson', 'Svanhals' and several breeding lines obtained from the NDSU barley program. Within the lines from the genebank and

Table 1. Mean values of *Fusarium* head blight severity measured by area under the disease progress curve (AUDPC) of 143 spring barley lines evaluated across 2 years, sorted in ascending order of AUDPC values. Lines are described by group (gr: E = European spring barley, A=spring barley obtained from North America, G = spring barley accession from IPK genebank) and spike morphology (sm: 2-row or 6-row).

Line	gr	sm	AUDPC
CIho 4196	A	2	26
PI 566203	A	2	30
Fredrickson	A	2	43
Svanhals	A	2	43
F102-61	A	2	49
F103-53	A	2	57
ND 16461	A	2	62
F103-79	A	2	70
F103-52	A	2	71
Lubicki	G	2	72
Misato Golden	G	2	75
Hellana	E	2	82
Chevron	A	6	83
GOB96DH	A	2	99
Pixel	E	2	100
HOR 1867	G	6	104
Bios 1	G	2	104
Pallas	G	2	106
Secura	E	2	107
Thuringia	E	2	109
Forum	E	2	118
Gobernadora	A	2	118
Amalia	E	2	120
Robust	A	6	122
MN Brite	A	6	124
Sissy	E	2	130
Atahualpa 92	A	2	133
HOR 10903	G	2	143
Sigrid	E	2	144
Baronesse	E	2	146
Shyri	A	2	146
Steffi	E	2	146
ND 18364	A	2	149
Ohara	E	2	149
Selecta	E	2	151
AC Oxbow	A	2	155
Prosa	E	2	156
Stander	A	6	165
HOR 1895	G	2	171
HOR 1391	G	2	171
Orthega	E	2	173
GS 1852	E	2	175
Sarah	G	2	183
ND 18366	A	2	185
Heris	E	2	189
Wolfe	G	6	189
Emir	G	2	192
Angi	E	2	192
HOR 10986	G	2	192
Peggy	E	2	192
Eunova	E	2	196
Henni	E	2	196
Taiga	E	2	197
Charmant	E	2	198
Ria	E	2	198
Elisa	E	2	199
Sudan	G	6	199
Adagio	E	2	199
Danuta	E	2	201
Neruda	E	2	202
Sally	E	2	204
Krona	E	2	212
M95-4	A	6	213
Jersey	E	2	218
Viskosa	E	2	218
Risk	G	2	220
HOR 5650	G	2	222
Baccara	E	2	222
M95-1	A	6	229
Amulet	E	2	231
Astoria	E	2	231
Barke	E	2	232
Derkado	E	2	233
Penelope	E	2	235
GS 1916	E	2	236
HOR 10860	G	2	239
Delibes	E	2	244
Chime	E	2	247
Brenda	E	2	250
GS 1914	E	2	251
Madras	E	2	251
Meltan	E	2	253
Bizet	E	2	257
Tolar	E	2	260
Morgenrot	G	2	268
GS 1899	E	2	269
Nordus	E	2	272
Jacinta	E	2	272
Akzent	E	2	275
STRG 669/98	E	2	276
Kompakt	E	2	277
GS 1915	E	2	279
Hanka	E	2	279
Prominant	E	2	284
Annabell	E	2	286
Maresi	E	2	291
Sabel	E	2	291
Viva 1	E	2	296
Helmi	G	2	304
Century	E	2	305
Ortoli	E	2	306
Madonna	E	2	308
Decanter	E	2	309
HOR 1868	G	6	316
GS 1897	E	2	317
Scarlett	E	2	317
Olbram	E	2	324
HOR 766	G	6	330
HOR 465	G	6	331
Chalice	E	2	333
Aspen	E	2	336
Alliot	E	2	341
HOR 2849	G	2	341
Model	E	2	342
Chariot	E	2	342
Video	E	2	344
Betty	E	2	345
Hydrogen	E	2	345
GS 1913	E	2	346
Madeira	E	2	348
Charlotte	E	2	348
HOR 9551	G	6	350
Pasadena	E	2	368
Extract	E	2	374
HOR 3715	G	6	379
GS 1898	E	2	381
Ricarda	E	2	388
HOR 2567	G	6	401
HOR 1886	G	6	402
GS 1854	E	2	402
Saloon	E	2	405
Pejas	E	2	405
Ogalitsu	G	6	421
Tavern	E	2	433
Bartok	E	2	438
Chantal	E	2	439
HOR 1475	G	6	441
HOR 9726	G	6	463
HOR 7447	G	6	474
Fontana	E	2	476
2-4	G	6	539
Rupee	G	6	540
HOR 214	G	6	704
Mean			**244**
LSD 5%			144

the current European spring barleys a wide range of variation was evident. The two-row genebank lines 'Lubicki' and 'Misato Golden' were among the moderately resistant lines. Within the European spring barley collection 'Hellana', 'Pixel', 'Secura' and 'Thuringia' were those with relatively low FHB severity, however with 2-3 times larger AUDPC values than the most resistant lines. The least diseased six-row lines were 'Chevron', which is an old Swiss cultivar and 'HOR 1867', which is the old hulless barley cultivar 'Boehmische Nackte' from Bohemia (the Czech Republic). The general trend that six-row barley tended to be more diseased than two-row barley is in agreement with other studies (MESFIN *et al.* 2003).

The barley lines showed considerable variation in flowering date. Several lines from the American breeding programs and the genebank tended to be earlier in flowering than the majority of the European lines. Although the inoculation period stretched over almost 3 weeks there was no significant bias of the FHB severity data in favor of early or late flowering lines. We therefore do not expect serious difficulties in combining earliness with FHB resistance, at least for central European growing conditions. This result is in disagreement with studies conducted in the United States, which reported that late heading was frequently associated with lower FHB severity (STEFFENSON *et al.* 1996; URREA *et al.* 2002).

Plant height was significantly correlated with FHB severity. Taller lines tended to be less diseased than shorter lines. This can partly be explained by the fact that many of the overseas resistant lines were tall, but the more susceptible European lines were shorter on average. In addition the significant correlation between FHB severity and plant height was also evident within the European material. This phenomenon appears to be a common feature reported in several studies (DE LA PENA *et al.* 1999; ZHU *et al.* 1999; MA *et al.* 2000; URREA *et al.* 2002; MESFIN *et al.* 2003).

Certainly the most important issue in FHB resistance breeding is the development of lines with low FHB severity and low mycotoxin contamination. The mycotoxin levels observed here were very high. This may be explained by the fact that artificial inoculation led to a severe disease pressure. In addition the isolate used is known to be a potent trichothecene producer. Even though the most resistant line 'CIho 4196' showed an average of only 3% FHB infected spikelets, it contained 2.9 mg kg^{-1} DON in 2002, which is well above acceptable levels. The hulless barley line 'HOR 1867' (='Boehmische Nackte'), which was among the moderately resistant lines, had the lowest toxin contamination (1.6 mg kg^{-1} DON). LEGZDINA & BUERSTMAYR (2004) reported that hulless barley lines appeared to accumulate less mycotoxins in the harvested grain than hulled barley lines. The correlation between FHB severity and the content of trichothecene toxin DON was high. Selection for low disease symptoms should lead to a simultaneous reduction in contamination with DON.

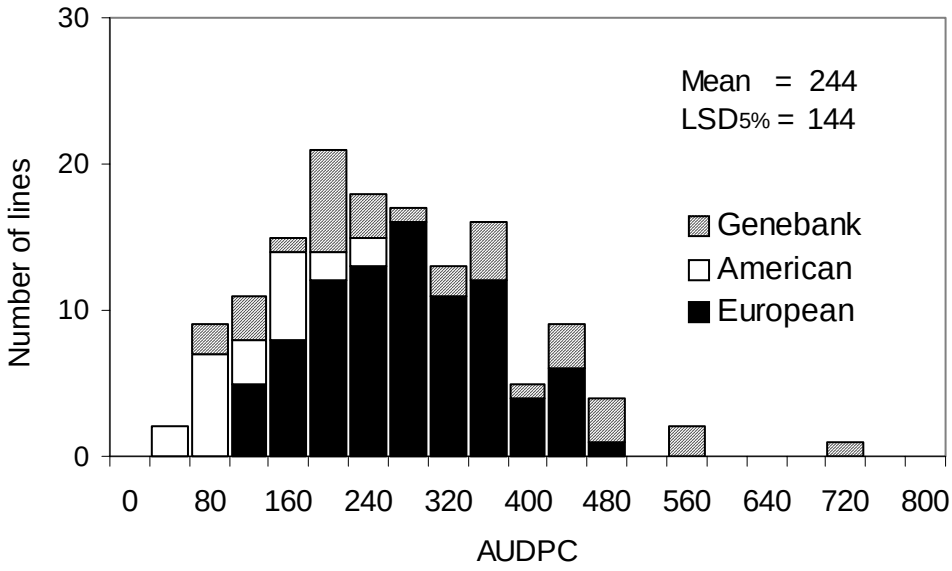


Figure 1. Histogram of distribution of barley lines for FHB severity measured by AUDPC mean values across two years

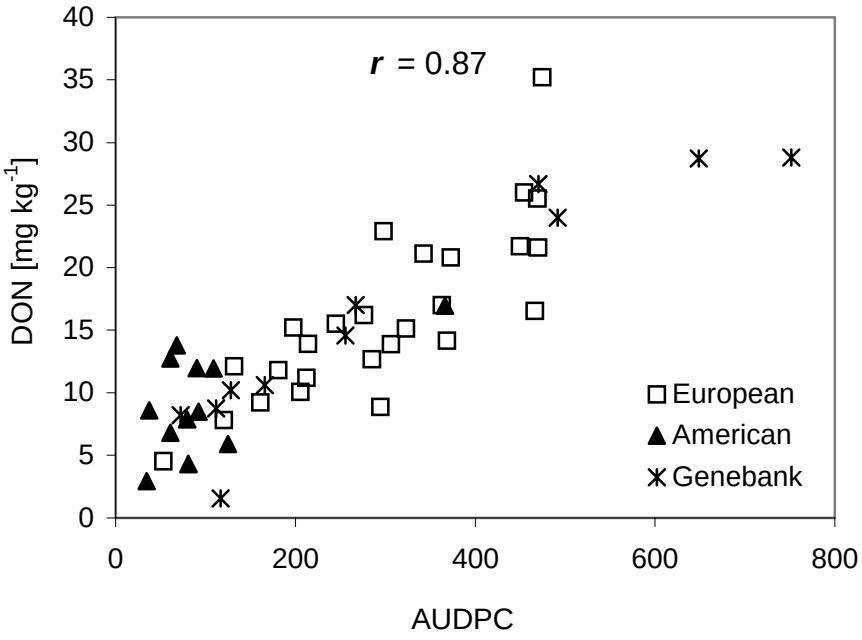


Figure 2. Correlation between AUDPC and DON content (in mg kg^{-1}) among 49 spring barley lines evaluated in 2002

Acknowledgements
We gratefully acknowledge Ruth Dill-Macky (University of Minnesota, USA), Claus Einfeldt (Saatzucht Ackermann, Germany), Andreas Fleck (Saatzucht Donau, Austria), Jerry Franckowiak (North Dakota State University, USA), Andreas Graner (Genebank IPK Gatersleben, Germany), and Uwe Scholz (IFA-Tulln, Austria) for supplying the barley lines. We also thank Matthias Fidesser for his skillful technical assistance on the field, and we gratefully acknowledge Rudolf Krska and Alexandra Parich for their guidance in the mycotoxin analysis. We also like to thank Diane Mather (McGill University, Canada) for her review and suggestions in improving the manuscript.

References

BUERSTMAYR H., STEINER B., LEMMENS M., RUCKENBAUER P. (2000): Resistance to Fusarium head blight in two winter wheat crosses: heritability and trait associations. Crop Sci., **40**: 1012-1018.

BUERSTMAYR H., LEMMENS M., HARTL L., DOLDI L., STEINER B., STIERSCHNEIDER M., RUCKENBAUER P. (2002): Molecular mapping of QTLs for Fusarium head blight resistance in spring wheat. I. Resistance to fungal spread (Type II resistance). Theor. Appl. Genet., **104**: 84-91.

BUERSTMAYR H., LEGZDINA L., STEINER B., LEMMENS M. (2004): Variation for resistance to Fusarium head blight in spring barley. Euphytica (*in press*).

DE LA PENA R.C., SMITH K.P., CAPETTINI F., MUEHLBAUER G.J., GALLO-MEAGHER M., DILL-MACKY R., SOMERS D.A., RASMUSSON D.C. (1999): Quantitative trait loci associated with resistance to Fusarium head blight and kernel discoloration in barley. Theor. Appl. Genet., **99**: 561-569.

KRSKA R., BAUMGARTNER S., JOSEPHS R. (2001): The state – of – art in the analysis of type-A and –B trichothecene mycotoxins in cereals. Fresenius Journal of Analytical Chemistry, **317**: 285-299.

LEGZDINA L., BUERSTMAYR H. (2004): Comparison of hulless and covered barley for infection with Fusarium head blight and accumulation of mycotoxins in grain. J. Cereal Sci. (*in press*).

MA Z., STEFFENSON B.J., PROM L.K., LAPITAN N.L.V. (2000): Mapping of quantitative trait loci for Fusarium head blight resistance in barley. Phytopathology, **90**: 1079-1088.

MESFIN A., SMITH K.P., DILL-MACKY R., EVANS C.K., WAUGH R., GUSTUS C.D., MUEHLBAUER G.J., (2003): Quantitative trait loci for Fusarium head blight resistance in barley detected in a two-rowed by six-rowed population. Crop Sci., **43**: 307-318.

NYQUIST W.E. (1991): Estimation of heritability and prediction of selection response in plant populations. Crit. Rev. Pl. Sci., **10**: 235-322

PROM L.K., STEFFENSON B., SALAS T.G., FETCH JR., CASPER H.H. (1996): Evaluation of selected barley accessions for resistance to Fusarium head blight and deoxynivalenol concentration. In: SLINKARD A. *et al.* (ed.): Proc. 7th Int. Barley Genetics Symp. Univ. Extension Press, Saskatoon, Saskatchewan: 764-766.

SAS INSTITUTE INC. (1989): SAS/STAT User's Guide, Version 6, 4th Edition, Cary, NC.

SCHOLZ, U., STEFFENSON B., URREA C., HORSLEY R. (1999): Evaluation of six rowed spring barley accessions for resistance to Fusarium head blight. In: WAGESTER J. *et al.* (ed.): Proc. 1999 National FHB Forum. University Printing, East Lansing, MI, USA: 137-139.

SKOGLUND, L.G., MENERT J.L. (2002): Evaluation of the national small grains collection of barley for resistance to Fusarium head blight and deoxynivalenol accumulation. In: CANTY S. *et al.* (ed.): Proc. 2002 National FHB Forum. University Printing, East Lansing, MI, USA: 213-216.

STEFFENSON B.J., SCHOLZ U. (2001): Evaluation of *Hordeum* accessions for resistance to Fusarium head blight. In: CANTY S. *et al.* (ed.): Proc. 2001 National FHB Forum. University Printing, East Lansing, MI, USA: 208-211.

TAKEDA K. (1992): Current topics on the scab disease resistance in barley and wheat. Proc. Jpn. Assoc. Mycotoxicol., 3**6**: 13.

URREA C.A., HORSLEY R.D., STEFFENSON B.J., SCHWARZ P.B. (2002): Heritability of Fusarium head blight resistance and deoxynivalenol accumulation from barley accession CIho 4196. Crop Sci., **42**: 1404-1408.

ZHU H., GILCHRIST L., HAYES P., KLEINOFS A., KUDNRA D., LIU Z., PROM L., STEFFENSON B., TOOJINDA T., VIVAR H. (1999): Does function follow form? Principal QTLs for Fusarium head blight (FHB) resistance are coincident with QTLs for inflorescence traits and plant height in a doubled-haploid population of barley. Theor. Appl. Genet., **99**: 1221-1232.

Evaluation of Barley Resistance to *Fusarium* Head Blight Infection and Mycotoxin Contamination of Grain

V. Šíp[1], L. Tvarůžek[2], J. Chrpová[1], S. Sýkorová[1] , L. Leišová[1] , L. Kučera[1] and J. Ovesná[1]

[1]Research Institute of Crop Production, Drnovská 507, CZ-161 06 Prague –Ruzyne, Czech Republic
[2]Agricultural Research Institute, Havlíčkova 2787, CZ- 767 01 Kroměříž, Czech Republic

Abstract

The results are based on field infection trials with six spring barley cultivars registered in the Czech Republic and resistance sources Chevron and CI 4196. In experiments at two locations (Ruzyně, Kroměříž) lasting for two years two inoculation techniques were applied (spraying and brushing of inoculum). Four isolates of *Fusarium culmorum* and *Fusarium graminearum* were used for inoculations by brushing technique at the location Ruzyně. Highly significant and positive correlations between determinations of DON content by ELISA and GC method were obtained for all isolates. One isolate of *F. graminearum* was predominant nivalenol producer, while the other isolates were deoxynivalenol (DON) producers. From the other mycotoxins 3-AcDON was found in grain at relatively higher concentration, particularly after inoculation with *F. culmorum* isolates. Significant cultivar differences in DON content, examined yield traits, percentage of *Fusarium* colonies and percentage of non-sprouting grains were detected after spraying of inoculum in two terms and mist irrigation of infected plots. After inoculation with aggressive isolate of *F. culmorum* Chevron and CI 4196 showed high resistance and Jersey, Olbram and Scarlett moderate resistance to accumulation of DON in grain. Treatment with fungicide Horizon 250 EW (active ingredient tebuconazole) led on average to 52.5 % reduction of DON content, but the effectiveness of fungicide treatment was highly influenced by year and cultivar. Fungicide treatment did not have significant effect on grain weight per spike and, in general, affection of examined yield traits by infection was in these experiments low. Low DON content was associated with low percentage of *Fusarium* colonies in grains, but this trait was highly affected by location and year. It was detected close correspondence between accumulation of DON in grain and parameter C_T Fus (transformed) from quantitative real time PCR analysis. It was possible with the developed PCR system to specify clearly cultivar response to infection and effects of fungicide treatment. Due to often unclear symptoms of infection and inconsistent effects on yield traits in barley, this analysis, which is able to identify causal organism, can be reckoned as very helpful to screening for disease resistance and disease control in practice.

Keywords: spring barley; *Fusarium culmorum*; *Fusarium graminearum;* head blight; DON content; yield traits; cultivar resistance; fungicide treatment; quantitative PCR analysis

Introduction

Since the beginning of the twenties century *Fusarium* head blight (FHB) of barley has been recognized as a devastating disease in periods of epidemics. Significant losses to barley producers were reported in several states of the USA (JONES & MIROCHA 1999) and Canada (TEKAUZ *et al.* 2000; CAMPBELL *et al.* 2000). This pathogen has reduced both the yield and quality of barley and has raised food safety problems due to the contamination of grain by mycotoxins. FHB significantly affects the malting and brewing quality of barley

grain. Perhaps the most insidious problem caused by *Fusarium* is beer gushing (STEFFENSON 1998).

In some regions the species *Fusarium graminearum* was found to be the principal pathogen of barley heads (SALAS *et al.* 1999). This toxicogenic pathogen now becomes more important also in conditions of Central and Northern Europe (SÝKOROVÁ *et al*. 2003; WAALWIJK *et al*. 2003), besides *Fusarium culmorum*. Both species were reported to be prevalent producers of deoxynivalenol (DON), but also the ability to produce nivalenol (NIV) or acetyldeoxynivalenol (3-AcDON or 15-AcDON) has been recognized in some isolates of both *F. culmorum* and *graminearum* (MIROCHA *et al*. 1994; PERKOWSKI *et al*. 1997; SALAS *et al.* 1999; CHELKOWSKI *et al*. 2000).

Four primary strategies for the control of FHB include biological, cultural, chemical, and genetic (STEFFENSON 1999). Development of a biological agent to effectively control FHB (suppress the pathogen) is undoubtedly a difficult task and also cultural practices that rely on reduction of the amount of *Fusarium* inoculum residing in crop debris cannot guarantee sufficient protection. Fungicides based on tebuconazole and metconazole were reported to suppress FHB and accumulation of mycotoxins (MAGAN *et al.* 2002), however, the protection by this means is generally not yet sufficient both in barley and wheat (Jones 2000; MESTERHÁZY *et al.* 2003; ŠÍP *et al.* 2004). The deployment of barley cultivars with genetic resistance is the most cost effective and environmentally sound means of controlling FHB. It is important that valuable sources of resistance to FHB and the accumulation of DON have been detected when evaluating barley germplasm. Particularly Chevron, a six rowed, nonmalting barley originating from Switzerland, CI 4196 (a landrace from China) and Svanhals (a landrace from Sweden) exhibited low levels of FHB and DON under epidemic conditions of North Dakota (STEFFENSON 1999). CHELKOWSKI *et al.* (2000) detected the line MP7 from a doubled haploid set of lines with the lowest mycotoxin accumulation and reduction in yield traits. Survey on different resistance sources and results of breeding barley for FHB resistance was given by RUDD *et al.* (2001). Accelerated FHB resistance breeding programs that use the detected resistance sources have been established in different parts of the world including Europe. Introduction of high resistance into modern barley cultivars has not yet been successful, but new moderately resistant materials have been developed within these programs. Greater problems than in wheat may arise in barley in field resistance tests, because cultivar resistance is not so easy to identify properly with the use of simply determined parameters (JONES & MIROCHA 1999). Particularly the assessment of disease amount by the number of blighted kernels is in barley complicated due to frequent misidentifications of FHB lesions in grain samples (PROKINOVÁ 1999; SALAS *et al.* 1999). However, flanking molecular markers have already been identified and it can be expected that in near future marker assisted selection will help to facilitate the transfer of resistance into adapted germplasm. Further on, the analyses on molecular level, able to determine the quantity of pathogen DNA, are expected to contribute significantly to better identification of resistance level and understanding of factors that influence FHB, which is important for eliminating the risk of mycotoxin contamination of grains and foodstuffs (NICHOLSON *et al.* 2003).

We aimed to develop a procedure that would enable quantification of pathogen in barley grains, using real-time PCR approach, and use this method to study the genotypic and fungicide treatment effects on accumulation of DON in grain and disease severity parameters. Another objective was to examine spikes infected with different isolates of *F. culmorum* and *F. graminearum* for mycotoxin content and analyze variation in different traits in artificially inoculated spring barley genotypes.

Material and Methods

Plant Material. Material for this study comprised eight spring barley cultivars with varying level of resistance. The line CI 4196 (two-rowed landrace from China) and cultivar Chevron (six-rowed landrace from Switzerland) were included as the most resistant materials to both FHB and DON accumulation in grain (STEFFENSON 1999). The other six cultivars Jersey, Scarlett, Olbram, Akcent, Tolar and Kompakt, registered in the Czech Republic (Bulletin of the Central Institute for Supervising and Testing in Agriculture, Brno, 2003) and widely used in practice, are two-rowed spring barleys that were found in previous experiments (unpublished data) highly variable in FHB resistance and DON content. All these cultivars are medium early (Olbram, Scarlett and Kompakt) or medium late (Jersey, Akcent and Tolar) malting barleys. Cultivar differences in heading date did not exceed five days.

Description of Field Experiments. This study is based on two types of experiments, which included eight spring barley materials. In the first experimental series carried out at the location Prague-Ruzyně for two years (2002-2003) 30 spikes were in each cultivar at the stage of full flowering inoculated by brushing 2ml of conidial suspension (5×10^6 in l ml) of the fungus (CHELKOWSKI *et al.* 2000). The following isolates were used for inoculation: 1/ isolate A of *F. culmorum* (CZ); 2/ isolate B (Stupice) of *F. culmorum* (CZ); 3/ /isolate 821 of *F. graminearum* (PL) 4/ isolate 608 of *F. graminearum* (CZ). The isolates were selected on the basis of previous experiments with wheat (ŠÍP *et al*. 2003), in which data were obtained on their aggressiveness and chemotype. After inoculations spikes were covered with polythene bags for 48 hours.

In the second experimental series at two locations (Prague-Ruzyně and Kroměříž) and in two years (2002-2003) cultivars were grown on 2.5 m^2 plots in three replicates of four variants: 1/ Infection variant (I), 2/ Infection variant treated with fungicide (IF) 3/ Control uninfected variant (C) and 4/ uninfected variant treated with fungicide (CF). Infection and control variants were grown in two separate blocks isolated by five meter wide wheat stand that was kept free from diseases by protective chemicals. Highly pathogenic isolate (B) of *Fusarium culmorum* (ŠÍP *et al.* 2002a) was used for inoculation. The spore mixture (0.8×10^7 ml^{-1}) was applied with the use of hand sprayer directly onto the whole plot in two terms: first at full flowering (>50% of flowering spikes) and second one week later. Fungal infection was promoted by mist irrigation of plots (applied in all variants). In both years and at both sites fungicide Horizon 250 EW (active ingredient Tebuconazole; supplier Bayer, Aktivengesellschaft, Leverkusen, Germany) was applied in IF and CF variants following manufacturer instructions. Inoculation with *Fusarium* conidia suspension followed in IF variant after 24 hours, when positive occurrence of fungicide in plant tissue was assured.

Determination of FHB Resistance Traits. In the first experimental series both the inoculated spikes and control uninfected spikes were analyzed for number of grains per spike (GNS), thousand grain weight (TGW) and grain weight per spike (GWS). Determination of resistance traits in the second series of experiments was based on seed samples obtained in each plot from randomly selected 50 spikes. In all variants the traits grains per spike, thousand grain weight and grain weight per spike were determined and tolerance to the infection was expressed as percent reduction (R) from uninfected, control variant C in these traits. In both series of experiments spikes were threshed at a low wind not to lose light infected scabby kernels.

Analysis of extent of infection penetration into grain was based on a procedure described by AMELUNG (1996), determining the percentage of *Fusarium* colonies and percentage of non-sprouting kernels from samples that contained 100 randomly selected seeds.

Chemical Analyses. In the first experimental series seed samples of each cultivar obtained after inoculations with four isolates were analyzed for content of trichothecene mycotoxins and zearalenone using either GC/ECD and clean-up on MycoSep 225 column (Institute of Chemical Technology, Dept. of Food Chemistry and Analysis, Prague) or ELISA (Research Institute of Crop Production, Prague) by Ridascreen Fast DON kits (SÝKOROVÁ *et al.* 2003). In the second experimental series the content of deoxynivalenol (DON) was determined by ELISA on RIDASCREEN^{R} FAST DON kits from R- Biopharm GmbH, Darmstadt, Germany. A representative sample was ground and thoroughly mixed. After that 5 g of ground sample was shaken with 100 ml of distilled water and filtered. 50 µl of the filtrate was used for the test. Samples and standards were applied according to manufacturer instructions. The kit was measured at 450 nm. RIDAWIN^{R} software was used for the data processing (ŠÍP *et al*. 2002a).

DNA Isolation. DNAs were isolated using DNeasy Plant Mini Kit (QIAGEN) from mycelia and from infected/non infected plant tissues. DNA concentrations and qualities were checked electrophoretically and spectrophotometrically in Gene Quant Pro instrument.

Taq Man and Primers for Real Time PCR. The PCR primers and TaqMan probe used for the real time PCR were designed using Primer Express software (Applied Biosystems). The input sequences were obtained from amplified DNA regions with species specific primers (DOOHAN *et al.* 1998). The TaqMan probe was labelled at the 5´-end with the FAM reporter dye and at the 3´-end with the MGB quencher. A 92-bp long DNA fragment was amplified.

Real Time Quantitative PCR. Real time PCR assay consisted of 50µl PCR mixture containing 12.5µl of TaqMan universal PCR master mix (Applied Biosystems) composed of 5mM $MgCl_2$, ROX as an internal reference, 200 µM each dATP, dCTP and dGTP, 400 µM dUTP, 1U AmpliTaq Gold DNA polymerase and 1U AmpErase uracil-N-glycosylase. The concentrations of primers were respectively 200nM and 300nM. Program consisting of 2 min. at 50°C 10min. at 95°C, followed by 40 cycles of 15sec. at 95°C and 1 min. at 60°C was used. Amplifications and detections were performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems).

Post-PCR data analysis was performed using the Sequence Detector Software (Applied Biosystems). The threshold cycle (C_T) indicates the fraction cycle number at which the amount of amplified target reaches a fixed threshold. C_T value for *Fusarium* target amplicons (C_T Fus) was transformed in the following way:

C_T Fus transf = $10^7 * 2^{-CT\ Fus}$.

Statistical Analyses. The UNISTAT 5.0 package (UNISTAT Ltd., London W9 3DY, UK) was used for statistical analyses of the data.

Results and Discussion

Mycotoxin Assays on Inoculated Spikes. Results of GC analyses of mycotoxin spectra in grain of spikes inoculated with the use of "brushing" method (Table 1) showed prevalent production of mycotoxin DON in three of four isolates (range 1537 to 4784 µg/kg), similarly as in experiments with winter wheat (ŠÍP *et al*. 2003). Isolate 608 of *F. graminearum* was found predominant NIV producer (475 µg/kg). Isolates of *F. culmorum* (particularly isolate A) produced relatively higher quantities of DON and isolate *F.g.* – 821, which showed in experiments with wheat the highest aggressiveness and DON content, reached with barley genotypes below average DON content. From the other thrichothecene mycotoxins 3-AcDON

had relatively higher content (range 49 to 478 μg/kg), particularly after infection with *F. culmorum* isolates. The examined isolates also produced in small quantities 15-AcDON (range 7 to 43 μg/kg), T-2 tetr. (range 7 to 21 μg/kg), HT-2 tox. (range 3 to 37 μg/kg) and zearalenone (ZEA)(range 3 to 53 μg/kg). The content 53 μg/kg of the highly deleterious mycotoxin ZEA was detected after inoculation with isolate B of *F. culmorum*. No mycotoxins were detected in the control samples.

Table 1. Average values (μg/kg) for trichothecene mycotoxins and zearalenol in grain of 8 cultivars inoculated with four *Fusarium* isolates (F.c. - *F. culmorum*; F.g. . *F. graminearum*) And coefficients of correlation between DON-Elisa (*) and toxins determined by GC

Isolate	F.c. – A	F.c. - B	F.g. - 821	F.g. - 608
DON-Elisa	6337.50	3775.00	3164.50	478.13
DON-GC	6157.15	3348.69	1977.69	86.18
NIV	36.65	26.21	37.36	474.85
3-AcDON	477.58	290.23	196.08	49.17
HT-2 tox.	13.20	2.50	37.19	2.50
T-2 tetr.	8.88	7.03	15.35	20.78
15-AcDON	42.74	27.87	13.37	8.60
ZEA	4.13	53.40	2.64	2.54
*/DON-GC	0.94***	0.88***	0.99***	0.77**
*/3-AcDON	0.91***	0.57	0.95***	0.05
*/NIV	0.01	0.35	0.68*	0.54

There is ample evidence of occurrence of DON in infected barley grains, but the production of other toxins was found highly dependent on *Fusarium* species and isolate used (PERKOWSKI *et al*. 1997; SALAS *et al.* 1999; CHELKOWSKI *et al*. 2000). The studies on isolate chemotype are, therefore, highly desirable.

The results of DON determination by the ELISA method were compared with the results of the gas chromatography and highly significant and positive correlations were obtained for all four isolates (Table 1). Comparative testing with GC method showed relatively higher average DON content with the use of ELISA method, which could be explained by high specificity of the used antibodies and consequently by high recovery (106%) of ELISA („Reference Material No 379 – Deoxynivalenol in wheat – medium level"; Commission of the European Communities, Community Bureau of Reference BCR No 00462). Besides this, it could not be possible by immunochemical method to completely exclude the occurrence of cross reactivity with some other trichothecene substances (occurring in very low quantities). As shown in Table 1, coefficients of correlation between DON content determined by ELISA method and contents of other "more important" toxins (NIV and 3-AcDON) were, however, significant only sporadically, which indicates that specific studies on accumulation of different mycotoxins are needed with respect to isolate used.

Analyses of Variation for DON Content and Examined Disease Severity Traits. Variance analyses with aim to detect cultivar, year (location), fungicide treatment and interaction effects on DON content and examined disease severity traits were performed in both experimental series. The results are given in Table 2. It is necessary to mention that "the brushing" series of experiments (series 1) with the use of isolate B of *F. culmorum* resulted in relatively lower average DON content determined by ELISA (3.8 mg/kg) than experiments 2

with spraying of inoculum onto the whole plot followed by mist irrigation of infected plants (14.1 mg/kg). In series 1, with lower disease incidence, significant cultivar differences were not detected, but year, isolate and year by isolate interactions explained highly significant effects on accumulation of DON. In the experiments with spraying of inoculum (2) cultivars significantly differed in accumulation of DON, however, the effects of years/locations and fungicide treatment were relatively larger. The interactions between these factors were also highly significant. Similar pictures gave ANOVAs for the traits % of *Fusarium* colonies and % of non sprouting grains. Effect of environment (year/location) was particularly large in the trait % of *Fusarium* colonies.

Table 2. ANOVA mean squares for different traits in two series of experiments
(1 - inoculation by "spraying" technique; 2 - inoculation by "brushing" technique)

	Source of variation	df	DON content (mg/kg)	Grain number/ spike- reduction	Thousand grain wt. reduction	Grain wt./spike - reduction	% of *Fusarium* colonies	% of non-sprouting grains
1	Cultivar	7	577***	327***	938***	2040***	670***	3030***
	Year/location (YL)	3	1253***	488***	6147***	10877***	14234***	6234***
	Treatment (I/IF)	1	1997***	56*	127*	46	2327***	2262***
	Cultivar x YL	17	158**	784***	770***	2755***	763***	1106***
	Cultivar x treatment	7	381***	54***	88***	238***	172	317*
	YL x treatment	3	187*	299***	1143**	2699***	1598***	401*
	Error	81	63	11	14	44	154	132
2	Cultivar	7	8	93*	104**	233*		
	Year	1	78***	144	283**	722**		
	Isolate	3	33**	85	95**	152		
	Cultivar x Year	7	6	125*	86**	238*		
	Cultivar x Isolate	21	4	42	21	83		
	Year x Isolate	3	35***	20	6	21		
	Error	21	4	36	19	68		

It is clear from Table 3, that the examined grain yield traits showed on average very little differences between infected and control variants. It can be deduced from comparisons with the other examined parameters (particularly DON content) that the infection with *Fusarium culmorum* affected in these experiments mainly grain quality. ANOVAs for reductions of yield traits showed significant cultivar and environmental effects, but the effects of fungicide treatment, isolate used and specific interactions were not clear. There were not found significant fungus isolate and fungicide treatment effects on the reduction of grain weight per spike.

The detection of lower contents of DON with the use of "brushing techniques" in barley was analogous to single floret inoculation experiments with wheat (ŠÍP *et al.* 2003). Experiments using spraying (atomizing) of inoculum onto entire spike surface , that reflect to resistance of type I, evidently created in mist-irrigated plots more advantageous conditions for development of the disease and enabled detection of cultivar differences in the examined traits. Spraying systems or grain spawn methods are widely used for FHB resistance screening in barley nurseries (RUDD *et al*. 2001).

Table 3. Trait means in variants I (infection), IF (infection and fungicide), C (control, uninfected) and CF (control and fungicide) and comparison of I and IF for single traits

Variant	DON (mg/kg)	% of *Fusarium* colonies	% of non-sprouting grains	Grain number/ spike	Thousand grain wt. (g)	Grain wt./spike (g)
I	14.14	43.68	39.46	23.24	46.33	1.07
IF	6.71	29.28	30.43	24.13	48.09	1.13
C	0.31	9.25	5.58	23.65	47.14	1.13
CF	0.21	6.58	1.25	24.33	48.50	1.18
% reduction (increase*)^	52.5	33.0	22.90	3.7*	3.7*	5.4*
I-2002	14.61	48.32	22.87	23.09	45.65	1.05
IF-2002	9.75	29.77	9.05	23.28	46.80	1.07
% reduction (increase*)^ - 2002	33.2	38.4	60.4	0.8*	2.5*	1.9*
I-2003	13.67	35.07	39.93	23.39	47.01	1.08
IF-2003	3.67	20.63	30.7	24.98	49.38	1.19
% reduction (increase*)^ - 2003	73.2	41.1	23.1	4.8*	6.3*	9.5*

^ 100 -IF/I (I/IF*) x 100

Effects of Genotype and Fungicide Treatment on Different Traits. Genotypic differences in DON content between cultivars in infection variant are shown in Fig. 1. High resistance to accumulation of DON was detected in Chevron and CI 4196, but in the first "resistant" group also the cultivars Jersey, Olbram and Scarlett could be included. Similar genotypic

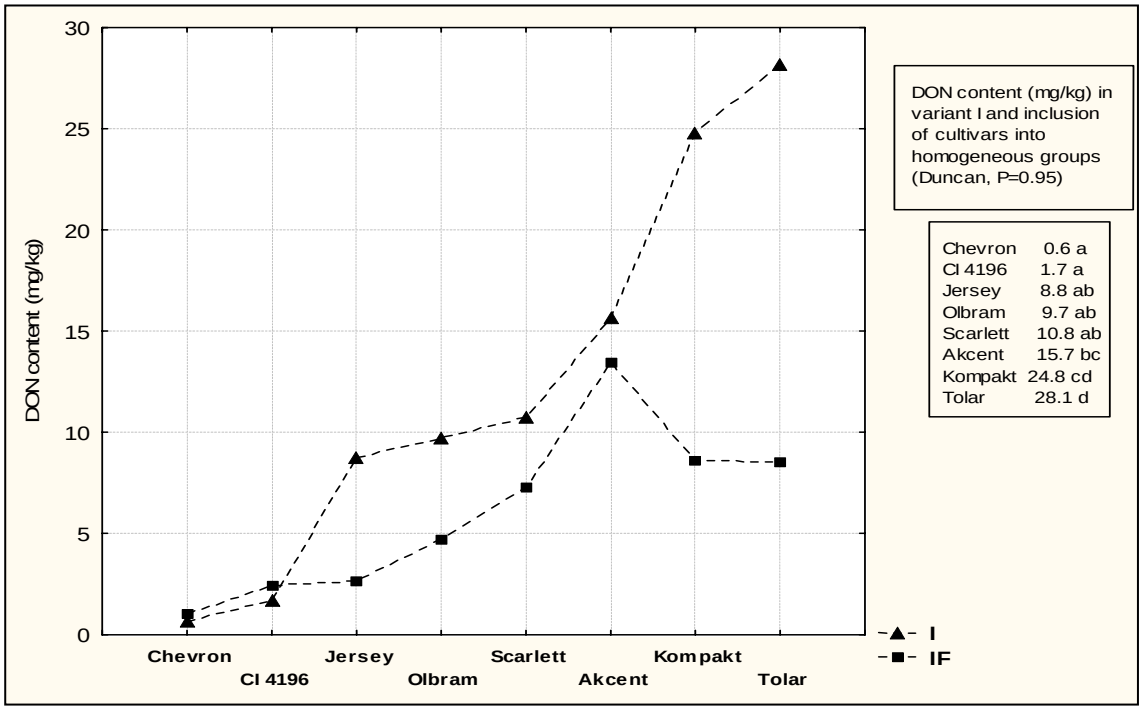


Fig. 1. Average DON content (from two years and two sites) of 8 spring barley cultivars in variants I (infection) and IF (infection and fungicide) and results of multiple comparisons

classification as for DON was obtained for percentage of *Fusarium* colonies (r=0.74; P<0.01). In this trait Chevron and CI 4196, with 4.9% and 17.1%, were clearly separated from the other cultivars (which showed the range between 32% and 53%). Classification of genotypes in other traits (percentage of non-sprouting grains and yield traits) was not related to classification according to DON content.

It comes from Tables 2 and 3 that fungicide treatment had significant effect on reduction of DON content in grain and also on percentage of *Fusarium* colonies and percentage of non-sprouting grains. Mean fungicide efficacy for DON content was 52.5 %, which indicates that prospects for chemical control of FHB in barley are limited (JONES 2000) as in wheat (MESTERHÁZY *et al.* 2003). Similarly conducted experiments with winter wheat (ŠÍP *et al.* 2004) showed for the years 2002 and 2003 on average 49% reduction of DON content due to fungicide treatment. There were, however, both in wheat and barley large year effects on fungicide effectiveness. While in 2002 the efficacy reached in barley 33.2 %, in 2003 it exceeded 70 %. When compared with other years (period 1992-2003 - ŠÍP *et al.* 2002b, 2004), the year 2003 was characteristic by short disease development as a consequence of high temperatures following inoculation at water availability on mist irrigated plots. Shortness of fungicide action could contribute to higher effectiveness in comparison with 2002. Fungicide treatment led to 33 % decrease of percentage of *Fusarium* colonies and 23 % decrease of percentage of non-sprouting grains. Low (5%) was the effect of fungicide treatment on increase of grain yield per spike, but it is necessary to take into consideration that in these experiments infection with *Fusarium culmorum* had on average low effect on yield traits (Table 3). The same grain yield per spike was detected in variants IF (infection and fungicide) and C (control variant). On contrary "wheat" experiments showed in 2002 and 2003 experiments 33% fungicide treatment efficacy for grain weight per spike (ŠÍP *et al.* 2004).

As shown in Fig. 1, fungicide treatment was not similarly effective in all cultivars. While in susceptible cultivars Kompakt and Tolar application of fungicide led to 60-70 % reduction of DON content, practically ineffective was this treatment in cultivar Akcent, which was also documented by the real time PCR analyses (Fig. 2). In this cultivar the fungicide treatment evidently did not result in substantial reduction of the disease (pathogen DNA). Fungicide treatment was with this cultivar ineffective also in 2001 (data not presented here). Explanation of this specific reaction was not found in these experiments because this cultivar, registered in the Czech Republic, was always treated similarly as the other cultivars. To explain differential effects of fungicides on cultivars and *Fusarium* species, specific detailed studies of host-pathogen interactions on molecular level are evidently needed (DOOHAN *et al.,* 1999; NICHOLSON *et al.* 2003). However, it comes from the obtained confounding results that in agricultural practice only "double protection" consisting in growing resistant cultivars and fungicide treatment may guarantee sufficient protection (MIELKE & WEINERT 1996), particularly in "endangered crops" (when maize is the preceding crop and with application of reduced tillage practices).

Significance of Different Traits for Depiction of Harmfulness of FHB Infection. In these experiments DON content in grain could be considered as decisive for classification of the severity of the disease and genotype and fungicide effects, because affection of yield traits was low. However, deleterious effects of *Fusarium* infection on barley yield cannot be neglected, as reported from the USA and Canada (JONES & MIROCHA 1999; TEKAUZ *et al*. 2000).

Table 4. Phenotypic coefficients of correlation between examined traits in 2002 (below diagonal) and 2003 (above diagonal)

	DON	FUC	NSG	GNS-R	TGW-R	GWS-R
C_T	0.92***	0.32	0.25	0.32	0.48**	0.42*
DON	---	0.38*	0.29	0.33	0.50**	0.43*
FUC	0.50**	---	0.68***	-0.34	-0.26	-0.33
NSG	0.04	0.82***	---	-0.27	-0.32	-0.16
GNS-R	-0.07	-0.30	-0.12	---	0.81***	0.95***
TGW-R	-0.07	0.00	-0.02	0.33	---	0.95***
GWS-R	-0.15	-0.22	-0.14	0.84***	0.78***	---

C_T = C_T Fus transformed, DON= DON content, FUC= percentage of *Fusarium* colonies, NSG=percentage of non-sprouting grains, GNS-R= reduction of grain number per spike, TGW-R= reduction of thousand grain weight, GWS-R= reduction of grain weight per spike
*** P<0.001, **P<0.01, *P<0.05

Low DON content was in both years associated with low percentage of *Fusarium* colonies in examined grains and in 2003 also with lower reductions of thousand grain weight and grain weight per spike (Table 4). The value of percentage of non-sprouting grains from these aspects could not been justified by these experiments, because relations with this trait were insignificant (except for relations with % of *Fusarium* colonies).

These results are supported by the findings of JONES & MIROCHA (1999) who found that DON concentration in barley could not be effectively estimated by yield traits, visual index or by discolorations of grain. We also tried in these experiments to determine visually the grain infection score, but due to grain discolorations observed also in control variants and in materials resistant to FHB, this trait could not be used as indicator of FHB disease. Ample simultaneous discolorations due to occurrence of *Bipolaris sorokiniana* and *Alternaria* spp., as well as *Cladosporium* or *Epicoccum* spp. have been observed, similarly as in experiments of PROKINOVÁ (1999) and SALAS *et al.* (1999). Visual scoring of symptoms (used without problems e.g. in wheat) could not in barley be performed because of predominant "latency" of the disease. Therefore, in comparison with wheat, in barley there was not substantiated clearly by the experiments to rely on easily determined traits when evaluating genotype resistance to FHB and accumulation of mycotoxins in grain. Significant relationships between mycotoxin content and different traits were observed (ADAMSKI *et al.* 1999; CHELKOWSKI *et al.* 2000), but it is evident that in general observations on yield traits or visual assessments of the disease cannot substitute in barley for direct determinations of mycotoxin content.

Importance of real time PCR assays for quantification of FHB causal agents in barley. Due to the fact that FHB causes in barley very miscellaneous and often unclear symptoms of infection, it was suggested that PCR analysis could be particularly helpful for quantifying FHB and for evaluating the efficacy of the fungicides. The method based on real time PCR has been developed in RICP Prague- Ruzyně and the results concerning 2003 experiments with barley are presented here. This system was based on the detection and quantification of a fluorescent reporter. This signal increased in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it was possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates with the initial amount of target template (HEID *et al.* 1996). The obtained C_T values were transformed (see Material and Methods), which allowed for positive and linear relationship with DON content (Fig. 3).

There was found very close relationship between detection of DON by ELISA and DNA content of the pathogen in analyzed samples estimated by C_T Fus transformed (r= 0.92, P<0.001)(Fig. 3). Besides DON, C_T significantly positively correlated also with reductions of thousand grain weight and grain weight per spike (Table 4). Cultivar differences in effects of fungicide treatment on reduction of DON content in grain were clearly described by C_T values as well (Fig. 2).

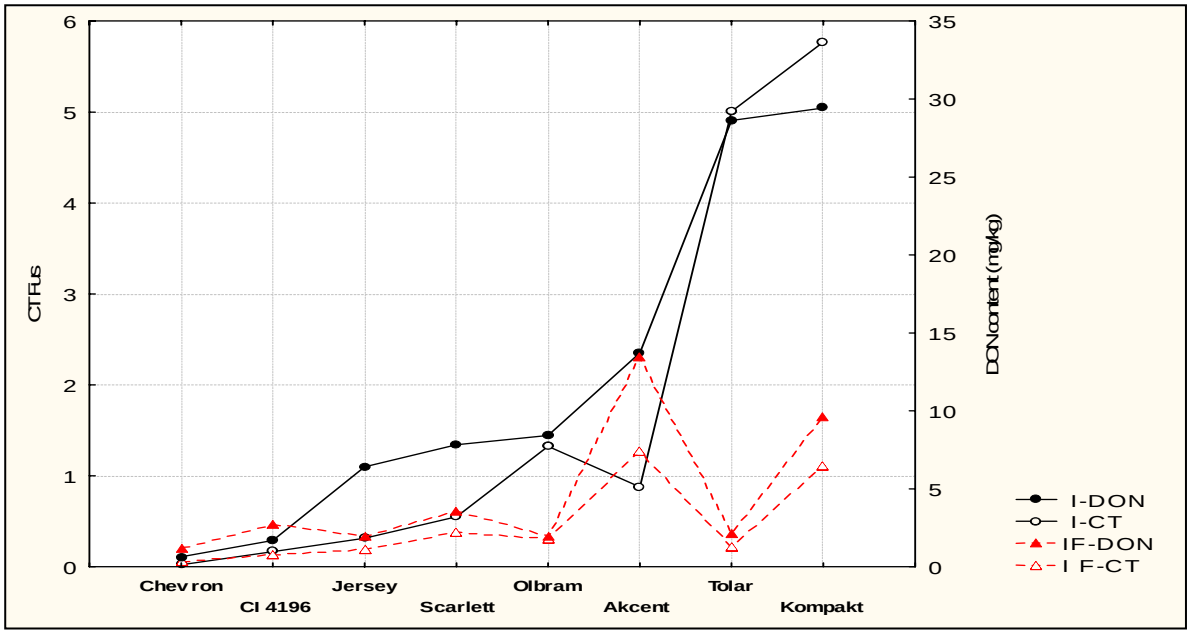


Fig. 2. Correspondence between DON content in grain of 8 barley cultivars and C_T Fus transformed (real time PCR) after infection with *Fusarium culmorum* (I) and fungicide treatment (IF) in 2003

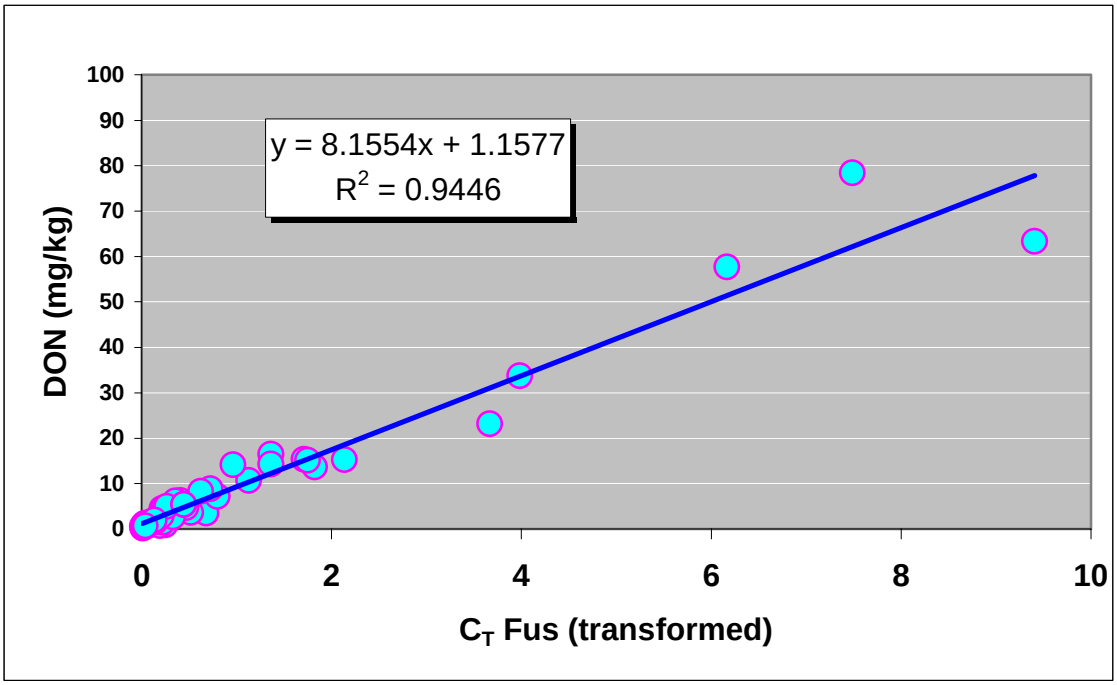


Fig. 3. Relation between DON content and C_T Fus (transformed) obtained from real time PCR analysis across all tested samples

The obtained results indicate that C_T can give reliable evidence of both the cultivar resistance to accumulation of DON and FHB, and efficacy of fungicide treatment. It is very important that accumulation of DON in grain was found closely related to the pathogen presence in infected grain. Therefore, PCR analysis is expected to provide from these aspects greater resolution than any other trait. Further on, it is possible with this technique to detect and quantify different metabolite (toxin) profiles. As shown, the analysis also allowed discrimination in fungicide efficacy that was not apparent from examined yield traits. DOOHAN *et al.(*1999) reported that only PCR analysis enabled in wheat to determine fungicide effects on reduction of colonization of tissues by both *F.culmorum* and *F.poae*. Great advantage of this technique lies in the fact that it enables identification of the causal organism and therefore, it is superior to analyses measuring infection effects. It can be expected that molecular assays could substitute for examinations of many characters, which are due to complicated character of the disease needed in practice and breeding for evaluating genotype resistance and efficacy of treatments. As described by NICHOLSON *et al.* (2003), the PCR offers a sensitive and potentially specific means to detect, identify and quantify different species present within plant tissues, as well as to differentiate between chemotypes within a single species. A robust, multiplex PCR was developed in the Netherlands (WAALWIJK *et al.* 2003), which enabled to screen a large series of isolates and detect major changes in *Fusarium* spp. on wheat. Different uses of competitive and real time PCR assays were lately reviewed by NICHOLSON *et al.* (2003). These authors gave evidence about usefulness of these assays to increase our understanding of factors that influence FHB and ability to control the disease. In barley, where assessment of the disease according to symptoms and other measurements is even more complicated than in wheat, these assays may significantly help to increase the effectiveness of disease control in this crop both by genetic means and chemicals.

Acknowledgements
The authors would like to express thanks to Prof. B.J. Steffenson, North Dakota State University, for providing material resistant to FHB. The work was supported by the Grant Agency of the Czech Republic, project No. 521/02/0099 and Ministry of Agriculture of the Czech Republic, project 0002700602.

References

ADAMSKI T., CHELKOWSKI J., GOLINSKI P., KACZMAREK Z., KOSTECKI M., PERKOWSKI J., SURMA M., WISNIEWSKA H. (1999): Yield reduction and mycotoxin accumulation in barley doubled haploids inoculated with *Fusarium culmorum* (W.G.Sm) Sacc. J. Appl. Genet., **40**: 73-84.

AMELUNG D. (1996): Experience with the isolation of plant pathogenic fungi: In: Proc. of the 4th Internat. Symposium of the European Foundation for Plant Pathology "Diagnosis and Identification of Plant Pathogens" (DAHNE et al. eds.), 9-12 September 1996, Bonn, Germany: 35-36.

CAMPBELL H., CHOO T.M., VIGIER B., UNDERHILL L. (2000): Mycotoxins in barley and oat samples from eastern Canada. Can. J. Plant Sci., **80:** 977-980.

CHELKOWSKI J., WISNIEWSKA H., ADAMSKI T., GOLINSKI P., KACZMAREK Z., KOSTECKI M., PERKOWKI J., SURMA M. (2000): Effects of *Fusarium culmorum* head

blight on mycotoxin accumulation and yield traits in barley doubled haploids. J. Phytopathology, **148**: 541-545.

DOOHAN F.M., PARRY D.W., JENKINSON P., NICHOLSON P. (1998): The use of species-specific PCR-based assays to analyse *Fusarium* ear blight of wheat. Plant Pathology, **47**: 197-205.

DOOHAN F.M., PARRY D.W., NICHOLSON P. (1999): *Fusarium* ear blight of wheat: the use of quantitative PCR and visual disease assessment in studies of disease control. Plant Pathology, **48**: 209-217.

HEID C.A., STEVENS J., LIVAK K.J. WILLIAMS P.M. (1996): Real time quantitative PCR. Genome Res., **6**: 986-994.

JONES R.K. (2000): Assessments of Fusarium head blight of wheat and barley in response to fungicide treatment. Plant Dis., **84:** 1021-1030.

JONES R.K., MIROCHA C.J. (1999): Quality parameters in small grains from Minnesota affected by Fusarium head blight. Plant Dis., **83**: 506-511.

MAGAN N., HOPE R., COLLEATE A., BAXTER E.S. (2002): Relationship between growth and mycotoxin production by *Fusarium* species, biocides and environment. European Journal of Plant Pathology, **108**: 685-690.

MESTERHÁZY A., BARTÓK T., LAMPER C. (2003): Influence of wheat cultivar, species of *Fusarium*, and isolate aggressiveness on the efficacy of fungicides for control of Fusarium head blight. Plant Dis., **87**: 1107-1115.

MIELKE H., WEINERT J. (1996): Investigations on the effect of various fungicides on the pathogen of partial head blight (Fusarium culmorum (W.G.Sm.) Sacc.). Nachrichtenblatt des Deutschen Pflanzenschutzdienstes, **48:** 93-95.

MIROCHA C.J., XIE W., XU Y., WILCOXSON R.D., WOODWARD R.P., ETEBARIAN R.H., BECHELE G. (1994): Production of thrichothecene mycotoxins by *Fusarium graminearum* and *Fusarium culmorum* on barley and wheat. Mycopathologia, **128**: 19-23.

NICHOLSON P., CHANDLER E., DRAEGER R.C., GOSMAN N.E., SIMPSON D.R., THOMSETT M., WILSON A.H. (2003): Molecular tools to study epidemiology and toxicology of fusarium head blight of cereals. European Journal of Plant Pathology, **109**: 691-703.

PERKOWSKI J., KIECANA I., SCHUMACHER U., MŰLLER M., CHELKOWKI J., GOLINSKI P. (1997): Head infection and accumulation of *Fusarium* toxins in kernels of 12 barley genotypes inoculated with *Fusarium graminearum* isolates of two chemotypes. European Journal of Plant Pathology, **103**: 85-90.

PROKINOVÁ E. (1999): Necrosis of barley grains, the germination and fungi isolated from grains. Rostlinná Výroba, **45:** 133-144.

RUDD J.C., HORSLEY R.D., McKENDRY A.L., ELIAS E.M. (2001): Host plant resistance genes for Fusarium Head Blight: Sources, mechanisms and utility in conventional breeding systems. Crop Sci., **41**: 620-627.

SALAS B., STEFFENSON B.J., CASPER H.H., TACKE B., PROM L.K., FETCH T.G., Jr., SCHWARZ P.B. (1999): *Fusarium* species pathogenic to barley and their associated mycotoxins. Plant Dis., **83**: 667-674.

ŠÍP V., SÝKOROVÁ S., STUCHLÍKOVÁ E., CHRPOVÁ J. (2002a): The effect of infection with *Fusarium culmorum* L. on deoxynivalenol content in grain of selected winter wheat varieties. J. Appl. Genet., **43A**: 319-332.

ŠÍP V., STUCHLÍKOVÁ E., CHRPOVÁ J. (2002b): Evaluation of the response of selected winter wheat cultivars to artificial infection with *Fusarium culmorum* in field conditions. Petria, **12:** 287-291.

ŠÍP V., CHRPOVÁ J., SÝKOROVÁ S. (2003): Analysis of factors that influenced accumulation of *Fusarium* mycotoxins in wheat grain. In: Proc. of the Tenth International Wheat Genetics Symposium (POGNA N.E. et al., eds.), 1-6 September 2003, Paestum, Italy: 1254-1256.

ŠÍP V., CHRPOVÁ J., SÝKOROVÁ S. (2004): Effect of genotype, environment and fungicide treatment on severity of *Fusarium* head blight infection and accumulation of DON in grain of winter wheat: *in press*.

STEFFENSON B.J. (1998): Fusarium head blight of barley: epidemics, impact, and breeding for resistance. Technical Quarterly, MBAA TQ, **35:** 177-184.

STEFFENSON B.J. (1999): Combating *Fusarium* head blight: an emerging threat to malting barley quality throughout the world. EBC Congress 1999: 531-538.

SÝKOROVÁ S., ŠÍP V., NEVRKLOVÁ M., SYPECKÁ Z., HAJŠLOVÁ J., HÝSEK J. (2003): The survey of *Fusarium* mycotoxins content in grain of winter wheat cultivars collected from different regions of Czech Republic. In: Proc. of the Tenth International Wheat Genetics Symposium (POGNA N.E. et al., eds.), 1-6 September 2003, Paestum, Italy: 1266-1268.

TEKAUZ A., McCALLUM, B., GILBERT J. (2000): *Fusarium* head blight of barley in western Canada. Can. J. Plant Pathol., **22:** 9-16.

WAALWIJK C., KASTELEIN P., deVRIES PhM., KERÉNYI Z., van der LEE, T.A.J., HESSELINK, T., KÖHL J., KEMA G. (2003): Major changes in *Fusarium* spp. in wheat in the Netherlands. European Journal of Plant Pathology, **109**: 743-754.

Breeding for Virus Resistance of Barley: Amalgamation of Classical and Biotechnological Approaches

W. Friedt[1] and F. Ordon[2]

[1]Institute of Crop Science and Plant Breeding I, Justus-Liebig-University, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany, wolfgang.friedt@agrar.uni-giessen.de;
[2]Institute of Epidemiology and Resistance, Federal Centre of Breeding Research for Cultivated Plants, Theodor-Roemer-Weg 4, 06449 Aschersleben, Germany

Abstract

Soil-borne barley yellow mosaic viruses (BaMMV, BaYMV, BaYMV-2) and the aphid transmitted barley yellow dwarf virus (BYDV) are serious pathogens on winter barley. Resistance to barley yellow mosaic virus disease has been identified in extensive screening programmes and several recessive resistance genes have been mapped. In contrast to barley yellow mosaic viruses, no complete resistance to BYDV is known in the barley gene pool. But tolerant accessions have been identified and QTL for BYDV-tolerance have been detected on chromosomes 2HL and 3HL. Today, the process of breeding virus resistant or tolerant barley cultivars, respectively, can be considerably enhanced and accelerated by creating anther- or microspore-derived doubled haploid (DH) lines. Furthermore, the breeding efficiency can be improved by molecular markers as they facilitate (i) reliable selection on a single plant level independent from symptom expression in the field, (ii) the acceleration of back crossing procedures, (iii) the pyramiding of resistance genes, (iv) the detection of QTL and marker based combination of positive resistance alleles as well as the combination of resistances to different (virus) diseases.

Introduction

Barley mild mosaic virus (BaMMV) and barley yellow mosaic virus (BaYMV), belonging to the bymoviruses (USUGI *et al*. 1989) are the causal agents of barley yellow mosaic disease. These viruses either separately or in mixed infections (HUTH & ADAMS 1990) cause severe yield losses which cannot be prevented by chemical means due to transmission by the soil-borne fungus *Polymyxa graminis* (TOYAMA & KUSABA 1970). Therefore, breeding for resistance to these viruses is of special importance. With regard to pathogenicity, seven strains of BaYMV and two of BaMMV have been distinguished in Japan (NOMURA *et al*. 1996). In Europe only two strains of BaYMV (BaYMV-1 and BaYMV-2) were earlier described by HUTH (1989) in addition to BaMMV, but a second strain of BaMMV has been recently detected in France (HARIRI *et al*. 2003).

In Germany, the resistance of commercial cultivars to barley yellow mosaic disease is mainly based on the recessive resistance gene *rym4*, derived from the Dalmatian landrace 'Ragusa'. This gene was assigned to the long arm of chromosome 3H using telo-trisomic and RFLP analysis (KAISER & FRIEDT 1989, 1992; GRANER & BAUER 1993). The *rym4* gene confers resistance to both BaMMV and BaYMV-1 but is not effective against BaYMV-2 (HUTH 1989). Therefore, *rym5* detected in the Chinese landrace ´Mokusekko 3´ (KONISHI *et al.* 1997; GRANER *et al*. 1999) displaying resistance to all German yellow mosaic inducing viruses, has been introduced into a number of barley cultivars, e.g. 'Anastasia' and 'Kamoto' (ANONYMOUS 2003). Attempting to diversify the sources of resistance to barley yellow mosaic disease, extensive screening programmes were carried out on barley germplasm permitting the identification of numerous recessive resistance genes (GÖTZ & FRIEDT 1993; ORDON *et al*. 1993; ORDON & FRIEDT 1993) that were then

subjected to an intensive molecular mapping (cf. ORDON *et al*. this volume).

On the world-wide level barley yellow dwarf virus causes one of the economically most important diseases of cereals (e.g. LISTER & RANIERI 1995). The isometric virus particles are persistently transmitted by aphids. Because of different prevalent vectors several serotypes of BYDV have been distinguished. In Northern Europe *Rhopalosiphum padi* and *Macrosiphum* (*Sitobion*) *avenae* are the prevalent vectors and therefore serotypes BYDV-PAV and BYDV-MAV are of special importance in this region (PLUMB & JOHNSTONE 1995). For economical and ecological reasons cultivation of highly BYDV-tolerant cultivars with satisfactory grain yield would be advantageous. The *Ryd2* gene (RASMUSSON & SCHALLER 1959) conferring tolerance to BYDV which is additionally influenced to some extend by the genetic background, the virus isolate and the environmental conditions (SCHALLER 1984) has been introduced, e.g to the cultivar 'Vixen' (PARRY and HABGOOD 1986) as well as into new breeding lines (BURNETT *et al*. 1995, DELOGLU *et al*. 1995) and cultivars like ´Naturelle´. Besides this, extensive QTL analysis in different crosses have revealed the presence of quantitative trait loci (QTL) on chromosomes 3H and 2H (ORDON *et al*. this volume) which can be exploited in barley breeding.

The Present Situation of Barley Production and Breeding

The barley area harvested world-wide was about 70 million hectares (ha) in the early 1970s, it reached a maximum of more than 80 million ha in the late 1970s and has then dropped to about 55 x 10^6 ha in the beginning of the 21st century. For comparison, the cultivation area in Western Europe went down from approx. 16 to 14 x 10^6 ha whereas the cultivation area in Germany (2.1-2.2 x 10^6 ha) remained comparatively stable. Here, the average grain yield of spring barley harvested by farmers has grown from about 2.0 t/ha in the early 20th century to a current level of almost 6 t/ha (Table 1, FAOSTAT AGRICULTURE DATA, http://apps.fao.org/). The continuously increasing yield potential of modern cultivars and improved cultivation practices tend to lead to a permanent increase of barley production (Table 1). For example, over the last three decades the barley yield in Germany has increased by 50% and by 42% in Western Europe. Corresponding relative yield increases world-wide amount to 33%. According to GRAUSGRUBER *et al*. (2002) the yield potential of modern European barley cultivars is 4.4 versus 3.3 t/ha achieved by first varieties selected from landraces in the early 20th century. At present, more than 200 barley varieties are registered at the Community Plant Variety Office (CPVO), Angers/France. Current leading winter barley varieties in Europe are 'Esterel' (6-row malting barley, France), 'Perl' (2-row malting barley, England), 'Franziska', 'Lomerit' and 'Merlot' (6-row feed barleys, Germany). For more details, cf. FRIEDT and RASMUSSEN (2003), FRIEDT *et al.* (2000, 2002), and CPVO (http://www.cpvo.eu.int/).

Table 1. Comparison of global and European barley production figures, 1970-2003 (FAO, 2004)

	World		Western Europe		
Period [1)]	Harvest (10^6 Mt)	Yield (t/ha)	Harvest (10^6 Mt)	Grain Yield (t/ha) W Europe	Germany
1970-74	135.9	1.89	45.0	3.21	3.83
1975-79	157.7	1.94	52.5	3.30	3.97
1980-84	159.3	2.01	58.0	3.69	4.43
1985-89	170.5	2.21	58.7	3.94	4.96
1990-94	168.8	2.28	51.1	4.05	5.28
1995-99	143.9	2.27	50.9	4.41	5.75
2000-03	138.1	2.51	49.6	4.55	5.73
Change (%)[2)]	+2	+33	+11	+42	+50

[1)]Averages of 5 or 4 consecutive years; [2)] Relative change 2000-2003 vs 1970-74. Area harvested (10^6 ha) in 2000-2003: World 55.0 (1970-1974: 71.8), Western Europe 10.9 (14.0), Germany 2.1 (2.2). Therefore, the world barley acreage has declined in this period by about 23% (W. Europe –22%).

Breeding for resistance in barley is a very important task on a global level, since average yield losses world-wide ranging from 20 to 30% have to be faced due to fungal and viral diseases along with insect pests. Therefore, in order to avoid necessary applications of chemicals for the prevention of yield losses and to ensure an economic grain production, future barley breeding will have to pay even more attention to disease and pest resistances. The increased level of disease resistance in cultivars also reflects a general tendency in agricultural practice, as farmers are under a constantly increasing pressure to reduce the use of agro-chemicals for environmental reasons, and thus enforcing focus on resistance properties of varieties. In this respect resistance and tolerance to viral diseases has gained evident importance during the last decades.

Breeding for Virus Resistance in Barley

Present Status of Variety Development

In Europe cultivars being resistant to BaYMV and BaMMV, e.g. ´Birgit ´ and ´Ogra´ were identified within the set of cultivars being released by the German Plant Variety Office soon after the first discovery of the disease in Germany in 1978. But these cultivars showed inferior agronomic performance and did not gain evident importance, therefore. However, already at the beginning of the 1990s several of the most important cultivars like 'Jana' combined superior agronomic performance with resistance to BaYMV and BaMMV, and today it can be stated that resistance to BaMMV and BaYMV has been fully combined with outstanding yieldability). With the exception of the two-rowed cv. ´Reni´ all important cultivars (seed propagation area >1000ha) are resistant to BaMMV/BaYMV (cf. ANONYMOUS 2003). Furthermore, extensive screening and genetic analysis enabled the release of the cultivar 'Tokyo' (= [(Fallon x 13060) x 87-5381 B] x Swift] (R. Hemker pers. com.), only a few years after the first detection of the resistance-breaking strain BaYMV-2 in Europe. The resistance of 'Tokyo' was derived from line 13060 which had 'Mokusekko 3', the donor of *rym5*, in its pedigree. Due to its agronomic shortcomings, susceptibility to scald (*Rhynchosporium secalis*) and the at that time limited distribution of BaYMV-2, this cultivar did not achieve great acceptance by growers. However, resistant lines derived from 'Tokyo' with better agronomic performance have been developed meanwhile. Currently listed BaYMV-2 resistant 2-rowed cultivars are 'Kamoto' and 'Kyoto' whereas 'Anastasia' and 'Structura' are corresponding 6-rowed varieties (Table 2). It is obvious that fully resistant cultivars are still inferior to the top varieties regarding total grain yield. However, this is not necessarily the case for marketable yield as demonstrated by the 6-rowed types.

Table 2. Comparison of relevant German winter barley varieties; currently leading cultivars are 'Franziska' and 'Reni'

Variety		Grain yield	Market yield (>2.5 mm)	Malt Extract
6-rowed				
Anastasia	r2*)	6	8	-
Franziska	r1	8	8	-
Merlot	r1	9	8	-
Structura	r2	6	8	-
2-rowed				-
Carrero	S	7	8	7
Kamoto	r2	6	7	-
Kyoto	r2	6	7	-
Reni	S	7	8	6

*) r1 = BaMMV/BaYMV1-resistance, r2 = BaYMV-2 resistant in addition, S = susceptible; Scale: 1-9 (1 = minimum and 9 = maximum trait expression, i.e. grain or malt extract yield, respectively)

Furthermore, current two-rowed BaYMV-2 resistant cultivars like 'Kyoto' do not possess suitable malting quality, as their non-resistant competitors (e.g. 'Carrero') have. However, it may be expected that in the near future - like in the case of *rym4* – the *rym5*-resistance will be combined with outstanding agronomic performance and quality, too.

Nevertheless, it has to be expected that new viruses or virus strains may again appear in the future, since in France a new strain of BaMMV has recently been detected overcoming *rym5* (HARIRI *et al*. 2003). Therefore, rapid introgression of additional resistance genes or the combination of different functional resistance genes is urgently needed.

Biotechnology-Assisted Barley Breeding

Combinations of different resistance genes, or the introgression of novel resistance genes from non-adapted germplasms, respectively, are usually achieved by sexual recombination, i.e. crosses of parental lines followed by phenotypic selection in the segregating offspring. In this case the success of breeding entirely depends on extensive field and/or glasshouse tests for resistance to the respective pathogen(s). However, since barley is damaged by many pathogens which often show a rapid adaptation to their hosts' resistance genes, breeding for resistance is a very complex task, and the identification of desired recombinants by phenotypic selection only, e.g. following pedigree selection schemes, has almost reached the limits of manageability. Thus, methods of plant biotechnology like anther and microspore culture which allow the rapid production of homozygous doubled haploid (DH) plants and cultivars offer new opportunities for a more efficient resistance breeding (FOROUGHI-WEHR & FRIEDT 1983; FRIEDT *et al*. 2003). For example, via anther culture the spring barley cv. 'Henni' (D, 1995), the 2-row winter barley 'Anthere' (D, 1995) and the 6-row cultivars 'Uschi' (D, 1997), 'Sarah' (D, 1997), 'Carola' (F, 1997), 'Nelly' (D, 1998), and 'Merlot' (D, 2002) – some of them being resistant to BaMMV and BaYMV - have been released and became widely grown in Europe (E. Laubach, pers. comm.). Recently, the 2-rowed DH-cultivars 'Annika' (2004) and 'Finita' (2004) have been released in Germany. They particularly combine BaMMV/BaYMV resistance with pronounced winter hardiness, especially 'Annika' which has a winter-kill score of 3 on a 1-9 scale (http://www.bundessortenamt.de/internet20/).

Year 1
May-June: Crossing - Transfer to the laboratory for anther culture

Year 2
Spring: Planting of doubled haploids (100 -200 plants per cross)
Field observation at 3 locations for major diseases etc.
Harvest: ca. 4 -5 kg of each DH line evt. winter generation in the south for acceleration

Year 3
Yield trials at 5 locations in Germany

Year 4
Yield trials at 8 locations
Application for registration by the Plant Variety Office

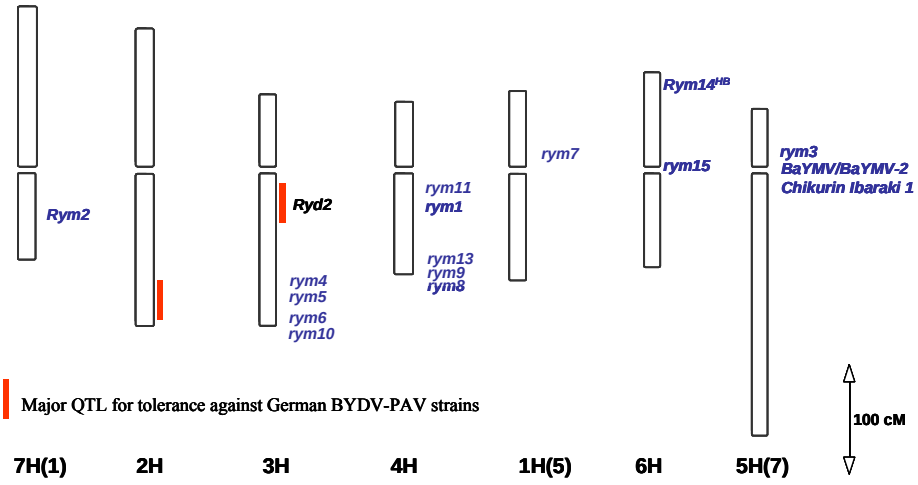
Fig. 1. Breeding scheme for spring and winter barley by application of the „haploid method" (E. Laubach, pers. comm.)

It is obvious that a significant time gain can be achieved by application of the haploidy method in barley (Fig. 1). Consequently, this breeding method is more widely applied now and has gained great importance in barley breeding programmes as demonstrated by the growing number of released DH varieties. Today, a substantial part of new barley variety applications in Germany actually represent DH lines. In the future, the efficiency of DH-breeding is expected to be enhanced due to the successful application of isolated microspore culture technique.

Marker-Assisted Breeding

Besides using cell- and tissue-culture techniques molecular markers have to be considered as efficient tools in breeding virus resistant barley, today. Molecular markers to some extent allow the transfer of selection from the phenotypic to the genotypic level. An overview on mapped resistance genes against barley yellow mosaic virus disease as well as on QTL indentified for tolerance against BYDV (Fig. 2) is given by ORDON *et al*. (this volume). With respect to barley yellow mosaic virus disease these markers facilitate effective selection on a single plant level in an early developmental stage independent from symptom development in the field (ORDON *et al*. 2003), e.g. selection may be carried out directly *in vitro* on plants derived from anther or microspore culture, thereby reducing the number of plants to be transferred to the greenhouse. For BYDV respective markers are a least suited for marker based pre-selection of more tolerant genotypes as the QTL identified in independent crosses explain about 50% of the phenotypic variance for the relative grain yield after infection with a German strain of BYDV-PAV (ORDON *et al*., this volume). Thereby, the number of plants to be tested by artificial inoculation with virus-bearing aphids which is difficult to implement in barley breeding programmes can be significantly reduced. Because respective QTL-flanking markers are polymorphic on cultivars carrying *rym4*, like ´Hanna´ a.o., and *rym5* (e.g. 'Tokyo´), they furthermore offer the opportunity for a simultaneous marker-based selection of genotypes with multiple virus resistance (SCHEURER et al. 2001).

Besides the use in marker assisted selection procedures directly aiming at breeding of new cultivars, respective markers are useful tools in backcross (BC) programmes needed to incorporate resistance genes derived from non-adapted germplasm (ORDON & FRIEDT 1994), e.g. *rym11*, *rym13*, *rym15,* into high yielding breeding lines. Because all the resistance genes against the soil-borne mosaic inducing viruses are inherited recessively (ORDON & FRIEDT 1993) a selfing generation is needed after each backcrossing step for the phenotypic identification of resistant plants (homozygous recessive) suitable for the next cycle. However, by co-dominant markers like microsatellites (SSRs) heterozygous carriers of the resistance encoding allele which can be used in the next BC step can be detected directly after backcrossing, thereby saving one year for each BC cycle (ORDON *et al*. 1999). The same holds true for dominant markers generating an additional fragment indicative for the recessive resistance encoding allele.



Modified according to Graner et al. (2000)

Fig. 2. Barley chromosomes and genetic loci involved in yellow mosaic virus resistance (rym) or BYDV-tolerance (quantitative trait loci, QTL)

Another important application of molecular markers is their use in strategies aiming at the combination of different resistance genes in one breeding line ('pyramiding', cf. Fig. 3). This approach may lead to longer lasting and broad-spectrum resistance, thereby probably avoiding the selection of new virus strains as already reported from Japan, where seven strains of BaYMV and two of BaMMV were described (NOMURA *et al*. 1996). Due to the lack of differentiating virus strains this task cannot be achieved by phenotypic selection. Therefore, molecular markers have to be considered as an essential tool for this purpose. As homozygous recessive genotypes are more frequent among doubled haploid lines (DHs) than in F_2 populations and dominant markers are as informative as co-dominant ones due to the lack of heterozygous genotypes, strategies for pyramiding of respective resistance genes are more efficient using DHs. In this respect it has to be noted that *rym4* and *rym5* seem to be non-combinable due to their chromosomal localisation and allelism concerning BaMMV-resistance (cf. GRANER et al. 1999). Starting from single crosses e.g. *rym4* x *rym9*, *rym4* x *rym11*, *rym5* x *rym11*, *rym5* x *rym9*, *rym9* x *rym11* two different strategies were followed (WERNER 2003). On the one hand DH-lines were directly produced in F_1 and plants carrying both genes in a homozygous recessive state were identified with corresponding PCR-based markers. Intercrossing of these genotypes followed by DH-line production and marker analysis led to the identification of genotypes being homozygous for two or three resistance genes. On the other hand, inter-crossing of F_1-progenies having one resistance gene in common, e.g. [(*rym4* x *rym9*) (*rym4* x *rym11*)] led to genotypes having the gene in common fixed homozygous-recessively and being heterozygous at the other resistance loci (*rym4/rym4, rym9/Rym9, rym11/Rym11).* This genotype which is theoretically present at a frequency of 6.25% can be identified by molecular markers and used for DH-line production leading to an offspring of 25% having three resistance genes and 50% having two genes fixed homozygous recessively.

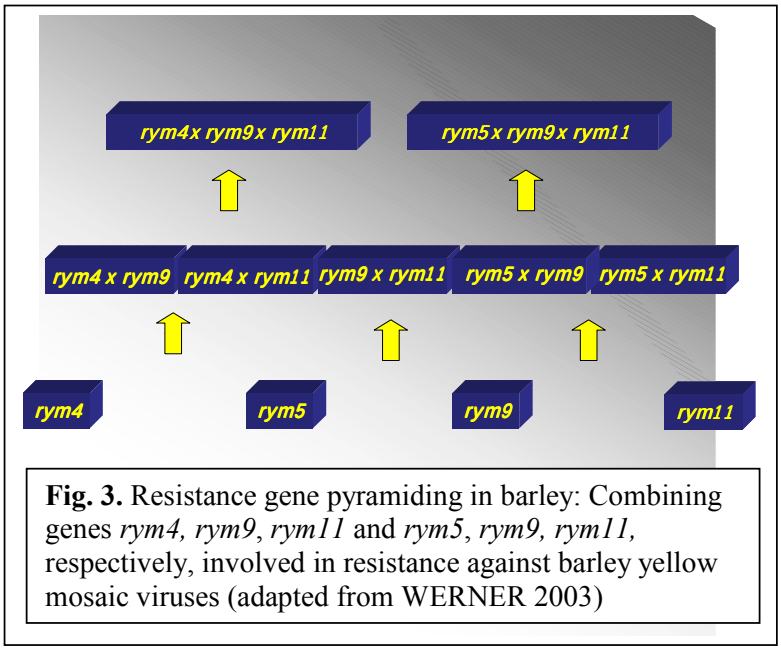


Fig. 3. Resistance gene pyramiding in barley: Combining genes *rym4, rym9*, *rym11* and *rym5*, *rym9, rym11,* respectively, involved in resistance against barley yellow mosaic viruses (adapted from WERNER 2003)

In comparison to the first strategy the second one is faster and cheaper as it involves only one haploid step, but it has to be taken into consideration that a minimum of 100 F_1 inter-cross seeds are needed in order to identify the desired rare genotype (theoretically 6.25%) with sufficient probability. In our own experiments between 1 and 11 of these genotypes were identified and after using them for DH-line production genotypes with three and two resistance genes homozygously fixed have meanwhile been identified according to the expected frequencies (Fig. 3, WERNER 2003). Future studies will show whether these gene

combinations provide better or more durable resistance(s) against barley yellow mosaic inducing viruses.

Conclusions and Perspectives

Barley breeding in Europe has been extremely successful throughout the last century. Cycles of cross breeding which first made use of hybridisation between European land-races, later exploiting more distant germplasms providing valuable disease resistances, and finally combining high-yield and high-quality varieties, in the end led to highly productive modern cultivars. In most cases, the practical breeding techniques com-prised little more than manual hybridisation, careful observation, precise testing and conscious selection. More recently, breeding programmes have been enhanced by the implementation of modern biotechnology tools, like the "haploid method" via anther culture *in vitro*. In the future, more efficient isolated microspore culture technique may replace the culture of whole anthers. In addition to that, it is expected that marker-assisted selection procedures – particularly PCR-based techniques combined with fast and high through-put analysis - will further enhance the process of selecting resistant barley varieties with superior agronomic performance in the future. Marker-assisted selection has already been widely used in virus resistance breeding, especially regarding the *rym4*-and *rym5* resistance. The same is expected in the future with regard to "new" yellow mosaic virus resistances, like *rym13* and *rym15*. Besides this, marker-assisted breeding may implement and use new strategies, e.g. as demonstrated by the example of "gene pyramiding". Above this, a better understanding of gene and genome functions will enable more straightforward breeding approaches.

Acknowledgements

We wish to cordially thank Dr. Eberhard LAUBACH, Nordsaat/Germany for variety and pedigree information. Thanks are due to the German Research Foundation (DFG) and the German Breeders Association (GFP) for project funding and support.

References

ANONYMOUS (2003): Bundessortenamt (ed.), Beschreibende Sortenliste 2003, Landbuch-Verlag, Hannover.

BURNETTT P.A., COMEAU A., QUALSET CO. (1995): Host plant tolerance or resistance for control of barley yellow dwarf. In: D'Arcy CJ, Burnett PA (eds.) Barley yellow dwarf. 40 years of progress, 321-343. APS Press, St. Paul, Minnesota

DELOGU G, CATTIVELLI L., SNIADRO M., StANCA A.M. (1995): The *Yd2* gene and enhanced resistance to barley yellow dwarf virus (BYDV) in winter barley. Plant Breeding, **114**: 417-420

FOROUGHI-WEHR B., FRIEDT W. (1983): Rapid production of recombinant barley yellow mosaic virus resistant *Hordeum vulgare* lines by anther culture. Theor. Appl. Genet. **67**: 377-382.

FRIEDT, W., RASMUSSEN M. (2003): Modern European barley cultivars: Genetic progress in resistance, quality and yield. Proc. EUCARPIA Cereal Section Meeting, Salsomaggiore/Italy 2002, 73-78.

FRIEDT, W., WERNER K., ORDON F. (2000): Genetic progress as reflected in highly successful and productive modern barley cultivars. Proc. 8th Int. Barley Genet. Symp, Adelaide, Vol I, inv. papers: 271-279.

FRIEDT, W., WERNER K., PELLIO B., WEISKORN C., KRÄMER M., ORDON F. (2002): Strategies of breeding for durable disease resistance in cereals. Progress in Botany **64**: 138-167.

FRIEDT W., SCHEURER K.S., HUTH W., HABEKUSS A., ORDON, F. (2003): Genetic analysis on BYDV-tolerance in barley (*Hordeum vulgare* L.) J. Plant Diseases and Protection, **110**: 278-286.

GÖTZ R., FRIEDT W. (1993): Resistance to the barley yellow mosaic virus complex – Differential genotypic reactions and genetics of BaMMV-resistance of barley (*Hordeum vulgare* L.). Plant Breeding, **111**: 125-131.

GRANER A., BAUER E. (1993): RFLP mapping of the ym4 virus resistance gene in barley. Theor. Appl. Genet., **8**6: 689-693.

GRANER A., STRENG S., KELLERMANN A., SCHIEMANN A., BAUER E., WAUGH R., PELLIO B., ORDON F. (1999): Molecular mapping of the *rym5* locus encoding resistance to different strains of the Barley Yellow Mosaic Virus Complex. Theor. Appl. Genet., **98**: 285-290.

GRANER A., MICHALEK W., STRENG S. (2000): Molecular Mapping of Genes Conferring Resistance to Viral and Fungal Pathogens. Proc. 8th International Barley Genetics Symposium, Adelaide, Australia. Vol I, inv. papers: 45-53.

GRAUSGRUBER, H., H. BOINTNER, R. TUMPOLD, RUCKENBAUER P. (2002): Plant Breeding 121, 411-416.

HARIRI D., MEYER M., PRUD'HOMME H. (2003): Characterization of a new barley mild mosaic virus pathotype in France. Eur. J. Pathol., **109**: 921-938.

HUTH W. (1989): Ein weiterer Stamm des Barley Yellow Mosaic Virus in der Bundesrepublik Deutschland. Nachrichtenbl. Deut. Pflanzenschutzd., **40**: 49-55.

HUTH W., ADAMS M.J. (1990): Barley Yellow Mosaic Virus (BaYMV) and BaYMV-M: two different viruses. Intervirology, **31**: 38-42.

KAISER R, FRIEDT W. (1989): Chromosomal resistance to barley yellow mosaic virus in German winter-barley identified by trisomic analysis. Theor. Appl. Genet., **77**:241-245

KAISER R., FRIEDT W. (1992): Gene of resistance to barley mild mosaic virus in German winter barley located on chromosome 3L. Plant Breeding, **108**:169-172

KONISHI T., BAN T., IIDA Y., YOSHIMI R. (1997): Genetic analysis of disease resistance to all strains of BaYMV in a Chinese barley landrace, Mokusekko 3. Theor. App.l Genet., **94**: 871-877.

LISTER R.M., RANIERI R. (1995): Distribution and economic importance of barley yellow dwarf. In D'Arcy CJ, Burnett PA (eds.) Barley yellow dwarf. 40 years of progress, 29-53. APS Press, St. Paul, Minnesota

NOMURA K., KASHIWAZAKI S., HIBINO H., INOUE T., NAKATA E., TSUZAKI Y., OKUYAMA S. (1996): Biological and Serological Properties of Strains of Barley Mild Mosaic Virus. J. Phytopathol., **144**: 103-107.

ORDON F., FRIEDT W. (1993:) Mode of inheritance and genetic diversity of BaMMV resistance of exotic barley germplasms carrying genes different from 'ym4'. Theor. Appl. Genet., 86: 229-233.

ORDON F., FRIEDT W. (1994): Agronomic traits of exotic barley germplasms resistant to soil-borne mosaic-inducing viruses. Genetic Resources and Crop Evolution, **41**: 43-46.

ORDON F., GÖTZ R., FRIEDT W. (1993): Genetic stocks resistant to barley yellow mosaic viruses (BaMMV, BaYMV, BaYMV-2) in Germany. Barley Genet. Newsl., **22**: 46-49.

ORDON F., SCHIEMANN A., PELLIO B., DAUCK V., BAUER E., STRENG S., FRIEDT W., GRANER A. (1999): Application of molecular markers in breeding for resistance to the barley yellow mosaic virus complex. J. Plant Diseases and Protection, **106**: 256-264.

ORDON F., PELLIO B., WERNER K., SCHIEMANN A., FRIEDT W., GRANER A. (2003): Molecular breeding for resistance to soil-borne viruses (BaMMV, BaYMV, BaYMV-2) of barley (Hordeum vulgare L.) J. Plant Diseases and Protection **110**: 287-295.

PARRY A.L., HABGOOD R.M. (1986): Field assessment of the effectiveness of a barley yellow dwarf resistance gene following its transference from spring to winter barley. Ann. Appl. Biol., **108**: 395-401.

PLUMB RT, JOHNSTONE G.R. (1995): Cultural, chemical and biological methods for the control of barley yellow dwarf. In: D'Arcy CJ, Burnett PA (eds.) Barley yellow dwarf. 40 years of progress, 107-127. APS Press, St. Paul, Minnesota

RASMUSSON D.W., SCHALLER C.W. (1959): The inheritance of resistance in barley to the yellow dwarf virus. Agron J., **51**: 661-664.

SCHALLER C.W. (1984): The genetics of barley yellow dwarf virus in barley. In: Burnett PA (ed) Barley yellow dwarf. Proc. Workshop CIMMYT, Mexico, 93-99

SCHEURER K.S., FRIEDT W., HUTH W. WAUGH R., ORDON F. (2001): QTL analysis of tolerance to a German strain of BYDV-PAV in barley (*Hordeum vulgare* L.). Theor. Appl. Genet., **103**: 1074-1083.

TOYAMA A., KUSABA T. (1970): Transmission of soil-borne barley yellow mosaic virus. 2. *Polymyxa graminis* Led. as vector. Ann. Phytopath. Soc. Japan, **36**: 223-229.

USUGI T., KASHIWAZAKI S., OMURA T., TSUCHIZAKI T. (1989): Some properties of nucleic acids and coat proteins of soil-borne filamentous viruses. Ann Phytopath Soc Japan, **55**:26-31.

WERNER K. (2003): Kartierung und Pyramidisierung von Resistenzgenen gegen die Gelbmosaikvirose (BaMMV, BaYMV, BaYMV-2) der Gerste (*Hordeum vulgare* ssp. *vulgare*). Diss. Univ. Giessen. Verlag Shaker Aachen, 125p.

S 9 – ABIOTIC STRESS

Genetic Bases of Barley Response to Abiotic Stresses

L. Cattivelli[1], C. Crosatti[1], C. Marè[1], S. Barilli[1], S. Belloni[1], E. Mazzucotelli[2] and A.M. Stanca[1]

[1]Experimental Institute for Cereal Research, Section of Fiorenzuola d'Arda (PC), Italy;
[2]Experimental Institute for Cereal Research, Section of Foggia, Italy

Introduction

Barley, one of the world largest crops, is grown from northern countries till the limits of the desert. Such a great diffusion, despite differences in the climatic conditions, already suggests that the barley gene pool should contain characters for wide environmental adaptability and good stress resistance.

Genetic variability plays a primary role in determining positive adaptation to environmental stresses and, hence, in supporting the spread of various barley genotypes to extreme climatic conditions. In barley, plant growth habit and heading-data are the basic traits involved in plant adaptation to environments because they allow to synchronise the plant life cycle with seasonal changes. However, adaptation to environments involves also the ability of plants to cope with stress factors such as frost, drought and low soil fertility. Compared to other cereals, barley is well adapted to poorly fertile soils or drought environments, although it is less frost resistant than other winter cereals (CATTIVELLI *et al*. 2001).

Winter Hardiness

Winter hardiness can be defined as the ability to survive throughout the winter. By the virtue of the wide range of stressful conditions that a plant may experience during the cold season, winter hardiness is a complex trait. Freezing temperature is the most relevant stress factor, although other stress situations, such as anoxia due to excess water or to ice encasement and photoinhibition due to the combination of light and low temperature, may also occur.

The adaptation to cold climate can be achieved either by the development of a powerful frost tolerance ability or by limiting the life cycle to the short summer season. Winter barley varieties are generally less hardy than winter wheat, rye and triticale. Nevertheless, barley is grown beyond the Polar Circle because early maturing spring cultivars are able to run their life cycle in the short summer season (STANCA *et al.* 2003). Plant growth habit and heading date can, therefore, be considered as the basic traits involved in barley adaptation to environments since they allow synchronisation of the plant life cycle with seasonal changes (BOYD *et al*. 2003).

Growth habit is the best known genetic trait affecting the winter hardiness of barley cultivars, the spring type being frost-sensitive and the winter type frost-tolerant. Winter growth habit is due to the requirement of an external signal to the plant to shift from vegetative to reproductive growth: this signal can be completion of a vernalization requirement (LIMIN and FOWLER 2002) and/or daylength of sufficient duration (KARSAI *et al*. 1999). In barley three *Vrn-H* (formerly *Sh*) loci determine vernalization requirement: *Vrn-H1* on chromosome 5H, *Vrn-H2* on 4H and *Vrn-H3* on 1H. Alleles at these loci interact epistatically, such that a vernalization requirement occurs in plants homozygous recessive at *Vrn-H1* and *Vrn-H3* and with at least one dominant allele at *Vrn-H2*. The inter-relationships vernalization,

photoperiod, and low temperature are most likely attributable to linkage rather than pleiotropy. In the 'Dicktoo' x 'Morex' population, low temperature tolerance and photoperiod sensitivity QTLs map to the same region as the *Fr-A1* and *Vrn-A1* loci, which SUTKA *et al.* (1999) demonstrated were distinct loci *via* physical mapping.

Resistance to low temperature is necessary for winter habit genotypes grown in areas with subzero winter temperatures. Maximum low temperature tolerance is achieved after induction - "hardening" *i.e.* exposure to moderately low temperatures - and it is achieved at vegetative growth stages. Vernalization, low temperature tolerance, and photoperiod sensitivity are inter-related (LIMIN and FOWLER 2002). The same conditions, essential for vernalization of winter genotypes, promote in both spring and winter types the hardening process. Since winter type plants are generally hardier than spring ones a major debated question is whether vernalization response and low temperature tolerance are pleiotropic effects of the same genes or determined by separate loci (LIMIN & FOWLER 2002). Nevertheless, in a large sample of barley germplasm the three phenotypes for vernalization, low temperature tolerance, and photoperiod sensitivity were observed to occur in all possible combinations (KARSAI *et al*. 2001).

Molecular analysis of the cold acclimation process has led to the isolation of many cold-regulated (*cor*) genes (CATTIVELLI *et al.* 2002; FACCIOLI *et al.* 2002). The level of hardiness among barley plants grown in different temperature environments was found to be strongly correlated with the expression level of *cor* genes (PEARCE *et al.* 1996).

Although cold acclimation in barley involves the expression of many cold-regulated genes, genetic studies have proved that only few chromosome regions (mainly on chromosome 5H) carry loci that play an important role in frost tolerance. On the long arm of chromosome 5H there are QTL/loci for vernalization response, low temperature tolerance, and photoperiod sensitivity (PAN *et al*. 1994; CATTIVELLI *et al*. 2002). These QTL effects have, in some reports, been described as the effects of single loci. Thus, low temperature tolerance loci on the wheat chromosomes 5A, 5B and 5D have been assigned the locus designations *Fr-A1*, *Fr-B1* and *Fr-D1*, respectively (TOTH *et al*. 2003) and by inference based on molecular markers in common to the various linkage maps, the H genome homoeolog to the *Fr* loci is the low temperature tolerance QTL first reported in the 'Dicktoo' x 'Morex' population (HAYES *et al*. 1993).

By using the highly syntenic wheat genome it has been demonstrated that on the homoeologous group 5, in genetic linkage with the growth habit locus, there are two loci which control the expression of several *cor* genes (VAGUJFALVI *et al*. 2000, 2003; FRANCIA *et al*. 2004) demonstrating a genetic linkage between frost resistance and the expression of *cor* genes. Among the *cor* genes described in wheat and barley, the cold-regulated gene *cor14b* is one of the best characterized. *Cor14b* encodes for a chloroplast localised protein of 14 kDa accumulated in higher amount in winter cultivars than in spring ones (CROSATTI *et al*. 1999, DAL BOSCO *et al.* 2003; GIORNI *et al*. 1999).

A notable advance in plant cold tolerance research was the discovery of the family of transcription factors known as C-Repeat binding factors (CBFs) or dehydration responsive element binding factors (DREBs) that controls, in ABA-independent manner, the expression of genes cold responsive. The CBF cold-response pathway, first identified in *Arabidopsis,* appears to be conserved in many species including the *Triticeae*, suggesting that regulation of the CBF transcriptional cascade during low temperature stress is an important strategy for plant frost tolerance (THOMASHOW *et al*. 2001). Genes with *CBF* signature sequences have been reported in barley and have been characterized in terms of their map location, coding sequence, and expression profile (CHOI *et al*. 2002; XUE 2003; FRANCIA *et al*. 2004). Recently, *HvCbf3* and *HvCBF2* genes were isolated and characterised. *HvCBF2*, is expressed in barley leaves under non-stress conditions while a transient increase in the transcript level

was observed during cold treatment. *HvCBF2* was shown to be capable of acting expression of a low-temperature and drought-responsive *HVA1s* barley promoter (XUE 2003). *HvCbf3* is transiently up-regulated by chilling treatment and the corresponding transcripts begin to rise after 15 min of cold stress, reaching a maximum level of transcripts after 2 h of treatment.

HvCbf3 is located together with other *Cbf*-like sequences on barley chromosome 5H but far (approximately 40-50 cM) from the major QTL for winter hardiness where the loci for vernalization requirement (*Vrn1*) and freezing tolerance (*Fr1*) are located (CHOI *et al*. 2002). When the level of *cor14b* mRNA expression or protein accumulation was used as a genetic tool to understand the relationship between the expression of *cor* genes and frost resistance, a single QTL for *cor14b* expression was found in the same position where the *Cbf* locus is located (VÀGÙJFALVI *et al.* 2003; FRANCIA *et al*. 2004).

Drought Tolerance

Drought is a multidimensional stress which is difficult to characterise. It is a function of the genotype, rainfall, temperature, and soil water holding capacity. Its occurrence and severity vary at any site from year to year. Drought is the most common abiotic constraint for stable barley production in rainfed areas. Under Mediterranean conditions, water stress is particularly common at the end of barley life cycle as grain filling usually occurs under relatively high evapotranspirative demands and low precipitation.

Variation in heading date is the primary cause for yield differences at different water regimes during grain filling (PASSIUORA 1996). Thus, the earliest cultivars generally perform better in rainfed low-yielding environments (ABAY & CAHALAN 1995), escaping the harshest conditions at the end of the growing season. There is a great deal of genetic diversity for traits related to the heading date. A number of well defined loci is known to control the flowering time following the interaction with environmental signals (temperature and day-length). Three different genetic components are known: photoperiod response (depending from day-length), vernalization response (depending from temperature), and "earliness *per se*" largely independent from both day-length and low temperature.

Grain yield in water-limited environments is directly related to the plant ability to capture water (reflected by the amount of water transpired) -T-; water use efficiency (the ratio of above ground biomass produced by unit water transpired) -WUE-; and harvest index -HI- (PASSIUORA 1996). Little is known about how much genetic diversity is present for T or WUE. For barley, the results of genetic gains in total plant biomass (the product of these two components) with time are contradictory. Some studies suggest that there is not much genetic variation for this trait since biomass production has varied little in recent decades (JEDEL & HELM 1994). On the contrary, RIZZA *et al.* (in press) have found a significant variation for WUE under moderate drought stress among a collection of 89 barley genotypes. This work has led to the identification of few cultivars with high WUE and low G x E interaction showing a high range of adaptability under drought and non drought environments. Increasing the amount of water extracted by the crop from the soil is meaningful if provided soil water is still present at maturity. In rice, genetic factors controlling the root characteristics were found to be associated with field drought tolerance (CHAMPOUX *et al.* 1995). Genetic variability for root characteristics has also been shown for barley (GRANDO & CECCARELLI 1995).
Induced osmotic adjustment may allow plants to extract water from the soil under drought, and therefore may be an important component of drought resistance in barley (BLUM 1989). In this context, increased accumulation of an osmoprotectant such as glycine-betaine has been associated with drought stress tolerance (ISHITANI *et al.* 1995).
ARNAU *et al.* (1997) studied the effect of water stress on plant water status and net photosynthetic gas exchange (P-N) in barley cultivars and landraces differing in productivity

and drought tolerance. Variability for some parameters like osmotic adjustment capacity and P-N was observed among drought tolerant genotypes and susceptible ones. In C_3 plants, the ratio of $^{13}C/^{12}C$ and of $^{15}N/^{14}N$ in plant tissues is different than in the surrounding air or soil, due to a selective preference towards ^{12}C and ^{14}N in the dry matter synthesis. The carbon isotope discrimination ('delta', $\Delta^{13}C$) is directly related to the water use efficiency, while the nitrogen isotope discrimination ('delta', $\Delta^{15}N$) may reflect the extent to which N can be retained within plant when stressed. When $\Delta^{13}C$ and $\Delta^{15}N$ were measured in 30 ecologically different genotypes of *H. vulgare* ssp. *spontaneum* and subjected to mild short-term drought, a large diversity was found. Notably, the most stress-tolerant genotypes were characterised by the less negative $\Delta^{13}C$ and by the most negative $\Delta^{15}N$ (ROBINSON *et al.* 2000).

The development of QTL analysis has allowed the identification of the genetic bases of drought tolerance, and different alleles (with either positive or negative effects) have been identified at the drought tolerance loci. TEULAT *et al.* (1998) identified two regions on the chromosome 6H and 7H where most of the loci controlling drought related traits are located. On the long arm of chromosome 4H are located the loci for water use efficiency and for adaptation to droughted environments (FORSTER *et al*. 2000). Different genomic regions are implicated in the total phenotypic variation of relative water content (RWC), in particularly the long arm of chromosome 6H contains the most-consistent QTL, region previously identified as controlling leaf osmotic potential under water stress and osmotic adjustment (TEULAT *et al.* 2003).

Plant exposure to drought promote a molecular response involving the induction of many dehydration-responsive genes (CATTIVELLI *et al.* 2002; OZTUR *et al.* 2002), the majority belonging to the class 2 LEA (Late Embryogenesis Abundant) gene family. LEA 2 proteins, known as dehydrins, are characterised by one or more conserved lysine-rich 15-amino acid sequences near the C-terminus and, in most cases, by a stretch of serine residues. Their hydrophilic characteristics suggest an osmoprotective role during cell dehydration (CHOI *et al.* 1999). Molecular diversity for drought-induced proteins has also been found in barley cultivars with different drought-tolerance (GROSSI *et al.* 1992). Other drought-responsive genes cloned in barley are known to encode for proteins with enzymatic activities to yield osmoprotective molecules, such as aldose reductase (BARTELS *et al.* 1991) or betaine aldehyde dehydrogenase (ISHITANI *et al.* 1995). The expression of many dehydration responsive genes is determined by the osmotic component of drought. It is therefore not surprising that some of these genes are also induced in other stress conditions characterized by an osmotic component as cold, salinity, etc..

Salinity Tolerance

Soil salinity and soil sodicity are common problems in arid and semi-arid areas.

In plants there is no specific carrier for Na^+ uptake; however, Na^+ enters by competition with other cations, particularly K^+. Regulation of cellular Na^+ is achieved by effluxing Na^+ through an Na^+/H^+ antiporter, driven by the electrochemical H^+ gradient across the plasmalemma. Intracellular compartmentalization can also occur due to the work of the vacuolar Na^+/H^+ antiporter driven by the electrochemical H^+ gradient across the tonoplast (SCHACHTMAN & LIU 1999). Salt tolerance in cereals is known to be associated with the control of shoot Na^+ content. Tolerant lines have more efficient systems to exclude Na^+ from their cells. Loci involved in salt tolerance have been identified on chromosomes 4H and 5H of *H. vulgare* and $1H^{ch}$, $4H^{ch}$ and $5H^{ch}$ of *H. chilense* (FORSTER *et al.* 1990). The gene pool of *H. spontaneum* may also represent an interesting source of new loci for salt tolerance and several QTLs were detected on chromosomes 7H, 4H, 1H and 6H (ELLIS *et al.* 1997).
MANO *et al.* (1997) evaluated a world collection of barley accessions for salt tolerance at

germination. Cultivar variation showed a normal distribution; the most sensitive varieties failed to germinate in 1% NaCl solution and the most tolerant ones could germinate in sea water. Tight correlation (r=0.789) between germinability in NaCl solution and in polyethylene glycol solution with comparative osmotic pressure suggested that salt inhibits seed germination primarily by osmotic effect. The QTLs for salt tolerance at germination are located on chromosome 1H, 4H, 5H and 6H, for seedling tolerance of salt on chromosome 1H, 2H, 5H and 6H. In a previous study, MANO and TAKEDA (1995), evaluating many barley cultivars for salt tolerance at seedling stage, showed also that there was no correlation between salt tolerance at germination and at seedling stage (r=-0.061), suggesting that the mechanisms of tolerance are different at the different stages. As mentioned before, salinity sensitivity at germination is primarily due to an osmotic effect, and the sensitivity at seedling stage may be due to sodium toxicity.

Acid and Alkaline Soils and Tolerance to Heavy Metals

Barley, like other plant species, suffers when grown in soils with non-neutral pH conditions. Both alkaline and acid soils can produce unfavourable conditions. In some cases a pure pH effect is present, related to hydrogen concentration, but mostly the effects are indirect and depend on the availability of other ions in the soil.

When plants are grown in alkaline soils, a manganese (Mn) deficiency may occur. Mn, although present in these soils, is usually in a complex form not available to plants. Among cereals, rye absorbs about five times as much Mn as wheat or barley and barley is more tolerant to Mn deficiency than durum wheat (SABERI *et al.* 1999). In barley, a genetic analysis of tolerance to Mn-deficiency has revealed the presence of an Mn-efficiency locus (*MelI*) located on chromosome 4H (JEFFERIES *et al.* 2000).

Acid soils are common in several areas worldwide. Lower soil pH affects soil structure, microflora, and the availability of mineral nutrients. Situations of mineral deficiency and mineral toxicity are both present in acid soils. Generally, barley is regarded as a species tolerating a very wide range of soil pH values.

A low soil pH also increases the availability of toxic heavy metals to the plant roots. In a screening of different grass species for phytoremediation purposes, barley, together with oat, was found to be able to tolerate relatively high concentrations of copper (Cu), cadmium (Cd) and zinc (Zn). These results indicated the potential role of barley in the phytoremediation of contaminated soils (EBBS & KOCHIAN 1998).

Upon exposure to heavy metals, barley cells activated the expression of a set of stress-responsive genes. TAMAS *et al.* (1997) showed that Cu, Cd and cobalt (Co), as well as aluminium (Al), induced extracellular accumulation of several polypeptides originally described as PR (pathogenesis-related) proteins. A genetic diversity in the molecular response to heavy metal stress was also found between resistant and sensitive cultivars. After treatment with Cu, Cd or Co, the Al-sensitive barley cultivar *'*Alfor*'* was found to accumulate two cytoplasmic polypeptides absent in the Al resistant cv. *'*Bavaria*'* (TAMÁS *et al.* 2000).

In acid soils with pH values below 5.5, the presence of available Al probably represents the most important growth-limiting factor. Among winter cereals barley is the most sensitive to the excess of soluble or exchangeable Al and a limited genetic diversity for Al tolerance has been found (GALLARDO *et al.* 1999). The cultivar Dayton has been referred to exhibit the highest level of Al tolerance. The inheritance of Al tolerance in barley is reported to be conferred by a single gene designated *Alp* located to the long arm of chromosome 4H (MINELLA & SORRELLS 1997). Recently, microsatellite markers flanking *Alp* have been identified in a mapping population from a cross between Dayton and Harlan Hybrid (RAMAN *et al.* 2003). The limited genetic variability found so far suggests that new and still

uncharacterised germoplasm and related species should be investigated in the future in a attempt to improve Al tolerance in barley.

References

ABAY F., CAHALAN C. (1995): Evaluation of response of some barley landraces in drought prone sites of Tigray (Northern Ethiopia). Crop Improv., **22**: 125-132.

ARNAU, G., P. MONNEVEAUX, D. THIS, L. ALEGRE (1997): Photosynthesis of six barley genotypes as affected by water stress. Photosynthetica, **34**: 67-76.

BARTELS, D., K. ENGELHARDT, R. RONCARATI, K. SCHNEIDER, A.M. ROTTER, F. SALAMINI (1991): An ABA and GA modulated gene expressed in the barley embryo encodes an aldose reductase related protein. EMBO J., **10**: 1037-1043.

BLUM A. (1989): Osmotic adjustment and growth of barley genotypes under drought stress. Crop Sci., **29**: 230-233.

BOYD W.J.R., LI C.D., GRIME C.R., CAKIR M., POTIPIBOOL S., KAVEETA L., MEN S., JALAL KAMALI M.R., BARR A.R., MOODY D.B., LANCE C.M., LOGUE S.J., RAMAN H., READ B.J. (2003): Conventional and molecular genetic analysis of factors contributing to variation in the timing of heading among spring barley genotypes grown over a mild winter growing season. Australian J. Of Agricultural Research, **54**: 1277-1301.

CATTIVELLI L., BALDI P., CROSATTI C., GROSSI M., VALÈ G., STANCA A.M. (2001): Genetic bases of barley physiological response to stressful conditions. In: A. SLAFER, J.L. MOLINA-CANO, J.L. ARAUS, R. SAVIN AND I. RAMAGOSA (eds), Barley Science: Recent Advances from Molecular Biology to Agronomy of Yield and Quality. Food Product Press, New York, USA: 307-360.

CATTIVELLI L, BALDI P, CROSATTI C, DI FONZO N, FACCIOLI P, GROSSI M, MASTRANGELO A.M., PECCHIONI N, STANCA A.M. (2002): Chromosome regions and stress-related sequences involved in resistance to abiotic stress in *Triticeae*. Plant Mol Biol, **48**: 649-665.

CHAMPOUX M.C., WANG G., SARKARUNG S., MACKILL D.J., O´TOOLE J.C., HUANG N., MCCOUCH S.R. (1995): Locating genes associated with root morphology and drought avoidance in rice via linkage to molecular markers. Theor. Appl. Genet., **90**: 969-981.

CHOI D.W., ZHU B., CLOSE T.J. (1999): The barley (*Hordeum vulgare* L.) dehydrin multigene family: sequences, allele types, chromosome assignments and expression characteristics of 11 *Dhn* genes of cv. Dicktoo. Theor. Appl. Genet., **98**: 1234-1247.

CHOI D.-W., RODRIGUEZ E.M., CLOSE T.J. (2002): Barley *Cbf3* gene identification, expression pattern, and map location. Plant Physiol., **129**: 1781-1787.

CROSATTI C., POLVERINO DE LAURETO P., BASSI R., CATTIVELLI L. (1999): The interaction between cold and light controls the expression of the cold-regulated barley gene *cor14b* and the accumulation of the corresponding protein. Plant Physiol., **119**: 671-680.

DAL BOSCO C., BUSCONI M., GOVONI C., BALDI P., STANCA A.M., CROSATTI C., BASSI R., CATTIVELLI L. (2003): *Cor* gene expression in barley mutants affected in chloroplast development and photosynthetic electron transport. Plant Physiol., **131**: 793-802.

EBBS S.D., L.V. KOCHIAN (1998): Phytoextraction of zink by oat (*Avena sativa*), barley (*Hordeum vulgare*) and Indian mustard (*Brassica juncea*). Environ. Sci. Technol., **32**: 802-806.

ELLIS R., FORSTER B.P., WAUGH R., BONAR N., HANDLEY L.L., ROBINSON D., GORDON D.C., POWELL W. (1997): Mapping physiological traits in barley. New Phytol., **137**: 149-157.

FACCIOLI P., LAGONIGRO M.S. DE CECCO L., STANCA A.M., ALBERICI R., TERZI V. (2002): Analysis of differential expression of barley ESTs during cold acclimatization using microarray technology. Plant Biol., **4**: 630-639.

FORSTER B.P., PHILLIPS M.S., MILLER T.E., BAIRD E., POWELL W. (1990): Chromosome location of genes controlling tolerance to salt (NaCl) and vigour in *Hordeum vulgare* and *H. chilense*. Heredity, **65**: 99-107.

FORSTER B.P., ELLIS R.P., THOMAS W.T.B., NEWTON A.C., TUBEROSA R., THIS D., EL-ENEIN R.A., BAHRI M.H., BEN SALEM M. (2000): The development and application of molecular markers for abiotic stress tolerance in barley. J. of Exp. Bot., **51**, 342: 19-27.

FRANCIA E., RIZZA F., CATTIVELLI L., STANCA A.M., GALIBA G., TOTH B., HAYES P.M., SKINNER J.S., PECCHIONI N. (2004): Two loci on chromosome 5H determine low-temperature tolerance in a "Nure" (winter) x "Tremois" (spring) barley map. Theor. Appl. Genet., **108**: 670-680.

GALLARDO F., BORIE F., ALVEAR M., VON BAER E. (1999): Evaluation of aluminium tolerance of three barley cultivars by two short-term screening methods and field experiments. Soil Sci. Plant Nutr., **45**: 713-719.

GIORNI E., CROSATTI C., BALDI P., GROSSI M., MARÈ C., STANCA A.M. AND CATTIVELLI L. (1999): Cold-regulated gene expression during winter in frost tolerant and frost susceptible barley cultivars grown under field conditions. Euphytica, **106**: 149-157.

GRANDO S., S. CECCARELLI (1995): Seminal root morphology and coleoptile length in wild and cultivated barley. Euphytica, **86**: 73-80.

GROSSI M., CATTIVELLI L., TERZI V., STANCA A.M. (1992): Modification of gene expression induced by ABA, in relation to drought and cold stress in barley shoots. Plant Physiol. Biochem., **30**: 97-103.

HAYES P.M., BLAKE T., CHEN T.H.H., TRAGOONRUNG S., CHEN F., PAN A., LIU B. (1993): Quantitative trait loci on barley (*Hordeum vulgare* L.) chromosome-7 associated with components of winterhardiness. Genome, **36**: 66-71.

ISHITANI M., NAKAMURA T., HAN S.Y., TAKABE T. (1995): Expression of the betaine aldehyde dehydrogenase in barley in response to osmotic stress and abscisic acid. Plant Mol. Biol., **27**: 307-315.

JEDEL P.E., HELM J.H. (1994): Assessment of western Canadian barleys of historical interest. I. Yield and agronomic traits. Crop Sci., **34**: 922-927.

KARSAI I., MÉSZÁROS K., SZUCS P., HAYES P., LÁNG L., BEDO Z. (1999): Effects of loci determining photoperiod sensitivity (*Ppd-H1*) and vernalization response (*Sh2*) on agronomic traits in the Dicktoo x Morex barley mapping population. Plant Breeding, **118**: 399-403.

KARSAI I., MÉSZÁROS K., LÁNG L., HAYES P.M., BEDÕ Z. (2001): Multivariate analysis of traits determining adaptation in cultivated barley. Plant Breeding, **120**, 3: 217-222.

LIMIN A.E., FOWLER D.B. (2002): Developmental traits affecting low-temperature tolerance response in near-isogenic lines for the Vernalization locus *Vrn-A1* in wheat (*Triticum aestivum* L. em Thell). Annals of Botany, **89**: 579-585.

MANO Y., TAKEDA K. (1995): Varietal variation and effects of some major genes on salt tolerance in barley seedlings. Bull. Res. Inst. Biores., Okayama University, **3**: 71-81.

MANO Y., TAKEDA K. (1997): Mapping quantitative trait loci for salt tolerance at germination and the seedling stage in barley (*Hordeum vulgare* L.) Euphytica, **94**: 263-272.

MINELLA E., M.E. SORRELLS (1997): Inheritance and chromosome location of *Alp*, a gene controlling aluminium tolerance in 'Dayton' barley. Plant Breed., **116**: 465-469.

OZTUR Z.N., TALAME V., DEYHOLOS M., MICHALOWSKI C.B., GALBRAITH D.W., GOZUKIRMIZI N., TUBEROSA R., BOHNERT H.J. (2002): Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. Plant Mol Biol., **48** (5-6): 551-73.

PAN A., HAYES P.M., CHEN F., CHEN T.H.H., BLAKE T., WRIGHT S., KARSAI I., BEDÕ Z. (1994): Genetic analysis of the components of winterhardiness in barley (*Hordeum vulgare* L.). Theor. Appl. Genet., **89**: 900-910.

PASSIUORA J.B. (1996): Drought and drought tolerance. Plant Growth Reg., **20**: 79-83.

PEARCE R.S., DUNN M.A., RIXON J., HARRISON P., HUGHES M.A. (1996): Expression of cold-inducible genes and frost hardiness in the crown meristem of young barley (*Hordeum vulgare* L. cv. Igri) plants grown in different environments. Plant Cell Environ., **19**: 275-290.

RAMAN H, KARAKOUSIS A., MORONI J.S., RAMAN R., READ B.J., GARVIN D.F., KOCHIAN L.V., SORRELLS M.E. (2003): Development and allele diversity of microsatellite markers linked to the Al tolerance gene *Alp* in barley. Australian J. Of Agricultural Research, **54**: 1315-1321.

RIZZA F., BADECK F. W., CATTIVELLI L, LIDESTRI O., DI FONZO N., STANCA A.M. (2004): Use of a water stress index to identify barley genotypes adapted to rainfed and irrigated conditions. Crop Sci., in press.

ROBINSON L.L., HANDLEY, SCRIMGEOUR C.M., GORDON D.C., FORSTER B.P., ELLIS R.P. (2000): Using stable isotope natural abundances (delta N-15 and delta C-13) to integrate the stress responses of wild barley (*Hordeum spontaneum* C. Koch) genotypes. J. Exp. Bot., **51**: 41-50.

SABERI H.K., GRAHAM R.D., RATHJEN A.J. (1999): Inheritance of manganese efficiency in durum wheat. J. Plant Nutr., **22**: 11-21.

SCHACHTMAN D., LIU W. (1999). Molecular pieces to the puzzle of the interaction between potassium and sodium uptake in plants. Trends Plant Sci., **4**: 281-287.

STANCA A.M., ROMAGOSA I., TAKEDA K., LUNDBORG T., TERZI V., CATTIVELLI L. (2003): Diversity in abiotic stresses. In: VON BOTHMER R., VAN HINTUM T., KNUPFFER H., SATO K. (eds.) Diversity in barley (*Hordeum vulgare*) Elsevier Publ. Pp 179-199.

SUTKA J, GALIBA G, VAGUIFALVI A, GILL B.S., SNAPE J.W. (1999): Physical mapping of the *Vrn-A1* and *Fr1* genes on chro-mosome5A of wheat using deletion lines. Theor. Appl. Genet., **99**: 199–202.

TAMÁS L., HUTTOVÁ J., ŽIGOVÁ Z. (1997): Accumulation of stress-proteins in intercellular spaces of barley leaves induced by biotic and abiotic factors. Biol. Plant., **39**: 387-394.

TAMÁS L., HUTTOVÁ J., MISTRÍK I., HAJASOVA L. (2000): Accumulation of two cytoplasmic polypeptides in roots of Al-sensitive and Al-resistant barley cultivars during Al, pH and metal stress. Biologia, **55**: 259-265.

TEULAT B., THIS D., KHAIRALL M., BORRIES C., RAGOT C., SOURDILLE P., LEROY P., MONNEVEUX P., CHARRIER A. (1998): Several QTLs involved in osmotic-adjustment trait variation in barley. Theor. Appl. Genet., **96**: 688-698.

TEULAT, B., ZOUMAROU-WALLIS N., ROTTER B., BEN SALEM M., BAHRI H., THIS D. (2003): QTL for relative water content in field-grown barley and their stability across Mediterranean environments. Theor. Appl. Genet., **108**: 181-188.

THOMASHOW M.F., GILMOUR S.J., STOCKINGER E.J., JAGLO-OTTOSEN K.R., ZARKA D.G. (2001): Role of *Arabidopsis* CBF transcriptional activators in cold acclimation. Plant Physiol., **112**: 171-175.

TOTH B, GALIBA G, FEHÉR E, SUTKA J, SNAPE J.W. (2003): Mapping genes affecting flowering time and frost resistance on chromosome 5B of wheat. Theor. Appl. Genet., **107**: 509-514.

VAGUJFALVI A., CROSATTI C., GALIBA G., DUBCOVSKY J., CATTIVELLI L. (2000): Two loci on wheat chromosome 5A regulate the differential cold-dependent expression of the *cor14b* gene in frost tolerant and sensitive genotypes. Mol. Gen. Genet., **263**: 194-200.

VÀGÙJFALVI A., GALIBA G., CATTIVELLI L., DUBCOVSKY J. (2003): The cold-regulated transcriptional activator *Cbf3* is linked to the frost-tolerance locus Fr-A2 on wheat chromosome 5A. Mol. Gen. Genom., **269**: 60-67.

XUE G.P. (2003) : The DNA-binding activity of an AP2 transcriptional activator HvCBF2 involved in regulation of low-temperature responsive genes in barley is modulated by temperature. The Plant J., **33**: 373-383.

Drought Tolerance in Barley

S. Grando, M. Baum and S. Ceccarelli

Germplasm Program, International Center for Agricultural Research in the Dry Areas (ICARDA), P.O. Box 5466, Aleppo, Syria

Abstract

Since agriculture began, drought has been one of the major plagues limiting crop production worldwide. Although it reaches the front pages of the media only when it causes famine and death, drought is a permanent constraint to agricultural production in many areas of the world. Drought causes marginalization and poverty, and its negative effects are likely to increase as water resources decline globally. Climate changes will increase the frequency of droughts, particularly in Southeast Asia and Central America; by 2050 water shortages are expected to affect 67% of the world's population.

Drought resistance has been always a challenge to plant breeders. Physiologists, biochemists, geneticists and breeders have dissected drought resistance into individual components, with the aim of finding simple associated traits. Examples include proline accumulation, osmotic adjustment, stomatal conductance, canopy temperature, and various root characteristics. Today, most scientists agree that a drought resistance gene does not exist, and that differences in drought resistance are due to the effects of several genes, affecting different characters that interact with each other. This is because drought is unpredictable in its occurrence, severity, timing and duration; and because drought seldom occurs in isolation and often interacts with other stresses, mostly temperature extremes thus determining many combinations of stresses. Often cultivars successful in one dry year may fail in another, thus eluding the need of farmers for consistency of performance. Therefore, while drought is a global issue, its effects need to be addressed locally because every dry area has its own type of drought.

Future research to increase the level of drought tolerance in barley needs to address the interaction between a number of traits and assemble those combinations of traits that maximize economic yields per unit of water utilized, possibly with the use of molecular tools.

Introduction

Drought, or more generally limited water availability, is one of the major factors affecting crop production. It is a permanent constraint to agricultural production in many developing countries, and occasionally a cause of losses of agricultural production in developed ones. Despite many decades of research, it is still a challenge to agricultural scientists in general, and to plant breeders in particular.

Irrigation has often been seen as the way to alleviate drought. However, only 16% of the total cropland in the world is irrigated, with an increase of only 2% from 14% during the 10 years between 1977-79 and 1987-89. The major problem with irrigation is salinization. This is believed to affect one third of irrigated land (ASHRAF 1994) and is estimated to endanger half of the irrigated cropland (STRAUSS 1993).

The development, through breeding, of cultivars with higher yield under drought would be a major breakthrough (CECCARELLI & GRANDO 1996). However, the ability of some plants to give a higher yield under drought than others is a very elusive trait from a genetic point of view. This is because occurrence, severity, timing and duration of drought vary from year to year, and cultivars successful in one dry year may fail in another. To make matters worse, drought seldom occurs in isolation, it often interacts with other abiotic stresses (particularly

temperature extremes), and with biotic stress. At a given location, grain yield of barley, estimated by the mean of several breeding lines, can be very different in different years even with nearly the same amount of rainfall (Figure 1). Also, areas with a high risk of drought (and/or other abiotic stresses) generally have low-input agriculture.
Therefore crops exposed to drought will at the same time face a number of other yield-limiting factors, which modify the response to available water. One of the major challenges for the plant breeder is to select for the ability to tolerate various combinations of all these stresses.

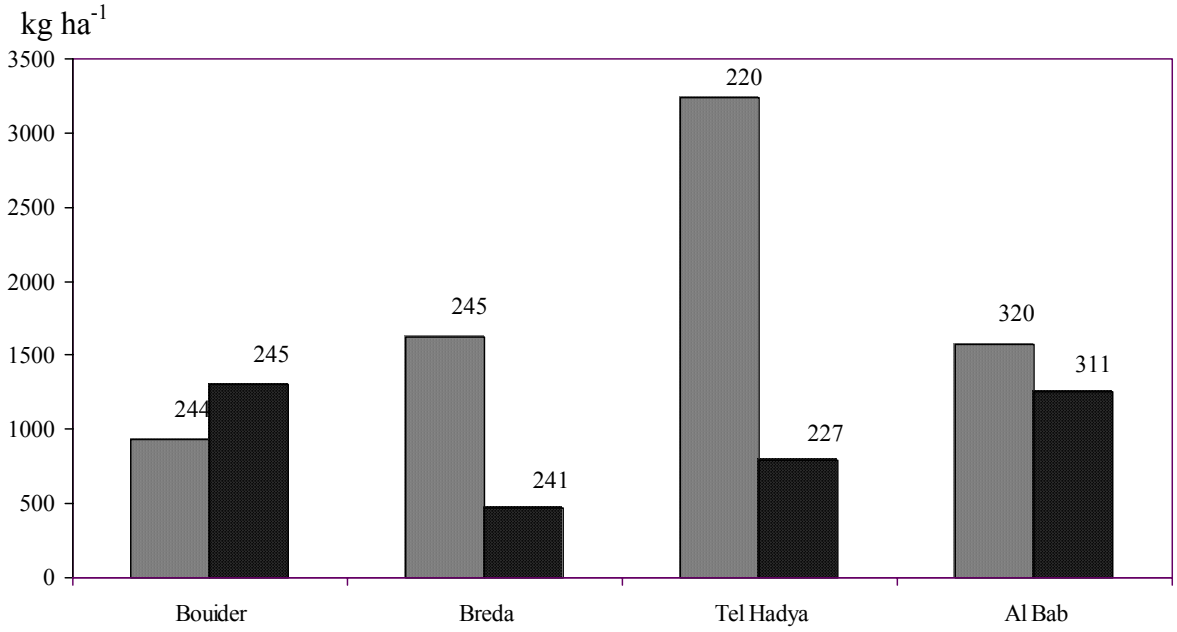


Figure 1. Similar amount of total rainfall (in mm above bars) from various locations in Syria may result in different grain yields because of other factors such as rainfall distribution, air temperature, and air humidity.

Breeding Philosophy for Drought Resistance

Two contrasting breeding philosophies have been used to breed crops for drought tolerance: the first uses selection under optimum growing conditions, and is based on the assumption that an increased yield potential will have a carry-over effect when the improved cultivars are grown under less favorable conditions. The second uses direct selection in the presence of drought, i.e. in the target environment, and can take two forms a) selection for physiological or developmental traits (analytical breeding), and b) direct selection for grain yield (empirical or pragmatic breeding).
The first philosophy has failed to produce convincing results about the existence of the spillover effect. In barley, we have consistently found a negative relationship between yield potential and yield under stress conditions (VAN OOSTEROM *et al*. 1993). The circumstantial evidence of the absence of the spillover effect is the fact that drought continues to affect negatively agricultural productions worldwide, despite the spectacular increases in crop yields obtained through breeding under optimum conditions.
Breeding for drought resistance based on traits associated with drought resistance, but easier to select for than grain yield, has been and still is very popular. Traits, which have been investigated, include physiological/biochemical traits (such as osmotic adjustment, proline content, stomatal conductance, epidermal conductance, canopy temperature, relative water content, leaf turgor, abscisic acid content, transpiration efficiency, water use efficiency, carbon isotope discrimination, and retranslocation), and developmental/morphological (such a

leaf emergence, early growth vigor, leaf area index, leaf waxiness, stomatal density, tiller development, flowering time, maturity rate, cell membrane stability, cell wall rheology, and root characteristics). In the case of barley, we have found that the traits more consistently associated with higher grain yield under drought are growth habit, early growth vigor, earliness, plant height under drought, long peduncle and a short grain filling duration (ACEVEDO & CECCARELLI 1989). The analytical approach has been very useful in understanding which traits are associated with drought tolerance and why, but it has been less useful in actually developing new cultivars showing an improved drought resistance under field conditions. This is because, as mentioned earlier, under field conditions, drought varies in timing, intensity and duration, and therefore it is the interaction among traits to determine the overall crop response to the variable nature of the drought stress, rather than the expression of any specific trait (CECCARELLI *et al.* 1991).

Although breeding for drought resistance based on direct selection for grain yield in the target environment (empirical or pragmatic breeding) appears intuitively as the most obvious solution, it has faced the criticism that being field-drought such a "moving target", the chances of progresses appear slow and remote.

Two of the major problems with selection in stress environments, are the precision of selection, and the existence of several target environments each characterized by its own specific type of drought, and more in general of combination of stress.

The issue of the precision of selection conducted under moisture stress conditions has been addressed by several papers, which have focused the attention on the magnitude of heritability. A literature review (CECCARELLI 1996a) showed the absence of a consistent relationship between grain yield (as a measure of the intensity of the stress) and the magnitude of heritability. Recently, VOM BROCKE *et al.* (2002) confirmed that in pearl millet it is possible to detect genetic differences even under severe moisture stress conditions. Furthermore considerable progresses have been made both in experimental design and statistical analysis that improve considerably the estimate of experimental error (SINGH *et al.* 2003). Therefore, it is possible to combine precision and relevance by conducting trials in the target environment even when this is a stress environment.

The second issue is how to deal with the multitude of target environments. This issue is intimately associated with that of broad and specific adaptation, which has been debated in plant breeding since the early 1920s and is still highly controversial. One of the causes of controversy is the definition of stress environment, which is different for different crops. For example, in a country like Syria, with a large spatial variability of rainfall within short distances, bread wheat, durum wheat and barley are grown in progressively drier environments with some overlapping. Therefore, a stress environment for bread wheat is moderately favorable for durum wheat, and a stress environment for durum wheat is moderately favorable for barley and lentil. At the drier end, barley is the only rainfed crops, and the other cereals are only grown under irrigation.

Another cause of controversy is the confusion between adaptation over time and adaptation over space, even though the distinction is of fundamental importance. Adaptation over time (also called stability or dependability) refers to the performance of a cultivar in a given location across several years; if the cultivar performs consistently better than a reference cultivar, it is said to be stable. Adaptation over space refers to the performance of a cultivar in several locations; if the cultivar performs consistently better than a reference cultivar, it is said to be widely adapted.

It can be argued that wide adaptation over time (also defined as stability) is much more important to farmers than wide adaptation over space. The latter is, for obvious reasons, the major concern of seed producer.

Breeding for specific adaptation is particularly important in the case of crops predominantly grown in unfavorable conditions, because unfavorable environments tend to be more different

from each other than favorable environments. Breeding for specific adaptation to unfavorable conditions is often considered an undesirable breeding objective because it is usually associated with a reduction of potential yield under favorable conditions. This issue has to be considered in its social dimension and in relation to the difference between adaptation over space vs. adaptation over time.

Choice of Germplasm

In many developing countries, and for crops grown in stress environments, landraces are still the backbone of agricultural production. The reasons why farmers prefer to grow only landraces or continue to grow landraces even after partial adoption of modern cultivars are not well documented, but include quality attributes such as food and feed quality, and seed storability. Another important reason is that landraces are often able to produce some yield even in difficult conditions where modern varieties are less reliable.

The value of landraces is well documented in the case of barley in Syria (GRANDO *et al.* 2001) and in several other crops (BRUSH 1999). The comparison between barley landraces and modern cultivars in a range of conditions from severe stress (low-input and low-rainfall) to moderately favorable conditions (use of inputs and high rainfall) (Table 1) have consistently indicated that 1) landraces yield more than modern cultivars under low-input and stress conditions, 2) the superiority of landraces is not associated with mechanisms to escape drought stress, as shown by their heading date; 3) within landraces there is considerable variation for grain yield under low-input and stress conditions, but all the landraces derived lines yield something whereas some modern cultivars fail; 4) landraces are responsive to both inputs and rainfall and the yield potential of some lines is high, though not as high as modern cultivars, and 5) it is possible to find modern cultivars which under low-input and stress conditions yield almost as well as landraces, but their frequency is very low. The data in Table 1 also suggest that selection conducted only in high input conditions is likely to miss most of the lines that would have performed well under low-input conditions

Table 1. Grain yields (kg ha^{-1}) of pure lines derived from Syrian landraces and modern cultivars at three levels of drought stress[a] in northern Syria (CECCARELLI 1996b).

Environment	Landraces (N=44)	Modern (N=206)	Difference	P[b]
Stress	1038	591	447	<0.01
Intermediate	3105	3291	-186	n.s.
Non-stressed	4506	6153	-1647	<0.01

[a] As defined by average precipitation and soil fertility

[b] Based on t-tests for groups of unequal size; n.s. = non significant

Decentralized Selection

Selection for specific adaptation to drought stress in barley has been implemented at ICARDA initially by expanding the evaluation of early segregating populations in dry sites and by modifying the breeding method from a classical pedigree method to a modified bulk (CECCARELLI 1996b). The most important results of this choice have been the re-evaluation of the role of landraces in breeding for drought tolerance described earlier, and the discovery of the importance of the wild progenitor of cultivated barley, *Hordeum vulgare* ssp. *spontaneum*, as a source of resistance to extreme levels of drought. Gas exchange observations made at anthesis in a wet site showed that *H. spontaneum* has widely open stomata higher net photosynthesis and lower predawn leaf water potential at this stage of development than cultivated barley (Table 2).

The ability of some accessions of *H. spontaneum* to tolerate extreme levels of drought stress was evident during the severe drought of 1987 when two lines of *H. spontaneum* were the only survivors in the breeding nurseries grown at a site which received only 176 mm rainfall (GRANDO *et al.* 2001).

Table 2. Gas exchange parameters of *H. spontaneum* accessions (means of 12 accessions) and ratio *H. spontaneum*/*H.vulgare* at Tel Hadya.

Parameter	*H.spontaneum*	*H. spontaneum*/*H. vulgare*
Net photosynthesis (μmol m^{-2} s^{-1})	16.90 ± 0.80	1.49
Leaf conductance (mol m^{-2} s^{-1})	0.40 ± 0.03	3.57
Transpiration efficiency	4.35 ±0.09	0.63
Leaf temperature (C^o)	25.50 ± 0.36	0.85
Predawn leaf water potential (Mpa)	0.89 ± 0.25	1.35

These lines had some photosynthetic activity early in the morning, even though six times less than in absence of stress, stomata were open and the predawn leaf water potential was negative. At the same time, the stomata of the black-seeded local landrace 'Arabi Aswad', considered by farmers to be very resistant to drought, were closed even though the pre-dawn leaf water potential was slightly higher than in *H. spontaneum*. By midday, the stomatal conductance of *H. spontaneum* decreased, the net photosynthesis became negative, while, the stomatal conductance of 'Arabi Aswad' was zero.

Decentralized selection, namely selection in the target environment, is mostly based on grain yield considered to be the integrated response of a number of physiological, morphological, and developmental attributes which allow that particular genotype or population to perform better than others to the particular type of drought encountered in a specific site and a specific year, and to the combination of that particular type of drought with other stresses. Genotypes or populations selected under this specific set of conditions are then tested for a second season in a number of sites, and if again successful, for a third season.

The process makes use of the large year-to-year variability that characterizes stress environments, so that in a short period of time such as three years, and in seven to ten sites, there is a high probability that a genotype or a population is exposed to a number of different types of drought in terms of occurrence, timing, duration and severity. Therefore, the process progressively accumulates favorable alleles for performance under various conditions.

The process relies heavily on grain yield, but we also consider other attributes such as early vigor, habit of growth, flowering time, plant height, tillering, and when extreme stress conditions occur, leaf rolling and leaf senescence. Therefore, the process combines the characteristics of the empirical and the analytical approach, and results in a slow but steady increase in yield under a combination of stresses. Figure 2 shows an example of this steady increase using a number of lines developed between 1985 and 2000: yield increased from less than 0.96 t ha^{-1} in 'Harmal' to 1.4 t ha^{-1} in 'Arta'. The latter however, has the major handicap of becoming very short under drought stress. A reduction of plant height is undesirable because it forces the farmers to harvest by hand which is much more expensive than combine harvesting. The use of *H. spontaneum* and of one of the tallest selections from the black seeded landrace, 'Zanbaka', has allowed reaching nearly 1.5 t ha^{-1} with a plant height only marginally lower than that of 'Zanbaka'.

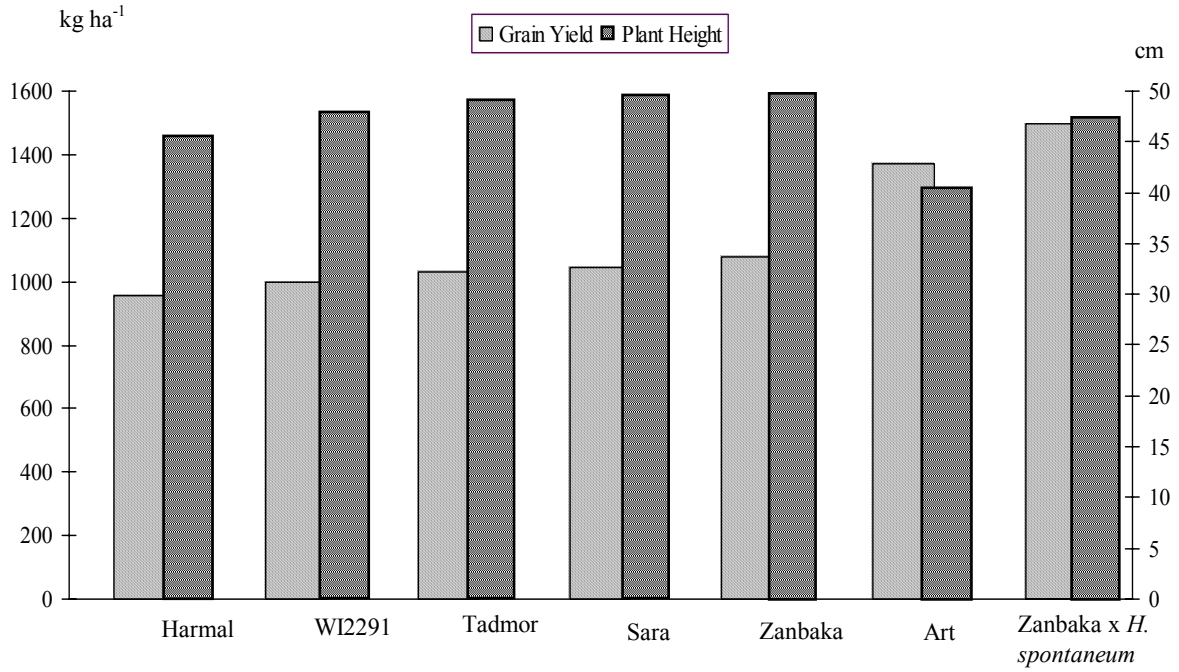


Figure 2. Changes in grain yield and in plant height in barley germplasm generated at ICARDA in a period of 15 years.

Participatory Plant Breeding

Selecting for specific adaptation has the advantage of adapting cultivars to the physical environment, and hence is more sustainable than other strategies which rely on changing the environment to fit new cultivars adapted to more favorable conditions. However, as mentioned earlier, one of the most serious limitations of decentralized selection for specific adaptation to unfavorable environments is in the large number of potential target environments. Moreover, the number of target environments increases if we consider that environment is not only climate, soil, agronomic practices, farming system, etc., but also people leaving in that physical environment, their perception of risk associated with yield variation over time, their uses of the crop, and the consequent importance of quality traits even if neutral in terms of adaptation to the physical environment. Clearly, selection for specific adaptation to unfavorable conditions needs a larger sample of selection environments than selection for favorable environments.

The participation of farmers in the very early stages of selection offers a solution to the problem of fitting the crop to a multitude of both target environments and users' preferences (CECCARELLI *et al.* 1996, 2000). Although decentralized selection and farmers' participation are unrelated concepts, the acceptance of the former as a breeding strategy almost inevitably leads to the acceptance of the latter as a tactical necessity. It is worth mentioning that, although farmer participation is often advocated on the basis of equity, there are sound scientific and practical reasons for farmer involvement to increase the efficiency and the effectiveness of the breeding program.

Participatory plant breeding has now been implemented on barley in a number of countries, and even though it was not specifically designed to breed for drought tolerance, the breeding material with the highest level of drought tolerance in Syria originated from this activity. In 2000, the total rainfall in most areas of Syria was below average, and crop yields were severely affected. In some areas the rainfall was so low that the crop did not even germinate, in many others the crop failed to produce grain. The driest sites where some new barley entries were able to produce some grain and/or some biomass received between 87 and 130 mm of rainfall. Average grain and biomass yield were very low but some lines were able

to produce between 300 and 500 kg ha^{-1} of grain (Figure 3) and between 500 and 3000 kg ha^{-1} of biomass yield.

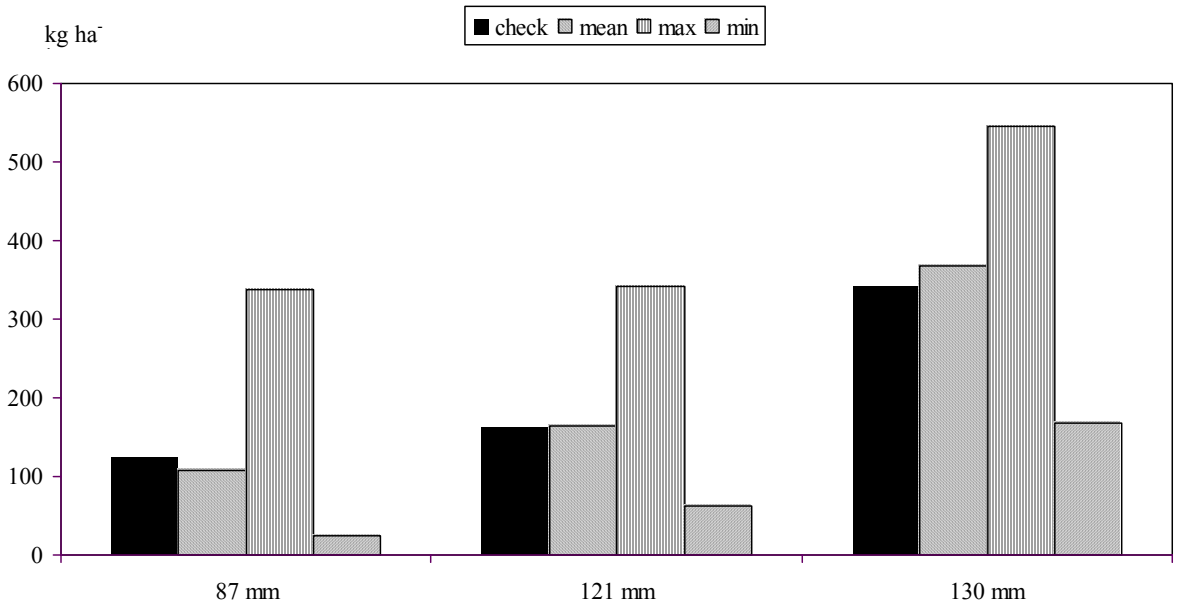


Figure 3. Grain yield (kg ha^{-1}) under drought stress in four locations in Syria in 2000.

Drought tolerance was assessed in the field when the plots where close to maturity with a score from 1 (the majority of plants in a plot with a spike and seed and no symptoms of leaf rolling or wilting) to 5 (absence of spikes, leaf desiccation and/or wilting). In all locations the crosses between *H. spontaneum-41* (one of *H. spontaneum* lines identified in 1987) and landraces were the most drought tolerant. This is a further indication that, if carefully selected, some *H. spontaneum* lines can contribute significantly to enhance the drought resistance of cultivated barley.

Molecular Approaches

The use of DNA molecular-marker techniques is now sufficiently well developed to be exploited in breeding programs to improve resistance/tolerance to both abiotic and biotic stresses, and can considerably reduce the complexity of combining in the same cultivar a number of desirable traits (GRANER *et al.* 1991). These techniques make it feasible to develop linkage maps, which are used to locate and estimate phenotypic effects of quantitative trait loci (QTL). One of the first molecular approaches has been the identification and the localization of those traits known to be associated with resistance to drought and with other stresses occurring in various combinations with drought (YIN *et al.* 1999, TEULAT *et al.* 1998, 2001, 2002).

The chromosomal locations of most of the traits described earlier in this paper are known. A major QTL affecting drought, induced abscisic acid accumulation, has been mapped on chromosome 5H, tightly linked to one of the dehydrin, the Dhn1/Dhn2 locus, suggesting a genetic linkage between abscisic acid accumulation and stress tolerance (QUARRIE *et al.* 1994). The chromosomal location of some other traits related to drought tolerance is known. TEULAT *et al.* (1997), using a barley population developed at ICARDA, found that the centromeric region and the long arm of chromosome 7H are involved in the control of relative water content, and ten QTLs for osmotic adjustment were identified (TEULAT *et al.* 1998). In the same population, two QTL for carbon isotope discrimination were identified on chromosomes 2H and 4H (TEULAT *et al.* 2002).

QTLs for agronomic traits related to drought resistance were localized with the help of a genetic linkage map in recombinant inbred lines (RILs) of the cross 'Arta' x *H. spontaneum* 41-1 (BAUM *et al.* 2003). For one of the most important characters, plant height under drought stress, QTLs on chromosomes 2H, 3H and 7H were detected in this population. The "plant height" QTLs, especially the one on 3H, showed pleiotropic effects on traits such as days to heading, grain yield and biological yield. QTLs were also identified for other traits associated with adaptation to Mediterranean environment such as cold tolerance (5H), days-to-heading (7H, 3H), and tiller number (3H).

A large number of genes have been isolated whose expression is affected by the application of drought stress or by more than one abiotic stress. One of the best known is the LEA (late embryogenesis-abundant proteins) gene family, induced by dehydration, cold, abscisic acid treatment, salt and osmotic stress. One class of LEA, the dehydrins, is the main group of stress-related proteins, and some are expressed during dehydration, others during cold treatment (ZHU *et al.* 2000).

Conclusion

Barley breeding for stress environments is possible provided it is conducted with strategies and methodologies that little have in common with those used in breeding for favorable environments. Adaptation over time can be improved by breeding for specific adaptation to a given type of stress environment. This can be achieved by taking advantage of the temporal variability of stress environments, which permits exposure of the same breeding material to variable combinations of stresses over a (relatively) short period. We are aware that this is fundamentally different from the modern trend of plant breeding towards broad adaptation over space. The difference represents the contrasting interests of farmers and seed companies. Farmers are interested in cultivars that are consistently superior on their farm, regardless of how they perform at other locations or in other countries.

Recent advances in plant genomics have enabled to dissect various molecular mechanisms involved in drought, cold and salt stress tolerance and in identifying various genes involved in these stress tolerance. Information generated in genomics should be integrated in the practical plant breeding. Various genes identified both in model plants and crop plants could be used for developing stress tolerant plants through either marker-assisted selection or direct gene transfer.

References

ACEVEDO E., CECCARELLI S. (1989): Role of physiologist-breeder in a breeding program for drought resistance conditions. In: BAKER F.W.G. (ed.): Drought Resistance in Cereals, CAB International: 117-139.

ASHRAF M. (1994): Breeding for salinity tolerance in plants. Critical Reviews in Plant Sciences, **13**: 17-42.

BAUM M., GRANDO S., BACKES G., JAHOOR A., SABBAGH A., CECCARELLI S. (2003): QTLs for agronomic traits in the Mediterranean environment identified in recombinant inbred lines of the cross 'Arta' x *H. spontaneum* 41-1. Theor. Appl. Genet., **107**: 1215-1225.

BRUSH S.B. (1999): Genes in the Field: On-Farm Conservation of Crop Diversity. IPGRI/IDRC/Lewis Publishers: 51-76.

CECCARELLI S. (1996a): Adaptation to low/high input cultivation. Euphytica, **92**: 203-214.

CECCARELLI S. (1996b): Positive interpretation of genotype by environment interactions in relation to sustainability and biodiversity. In: COOPER M., HAMMER G.L. (eds.): Plant Adaptation and Crop Improvement. CAB International, Wallingford. U.K., ICRISAT, Andra Pradesh, India, IRRI, Manila, Philippines: 467-486.

CECCARELLI S., GRANDO S. (1996): Drought as a challenge for the plant breeder. Plant Growth Regulation, **20**:149-155.

CECCARELLI S., GRANDO S., BOOTH R.H. (1996): International breeding programmes and resource-poor farmers: Crop improvement in difficult environments. In: EYZAGUIRRE P., Iwanaga M. (eds.). Participatory plant breeding. Proceeding of a workshop on participatory plant breeding, 26-29 July 1995, Wageningen, The Netherlands. IPGRI, Rome, Italy: 99-116.

CECCARELLI S., GRANDO S., TUTWILER R., BAHA J., MARTINI A.M., SALAHIEH H., GOODCHILD A., Michael M. (2000). A Methodological Study on Participatory Barley Breeding. I. Selection Phase. Euphytica, **111**: 91-104.

GRANDO S., VON BOTHMER R., CECCARELLI S. (2001): Genetic Diversity of Barley: Use of Locally Adapted Germplasm to Enhance Yield and Yield Stability of Barley in Dry Areas. In: COOPER H.D., SPILLANE C., HODGINK T. (eds.) Broadening the Genetic Base of Crop Production. CABI/FAO/IPRI: 351-372.

GRANER A., JAHOOR A., SCHONDELMAIER J., SIEDLER H., PILLEN K., FISCHBECK G., WENZEL G., HERRMANN R.G. (1991): Construction of an RFLP map of barley. Theor. Appl. Genet., **83**: 250 256.

QUARRIE S.A., GULLI M., CALESTANI C., STEED A., MARMIROLI N. (1994): Location of a gene regulating drought-induced abscisic acid production on the long arm of chromosome 5A of wheat. Theor. Appl. Genet., **89**: 794–800.

SINGH M., MALHOTRA R. S., CECCARELLI S., SARKER A., GRANDO S., ERSKINE W (2003): Spatial variability models to improve dryland field trials. Experimental Agriculture, **39**: 1-10.

STRAUSS M.S. (1993): The challenge ahead. In: International Crop Science I, Crop Science Society of America, Madison, WI: 401-405.

TEULAT B., MONNEVEUX P., WERY J., BORRIES C., SOUYRIS I., CHARRIER A., THIS D. (1997): Relationship between relative water content and growth parameters under water stress in barley: a QTL study. New Phytol., **137**: 99–107.

TEULAT B., THIS D., KHAIRALLAH M., BORRIES C., RAGOT C., SOURDILLE P., LEROY P., MONNEVEUX P., CHARRIER A. (1998): Several QTLs involved in osmotic-adjustment trait variation in barley. Theor. Appl. Genet., **96**: 688–698.

TEULAT B., BORRIES C., THIS D. (2001): New QTLs identified for plant water status, water-soluble carbohydrates, osmotic adjustment in a barley population grown in a growth-chamber under two water regimes. Theor. Appl. Genet., **103**: 161 170.

TEULAT B, MERAH O., SIRAULT X., BORRIES C., WAUGH R., THIS D. (2002): QTLs for grain carbon isotope discrimination in field-grown barley. Theor. Appl. Genet., **106**: 118.126.

VAN OOSTEROM E.J., CECCARELLI S., PEACOCK J.M. (1993): Yield response of barley to rainfall and temperature in Mediterranean environments. J. Agric. Sci. Camb., **121**: 307-314.

VOM BROCKE K., PRESTERL T., CHRISTINCK A., WELTZIEN E., GEIGER H.H. (2002): Farmers' seed management practices open up new base populations for pearl millet breeding in a semi-arid zone of India. Plant Breeding, **121**: 36-42.

YIN X., STAM P., DOURLEIJN J.C., KROPFF M.J. (999): AFLP mapping of quantitative trait loci for yield-determining physiological characters in spring barley. Theor. Appl. Genet., **99**:244 253.

ZHU B., CHOI D.W., FENTON R., CLOSE T.J. (2000): Expression of the barley dehydrins multigene family and the development of freezing tolerance. Mol. Gen. Genet., **264**: 145-153.

Freezing Injury and Breeding on the Frost Resistance in Continental Russia

V.M. Shevtsov

Krasnodar Lukyanenko Research Institute of Agriculture KNIISH, Krasnodar, 350012, Russia, e-mail: vshevtsov@inbox.ru

Abstract

Winter hardness is an important agronomic character which facilitates barley yield stability Resistance to freezing is a principal component of this trait. It was shown that the deeper formation of crown node can significantly improve barley over-wintering. Some accessions from the world collection possess high level of resistance to freezing. Also, there are barley samples with deep crown node. The identified sources were used in crosses to combine these characters. The method of experimental mutagenesis proved to be useful to induce a genetic variation on cold tolerance. A selection from the class of visible mutations was more effective Some macro mutants have become commercial cultivars after direct multiplication. Others being involved into hybridization have conditioned the great divergence including valuable transgressive recombinants. That resulted in developing productive varieties Radical, Bastion, Dobrynia-3, tolerant to stressful factors. The new winter barley varieties Fakir and Almaz carry the highest level of winter hardness in combination with the yield potential.

Introduction

In spite of the forecast on global warming, winter hardness still plays an important role in yield stabilization of winter cereals. In extreme years, like the last 2002-2003 season, plants of winter crops were frost killed or sever damaged and that led to big yield losses. It is essential for many European countries and especially for continental Russia, where winters with sever frosts occur very often. Winter cereals have a significant yield advantage in comparison with spring one (CATTIVELLI *et al.* 1996, 2000; SPUNAR 1996; MAKSIMOVIC 1996; SHEVTSOV *et al.* 1990, 2000). On the North Caucasus winter barley is higher in yield than spring barley 20-50 % and even more. During the last two seasons in Krasnodar region winter barley two times out-yielded spring one. Additionally, protein content in winter barley grain is less and varies usually in interval 9-11%, while in spring barley 11-13 %. Therefore, winter two-row barley with acceptable yield potential would be preferable crop for malting purposes in some countries of Europe and on the North Caucasus (SPUNAR 2000; FUCHS 1993; SCHILDBACH 1994; SHEVTSOV *et al.* 1992). As many years experiments showed, two-row germplasm of winter barley has less cold tolerance than six-row barley. Some useful mutants were reported to be developed by the method of experimental mutagenesis (RAPOPORT 1966; NILAN *et al.* 1976; LUNDQWIST 1986). It was shown that practically every trait can be changed with mutagen treatment, including biological type and cold tolerance (GUSTAFSSON *et al.* 1971, 1986; SIGURBJORNSSON 1983; MICKE 1989). It was shown that the deeper formation of crown node can significantly facilitate barley over-wintering.

The task of the paper is to summarize the results, achieved at the Krasnodar Research Institute of Agriculture during 40 years (1962-2003) in improvement of barley winter hardiness, and show the possibility to combine this character with high yield potential.

Material and Methods

Annually, a total of 1500-1800 entries from advanced and preliminary trials, observation nursery and collection are tested on cold tolerance. The evaluation was carried out in natural environments (field and concrete beds) in Krasnodar Research Institute of Agriculture (Russia) and its branch the North-Kuban experimental station and also under controlled conditions in Krasnodar (freezing in chambers under temperature – 12…-14 ^{o} C during 24 hours). Planting on special concrete beds, from which snow was taken away, was done in two dates. In order to study the depth of the crown node, the same volume of breeding material was planted in the field in trances with fixed depth of 8 cm. As for experimental mutagenesis the common procedure consists in the annual treatment of 4-5 promising lines or cultivars with 2-3 chemical mutagens in 6-9 dosages (3 concentrations and 2-3 expositions). Individual plants were selected in M^2–M^3 and in F4-F5 and then were tested in accordance with the traditional breeding procedure. The promising lines have been testing in different geographical locations with diverse agronomical conditions for the assessment of their ecological plasticity.

Results and Discussion

Among other winter cereals barley has a comparatively low polymorphism on cold tolerance. Inter generic crosses of barley with winter rye and wheat were so far not successful. Therefore, the only way-out for breeders is to develop a new initial cold tolerant material experimentally by means of germplasm exchange, mutations and recombination. For this purpose two approaches were used: 1) selection of productive breeding material in early generation and cold tolerance evaluation on the final stage in advanced trials, 2) cold tolerance control starting from F2 and all breeding nurseries and then selection of productive lines from cold tolerant germplasm. Both ways are possible, but the last one is more effective though it is more expensive. Traditional hybridization of parents of ecologically and geographically distant origin and method of mutations were used to produce a genetic variation and process a big volume of tested lines.

Table 1. Characteristics of selected sources of cold tolerance in winter barley

Variety	Depth of crown node, cm	Plant survival*2003, %			
		Krasnodar			North-Kuban
		Concrete beds	Artificial freezing	Field	Field
Mihailo-check	3.4	0	20.6	46.8	0
Dobrynia-3	4.2	0	45.6	80.6	48.6
Plaisant	3.8	0	0	0	0
26M8	6.2	0	80.5	100	76.5
MM-1	4.3	15.6	100	100	50.6
Dictoo	6.2	18.6	90.5	100	81.2
NE 93747	6.4	22.6	100	100	91.5
Almaz	4.1	46.2	100	100	100
Radical	3.8	10.2	75.3	100	50.2
Bastion	3.6	15.3	85.8	100	61.2
Fakir	3.6	33.5	100	100	100
LSD 0.05		9.3	8.7	6.8	9.5

*) Plant survival- percentage of survived plants after freezing in chambers or field

After artificial freezing in chambers annually about half million of M^2 and F2 plants with the selection pressure less than 1 % survived seedlings, a series of unique segregates and macro mutants has been isolated. A mutant line 26M8 = (Pallidum 48 x Nitrozo-etil-urea 0.13 % - 6

hours) has a deep crown node. Another macro mutant MM-1 (Paoly x Nitrozo-metil-urea 0.02 12 hours) possesses very high frost resistance. Very interesting varieties Dictoo, Kearney, 93760 and NE 93747 were earlier reported to be identified as sources of cold tolerance from International Uniform Barley Winter Hardness Nursery (UBWHN) (SHEVTSOV *et al.* 1 2000).They have got increased resistance to freezing and deep crown node (Table 1).
The testing and introduction of these varieties and lines showed that they could be transferred to farmer's fields provided they have acceptable yielding capacity. Therefore, our many years breeding efforts were directed to check if it is possible to combine the achieved winter hardness with high yield potential. The involvement into hybridization some ecologically remote accessions from the world collection and drastic macro-mutants, carrying desirable traits, has conditioned a real flash of transgressive variability, producing economically valuable genotypes. They include a released cultivar Radical=(Paoly x Mutant 52M1) x Novator and Bastion=Radical x Vavilon. The new cultivars thanks to good balance of economically important characters played a decisive role in a real break-through in barley grain production in Krasnodar krai. While growing these varieties, many farmers have succeeded to harvest from 8 to 9 tons per hectare. Four years ago the varieties have been changed by new released winter barley Dobrynia-3. It combines many advantages of the parental components. But its cold tolerance was not enough to withstand a sever frosts in the last season. Fortunately, this year the more reliable material (Fakir and Almaz) has been identified in the advanced yield trials as promising due to its high cold tolerance, resistance to the disease, plasticity and high yield potential. The successful balance of economically important characters makes the varieties the most suitable for cultivation in the zones with frequent frosty winters. The new variety Fakir has evident superiority in cold tolerance and resistance to snow mold. The yield trials, carried out in the Northern zone of Krasnodar region, showed very good adaptation of this variety to medium and low yielding environments, though its yield potential is high, on the level of 9 t/ha (Table 2).

Table 2. Performance of the new winter barley variety Fakir in the North-Kuban experimental station

Variety	Cold tolerance 2003, %	Yield after preceding crops, q/ha			Average	
		2002		2003		
		Wheat	Sunflower		q/ha	%
Dobrinya	30.6	52.9	87.8	20.5	53.7	100
Michailo	11.6	60.6	88.8	10.3	53.2	99
Fakir	86.7	73.8	89.1	54.5	72.4	132
LSD 0.05	9.8	3.5	4.2	4.4		

Some additional research is needed to find out optimal technological requirements for its cultivation.

So, there are reserves for barley improvement on cold tolerance by methods of the international germplasm exchange, mutagenesis and hybridization. The recombination of adaptive and productive characters is possible. High yielding and cold tolerant varieties Radical, Bastion and Dobrynia-3 were developed and introduced on farmer's fields. The new variety Fakir has good combination of high winter hardness with disease resistance, plasticity and high yield potential.

References

CATTIVELLI L., CROSATTI C., GROSSI M., RIZZA F., STANCA A. M. (1996): VII IBGS: 291-296.

CATTIVELLI L., BALDI P., BUSKONI M., CROSATTI C., DAL C., BASCO M., GROSSI A., MASTRAGELO C., FUCHS W. (1993): Arch. Acker-Pfl. Boden, **37:** 247-255.

GUSTAFSSON A., HAGBERG A., WIKLUND K. (1971): Theor.Appl. Gen., **41:** 239-241.

GUSTAFSSON A. (1986): In: Svalof, 1886-1986: 76-84.

LUNDQWIST U. (1986): In: Svalof, 1886-1986: 85-88.

MAKSIMOVICH D., KNEZIC D. PAUNOVIC A. (1996): VII IBGS. Canada: 26-28.

MARE C., RIZZA F., VANTINI F., STANCA A. M. (2000): VII IBGS. Australia: 265-266.

MICKE A. (1989): Mutation Breeding Newsletter, 34: 2-3.

NILAN R., KLENHOTS A., Sandler C (1976): III IBGS, Garching, 1975, 3: 113-122.

RAPOPORT I. A. (1966): Super mutagenes. Science. Moscow: 3-330.

SCHILDBACH R. (1994): Brauwelt, **4:** 292-310.

SIGURBJORNSSON B. (1983): Crop Breeding. Crop Science. Madison. USA.: 25-37

SHEVTSOV V. (1990): Rachis, **9:** 6-9.

SHEVTSOV V., TAHIR M., OTTEKIN A., TOSUN H., AKAR T. (1994): Rachis, **13:** 5-11.

SHEVTSOV V., TAHIR M. (1994): Barley Newsletter, **38:**121-123.

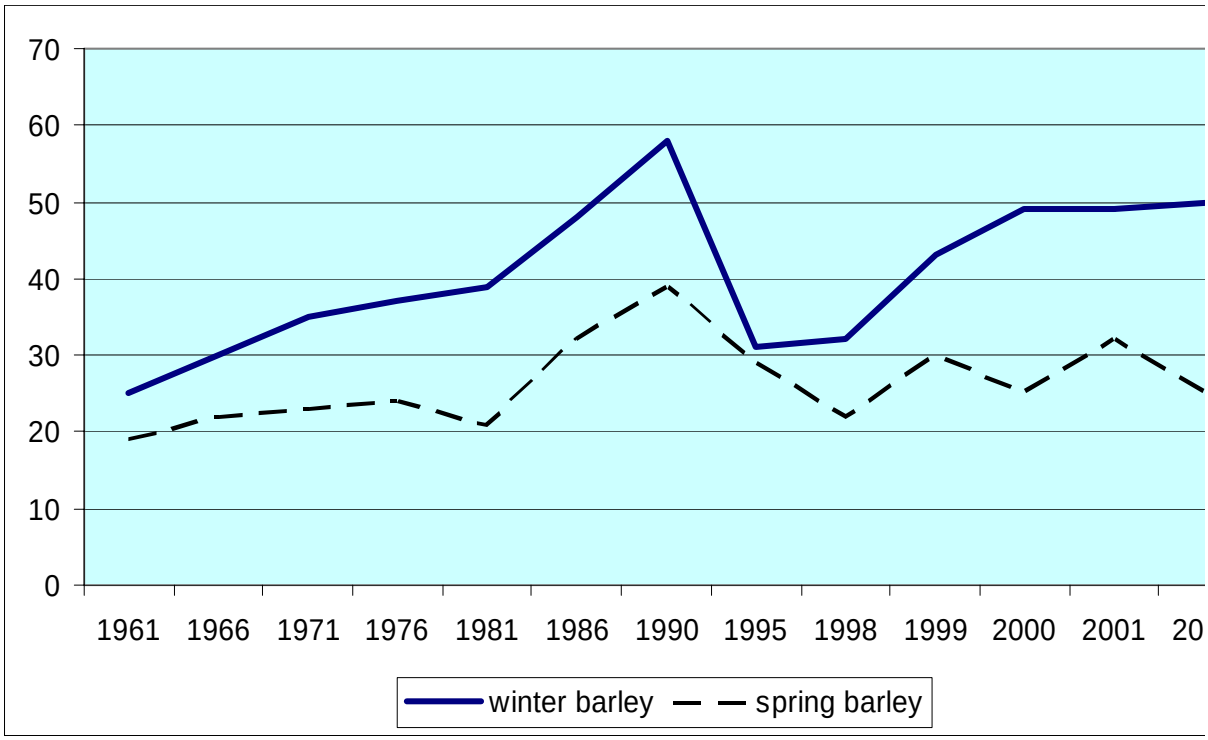
SHEVTSOV V., CECCARELLI S., GRANDO S., TAHIR M. (2000): VIII, IBGS: 284-286.

SHEVTSOV V., LUKOMETS V., GRUNTSEV U. (1992): Barley Newsletter, **36:** 177-180.

SPUNAR J., SPUNAR M.(1996): VII IBGS. Canada: 35-37.

SPUNAR J. (2000): Personal communication: 3-20.

Average barley yields in Krasnodar region (q/ha)



Average barley yields in Krasnodar Research Institute of Agriculture (q/ha)

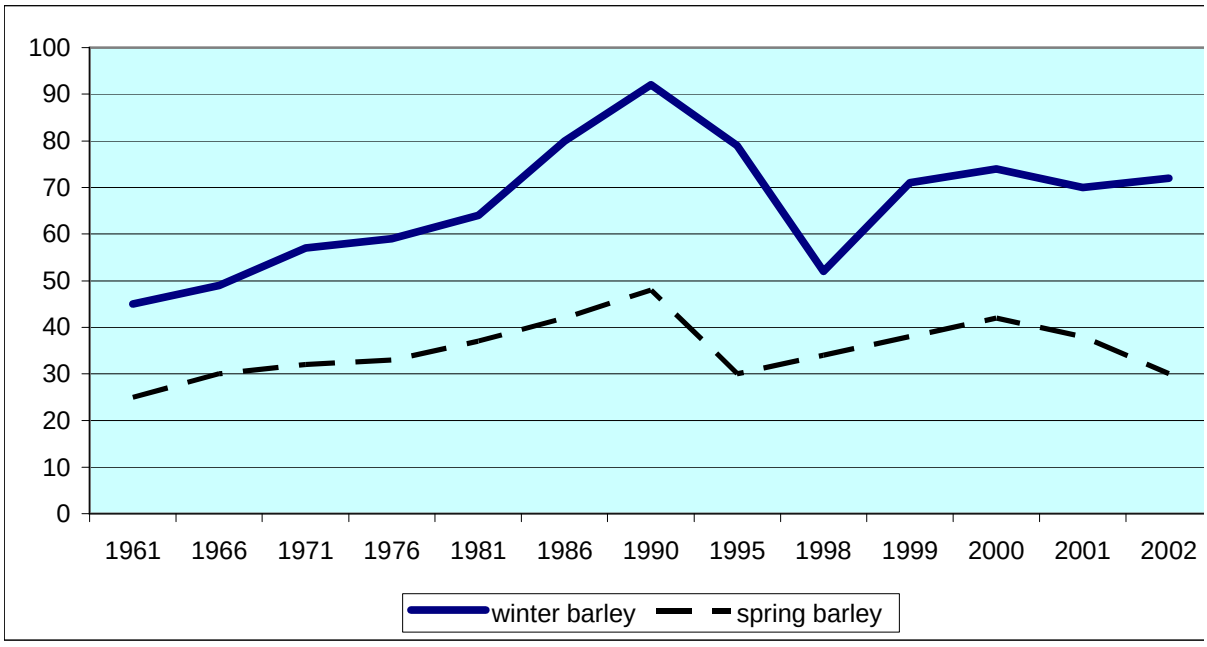


Figure 1. The advantage in yield of winter barley in comparison with spring barley

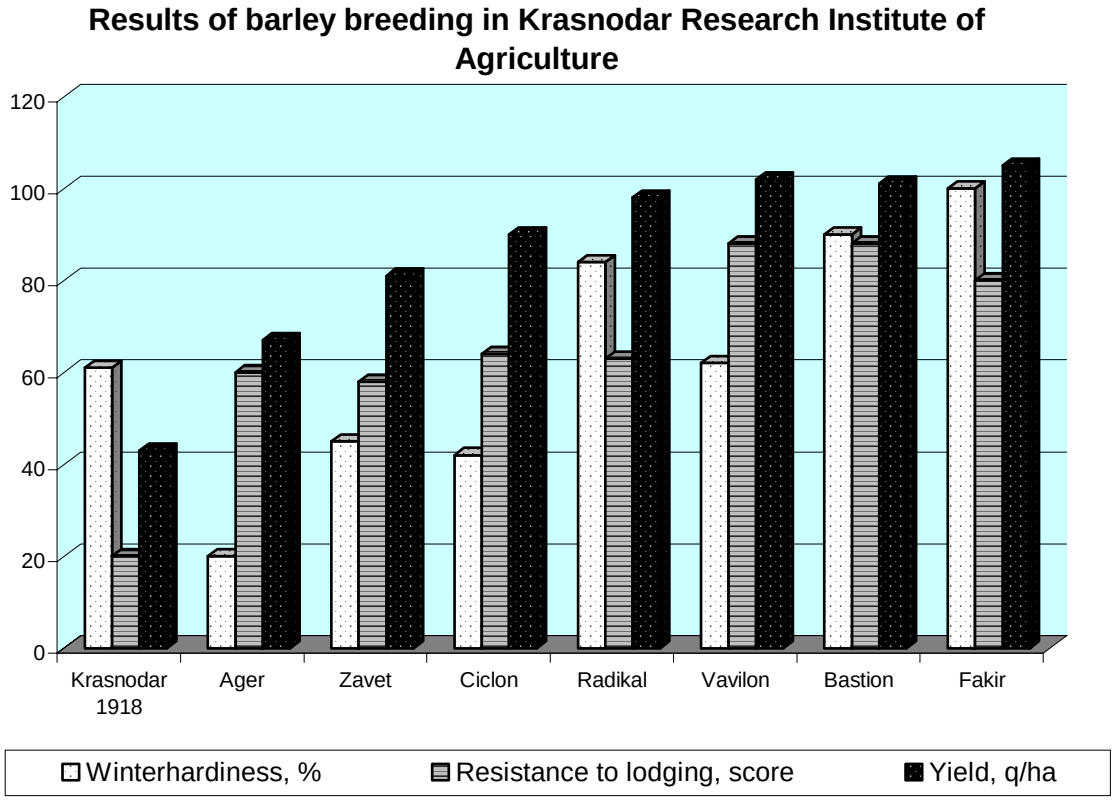


Figure 2. The improvement of barley winter hardiness and productivity

Versatile Barley Varieties for the Mexican Highlands

F. Capettini

ICARDA/CIMMYT Latin America Regional Program, Apdo. Postal 6-641, 06600, Mexico D.F., Mexico

Abstract
Barley production in the highlands of Mexico dates from the time of the Spanish conquest. Today, almost all the barley planted in the region is used for malting to produce beer. Globalization, most notably the North American Free Trade Agreement, is prompting farmers to look for new crops, such as forage barley, which appears to offer an economically viable alternative to malt barley production. Testing experiments were placed at farmers' fields of the State of Hidalgo between 2000 and 2003. Results obtained lead to release of forage barley that clearly outperformed the local malting barley variety 'Esmeralda', having better disease resistance and agronomic type. This variety is expected to give local growers an additional crop option without having to change very much their production systems.
Keywords: forage barley; production systems; crop conversion

Introduction

Barley has been grown in the highlands (over 2200 m above sea level) of Mexico since the Spaniards introduced the crop about 500 years ago. It is mostly used to make beer, production of which has increased significantly in the country in the last few years due to an increase in exports. In some Mexican states, such as Hidalgo, where 120,000 ha are grown annually, barley is the main crop. In other highland states, such as Mexico State, barley is the best alternative in years when the maize crop fails or cannot be planted due to adverse weather conditions. Because of the success of Mexican beer worldwide have led to an increase in imports of malting barley from the United States of America and Canada, within new trade agreements environments, specifically the North American Free Trade Agreement.

The environment in the Mexican highlands is diverse and variable, ranging from very dry, with less than 200 mm of precipitation in some years, to areas where annual rainfall reaches 500 mm. The ICARDA/CIMMYT (International Center for Agricultural Research in the dry Areas and International Maize and Wheat Improvement Center) Latin America Regional Program is helping in the development of barley varieties adapted to drought, as well as varieties adapted to more favorable environments. The program have had reported achievements in developing barley with multiple disease resistance in an acceptable agronomic background (VIVAR 2000; CAPETTINI *et al.* 2001).

In order to achieve success, the program has signed research collaboration agreements with the Agriculture Secretaries of Hidalgo. The strategy includes the generation of variability through targeted crosses at research stations, followed by selection under target conditions, followed by extensive on-farm testing throughout the target area. The experiments and variety demonstration plots are presented to farmers during field days, which also afford an opportunity to discuss aspects of barley production and market preferences.

Material and Methods

Experiments were planted in commercial barley fields at one location in 2000, and three locations in 2002 and 2003 at the State of Hidalgo. Locations were chosen to be representative of the environments present in the State. One location was not considered for yield in each of the 2002 and 2003 years because of uneven emergence due to extreme drought stress. The genotypes included in the experiments were 5, 18 and 50 in 2000, 2001 and 2002, respectively. The design used in 2002 and 2003 was an unreplicated trial with two checks repeated eight times and alternated every seventeen plots. Analysis of variance, estimation of the variance components, and spatial analyses were carried out at each location and combining locations in the last two years using ASREML statistical software (BURGUEÑO *et al.* 2000). Trials were laid out in a rectangular array of rows and columns then spatial analysis could be performed to improve the precision of estimates of variety effects and variety contrast. Plots were 10m long and 1.40 wide, used also as demonstration plots in field days.

Results and Discussion

The barley evaluated in this research was composed of elite core genotypes from the ICARDA/CIMMYT program, which most of them presented wide adaptation when tested in locations worldwide (VIVAR 2000). Nevertheless, the results obtained showed a wide range of yield results and genotype x location interaction (data not shown) under the specific conditions of the State of Hidalgo (Table 1). Interactions were expected knowing how variable environmental conditions can be in the State. However, it is important to remark that since one of the main experiment stations where the program is conducted (El Batán) is only at 100-200 km range from the testing areas, we would also have expected that differences in specific adaptation would not be so remarked. Significant differences among genotypes were found each year and in the combined analysis through years. The top yielding genotypes were significantly higher in yield in the last two years tested. The average yield for the two years more than duplicated the yield of the only malting barley 'Esmeralda', recommended and distributed by the malting and brewing industry in the State. Besides high yield, the 'P.STO/3/LBIRAN/UNA80//LIGNEE640/4/BLLU/5/PETUNIA1' line presented higher levels of resistance to the most prevalent diseases in the area, e.g. yellow rust (*Puccinia striiformis*), leaf rust (*Puccinia hordei*), scald (*Rhynchosporium secalis*), spot blotch (*Bipolaris sorokiniana*) and BYDV. It also presented better agronomic type, including higher lodging resistance (data not shown).

Results obtained are very encouraging, since the objective of offering new commercial cultivar options to the producers of the region were achieved in a relatively short timeframe. That was possible due to the presence of a breeding program that has higher degree of diversity and offers a large option of extensively tested germplasm, increasing the probability of finding varieties adapted to the target area.

Table 1. Sample of genotypes tested on farm in the locations of Almoloya, Apan and Tecocomulco in the State of Hidalgo in 2001-02

Genotype	Almoloya 2002 (t/ha)	Apam 2002 (t/ha)	Almoloya 2001 (t/ha)	Tecocomulco 2001 (t/ha)	Average 2000-02 (t/ha)
P.STO/3/LBIRAN/UNA80//LIGNEE640/4/BLLU/5/PETUNIA 1	3.63	9.07	8.27	7.55	7.13
ROBUST//GLORIA-BAR/COPAL/3/TOCTE/9/GLORIA-BAR/4/SOTOL//2762/BC-B/3/11012.2/…	5.44	3.76	6.79	5.91	5.47
PETUNIA 2/3/GAL/PI6384//ESC.II.72.607.1E.4E.5E	5.35	4.27	5.79	6.40	5.45
BELLA UNION	4.14	6.39			5.26
ABETO/5/GLORIA-BAR/4/SOTOL//2762/BC-B/3/11012.2/TERN-B//H272/6/SEN/7/CEN-B/…	4.32	4.02	6.92	5.80	5.26
PETUNIA 1/CALI92//BLLU	5.74	4.74			5.24
ALPHA-BAR/DURRA//CORACLE/3/ALELI/7/BOLDO/POLEO/4/RHODES//TB-B/CHZO/3/…	4.02	5.15	6.06	5.10	5.08
LINAZA-BAR/JAZMIN	3.58	3.22	6.84	6.17	4.95
IC93	4.56	5.33			4.95
DC-B/SEN/3/AGAVE/YANALA//TUMBO/4/CEN-B/2*CALI92	4.10	3.99	6.24	5.44	4.94
P.STO/3/LBIRAN/UNA80//LIGNEE640/4/BLLU/5/PETUNIA 1	5.24	4.60			4.92
GLORIA-BAR/4/SOTOL//2762/BC-B/3/11012.2/TERN-B//H272/5/LIGNEE640/6/S.P-B/7/…	3.72	3.97	6.10	5.82	4.90
TRIUMPH-BAR/TYRA//ARUPO*2/ABN-B/3/CANELA	4.21	4.30	5.97	4.90	4.85
ANCA/2469//VALERIANA/3/SHYRI/4/CERISE/LAUREL//ALELI/5/CANELA	3.45	4.88	6.36	4.38	4.77
ANTARTICA 6/CANELA//AZAF	3.37	4.88	5.91	4.85	4.75
TOCTE/3/MJA/BRB2//QUINA	4.13	5.29			4.71
CABUYA (Program Check)	**4.16**	**4.48**	**5.37**	**4.49**	**4.63**
PETUNIA 2/3/GAL/PI6384//ESC.II.72.607.1E.4E.5E	4.35	4.15	4.91	4.14	4.39
IC89	3.53	5.21			4.37
DC-B/SEN/3/AGAVE/YANALA//TUMBO/4/CEN-B/2*CALI92	2.96	2.22	6.31	5.71	4.30
IC91	4.36	4.11			4.24
IC90	3.37	4.98			4.18
PETUNIA 2/3/GAL/PI6384//ESC.II.72.607.1E.4E.5E	3.80	3.78	4.38	4.51	4.12
ARUPO/K8755//MORA	5.30	3.66	5.47	3.58	4.50
PETUNIA 2/3/GAL/PI6384//ESC.II.72.607.1E.4E.5E	3.06	4.03	3.56	3.66	3.58
ZIG ZIG	3.60	4.22	5.14	3.54	4.12
ESMERALDA (Commercial Check)	**4.17**	**3.08**	**3.30**	**2.50**	**3.26**
TOCTE	2.82	4.75	4.82	2.32	3.68
MARCO/FRAGIL//CALI92/6/GLORIA-BAR/4/SOTOL//2762/BC-B/3/11012.2/TERN-B…	2.93	2.42	4.72	3.67	3.44

One of the important additional aspects of this research was the possibility to closely interact with producers and exchange experiences about their needs and economic aspects, allowing the selection of the specific barley types. The dual-purpose large experimental plots served for yield testing and demonstration plots, were used in field days, technical discussions and helping farmers getting acquainted with the new varieties available.

The higher differences in yield between the local variety and the introduced ones were not surprising. Breeding of malting barley usually limits the degree of genetic diversity of the programs and then the opportunities to make gains in other agronomic traits, due to the need to maintain the quality requested by the industry. The rate of variety replacement is usually much lower for malting barley than for forage barley, for a given target area (HORSLEY *et al.* 1995). The introduction of different options (e.g. forage barley) into an area where only one malting barley variety was planted, added higher variability in the germplasm available, decreasing the risk of disease epidemics and giving producers new options of basically the same crop. Deeper economic evaluations will lead to decide about the advantage of planting

the new forage variety versus the old malting one. The higher level of yield, disease resistance and better agronomic type will probably give more advantages to the new variety in the production system. Barley growers are looking for new opportunities, and forage barley appears to be a good alternative. Although it cannot fetch a premium price for quality, high-yielding forage barley developed by the program has been proven to deliver a higher total income for growers than does malting barley. Farmers are able to sell the grain when the price is good, or use the barley to feed their lambs and goats when the grain price is low.

Additional testing is already underway to help in the decision of releasing additional varieties identified in the study, especially the hulless barleys. New similar research projects are going to be initiated with other states in Mexico.

Acknowledgements
I would like to thank Dr. Jose Crossa for his support in the planning and statistical analysis of the experiments.

I would also like to thank the Hidalgo Foundation in Mexico for supporting this research.

References

BURGUEÑO, J., A. CADENA, J. CROSSA, M. BÄZINGER, A.R. GILMOUR, B. CULLIS (2000): User's Guide for Spatial Analysis of Field Variety Trials Using ASREML. Mexico, D.F. CIMMYT.

CAPETTINI, F., H. VIVAR, L. GILCHRIST and M. HENRY (2001): Building Up Multiple Disease Resistance In Barley. Warren E. Kronstad Symposium. CIMMYT. Cd. Obregon, Mexico, May 15-17, 2001.

HORSLEY, R.D., P.B. SCHWARZ, and J.J. HAMMOND (1995): Genetic diversity in malt quality of North American six-rowed spring barley. Crop Sci., **35:** 113-118.

VIVAR, H. E. (2000): Building multiple disease resistance into high yielding platform. *In* Susan Logue ed. Proceedings of the 8th International Barley Genetics Symposium. October 22-27, 2000, Adelaide, Australia. P. 280-286.

Screening Barley Germplasm for Waterlogging Tolerance

M.X. Zhou[1], N.J. Mendham[1], G.P. Zhang[2], Q.Q. Shen[3], R.G. Xu[4], G.R. Zhang[5] and H. Chen[6]

[1]Tasmanian Institute of Agricultural Research, University of Tasmania, P.O. Box 46, Kings Meadows, Tas 7250, Australia; [2]Agronomy Department, Zhejiang University, Hangzhou, China; [3]Zhejiang Academy of Agricultural Sciences, Hangzhou, China; [4]Agronomy Department, Yangzhou University, Yangzhou, China; [5]Shanghai Academy of Agricultural Sciences, Shanghai, China; [6]Yancheng Institute of Agricultural Research, Yancheng, China

Abstract

Field experiments were conducted to screen barley germplasm for waterlogging tolerance at four Chinese Institutes/Universities during 2001/02 and 2002/03 growing seasons. More than 500 barley varieties or breeding lines from different Chinese breeding programs were selected. The death rate of seedlings was measured after waterlogging treatment. Other agronomic traits and yield components were also recorded. Varieties showed diverse responses to waterlogging. Based on the differences in yield and components between the treated and control plants, some varieties were found to be tolerant to waterlogging. Most of these were landrace varieties from the lower Yangtze River area of China. This paper details the response of different barley genotypes to waterlogging and the distribution of waterlogging tolerant varieties.

Keywords: barley; waterlogging; germplasm

Introduction

Barley can suffer severe damage from waterlogging. BANDYOPADHYAY and SEN (1992) reported more than 50 per cent loss in yield after 2 days and 80% loss in yield after 3 days of super-saturation treatment after six weeks normal growth in a coastal saline soil. Low grain quality was also reported due to an increase in husk under-development in response to a shortage of sunshine hours around heading date, and grain with ventral swelling because of high precipitation during the grain-filling stage (MATSUE *et al*. 2000).

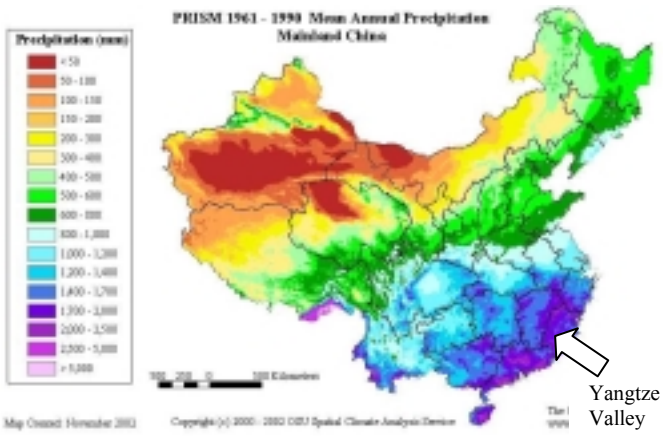


Figure 1. 1961 – 1990 mean annual precipitation in China

Tolerance to waterlogging exists in barley. TAKEDA and FUKUYAMA (1986) tested 3457 varieties in the world collection by submerging 50 sterilized grains of each in deionized water in a test tube for 4 days at 25 deg C and subsequently determining their germination percentage after 4 days on moistened filter paper at 25 deg C. The germination percentage ranged from 0 to 100. The collections from China, Japan and Korea contained many tolerant varieties (average germination 71.6, 66.3 and 60.5 %, respectively) while those from North Africa, Ethiopia and southwest Asia showed few tolerant (19.6, 13.8 and 13.2, respectively). The most tolerant varieties retained complete germinability after 8 days' soaking at 25 deg C. FUFA and ASSEFA (1995) reported some variation among

genotypes in their tolerance to waterlogging and suggested locally adapted landraces could be major sources of tolerance. China holds a substantial proportion of the world's barley germplasm with more than 20,000 accessions which include 1,500 wild barleys (ZHOU *et al*. 2001). A large number of Chinese barley accessions have been selected for waterlogging, saline soils and acid soil tolerance for centuries particularly in the lower Yangtze Valley (Figure 1). Zhejiang Academy of Agricultural Sciences, China, tested 3064 Chinese barley varieties for waterlogging tolerance (unpublished report, 1990) and identified 211 varieties which had medium tolerance to waterlogging. Varieties from the lower Yangtze Valley showed much better tolerance than those from other areas. After testing 4572 barley varieties, QIU and KE (1991) found 16 (0.4%) highly tolerant varieties and 188 (4.8%) tolerant varieties. Among those tolerant varieties, 79.2% were from lower Yangtze Valley. They also found that hulled barley had a relatively higher percentage of tolerant varieties (7.2%) than naked varieties (4.0%). There are still many barley varieties which have not assessed for waterlogging tolerance. This experiment aimed at studying the effect of waterlogging on barley and screening more Chinese varieties for waterlogging tolerance.

Material and Methods

More than 500 Chinese barley varieties were evaluated at Shanghai Academy of Agricultural Sciences, Yangzhou University and Yancheng Institute of Agricultural Research during 2001/2002 and 2002/2003 growing seasons. For the 2001/2002 season, most of the varieties were different at each site. After first year's preliminary evaluation, 244 (tolerant) and 13 (susceptible) were re-evaluated in the second year. Each variety was sown in a 1.5m x 0.27m plot. After germination, the number of seedlings was adjusted so that both waterlogging treated plots and control plots had a similar number of seedlings. Waterlogging treatments were applied when the plant reached the three leaf stage. Water level was kept 1cm above the soil surface for two to three weeks until the varieties showed significant differences. Two replications were waterlogged, with one replication as a control. Dead seedlings were counted soon after waterlogging. The agronomic traits recorded included: maturity, plant height, dry matter per plant, length of the main ear, ears per plant, number of grains per plant, 1000 grain weight, grain weight per plant in both growing seasons, ears per plot and yield per plot in 2002/03 growing season. Plant survival rate was calculated as the ratio between the seedlings after waterlogging and the seedlings before waterlogging. Raw data from 2002/2003 season were statistically analysed at the University of Adelaide, Australia. Some varieties were not included in final analysis, due to factors such as bird damage and low seed germinability

Three of the Chinese varieties identified at an early stage in the project (TX9425, YYXT and DYSYH) were sown in stainless steel tanks (filled with soil from a waterlogged site in Tasmania) during the 2003-4 summer at Mt Pleasant Laboratories in Launceston, Tasmania, Australia. Starting from the 3-leaf stage, all the varieties were subjected to waterlogging (keeping the water level just above the soil surface) for 10 days. The percentage of yellow leaf area was recorded immediately after the termination of waterlogging.

Results and Discussion

Effects of Waterlogging on Agronomic Traits and Yield Components

Waterlogging treatment caused severe damage to the plants of most varieties (Table 1). The average plant survival rate from three sites was 0.74 (0.51 – 0.98). The most affected traits were ears per plot and yield per plot with average percent loss of 42.2 and 37.3, respectively. The 1000-grain weight was the least affected trait. For some varieties, ears per plant, grain number per plant, 1000 grain weight and grain weight per plant were increased after waterlogging. These increases were mainly caused by the death of seedlings which left more spaces for the individuals.

Table 1. Effect of waterlogging on yield components (2002/2003 growing season)

		Plant Survival Rate (%)	Plant Height (cm)	Ears /Plant	Grains /Plant	1000 Grain Weight (g)	Grain Weight /Plant (g)	Ears /Plot	Yield /Plot (g)
Shanghai	Control	100.0	87.1	5.8	186.5	41.2	7.5	154.7	199.8
	Waterlogged	54.0	74.4	4.8	157.9	43.5	6.6	66.6	92.4
	Loss %	46.0	14.3	9.2	9.3	-7.0	2.0	53.2	49.4
Yangzhou	Control	100.0	95.0	6.1	201.1	42.5	8.1	205.2	271.4
	Waterlogged	77.7	94.3	6.1	217.8	43.5	8.8	148.6	211.0
	Loss %	22.3	0.8	-3.3	-9.9	-3.6	-12.5	26.5	19.6
Yancheng	Control	100.0	86.2	7.6	215.5	35.9	7.6	154.6	157.0
	Waterlogged	89.7	73.5	4.3	117.8	39.1	4.5	78.6	83.4
	Loss %	10.3	14.4	42.5	43.7	-10.9	37.8	46.9	42.9
Average Loss (3 sites) %		26.2	9.8	16.1	14.4	-7.2	9.1	42.2	37.3
Minimum Loss %		2.0	-5.7	-48.5	-50.6	-58.4	-77.5	12.9	-7.2
Maximum Loss %		49.0	22.8	47.3	52.2	16.0	49.7	68.7	70.2

Distribution of Varieties with Waterlogging Tolerance

According to the loss of yield per plot after waterlogging treatment, the varieties were classified into three groups: tolerant varieties (yield loss less than 25%); medium (yield loss between 25 – 75%) and susceptible varieties (yield loss more than 75%). Table 2 shows that landrace barley had a higher percentage of tolerant varieties (64%) than bred varieties (11%). 6-row barley showed a higher percentage (50%) of waterlogging tolerant varieties than 2-row varieties (13%), which was partly because most of the landrace varieties were 6-rowed. Naked barleys have a higher proportion of tolerant (42%) than the hulled (18%). Similar to previously reported (QIU and KE 1991), most of the tolerant varieties were from lower Yangtze River area (high rainfall, Figure 1) where the barley had been selected for waterlogging tolerance for centuries, particularly in padi situations as a winter crop in rotation with the main summer crop, rice.

Table 2. Waterlogging tolerance of barley for different type and origin

Yield Loss (%)	Total	Type				Origin	
		2-Row Hulled	2-Row Naked	6-Row Hulled	6-Row Naked	Landrace	Bred Varieties
< 25	38	15	2	12	9	23	15
25 – 75	100	81	3	7	9	11	89
> 75	34	29	0	2	3	2	32
Total	172	125	5	21	21	36	136

Correlations between Percent Loss of Different Traits

Significant correlations were shown between the percentage of yield loss and the loss of most other traits (Table 3). Greater correlation was shown between yield loss and the loss of grain weight per plant than between yield loss and plant survival rate, indicating that the tolerance of most varieties was due to the good recovery ability from waterlogging. For example, the landrace variety, Mimai, was affected by waterlogging at the early stage with only 70% of plants surviving. However, after waterlogging, the survivors recovered very well, with 1000-grain weight, ears per plant, grains per plant and grain weight per plant being 16%, 18%, 51% and 77%, respectively, higher than the control. The final yield per plot was even 7% higher than the control. There were also a few tolerant varieties which showed a better survival rate after waterlogging than susceptible varieties. For the susceptible varieties, both higher plant death rate and low recovery ability after waterlogging caused the greater yield loss.

Table 3. Correlation coefficients between percent loss of different traits

	Plant Survival Rate	Plant Height	Ears /Plant	Grains /Plant	1000 Grain Weight	Grain Weight /Plant	Ears /Plot	Yield /Plot
Plant Survival Rate	1.00							
Plant Height	0.01	1.00						
Ears/Plant	-0.19**	0.43**	1.00					
Grains /Plant	-0.14*	0.47**	0.92**	1.00				
1000 Grain Weight	-0.05	0.21**	0.11	0.15*	1.00			
Grain Weight/Plant	-0.11	0.48**	0.86**	0.94**	0.44**	1.00		
Ears/Plot	0.44**	0.42**	0.60**	0.58**	0.08	0.54**	1.00	
Yield/Plot	0.35**	0.45**	0.54**	0.67**	0.41**	0.74**	0.84**	1.00

Correlation between Years and Sites

Table 4 showed that significant differences existed among varieties for all the traits measured. Both waterlogging treatment and growing sites had very significant effects on all the traits. Strong interactions between variety and growing site and between site and treatment indicated low correlations between growing sites or that the results from different sites were not consistent. The ANOVA showed similar trend for same site from two years (data not shown), i.e. results from different years at the same site were also inconsistent. This is largely due to the variable waterlogging conditions, which makes the evaluation more difficult. The interactions between variety and waterlogging treatment (differences in the response of variety to waterlogging) were not significant for plant survival rate, 100 grain weight, ears per plot and yield per plot. However, when comparing individual varieties, some varieties showed consistent tolerance and others showed consistent susceptibility. For example, the variety, KA-4B was susceptible in all three sites in both years with yield loss from 46% to 49% in 2002/03 growing season and the loss in grain weight per plant in 2001/02 growing season from 52% to 68%. In contrast, the tolerant variety, Chengxian Hongjin Sileng showed consistent tolerance in different years and sites with the yield loss from –1% to 27% in 2002/03 growing season and the loss in grain weight per plant from –6% to 37% in 2001/02 growing season. Mimai, as mentioned above, showed very good tolerance to waterlogging in 2002/03 growing season. The grain weight per plant was also not affected by waterlogging in 2001/02 growing season. Varieties responded differently in grain weight per plant to waterlogging treatment (Table 4). Since the response of grain weight per plant was significantly correlated wit the response of plot yield to waterlogging (Table 3), the selection for waterlogging tolerance can be achieved by selecting grain weight per plant under waterlogging condition.

Table 4. ANOVA of the data from 2002/03 growing season (F Value)

Source of variance	df	Plant Survival Rate	Plant Height	Ears /Plant	Grains /Plant	1000 Grain Weight	Grain Weight /Plant	Ears/Plot	Yield /Plot
Variety	171	4.27**	29.42**	5.86**	9.32**	24.21**	3.06**	5.55**	2.85**
Treatment	1	928.92**	740.41**	333.20**	166.71**	98.89**	78.92**	1047.83**	543.26**
V x T	**171**	**0.69**	**1.23***	**1.38****	**1.54****	**0.90**	**1.37****	**1.15**	**0.96**
Site	2	495.55**	902.90**	39.57**	90.16**	245.13**	131.87**	346.93**	454.28**
V x S	342	1.90**	3.85**	2.26**	2.62**	2.05**	2.04**	1.81**	1.85**
S x T	2	141.41**	156.66**	171.25**	137.88**	8.10**	81.64**	16.29**	16.48**

Comparison of Waterlogging Tolerance under Controlled Environment

In the first year's waterlogging trial at Yangzhou University, YYXT, DYSYH and TX9425 (all Chinese varieties) had showed very good waterlogging tolerance while two Australian varieties, Franklin and Gairdner and one Japanese variety, Naso Nijo were susceptible. When these varieties were waterlogged under more precisely controlled conditions (tanks filled with same soil from waterlogging area), the Chinese varieties still showed much better tolerance. Dead leaf percentage under excess soil moisture was thought to be the best criterion for selection for flooding tolerance in early generations because its heritability values are relatively constant and it is easy to measure **(**HAMACHI *et al*. 1990**)** and was correlated with reduction of grain yield/plant and culm length (HAMACHI *et al*. 1989). The varieties showed significantly different levels of leaf chlorosis after waterlogging. Three Chinese varieties, TX9425 (12.6%), DYSYH (5.0%) and YYXT (7.0%) showed much less yellow leaf percentage than Franklin (43.9%), Gairdner (31.3%) and Naso Nijo (39.5%) (Figure 2). The Chinese varieties also showed better root and shoot growth under waterlogging conditions.

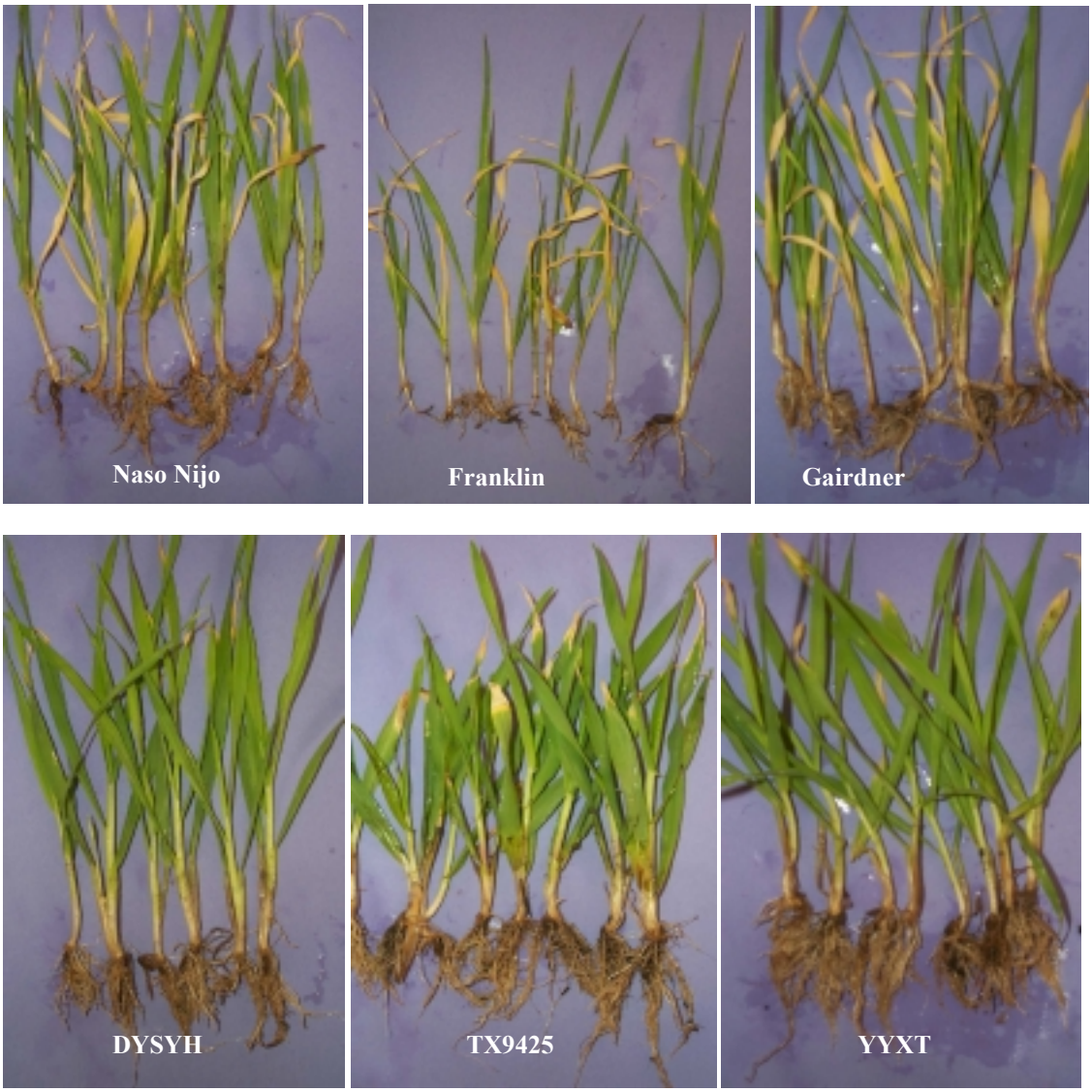


Figure 2. Effect of waterlogging on leaf chlorosis and root development of different varieties. Photos taken one week after waterlogging.

Screening Waterlogging Tolerance in Barley

Selection for waterlogging tolerance in barley is very difficult. As discussed above, the results from the yield trials carried out in China varied between sites and years due to variable field conditions. Thus, efforts have been done to find indices which can be easily selected for, which are closely related to yield. SUH (1978) reported that waterlogging tolerant varieties were generally shorter, more prostrate, produced more tillers and had longer roots when grown under waterlogged conditions, which lead to higher yields. MUSGRAVE and DING (1998) found that greenhouse measures of photosynthesis under waterlogging were weakly predictive of yield ($r^2 = 0.61$) but the yield was closely correlated with biomass ($r^2 = 0.84$) and root mineral content (Fe + Mn + P) ($r^2 = 0.94$). We have also carried out a series of studies to identify useful indices for selecting waterlogging tolerance and found that breeding for waterlogging tolerance could be facilitated by selecting genotypes with the development of a larger root system, the least pronounced reduction of photosynthetic rate, chlorophyll content and chlorophyll fluorescence under the condition of waterlogging, and rapid recovery after drainage (PANG *et al.* 2004a). Anatomical study of the adventitious roots showed that aerenchyma was formed in the cortex in both TX9425 and Naso Nijo after 3 weeks hypoxia treatment. The percentage of aerenchyma area in the root cross-section area was larger for TX9425 than the corresponding value in Naso Nijo (PANG *et al*. 2004b). A larger scale field experiment is also being conducted this year to study physiological or agronomic traits which could be used as selection indices. Several doubled haploid populations are being used to screen for molecular markers for waterlogging tolerance. This technique will make selection more effective in a breeding program.

Acknowledgements

This work has been supported by Grain Research and Development Corporation (GRDC) of Australia.

References

BANDYOPADHYAY B.K., SEN H.S. (1992): Effect of excess soil water conditions for a short period on growth and nutrition of crops on coastal saline soil. J. Indian Soc. Soil Sci., **40**: 823-827.

FUFA F., ASSEFA A. (1995): Response of barley to waterlogging: improved varieties versus local cultivars. IAR Newsl. Agric. Res., **10**: 6-7.

HAMACHI Y., FURUSHO M., YOSHIDA T. (1989): Heritability of wet endurance in malting barley. Jpn. J. Breed., **39**: 195-202.

HAMACHI Y, YOSHINO M., FURUSHO M., YOSHIDA T. (1990): Index of screening for wet endurance in malting barley. Jpn. J. Breed., **40**: 361-366.

MATSUE Y., YAMAGUCHI O., SATO H., BABA T., TANAKA K., FURUSHO M., OGATA T., FUKUSHIMA Y. (2000): Influence of abnormally warm and wet weather on the yield and apparent quality of wheat and two-rowed barley and its related factors in northern Kyushu in 1998. Jpn. J. Crop Sci., **69**: 102-109.

MUSGRAVE M.E., DING N. (1998): Evaluating wheat cultivars for waterlogging tolerance. Crop Sci., **38**: 90-97.

PANG J.Y., ZHOU M.X., MENDHAM N.J., LI H.B., SHABALA S. (2004a): Growth and physiological responses of six barley genotypes to waterlogging and subsequent recovery. Aust. J. Agric. Res., Submitted.

PANG J.Y., ZHOU M.X., MENDHAM N.J., SHABALA S. (2004b): Growth, morphological and anatomical responses of two barley genotypes to hypoxia. 9th International Barley Genetics Symposium. Brno, Czech Repulic.

QIU J.D., KE Y.A. (1991): Study of determination of wet tolerance of 4572 barley germplasm resources. Acta Agriculturae Shanghai, **7**: 27-32.

SUH H.S. (1978): Studies on the wet-injury resistance of wheat and barley varieties. III. Effect of various moisture levels on the top and root growth of the barley crop. J. Kor. Soc. Crop Sci., **22**: 80-92.

TAKEDA K., FUKUYAMA T. (1986): Variation and geographical distribution of varieties for flooding tolerance in barley seeds. Barley Genet. Newsl., **16**: 28-29.

ZHOU M.X., MENDHAM N.J. (2001): Developing collaborative studies with China. Proceedings of the 10th Australian Barley Technical Symposium, Canberra, Australia

S 10 – BREEDING SUCCESS STORIES

Progress in Malting Barley Breeding in SECOBRA

M. Madre

SECOBRA Recherches, Centre de Bois-Henry, 78580 Maule, France

SHORT HISTORY

Created in 1902 by the French Brewing industry, the first mission of SECOBRA Association has been to develop and improve the supply of good quality barley for industry.
As early as 1903, SECOBRA was conducting various projects aimed at isolating the different botanical strains of the period, existing as mixtures in indigenous varieties, and testing foreign varieties. After studies, tests and multiplication, some of these varieties were very quickly used at the industrial level (SARAH, COMTESSE, etc.)
The first crosses were made towards 1920. During the 1930s, work focused on the breeding, the study of agricultural practices and the production of pure barleys. In addition, work was conducted on varietal identification which has been used to establish the first official French catalogue. In 1947, the chairman of SECOBRA, Philippe KREISS set into motion the creation of the EBC (European Brewery Convention), initially representing 7 countries but rapidly incorporating many others.

Development continued up to the 1970s and included many regional experiments and study programs with publications and reports. A new stage was then begun with the creation of SECOBRA Recherches whose main mission is varietal breeding.
Under the impetus of French brewers and Maltsters, especially Kronenbourg, and of the Soufflet Group and Champagne Céréales Malteurop, a new breeding station was created in 1985 at the centre of an experimental farm. After this, three new breeding centres were opened in rapid succession, in Germany in 1992, in England in 1993 and in Spain in 1997.
In 1999, SYNGENTA SEEDS joined the Company.
Since the beginning, SECOBRA is managed by the French Malting and Brewing Industry which holds today 80 M of shares.

1. BARLEY SITUATION IN FRANCE

Starting from nearly 3 million hectares in the period between 1975 and 1980, there followed a great reduction of about 1 million hectares, the total area stabilising at about 1 800 000 ha until 1992 with an almost constant production of 10 million tons.
From 1993, the reform of the CAP led to further reduction in the area of barley (mainly the winter and feed types) so that in the last 10 years the area has varied from 1.5 to 1.6 million hectares, including 500 000 ha of spring barley *(Table I)* and 1 to 1.1 million hectares of 6 rowed and 2 rowed winter barley.
After a predominance of spring barley areas in the seventies, the development of 2 row winter barley areas in the eighties and then, a period of roughly equal proportions of the 3 main types of barley in 1985, the current proportions, excluding the years 2001 and 2003 which were affected by extreme weather conditions, are approximately as follows *(Table II)* :

35% spring barley
45% 6 row winter barley
et 20% 2 row winter barley (a decrease that started with the 1998 harvest).

The total annual production is about 10 million of tons and more than 75 % of this production go into commercial channels.

1.1. CHANGE IN YIELDS

While the cultivated areas were being reduced and changes were occurring in the proportion of the various types of barley, there was also a significant improvement in productivity, especially in spring barley *(Table III)*. In the last 25 years, there has been an average increase in the yields of spring barley of 0,12 ton/Ha/year with varieties that are almost all intended for brewing.
This progress can be attributed in equal proportions to genetic improvement in the form of new varieties and improvements in agricultural practices such as earlier sowing and the use of fungicides.
As far as winter barley is concerned (6 row and 2 row types combined) and where the proportion of varieties intended for animal feed is still significant, progress has been slightly slower (nearly 0,1 ton/Ha/year) but with a great final difference in productivity of more than 1 ton/Ha in favour of 6 row varieties.
The table shows a clear superiority in yield of the 6 row winter barleys compared to the 2 row winter ones, which yield approximates the one of the 2 row spring barley.
The following productivity classification : 6RW > 2RW > 2RS explains the various changes.
Alongside, changes in productivity, factors improving the reliability of yields have also greatly improved like resistance to fungal diseases, resistance to lodging, resistance to viruses (with generalised resistance to M & Y1 mosaïc viruses in winter barley).

1.2. CHANGES IN BREWING QUALITY

Since the 1980s, with the impulse of industrial maltsters and brewers, increasingly elaborate protocols for assessing the brewing quality of barleys have been gradually set up by official bodies of the Ministry of Agriculture (CTPS : Comité Technique Permanent de la Sélection) and malting / brewing professionals (CBMO : Comité Bière-Malt-Orge). These protocols include quality indices, the CTPS's list of barleys with brewing potential, functional indices, pilot tests, list of varieties preferred by French maltsters and brewers, etc. *(Table V)*.

These protocols, through a systematic study of the varieties, have allowed the breeders to make some progress in some technological characteristics. For example, for spring barley, the study on varieties registered since 1989, of quality criteria during registration, such as : extract, Kolbach index, Diastasic power, final attenuation, shows :

- A significant progress in the dry extract from the malt of 0,2 % /year (i .e. : 2.8 points in 14 years) *(Table IV)*
- A reduction in protein content (- 0.1 point every 2 years) and in viscosity
- A slight increase in the Kolbach index. The diastasic power and final attenuation have remained almost stable.

These changes should be interpreted with caution because the annual data include both a genetic effect (variety) and a year effect (the environment and the malting process). However, it shows that the number of spring barley varieties approved on the list of malting characteristics and having favourable properties increases strongly each year (2 or 3 by year at the beginning to more than 10 to 12 today).

On examining the multi-annual averages from 1989 to 2002 *(Table VI)* of varieties recorded on the CTPS's list of barleys with brewing potential, still more marked differences are seen in the varietal populations of the 3 types of barley.
For spring barleys, technical progress and productivity improvement have been rapid and significant. This species currently represents the best balance of technological criteria, because it has received the greatest attention from all breeders across the world. For 2-row winter barley, a species developed in Europe since 1972/75, and 6-row winter barley for brewing, grown almost exclusively in France, technological progress has been slower. The table shows an overall technological rank classification of 2RS>2RW>6RW, which is the opposite of the classification for yield.

1.3. SECOBRA VARIETIES IN FRENCH BARLEY CROP

Leaving aside very old varieties (BEKA 56, etc.), SECOBRA varieties for the last twenty years have contributed with others to the development of the brewing barley crop in France and Europe, including:

- spring barleys, such as the highly productive varieties VOLGA (88), NEVADA (93), ASTORIA (99), etc.
- 2-row winter barleys, such as MOGADOR (80), FLAMENCO (81), CLARINE (87), BOREALE (01), NECTARIA (02) etc.
- 6-row winter barleys, such as ESTEREL (95), which has increased productivity and resistance to mosaics while surpassing Plaisant in quality (extract yield, modification, diastasic power and α amylase, viscosity, etc.), and more recently REGALIA (03), ARTURIO (03), DOROTHEA (04) etc.

In Germany, the registration of new brewing varieties such as JOSEFIN (03), a very high quality spring barley, or DOROTHEA (04), a new 6-row brewing barley, strengthens the presence of SECOBRA.
In parallel to the brewing barley varieties, feed barleys have been very significantly developed in Europe:

- 2-row winter varieties PASTORAL (86), ANTONIA (98)
- 6-row winter varieties MANITOU (89), FEDERAL (94), SIBERIA (00) and above all the past and present leading varieties in Germany: JANA (90), THERESA (94), FRANZISKA (00), in addition to other new varieties such as STEPHANIE (02), MAXIMILIANE (04) etc.

As a result, SECOBRA varieties are currently grown on more than 40% of the barley cultivated areas in France and are present in many other European countries (Germany, Denmark, United Kingdom, Spain, Sweden, …)

2 . SECOBRA BREEDING

SECOBRA is currently developing very extensive barley breeding programs in 4 major cereal-growing countries: France, Germany, England and Spain.
These centres conduct independent, co-ordinated and complementary programs for each type of barley: 2 row Spring, 2 row Winter and 6 row Winter.

2.1. OBJECTIVES

To supply the various partners in the chain with conventional varieties combining Quality, Productivity and Regularity, adapted to the various agroclimatic conditions and markets: these objectives vary according to the types of barley.

- ***In spring barley*** :
    - The maintenance of a high or very high quality level, with an excellent balance between the technological properties of the barley (grain size, speed of germination) and of the malt (extract, Kolbach index, enzymatic potential and other specific characters …).
    - Improvement of yield and its reliability (earliness varieties, diversifying of the resistance to fungal and viral diseases, fusarium resistance …).

- ***In winter barley***, after the generalisation of resistance to M and Y1 mosaïc viruses, introduction of resistance to Barley yellow dwarf and Y2 mosaïc viruses.
    - In 6 row « escourgeon », a French speciality, the work has concentrated on improving general quality, where progress is possible on barley (in terms of grain size, dormancy …) and on malt (extract, modification speed, viscosity …), while maintaining a high or very high yield and improving resistance to fungal and viral disease and adverse weather conditions such as cold and lodging.

- In 2 row winter barley, an intermediate type developed in the 1970s in the main European countries, the improvement of quality will be continued (modification, viscosity …), which are already at a good level, but the yield still needs to be improved and the protein content reduced.

For all 3 types of barley, in parallel with yield and quality, improvement in the reliability of yield, by improving resistance to diseases and lodging, is still essential since it reduces the need for agricultural inputs such as fungicides and growth regulators and thus reduces the risk of residues or toxins in the crop. Other aspects such as the search for varieties which do not need to be highly azot fertilised is of great interest.

Besides, improvement of technological criteria that are of interest to malting industry, such as specific enzyme activities (amylases, lypoxygenases …) takes a great part in our breeding quality programs.

2.2. METHODS

SECOBRA uses conventional breeding methodology based on pedigree method after crossing, with optimisation of the method at each level through the various acceleration, screening and breeding tools available.
Each year it carries out more than 2500 single or complex crosses for the 3 types of barley.
This results in a high volume of material for each generation and requires carefully adapted tools. Breeding pressures vary between generations and crosses and depend on the objectives. The normal cycle duration for generating a new variety is 7 to 8 years maximum.
To improve the efficiency of selection, various strategies are developed to increase the rate of fixation or increase the quality and safety of choices.

- Acceleration of breeding time
  This is achieved by increasing the number of generations per time unit or accelerated genotype fixation.
  Under artificial conditions, use of the greenhouse for some generations (crosses, F1 …) or growth chambers to produce Single Seed Descent (3 generations a year for spring barley), shortens the duration of the general cycle.
  Under natural conditions, nurseries are set up in the Antipodes (e.g. New Zealand) and used in alternation with the normal generations.

  In addition, the double-haploid method with anther culture, is used for some types of crosses as a fast way of obtaining fixed homozygote genotypes that are then tested in field trials.

- Improving breeding quality:
  For each generation, depending on the objectives, the difficulty is always to select specimens with the required characters.
  - For single characters such as certain types of resistance (powdery mildew, mosaic virus, etc.), screening is carried out to eliminate sensitive or non-conforming plants.
  - On the other hand, complex characters such as yield or quality generally require agronomic experiments and complex technological analyses.

    It is for this reason that we carry out as many predictive tests as possible on the intermediate generations where large numbers of specimens are still available and where intra-family variability is still relatively low.
    For quality, tests are conducted on barley (grading, thousand kernel weight, germination, infrared analyses, etc.) or on micromalts of some tens of grammes with measurement of important criteria such as extract yield, diastatic power, Kolbach index, viscosity, etc.).
    Each year, more than 8000 infrared analyses and nearly 4000 micromaltings are therefore carried out.

- Use of the molecular marking
  Molecular marking, a new tool available to the breeder, is used in a very pragmatic way in the programs for specific criteria of an agronomic nature (resistance to viruses, etc.) or technological nature (enzymatic activities, etc.), enabling identification and positive selection of a character that can be usefully added to a standard selection schema.

2.3. RESULTS

Each year, the various SECOBRA centers produce a large and constantly renewed output of new varieties of classic barleys as well as original and innovative varieties resistant to BYDV or having specific enzymatic activity characteristics, or in the case of 6-row winter barley, improved technological qualities on a comparable level to those of spring barleys.
On average, 15 to 30 new lines emerge from the 3 main programs in France, Germany and England, for each winter and spring species. In other words, roughly 120 new varieties are tested each year in pre-application tests for more than 25 European and non-European countries.

CONCLUSION

SECOBRA, a research and breeding company for the French Malting-Brewing industry has, for a number of years, invested major resources in the improvement of barley.

Through its breeding programs, some of the most extensive in Europe, the company is working to create future varieties improved at both the technological level (specific qualities) and the agronomic level (resistance, etc.), meeting the demand of transforming industry as well as agriculture.

This is made possible through the continuous support, for more than 100 years, of the French Brewing and Malting industry (leading malt exporter in the world): a unique example in Europe of co-operation between a breeding company and its downstream industry.

GNIS : Groupement National Interprofessionnel des Semences - *French National Interprofessional Seed Group*
ONIC : Office National Interprofessionnel des Céréales - *National Interprofessional Cereal Office*
CTPS : Comité Technique Permanent pour la Sélection – Ministère de l'Agriculture - *Permanent Technical Committee for Registration – Ministry of Agriculture*
CBMO : Comité Bière-Malt-Orge - *Beer – Malt – Barley Committee*

Table I : **Global change in Barley cultivated areas in France**

Table II **: Change in cultivated Barley types in France**

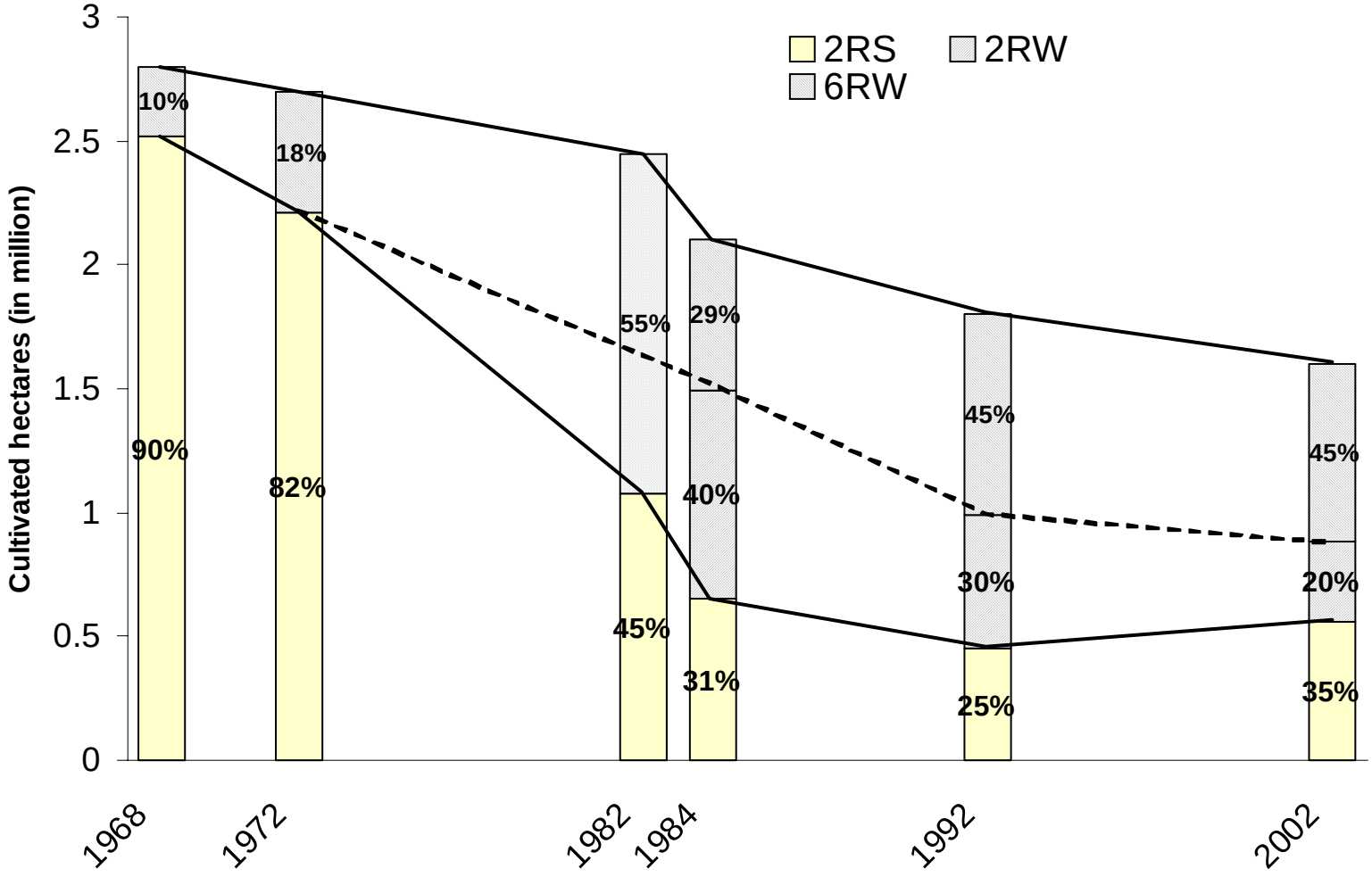


Table III : **Barley : Global change in yields in France**

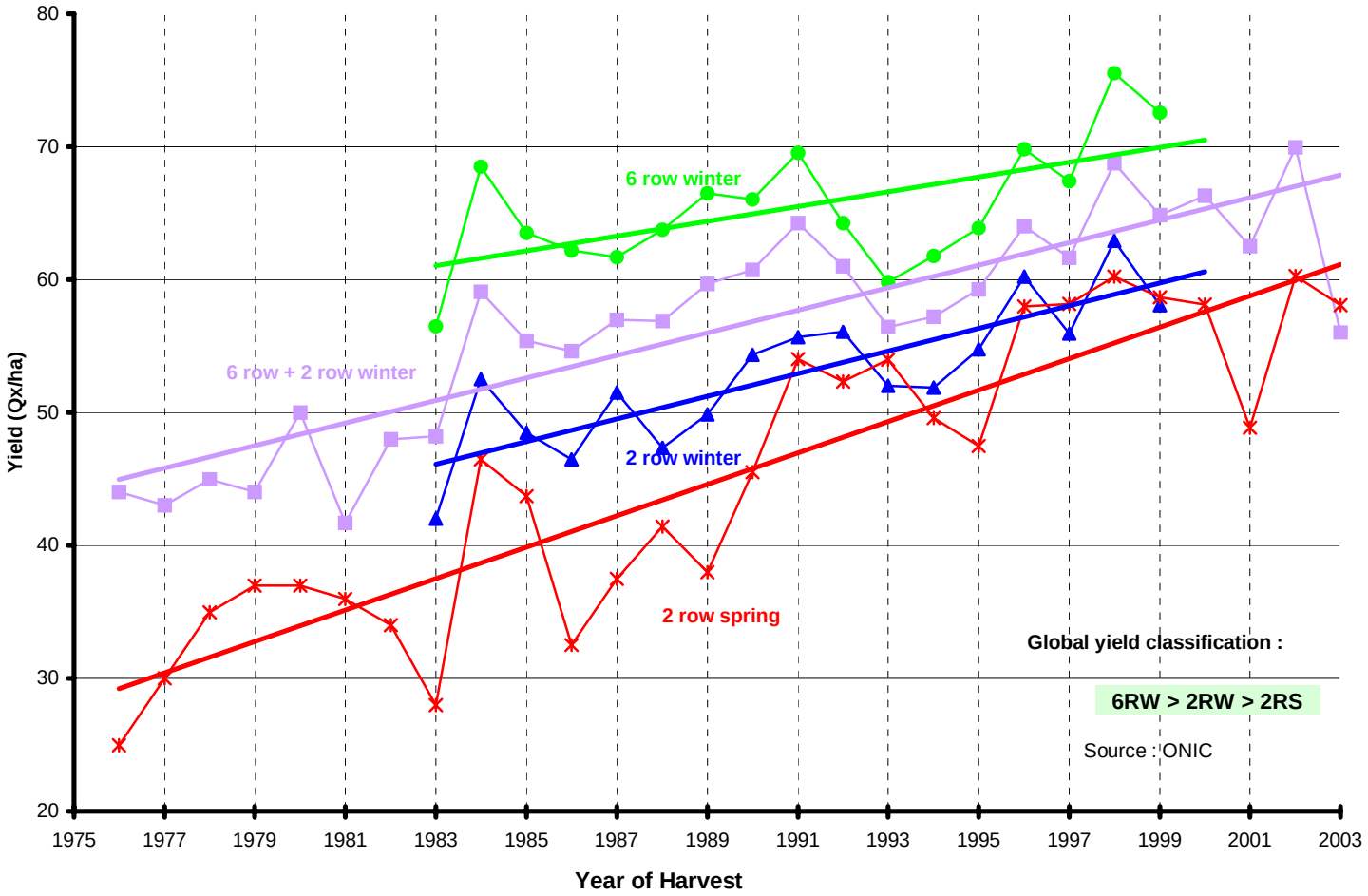


Table IV : Changes in quality : Malt extract – Average of the spring barley varieties included in the CTPS approved malting barley list

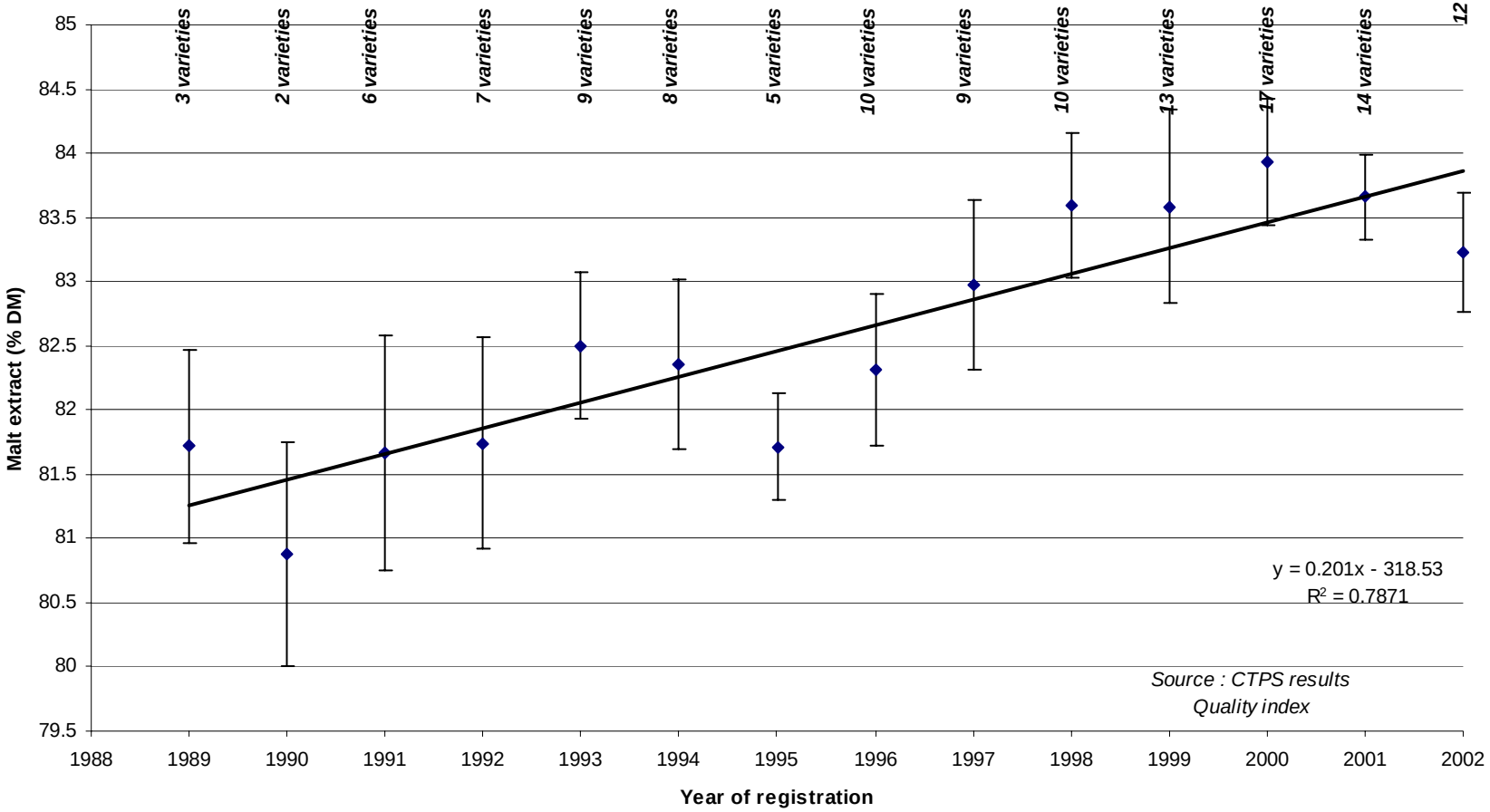
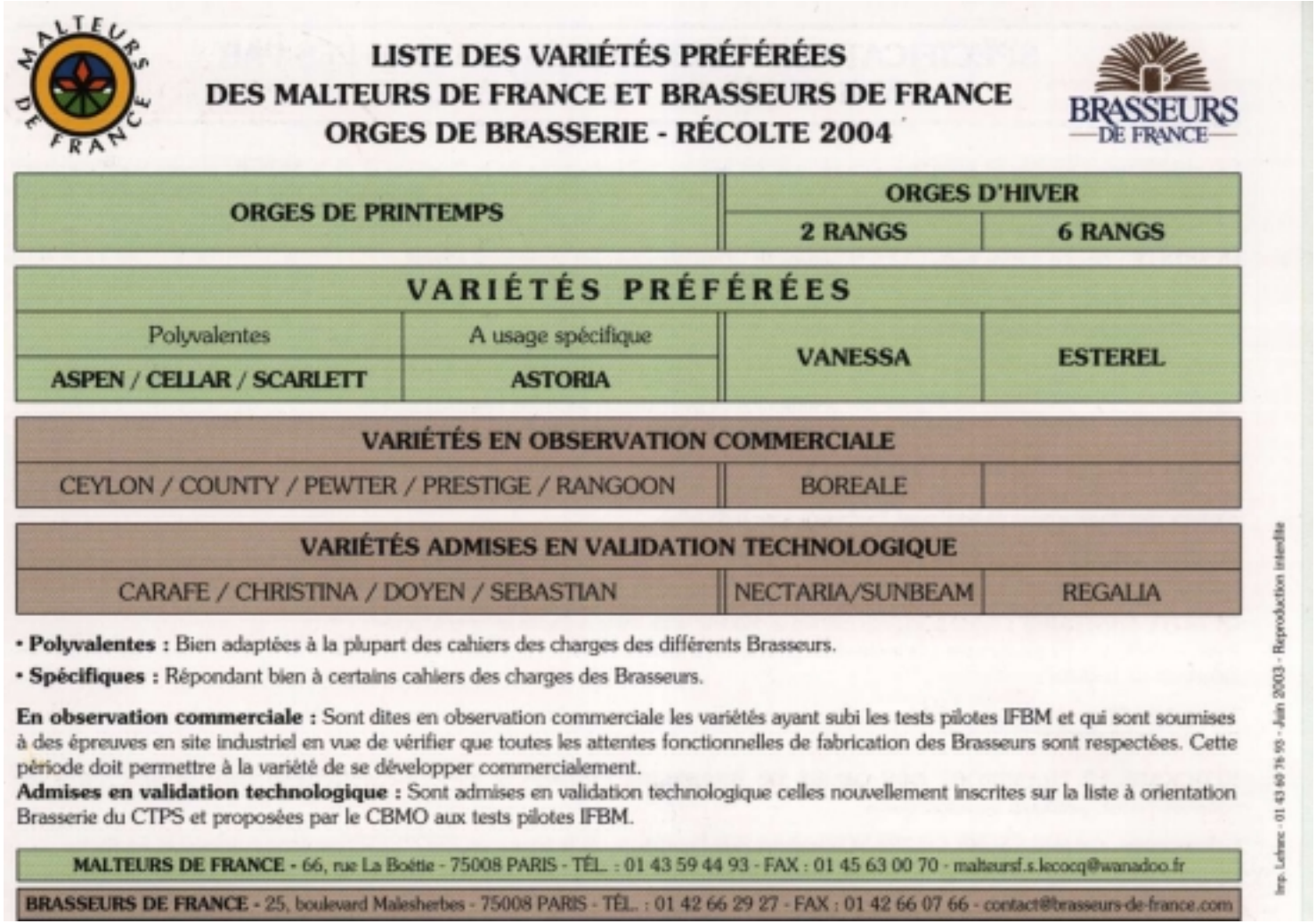


Table V **: « Malteurs et Brasseurs de France» official list of malting barley : Harvest 2004**



LISTE DES VARIÉTÉS PRÉFÉRÉES
DES MALTEURS DE FRANCE ET BRASSEURS DE FRANCE
ORGES DE BRASSERIE - RÉCOLTE 2004

BRASSEURS DE FRANCE

ORGES DE PRINTEMPS		ORGES D'HIVER	
		2 RANGS	6 RANGS
VARIÉTÉS PRÉFÉRÉES			
Polyvalentes	A usage spécifique	VANESSA	ESTEREL
ASPEN / CELLAR / SCARLETT	ASTORIA		
VARIÉTÉS EN OBSERVATION COMMERCIALE			
CEYLON / COUNTY / PEWTER / PRESTIGE / RANGOON		BOREALE	
VARIÉTÉS ADMISES EN VALIDATION TECHNOLOGIQUE			
CARAFE / CHRISTINA / DOYEN / SEBASTIAN		NECTARIA/SUNBEAM	REGALIA

• **Polyvalentes :** Bien adaptées à la plupart des cahiers des charges des différents Brasseurs.
• **Spécifiques :** Répondant bien à certains cahiers des charges des Brasseurs.

En observation commerciale : Sont dites en observation commerciale les variétés ayant subi les tests pilotes IFBM et qui sont soumises à des épreuves en site industriel en vue de vérifier que toutes les attentes fonctionnelles de fabrication des Brasseurs sont respectées. Cette période doit permettre à la variété de se développer commercialement.
Admises en validation technologique : Sont admises en validation technologique celles nouvellement inscrites sur la liste à orientation Brasserie du CTPS et proposées par le CBMO aux tests pilotes IFBM.

MALTEURS DE FRANCE - 66, rue La Boétie - 75008 PARIS - TÉL. : 01 43 59 44 93 - FAX : 01 45 63 00 70 - malteursf.s.lecocq@wanadoo.fr

BRASSEURS DE FRANCE - 25, boulevard Malesherbes - 75008 PARIS - TÉL. : 01 42 66 29 27 - FAX : 01 42 66 07 66 - contact@brasseurs-de-france.com

Table VI: **Change in malting quality – Global improvements**

Average of criteria : varieties included in the CTPS approved malting barley list 1989 to 2002

	Nber of varieties On CTPS List	Grain size	Extract	Proteins	Kolbach	D.P.	Viscosity	Final attenuation
SPRING BARLEY	125	-	82,56	9,86	43,01	311	1,50	83,11
2 ROW WINTER BARLEY	65	91,60	81,29	10,34	40,18	347	1,60	82,54
6 ROW WINTER BARLEY	21	83,88	80,47	9,57	40,94	293	1,67	82,59

Source CTPS results

Global quality classification :

➔ 2 RS > 2 RW > 6 RW

Breeding Malting Barley for the Canadian Prairies, A Greybeard's Perspective

B.L. Harvey

University of Saskatchewan, Saskatoon S7N 4J8, Canada

Abstract

Over the past 40 years many things have changed yet some important things have remained the same. In the early 60's cell phones, faxes, PC's and the internet were not available to us. Canada grew very little two-row barley and was a relatively small player in world trade of malt and malting barley. The USA and UK (before tariffs) were our major export markets. High diastase malt was in demand for whisky making in the UK and for domestic brewing in North America. In broad terms the objectives of our breeding programs were the same as now; quality suited to market demands, excellent field performance and resistance to diseases. The details have of course changed markedly. Canada is now a major player in world trade and two-rows are by far the largest portion of this. High enzyme levels and rapid modification still characterize our barleys which are well suited to high gravity and high adjunct brewing.

The mechanical, electronic, molecular and chemical tools we now have available to us were only dreamed of 40 years ago. We have become much more effective and efficient. During the intervening years we have increased yield potentials by 30% or more and built in better sources of resistance to a number of diseases. In the two-rows we have moved from an older European quality profile to our modern North American profile. We have increased enzyme levels, to a par with our six-rows, without sacrificing extract. Modification times have been reduced by over 20% and low beta glucan levels mean fast run-off times in the brewery. The reason for this success is truly focussing on quality as the number one priority and on close interaction with the malting and brewing industries to gather the intelligence needed to achieve world class quality.

Introduction

Over the past 40 years many things have changed and many things have remained constant. In the early sixties cell phones, faxes, PC's, and the internet, for example were not available to us. For malting purposes Canada grew six-row barley almost exclusively. The three major Canadian brewers used 100% six-rows in their malt blend. The major U.S. breweries, to which we were residual suppliers, were also primarily six-row users. Canada was not a significant player in international trade in malt or malting barley. Our major export markets were the U.S.A. and Europe (before tariffs). The amount of our barley production purchased for malting was well under a million tonnes. High Diastatic power was characteristic of these six-row varieties and in demand for whisky making and for the high adjunct brewing which typified the North American industry.

Canada is now a significant player in the export markets for both malt and malting barley. Due to prohibitive tariffs and subsidies Europe is no longer a market for us, while the U.S.A. has grown to be our major customer. Asian markets have grown substantially especially in China and Japan, as have markets in Latin America. Over 2.5 million tonnes of barley are typically marketed each year. More than two thirds of this is two-rows. Of the two remaining major Canadian brewers only one uses six-row in their blend. The U.S.A. is still the most important user of six-row malting barley and this is where most of our production goes. The two-rows we now grow are the equal of the six-rows in enzymatic activity while maintaining the higher extract that characterizes two-rows. Thus they are suited to markets which demand high diastase for high adjunct brewing as were our six-rows in the past.

Canadian Prairies

Virtually all of Canada's malting barley is spring sown and is produced on the Canadian prairies, two thirds of this in Saskatchewan. This is a vast area with extremely harsh growing conditions. From Steinbach, Manitoba in the Southeast to Beaverlodge, Alberta in the Northwest is 1800 kilometers. This is equivalent to the distance from New York to Miami, Edinburgh to Vienna or Brno to Madrid. The area includes regions of steppe, cool continental and sub-arctic climates. Vegetation types include prairie grasslands, aspen parklands, broadleaf deciduous and coniferous evergreen forests. The growing season varies from 90-120 days with frosts and snow recorded in every month of the year. Days are long and irradiance levels high. Temperatures vary from – 50 to 45 degrees Celsius. Average annual precipitation ranges from 254 mm to 483 with large year to year variation. Soil types include brown, dark brown, black and gray chernozemics and gray luvisolic. Texture varies from sand to heavy clay. PH's are primarily alkaline to neutral but with significant areas mildly acidic. Over 4,000,000 hectares are affected by salinity. Biotic pests which affect barley include a number of grasshopper species and an array of diseases which includes stem rust, net-blotch, scald, septoria, spot blotch, fusarium, several root rots and three species of smuts. Dry weather and abundant sunshine usually limit levels of infection however. Despite all this it is a very fertile and productive area.

Improvements

Major changes have occurred in the tools we have available to handle materials, data and evaluation. Huge improvements have been made in our field equipment, our computational instruments, our disease nurseries, our chemical analysis capability and our understanding of the barley genome. Mechanically we have gone from hand dibbing and single row push-seeders to automated multi-row seeders, from hoes to powered cultivators and sprayers and from sickles and stationary threshers to single and multi-row combine harvesters. We have added a variety of seed cleaning, handling and evaluation equipment. We have gone from mechanical calculators and main frame computers fed by punch cards to very powerful, networked desktop computers and hand-helds for note taking. Programs are readily available to not only process our data but also to generate field plans and labels and to read and collate data from a variety of measuring equipment. We have enhanced our capabilities of creating epidemics in our disease nurseries and identified molecular markers for a number of our important diseases.

In the laboratory we have evolved from tedious test tube, chemical analyses of barley parameters to highly automated micro-malters and automated wet chemistry and infra-red analyzers. These instruments allow us to conduct thousands of analyses in the time and effort it took to do hundreds in the sixties. We have elaborated our knowledge of the barley genome enormously and developed efficient molecular markers for important traits, which can be routinely applied in a practical breeding program. With these tools we can now easily handle 10 hectares of plots or more, we can analyze large amounts of data from a variety of sources almost instantaneously and with less error, we can analyze malting quality of thousands of lines on actual malt. Are we better breeders as a result of all this technology? Yes and no. Yes we can handle much more material and can do it more efficiently and accurately. No if we substitute technology for common sense, priority setting and a breeder's eye and in depth knowledge of his or her material.

Progress has been made on a number of fronts over these last four decades. We have increased grain yields with varieties like CDC Copeland and Newdale by 25-30% (Table 1) over Hannchen and Betzes the standards of the past. We have optimized straw height and considerably increased its strength. We have identified sources of resistance to all of our important diseases and have combined a number of these into newer varieties. We have significantly improved kernel plumpness and uniformity. We have increased alpha amylase and diastatic power by over 30% and extract by 1-2% points. We have reduced protein content and wort beta-glucans (Table 2). Our modern varieties modify much faster and more completely than their predecessors. Thus we have effectively increased the capacity of malt plants by more than 20%. We have also improved traits which are not as easily measured. Harrington for example is known for the excellent shelf life of beers made from it. CDC Kendall produces beer with excellent head retention properties. These characteristics are only revealed after commercialization of the variety. To that extent of course, they are serendipitous.

Table 1. Yield as a % of Harrington from Saskatchewan adaptation tests

Variety	**Area 1**	**Area 2**	**Area 3**	**Area 4**
Hannchen	92	90	91	88
Betzes	94	92	89	91
Klages	95	96	93	97
Harrington	100	100	100	100
Stein	105	107	113	115
Manley	107	122	125	125
CDC Kendall	101	112	114	118
CDC Copeland	109	117	120	120
CDC Select	104	114	113	116

Table 2. Comparison of malting quality parameters from pre-registration cooperation trials

Variety	Plump %	S/T	Fine Extr. %	Fine-70° Extr. %	αAmyl DU	DP °L	βglucan ppm
Hannchen	80.2	30.4	75.8	9.7	39.7	88	-
Betzes	84.0	31.8	77.5	9.9	31.2	93	-
Klages	84.7	41.8	79.1	6.2	51.6	125	-
Harrington	92.8	44.7	79.7	5.1	60.2	130	200
Stein	87.5	42.5	79.0	4.0	64.4	147	195
Manley	88.3	43.3	79.2	3.4	61.1	149	220
CDC Kendall	95.2	41.6	80.2	2.8	62.3	150	115
CDC Copeland	97.4	40.0	80.6	3.0	55.6	129	75
CDC Select	96.1	42.7	80.0	3.1	65.1	150	120

The Future

We will vastly increase our knowledge of the barley genome through research on functional genomics. This will allow us to use conventional and r-DNA technologies to make improvements that we have only dreamed about. Products of r-DNA technology will come to be fully accepted in the marketplace. We will be able to develop selection tools which will enable us to select or create genotypes with more durable resistance to insects and diseases and much more resistant to abiotic stresses. Winter barley with cold resistance equal to rye for the northern prairies should be achievable. We will better understand the biochemistry and molecular biology of malting and brewing and thus be able to select in early generations for quality traits. We will increase conversion by focusing on more than just amylolytic enzymes. We will have a better handle on factors such as taste, foam stability, fermentability, haze formation and resistance to oxidation and thus the selection tools which are developed will permit us to create new varieties with marked improvements in these traits. Imagine what we can do with this new knowledge.

Recommendations

What advice would I give to young malting barley breeders. 1. Use all the intelligence you can gather from as many sources as you can. Brewers, maltsters, farmers, grain companies, fellow scientists, your own staff, extension agents and grain marketers, the works. 2. Sift this information and interpret it with care. 3. Develop an in-depth knowledge of the malting and brewing industries. 4. Use this information and your interpretations of it to set your breeding objectives. Keep in mind that it is 12-15 years from the time you make a cross until a variety is in full commercial production. Thus you need to use the intelligence to predict the future. (Imagine where we would be if we had listened to our domestic industry in the 60's who told us in no uncertain terms not to work on two rows. There would be no Harrington or its successors.)

5. Use these objectives to choose your parents, the most critical decision in you program. In choosing parents remember that you are building, over time, a harmonious balance of traits that is easily upset. Thus one should aim to make incremental improvements to a sound base. Creating too much variability is counterproductive. 6. Make quality truly the first priority in selection. Nothing is more useless than a variety which the brewer does not want, no matter how well it performs in the field or how resistant it ids to diseases. 7. Use the tools we now have for all they are worth and add the new ones to your arsenal as they become available. Remember however that they are just tools and do not substitute for the considered use of the human brain. 8. Get into the field often and know your "children" intimately. You cannot breed barley from behind a desk or lab bench. 9. Enjoy and network with the fantastic global community of the barley world. 10. Enjoy the ultimate fruits of your labor in its many manifestations around the world. It is as good as it gets.

Breeding Malting Barley under Stress Conditions in South America

S.E. Germán[1]

[1]INIA La Estanzuela, C.C. 39173, Colonia, C.P. 70000, Uruguay

Abstract

The annual average area sown to barley (*Hordeum vulgare*) in South America during 1999-2003 was 795,000 ha, averaging 1.81 tons of grain per ha (FAO, 2004). In Argentina, Brazil, Chile and Uruguay, two-rowed spring cultivars are used mostly for malt production. Research has been developed in private malting companies and official institutions supported by the industry (ARIAS 1995). In Argentina, tolerance to drought and heat stress during grain filling are important in drier areas. Maltería Quilmes released eight high quality cultivars in the last 20 years (SAVIO, pers.comm.). In Southern Brazil, progress in grain yield, grain size, malting quality, early maturity, resistance to net blotch (*Pyrenophora teres*), powdery mildew (*Blumeria graminis* f.sp. *hordei*), and leaf rust (*Puccinia hordei*) has been achieved by EMBRAPA and malting companies. Higher tolerance to soil acidity and resistance to spot blotch (*Cochliobolus sativus*) are required (MINELLA 2001). Since 1976 malting barley breeding in INIA-Chile improved grain yield, grain size, beer production efficiency, and resistance to scald (*Rhynchosporium secalis*), net blotch, stripe rust (*Puccinia striiformis*), and leaf rust (BERATTO 2001). Uruguay produces high quality malt mainly exported to Brazil. Malting companies have released locally bred and introduced cultivars since the early 1970´s. Initiated in 1988, INIA-Uruguay breeding program improved yield, malting quality, and lodging and disease resistance. Fusarium head blight (mainly incited by *Giberella zeae*) is a new challenge for research in Brazil and Uruguay. Information regarding malting barley production, most important stresses in different areas of production, and breeding progress under South American conditions is provided.

Keywords: *Hordeum vulgare*; plant breeding; stress conditions; Argentina; Brazil; Chile; Uruguay

Introduction

The average annual area sown to barley in South America during the last five years (1999/2003) was 795,000 ha (Table 1). The average yield during that period was 1.81 tons of grain per ha, making a total annual production of 1,384,200 mt. In Argentina, Brazil, Chile, and Uruguay (Southern Cone of South America), barley is used mostly for malt production. In Bolivia, Colombia, Ecuador and Peru most barley is used for food and feed. Other countries in South America do not have adequate conditions to develop the barley crop at a commercial level (ARIAS 1995). Therefore, the information provided will be focused in malting barley breeding in Argentina, Brazil, Chile, and Uruguay.

In the Southern cone region, an average of 505,000 ha per year were planted to barley in the period 1999-2003, with an average yield of 2.5 tons per ha and a total production of 1,100,000 mt per year (Table 1). During the last 20 years, the barley area and total production increased in average rates of 14,385 ha per year and 38,294 mt per year, respectively. In the period 1998- 2002, Argentina exported barley and malt, Chile and Uruguay imported barley and exported malt and Brazil imported barley and malt, and exported more beer than the other countries.

Table 1. Average barley area, grain yield, total grain production (1999-2003) and commerce (1998-2002) in the countries of the southern cone of South America

	Barley					Malt		Beer	
	Harvested area.	Grain yield	Total production	Imports	Exports	Imports	Exports	Imports	Exports
Country	ha	kg/ha	mt	mt	mt	mt	mt	mt	Mt
Argentina	248687	2305	571009	6134	123045	16268	230109	17595	10903
Brazil	137727	2030	275355	125409	16832	647041	774	9201	39936
Chile	18812	3843	70745	47279	298	16873	53795	14897	7766
Uruguay	99220	1936	185592	58548	7768	5	169496	2406	4691
South. Cone*	504446	2529	1102701	237370	147943	680187	454175	44100	63296

Source: FAO, 2004

* Argentina, Brazil, Chile and Uruguay: total area harvested, production, imports and exports; average grain yield.

Two-rowed spring cultivars with malting quality planted during the fall or winter are almost exclusively used in the Southern cone. Most of the crop area is sown under contract with the industry.

Barley research in the region began in the 1910´s in the public sector, and then in private malting companies and official institutions supported by the industry (ARIAS 1995). Traditional breeding methods have been used by most programs. Anther culture for the production of double haploids was introduced in EMBRAPA (Brazil) breeding program recently. Some research in molecular mapping development of markers is in progress but marker assisted selection is not being used as a routine tool in the regional programs.

Important progress has been made in the adoption of technology at the commercial crop level. An average grain yield increase rate of 36.9 kg per ha per year during the last 20 years can be explained by the improvement of crop management practices and the adoption of new higher yielding cultivars. However, the average regional yield of about 2.5 tons per ha is considerably lower than the potential yield of the crop under adequate growing conditions indicating that large areas of production in the Southern cone occur under stress conditions. Therefore, breeding for stress tolerance is one of the most relevant objectives for malting barley breeding in the region.

Argentina

Approximately 250,000 ha per year have been planted with barley in Argentina in the last five years (Table 1). Average yield of the crop was 2.3 tons per ha, resulting in an annual total production of 571,000 ton during 1999-2003. The malting capacity of the Argentinean industry is 385,000 ton of barley per year.

Most barley production in Argentina takes place in Buenos Aires Province (latitudes 34^{o} to 39 ^{o} South), where annual rainfall varies from 350 to 800 mm from South West to North East. Four main areas of production are the Southeast, West and Southwest, and North Center, accounting for about 50, 5, 20 and 25% of the total production, respectively (CATTANEO 2001).

Four programs are currently breeding malting barley: INTA at Bordenave, Malteria Quilmes at Tres Arroyos, Malteria Pampa at Coronel Suarez, and more recently, Anheuser Bush at Balcarce (CATTANEO 2004, pers. comm.). Emphasis and progress has been made in the selection of high yielding, high quality cultivars that can compete with wheat in the most productive areas. Selection for drought tolerance (characteristic of the South West area) and high temperatures during grain filling has been indirect, through selection of high and stable grain yield and grain quality. Since diseases are not an important yield limiting factor in

Argentina, due to cool temperatures and low rainfall in the most important growing areas, selection for resistance is not a relevant objective for the breeding programs. The most prevalent diseases are net blotch (*Pyrenophora teres*) and scald (*Rhynchosporium secalis*) while minor diseases are leaf rust (*Puccinia hordei*), powdery mildew *(Blumeria graminis* f.sp. *hordei*), take all (*Gaeumannomyces graminis*), and Fusarium head blight (mainly incited by *Giberella zeae*) (CARMONA & BARRETO 1995).
In the 1950´s, European cultivars Beka and Union were used in half of the growing area. These high quality cultivars, as the European germplasm in general, were not adapted to Argentinean conditions (H. SAVIO 2003, pers. comm.). Other cultivars used were locally bred, had good adaptation but their malting quality was lower than required. Maltería Quilmes started a barley breeding program in 1974, when the first crosses were made. Cultivars released by this program were Quilmes Pampa (1983), Q. Alfa (1983), Q. 27-1 (1983), Q. Centauro (1985), Q. Sur (1987), Q. Palomar (1994), Q. Painé (1997), and Q. Ayelén (1998) (A.Aguinaga, Coms.Per.). This breeding program has focused in breeding for grain quality. In the lower yielding region, INTA Bordenave has made emphasis in breeding for drought tolerance.
Cultivars bred by Malteria Quilmes were predominant since Q. Pampa was widely adopted. Cultivars sown in 2000 were Q. Alfa (44% of the growing area), Q. Palomar (25%), B1215 developed by Anheuser Bush (16%), Scarlett developed by Breun (10%) and others (5%) (CATTANEO 2001). This varietal composition has shifted to 33% of the area being planted with Scarlett, 20% with Q. Ayelén, 16% with Q. Alfa, 12% with Q.Palomar, 5% with Q. Paynee and 4% with others (CATTANEO, coms pers).
Grain yield and malting quality have been improved. Higher yielding cultivars as Scarlett and B 1215 account for part of the average yield increase of 19.8 kg per ha per year obtained at the commercial level since 1984. Plumpness (measured as percentage of grains bigger than 2.5 mm) increased from 70% in 1973-1977 to 92% in 1990-1994, malt extract increased from 77 to 80-81% in the decade 1984-1994 (TOMASO 1994). One of the most difficult challenges that needs to be addressed is the unstable protein content of the harvested grain. Grain protein may vary from 7% in highly productive areas under no water stress to 16% where yield is limited, usually due to drought.

Brazil
The average barley area harvested in Brazil in the last five years was 137,700 ha (Table 1). The average yield was 2.0 ton per ha, resulting in a total production of 275,355 mt. The barley production provides 30 to 60% of the raw material required by the industry, which has a malting capacity of 420,000 ton per year (MINELLA 2004). Malt produced represents one third of the brewing industry requirement.
The most important production area is in the Southern states of Río Grande do Sul, Santa Catarina, and Paraná (latitude 24º to 31º South) in altitudes between 500 and 1100 m, where cooler temperatures are more favourable for the crop (MINELLA 2000). Average rainfall during the growing season in 700 mm in average. A small area is being developed in central Brazil (Latitude 15º South) under irrigation, in altitude higher than 800 m. Planting dates vary from May to mid June, according to the location. Highly variable rainfall and temperatures are the major crop limiting factors (MINELLA 2000). Soil acidity associated to high Al content, and also diseases can drastically decrease grain yield and quality, leading to the widespread use of fungicides.
Malting barley breeding started in 1920 (ARIAS 1995) based on the selection of introduced barley cultivars best adapted to Brazilian conditions. Crossing begun in the 1950´s. Brahma and Antartica brewing industries began barley breeding in the 1940´s and 1950´s, respectively (Minella, 2004). In 1976 EMBRAPA (National Agricultural Research Institution) initiated a

barley breeding program in Passo Fundo, RS. An agreement with the private sector (Antartica, Brahma and Cooperativa Agraria Entre Rios Ltda.) begun to operate in 1994, coordinating private and official research efforts and speeding breeding efforts and adoption of new cultivars. AmBev, a new company resulting from the fusion of Antarctica e Brahma in 2000, is now the biggest malting company in South America with malting plants also in Uruguay, Argentina, and Venezuela (ZSCHOERPER & SPEROTTO 2001).

Breeding objectives of Brazilian programs are high and stable grain yield and malting quality, early maturity, tolerance to soil acidity (Al), decreased grain sterility, tolerance to sprouting and high temperatures during grain filling, and resistance to lodging and to prevalent diseases: net botch, powdery mildew, leaf rust, spot blotch (*Cochliobolus sativus*), Fusarium head blight and *Pyricularia grisea* (MINELLA 2004). Germplasm used are locally developed cultivars and lines as sources of adaptation and other characteristics, and introduced two row malting materials mainly from Europe, Canada, USA, and Australia.

Different crossing and selection schemes are used according to the objectives of the crosses. Modified bulk or genealogy methods are used, as well as SSD (up to four generations per year) for specific crosses (MINELLA 2004). Double haploids obtained from anther culture are used in the program since 1998. A total of 3588 DH lines were obtained from 133 crosses done in the period 1998 – 2002 (BRAMMER *et al.* 2003). Some DH lines were included in yield tests and could be potentially recommended for planting in 1995. Quality testing is done by the industry (micromalting) before release. New cultivars are tested in demonstration plots and a malting and brewing test are performed at a pilot scale.

BR2 (released by EMBRAPA in 1989) was the most widely grown cultivar from 1994 (MINELLA 2000) to 2002. MN 698 (released by Brahma in 1997) increased its relative area up to 40% in 2002, and together with BRS 195 (released by EMBRAPA in 2000) were the most widely used cultivars in 2003 (30% of the growing area each), followed by EMBRAPA 127 and EMBRAPA 128 (both released in 1997) with 15% of the growing area each. Other cultivars released in the last decade were not as widely grown.

Genetic progress was assessed by testing eighteen cultivars released from 1969 to 1996 during two years (SÓ E SILVA, 1999). The three parameters studied were improved: grain yield increased 31.7 kg per year, protein content decreased 0.057% and malt extract increased 0.065% per year. The introduction of soil acidity tolerance in cultivars FM 404 and Antarctica 01 allowed the expansion of the crop, and early maturity was essential for double cropping. Grain yield potential has increased from the 1970´s (MINELLA 2004), and is responsible of part of the commercial yield increase of 35.5 kg per ha per year in the last 20 years. Quality parameters improved significantly since the release of cultivares MN 599, MN 656, EMBRAPA 127 and MN 698. Plumpness increased from 65 to 95%, and malt extract from 79 to 82% in the new cultivars (MINELLA 2001). The first net blotch resistant cultivar, BR 2, was released in 1989 and derives its resistance from Norbert (MINELLA 2004). EMBRAPA 43, released in 1995, is resistant to net blotch and powdery mildew, and EMBRAPA 127, released in 1997, is resistant to net blotch and leaf rust. BRS 195 and BRS 224, released in 2000 and 2002 respectively, combine resistance to net blotch, powdery mildew, and leaf rust, the most prevalent leaf diseases (MINELLA 2004). Challenges for the future are improving spot blotch resistance, Fusarium head blight resistance and lodging resistance. The level of tolerance to abiotic stresses (deficit/excess rainfall and Al) should also be improved. Possible sources of higher levels of tolerance to Al are the barley cultivar Dayton and some native *Hordeum stenostachys* lines (MINELLA & SILVA 1996; SAWASATO *et al.* 2003).

Chile

The barley crop occupied an average area of 18,800 ha per year in the last five years (Table 1). Average grain yield in the same period was 3.8 ton per ha. Higher yields than the rest of

the region are mostly explained by the more favourable weather conditions for the crop in the area where barley is grown in Chile (approximate latitude 34º to 42º South), mostly in the Southern areas where most production takes place. Planting times are July to September, from the Northern to the Southern barley areas (BERATTO *et al.* 1998). Barley produced in 1999-2003 was 70745 mt, 80% of which is used for malting and sown under contract with local industries.

Most prevalent diseases limiting yield and quality are scald, net blotch, leaf and yellow (*Puccinia striiformis*) rusts, take all, and BYDV (GILCHRIST 1989). The barley growing area with trumao soils have acid soils with toxic levels of Al.

Barley breeding before 1976 was based mostly in European introductions. INIA (National Agricultural Research Institute) installed a breeding program in 1976, and signed an agreement with one of the brewing industries (Compañía Cervecerías Unidas, CCU), in 1978, which is still operative (BERATTO 2000). The Campex Semillas, located in Temuco, also works in barley breding.

The most important breeding objectives are to release high yielding and high quality winter, facultative and spring types adapted to different regions of production and resistace to diseases (mainly scald and net blotch), lodging and shattering (BERATTO 1999).

Cultivars grown before the beginning of the agreement with CCU were of European origin, well adapted to Chilean conditions (cool temperatures and long days during grain filling). Breuns Wisa and Firlsbeck Union were used in 1978. Carina replaced Breuns Wisa in 1980. In 1983 Granifen INIA-CCU (first cultivar developed by INIA) and Aramir were released. Libra INIAwas introduced to the commercial crop in 1989, Leo INIA (a line from the ICARDA/CIMMYT program) in 1991, and Acuario INIA in 1995. By 1999, 100% of the area was sown with Acuario INIA, the highest yielding cultivar. Other characteristics of Acuario INIA explaining its wide use are its high lodging resistance, moderately susceptibility to scald, resistance to stripe rust, and high quality attributes (large grain size, low grain protein content, and high extract) (BERATTO *et al.* 1998).

Yield increase during the last 20 years has been the highest of the region (71.5 kg per ha per year) and reflects the genetic improvement of this trait. Progress has also been made by INIA breeding program in quality characteristics. There was a significant increase in grain size during 28 years of breeding. The quantity of grain required to produce 100 l of beer decreased from 18.5 kg of barley in 1984 to16 kg in 2000 (BERATTO 2000). Sources of genetic resistance to relevant diseases, mainly scald, have been identified. The Campex Semillas Baer program has selected barley for tolerance to the toxic effect of low pH and high Al soil content since 1992, releasing the malting cultivar Aurora B. with tolerance from the feed barley Carmen B. (VON BAER Y BORIE 1999).

Uruguay

Average barley area harvested in the last five years was 100,000 ha (Table 1). Average yield in the same period (1.9 ton per ha) was negatively influenced by the extremely bad 2001 harvest when yield was 0.9 ton per ha, due to problems associated with excess rainfall. Most barley produced in the country (average of 185,500 mt in 1999-2003) is exported as malt mainly to Brazil. The industry has a malting capacity of about 250,000 tons per year.

Barley is planted in the South West region of Uruguay between latitudes 32º and 35º South. Average annual rainfall is 1000 mm and the accumulated rainfall during the crop season is approximately 450 mm. Planting time is June and July, but later planting dates are frequent in years with higher than normal winter rainfall.

Most important factors limiting the crop production are diseases, favoured by moisture excesses during the spring, and abiotic stresses such as excess rainfall, high temperatures during grain filling, and drought during part of the growing cycle mostly in late planted crops.

Cultivars selected from heterogeneous landraces were the result of early barley breeding that began in 1914. Breeding was discontinued until 1968, when the private company FNC (now MOSA) established a breeding program making the first crosses in the country. In the 1980's, malting industries OMUSA and CYMPAY (now MUSA) started their breeding programs which have now fused. The National Agricultural Research Institute (INIA) and the Faculty of Agronomy began to work significantly in breeding in 1988 and 1991, respectively. The National Barley Board was created in 1991 as an agreement among the official programs (INIA, F.Agronomy and LATU, the Technological Laboratory of Uruguay) and the malting industries. Through this agreement the private sector supports public institutions resulting in increased research and coordination. INIA and the private programs develope commercial cultivars and the F.Agronomy and INIA work in germplasm development. Micromalting, malting and brewing tests are performed by LATU and the industry.

Objectives of the breeding effort are high and stable malting quality and productivity, and resistance to most prevalent diseases (net blotch, spot blotch, leaf rust, scald, Fusariun head blight), lodging and straw breakage. A new potential problem is the spot form of net blotch, identified in 2003 for the first time in samples from commercial fields (PEREYRA & GERMÁN 2004). Photoperiod response, tolerance to water logging during tillering and to high temperatures during the reproductive phase confer adaptation to Uruguayan conditions. High harvest index, slow early development and tiller synchrony is required to improve yield potential (CASTRO & KEMANIAN 1999).

Germplasm used in the programs are locally developed materials, cultivars and lines from the region and Australia, which confers adaptation in terms of maturity and photoperiod response. Germplasm from Europe provides excellent malting quality, and resistance to lodging, leaf rust, powdery mildew and scald. Dwarf European cultivars are late maturity and have small grain size, decreasing markedly in late planting dates. Germplasm from North Dakota adapts particularly well to the Northern growing area where spot blotch and high temperatures during grain filling are more common. They are generally susceptible to leaf rust and scald.

Traditional breeding methodologies are used, advancing generations in an irrigated summer nursery or in the greenhouse. Selection for some traits is done under specific conditions. Sources of resistance to diseases, advanced lines and commercial cultivars are field tested under conditions favourable for each disease, including adequate planting dates, artificial inoculation, and spreader rows (PEREYRA 1996). Spot blotch is also assessed in the Northern region, where it is more frequent and severe. Fusarium head blight tests are also performed in the greenhouse to assess type I and type II resistance (S. PEREYRA, pers. comm). Seedling resistance to net blotch, spot blotch and leaf rust is tested in the greenhouse. Control of diseases using genetic resistance may have a short duration due to the appearance of new virulent pathotypes of the pathogens. The presence of different pathotypes of *Pyrenophora teres* (GAMBA & TEKAUZ 2002), *Cochliobolus sativus* (GAMBA & ESTRAMIL 2002) and *Puccinia hordei* in Uruguay has been demonstrated. Some lines with adult plant resistance to leaf rust have been identified and are being used as sources of potentially longer lasting resistance to the disease. Photoperiod response is determined using extreme planting dates. Early maturity cultivars with photoperiod response are best adapted to Uruguayan conditions. This characteristic confers flexibility in planting dates (especially important due to rainfall excesses during sowing time) and concentrates anthesis at the end of September to mid October, when the probability of frost damage and high temperatures during anthesis and grain filling is lower.

In the last 10 years, 25 cultivars locally developed or introduced from Argentina, Brazil, Australia, Europe, North Dakota and Europe have been used in the industries' production programs. The main reason for the renewal of cultivars has been the susceptibility to diseases, and the increase of quality requirements by the Brazilian industry. Clipper was

planted in 20 to 40% of the growing area for a long period until 1999. In 2003, Norteña Daymán and N. Carumbé, both from North Dakota, were planted in 36% of the area. Perún from the Check Republic, Q. Ayelén from Argentina, MUSA 936 developed in Brazil, and CLE 202 developed by INIA-Urguay, had 10 to 15% of the grown area each.
INIA breeding program has improved different characters in three steps since 1988. Firstly, improvement of grain yield and straw strength, to make the crop competitive with wheat in the best areas for production (E. Quebracho released). The second step was to increase the level of resistance to diseases, developing lines resistant to leaf rust and net blotch, some lines resistant to scald, few lines resistant to spot blotch. The third step was to improve malting quality. CLE 202, a line from the cross (Defra/FNCI 22) has been released recently. It is resistant to net blotch, scald and leaf rust. It is a dwarf type (as Defra), but the late maturity associated with this characteristic is balanced with incorporated photoperiod response which determines a relative short maturity and better performance than the European germplasm in late planting dates. Other lines with similar characteristics are under the final testing process.
Emphasis is being made in obtaining earlier maturity lines, better adapted to the Northern growing area. The level of resistance to spot blotch and Fusarium head blight is still lower than required.

Acknowledgements

To Euclydes Minella, Dr. Gerardo Arias (EMBRAPA, Brasil), Ing. Edmudo Beratto (INIA Chile), Ing. Mario Cattaneo (Maltería Pampa, Argentina), Ings. Silvia Pereyra, Marina Castro, Juan Díaz (INIA, Uruguay), Dr. Ariel Castro, Ing. Fernanda Gamba (F.Agronomía, Uruguay), Ing. Sergio Pieroni (MUSA), Ing. Domingo Luizzi, and others that provided information or contributed in other ways to this review.

References

ARIAS G. (1995): Mejoramiento genético y producción de cebada cervecera en América del Sur. FAO, Santiago, Chile. 157 p.

ARIAS G., MINELLA E. (1998): Avaliação de genótipos de cevada quanto à resistência a doenças. In: MINELLA E. (ed.): Reunião Anual de Pesquisa de Cevada (19). EMBRAPA Trigo, Passo Fundo, Brasil: 314-323.

BERATTO E. (2001): Contributions of breeding and crop management to increasing barley yield and grain quality in Chile. In: VIVAR H.E. and MCNAB A. (eds.): Breeding Barley in the New Millenium: Proceedings of an International Symposium. CIMMT, Mexico, D.F.: 10-17.

BERATTO E. (1999): Investigación en Mejoramiento Genético de cebada en Chile. In: BERATTO, E. (ed.): Congreso de Cebada Maltera (2). Paginas, Temuco, Chile: 63-72.

BERATTO E., et al. (1998): In: Normas técnicas de producción de cebadas con calidad maltera. INIA Carillanca, Compañía de Cervecerías Unidas S.A:18p.

BRAMMER S.P. (2003): Resultados da cultira de anteras de cevada, durante o período de 1998 a 2002, como apoio ao programa de melhoramento gnético de cevada na EMBRAPA Trigo. In: MINELLA E. (ed.): Reunião Anual de Pesquisa de Cevada (23). EMBRAPA Trigo, Passo Fundo, Brasil:61-67.

CARMONA M., BARRETO D. (1995): Enfermedades fúngicas de la cebada cervecera en la Provincia de Buenos Aires (Argentina) in 1991. Fitopatol. Bras., **20**: 509-510

CASTRO A., KEMANIAN A. (1999): Aproximación a un modelo de crecimiento del cultivo de cebada optimizado para el ambiente de producción del uruguay. In: Congreso Latinoamericano de cebada. Resúmenes (3). Mesa Nacional de Entidades de Cebada Cervecera. Colonia, Uruguay: 57.

CATTANEO M. (2001): Información cosecha 2000/2001 – Argentina. In: MINELLA E. (ed.): Reunião Anual de Pesquisa de Cevada (21). EMBRAPA Trigo, Passo Fundo, Brasil: 113-119.

FAO (2004): Faostat agriculture data (online). Rome, FAO. Available at: http://apps.fao.org/page/collections?subset=agriculture. Verified: April 13 2004.

GAMBA F., ESTRAMIL E. (2002): Variation in virulence within an Uruguayan population of *Cochliobolus sativus*. In: International Workshop on Barley Leaf Blights. ICARDA, Aleppo, Syria: 20-21.

GAMBA F., TEKAUZ A. (2002): Physiologic specialization in Uruguayan isolates of *Pyrenophora teres*. In: International Workshop on Barley Leaf Blights. ICARDA, Aleppo, Syria: 15-16.

GERMAN S. (2000): Characterization of photoperiod response of barley genotypes from diverse origin. In: LOGUE S. (ed.): International Barley Genetics Symposium (8). Department of Plant Science, Waite Campus, Adelaide University, Adelaide, Australia: 267-268.

GILCHRIST L. (1989): Enfermedades de la cebada en Chile. In. BERATTO E. (ed.): Mejoramiento y producción de cebada en Chile. INIA Serie Carillanca, Temuco, Chile, **12**: 85-90.

MINELLA E. (2004): Melhoramento de cebada. In: BOREM A. (ed.): Melhoramento de plantas cultivadas. Viçosa, MG, UFV, 2nd Ed. In press.

MINELLA E. (2001): Desafios e potencialidades do melhoramento genético de cevada no Brasil. In: MINELLA E. (ed.): Reunião Anual de Pesquisa de Cevada (21). EMBRAPA Trigo, Passo Fundo, Brasil: 31-40.

MINELLA E. (2000): Adapting barley to unfavourable environments: results from Brazil. In: LOGUE S. (ed.): International Barley Genetics Symposium (8). Department of Plant Science, Waite Campus, Adelaide University, Adelaide, Australia: 267-268.

MINELLA E., SILVA M.S. (1996): Breeding for aluminum tolerance in Brazil. In: SLINKARD A., SCOLES G., ROSSNAGEL B. (eds.): International barley genetics symposium (7). Mister Print Productions Ltd., Saskatoon, Canada: 528-529.

PEREYRA S. (1996): Identificación de fuentes de resistencia a enfermedades de cebada en Uruguay. In. BERATTO E. (ed.): Congreso de Cebada Maltera (2). Páginas, Temuco, Chile:173-174.

PEREYRA S., GERMAN, S. (2004): First report of spot-type of barley net blotch caused by *Pyrenophora teres* f.sp. *maculata* in Uruguay. Plant Disease. In press.

SAWASATO J.T. et al. (2003): Detecção e análise da tolerancia ao alumínio em novas amostras populacionais de *Hordeum stenostachys* do Rio Grande do Sul. In. In: MINELLA E. (ed.): Reunião Anual de Pesquisa de Cevada (23). EMBRAPA Trigo, Passo Fundo, Brasil: 93-100.

SÓ E SILVA M. (1999): Contribuição do melhoramento genético no rendimento de grãos e na qualidade industrial de cevada cervejeira no Brasil. In: MINELLA E. (ed.): Reunião Anual de Pesquisa de Cevada (19). EMBRAPA Trigo, Passo Fundo, Brasil: 293-298.

TOMASO J.C. (1995): Cultivo de cebada cervecera en Argentina, producción e investigación genética. In: Reunión Latinoamericana de Cebada Cervecera (1). FAO SNAG, Cochabamba, Bolivia: 69-74.

VON BAER E., BORIE G. (1999): Obtención de cebadas tolerantes a aluninio y acidez. In: Congreso Latinoamericano de cebada. Resúmenes (3). Mesa Nacional de Entidades de Cebada Cervecera, FAO, Colonia, Uruguay: 12.

ZSCHOERPER O.P., SPEROTTO A.L. (2001): In: MINELLA E. (ed.): Reunião Anual de Pesquisa de Cevada (21). EMBRAPA Trigo, Passo Fundo, Brasil: 19-22.

Barley Breeding Progress in the Czech Republic

I. Langer

SELGEN Co., Plant Breeding Station Stupice, Czech Republic

Abstract

First spring barley varieties in Bohemia and Moravia were selected from old local populations. More than 100 varieties were bred that way, among them e.g. Proskowetz Hana Pedigree, Nolč-Dreger's Bohemia, Haná II, Kneifel, Valtický and others. Later breeders began to utilize crossing and selection within segregating populations. Some 80 varieties were bred by means of this method, e.g. Korál, Krystal, Karát, Rubín, Bonus, Zenit, Akcent, Forum, Atribut, Amulet, Tolar, Heris, Malz, Respekt. Internationally renowned Czech variety Diamant is mutant from Valtický.

The Czech breeders' effort during last 40 years resulted in steady increase of yield potential (70 $kg.ha^{-1}$ per year), decrease of the grain protein content (0.02% per year), increase of hot water extract (0.06% per year) and improving of other malting quality characters, too. Breeders were also successful concerning the disease resistance genes exploitation, viz. *Mla* locus alleles, *mlo*, *Rph3*, *Rph7* and others, but the field horizontal resistance was utilized as well.

Breeding programs of winter barley had only marginal importance till 1980. Then breeding of 6-row feed varieties was re-established and several valuable varieties were released (Lunet, Kromoz, Okal, Kamil, Kromir, Luxor, Luran).

Keywords: barley varieties; Czech Republic; breeding progress

History of Barley Growing and Barley Breeding in Bohemia and Moravia

Barley growing on the territory of present Czech Republic has a long tradition. The first field crop growers, living here more than 2000 years ago, Celtic tribes, raised probably only six-row spring barley on their grounds. Two-row types of spring barley were introduced to this region presumably some 1000 years later. During hundreds years of natural as well as unintentional human selection many local barley populations evolved. Under favorable climatic and soil conditions in particular regions of the country valuable landraces of spring barley emerged. Later, when beer brewing was introduced, barley populations suitable for the beer production were preferred. Resultant local barley varieties can be characterized by the right adaptation to the Central Europe conditions, good yields and good malting quality.

From the score of local and regional populations the most important landraces are Old-Haná from famous Haná region, Old-Bohemian from lowlands of Bohemia, and Moravian from south part of Moravia. They laid the basis for later breeding work at home and abroad as well. Especially barley genotypes from Haná played an important role in the breeding of malting barley varieties in many countries of the world. We can find some Haná barley in the genealogy of almost all malting varieties worldwide.

The intentional breeding was born in Moravia and Bohemia at the end of the 19th century and the beginning of the 20th century. During this first period of breeding mainly single plant and ear selection from landraces and local varieties were used. Probably the first variety obtained by this way was the Proskowetz Hana Pedigree barley (1884), widely used later in many breeding programs. Individual selection from local varieties was more or less practiced

till the mid of the 20th century. More than 100 varieties of such origin were released during this era. Let us name at least some of more important of them: Nolč-Dreger's Bohemia, Haná II, Kneifel, Valtický and many others.

After the Mendel's laws rediscovery in 1900, the crossing followed by selection in segregating populations drew more and more attention of barley breeders. The first known variety, bred by this method, was Tschermak's Hana x Hannchen barley (1910), followed by almost 80 varieties bred at the breeding companies on the territory of the present Czech Republic.

Progress Achieved by the Czech Breeders' Effort during Last Forty Years

Barley breeding together with improved agricultural practice caused considerable increase of yield and quality of barley grain. Progress in yield potential, malting quality as well as disease resistance during period since 1965 till now is presented bellow. Following graphs are based on data from annual reports of the Central Institute for Supervising and Testing in Agriculture (CISTA), Brno and annual reports of the Research Institute of Brewing and Malting (RIBM), Malting Institute (MI), Brno. Yield potential is expressed as the average yield of Czech registered varieties and candidate varieties tested in the official trials by CISTA under optimum conditions. Malting quality parameters were evaluated by RIBM, MI, Brno.

Yield Potential

Because of change in the methodology of the official trials the data were divided into two parts: years 1995 – 1993 and years 1994 – 2003. Figures 1 and 2 illustrate the gradual and steady increase of the yield potential at rate around 70 $kg.ha^{-1}$ per year.

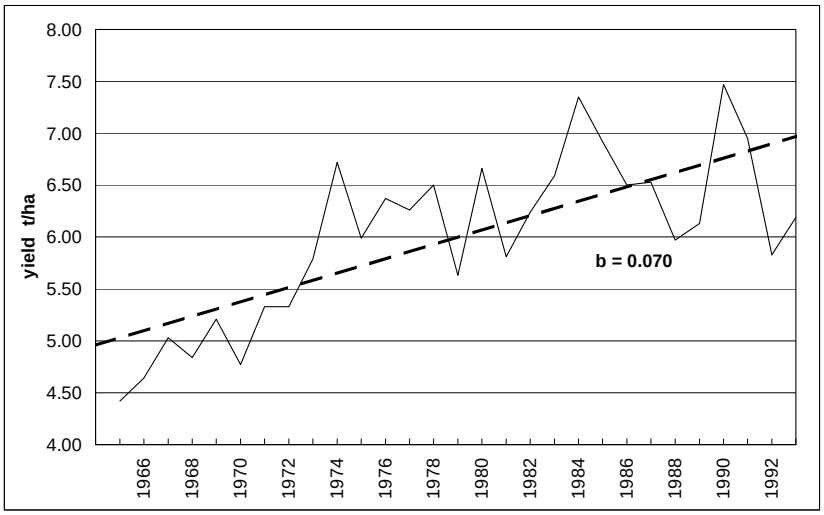


Figure 1. Yield potential 1965 – 1993

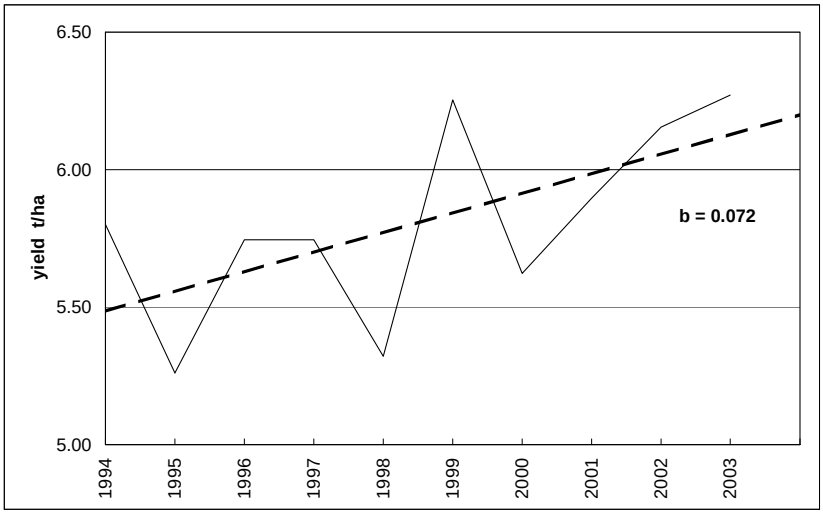


Figure 2. Yield potential 1994 – 2003

Barley Protein Content

Despite some annual fluctuation we can observe tendency of steady decrease of the protein content by 0.02% per year (Fig. 3). At present, most of varieties have achieved the optimum level of the protein at 10.5 – 11.2%.

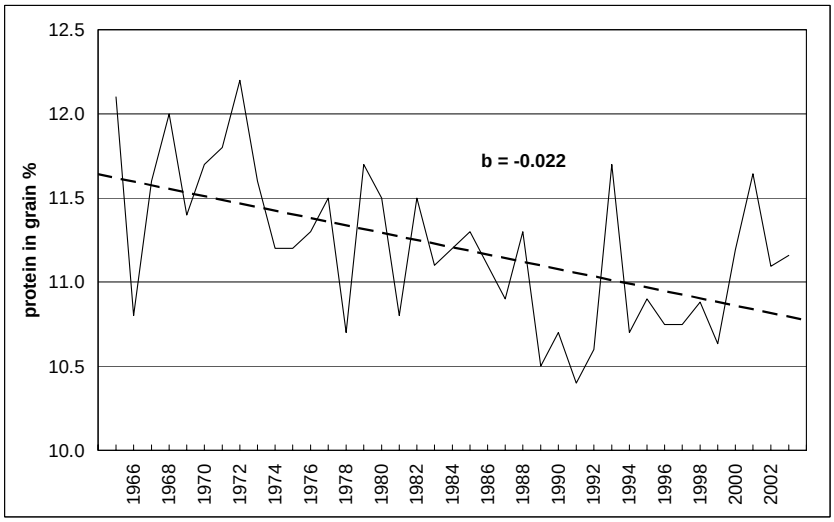


Figure 3. Protein content in barley grain

Hot Water Extract

This is the most important factor of malting quality. Czech breeders have managed to increase mean level of this character from 80% in 1965 to more than 82% in 2003 at rate 0.06% per year (Fig. 4).

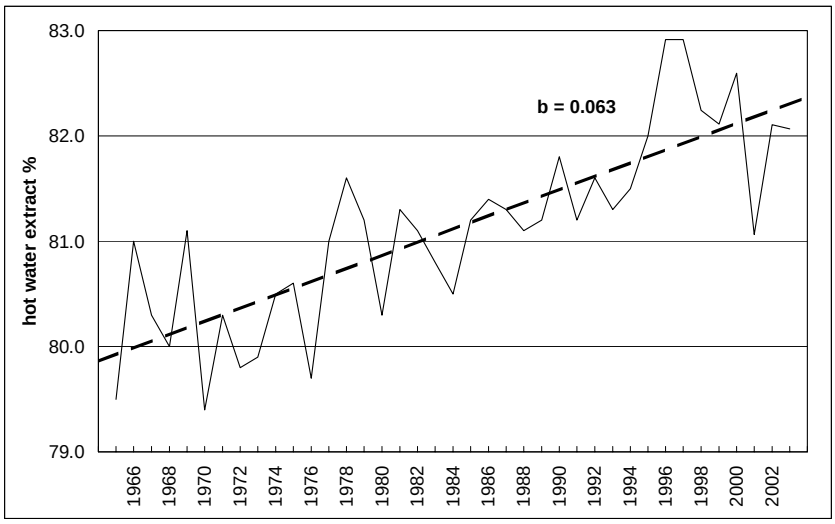


Figure 4. Hot water extract

Other Characters of Malting Quality

Relative extract at 45°C. Also this parameter has been steadily increasing from 37% to 41% at annual rate 0.1%. Because of demand of malts for production of the Czech type premium lager beer Czech breeders are selecting also genotypes with lower relative extract now.

Kolbach index. This character has been remarkably increasing during last 40 years – from 38 to almost 47 at annual rate 0.2 units per year, coming near the optimum level for brewers.

Diastatic power. After decrease during period 1965 – 1990 a steep rise occurred and the present level of this character lays between 360 and 380 Windisch-Kolbach units.

Final attenuation. Also final attenuation has been steadily improving (with some annual fluctuation) during last 40 years from 78% to the present day 82% at rate 0.1% per year.

Friability. This character was first time included in the barley quality evaluation reports in 1992 and during following twelve years its mean value considerably varied, nevertheless there can be noticed tendency to some increase at rate 0.05% per year. Mean values of this character vary around 82%.

Beta-glucans. Also beta-glucans were added to the evaluated characters in 1992 and also their content varied pretty much (between 120 and 290 $mg.l^{-1}$), but decreasing tendency at rate 0.8 $mg.l^{-1}$ per year can be presumed.

Disease Resistance

Concerning disease resistance the main attention was paid to the powdery mildew resistance. Since 1972 most of new varieties carried some allele of the *Mla* locus (*Mla6, Mla7, Mla9, Mla13, Mla12, Mla1, Mla3*), often combined with *Mlk* or *Mlg*. First variety with *mlo*

resistance was Olbram, followed by Atribut and others (Table 1). Together some 75 varieties having major gene for powdery mildew resistance were released (Table 2).

Table 1. Chronological review of the powdery mildew resistance genes in the Czech-bred spring barley varieties. First varieties with given gene or gene combinations are listed.

Year of Registration	**Variety Name**	**Resistances**
before 1972	Many	none or *Mlg*
1972	Ametyst	*Mla6*
1976	Atlas	*Mla7 Mlk1*
1977	Spartan	*Mla9 Mlk1*
1978	Korál	*Mla13*
1981	Karát	*Mla13 Rph3*
1981	Zefír	*Mla12 Mlg*
1982	Rubín	*Mla1*
1983	Mars	*Mla3 Mlg*
1984	Kredit	*Ml(Kr)*
1993	Forum	*Mlo*
1996	Atribut	*mlo Mla6* BYDV toler.
1996	Olbram	*mlo Mlk1*
1998	Heris	*mlo Rph7*
1999	Maridol	*MlaN81 MlLa*
2003	Respekt	*mlo, Sc*

Table 2. Number of the Czech-bred spring barley varieties with particular powdery mildew resistance genes (* often combined with other genes).

Gene	**No. of var.**
None	5
Mla1	2
Mla3	1
Mla6	8
Mla7	4
Mla9	2
Mla12	2
Mla13	13
Mlg *	12
MlLa *	13
Mlat *	5
Mlk1 *	5
MlaN81	1
Ml(Kr)	3
Mlo	4

With relation to other diseases, brown rust resistance genes *Rph3* and *Rph7* as well as field resistance against all diseases were exploited by the Czech breeders.

Significant Czech Spring Barley Varieties

Let us mention at least some of more important varieties registered since the 60's of the last century.

Without any doubt the most internationally renowned Czech barley variety is Diamant, mutant obtained after X-ray irradiation from local variety Valtický and released in 1965. Diamant was cultivar with high yield potential (its yield was more than 2 $t.ha^{-1}$ above then barley mean yield), altered plant development pattern, short straw and very good malting quality. This variety was widely used in breeding programs at home as well as abroad. A lot of notable varieties have Diamant in their pedigree, among them e.g. Trumpf, Alexis and many others.

Variety Korál, registered in 1978, represents the first variety with powdery mildew resistance gene *Mla13*, followed in 1981 by Krystal and Karát with the same resistance gene. Besides it, Karát was the first variety with combination of *Mla13* powdery mildew resistance gene and *Rph3* brown rust resistance gene. Very important role in the domestic barley grain production played Krystal (1981-1999).

Another significant variety was Rubín (1982-1999) distinguished by excellent malting quality. For many years it was the leading malting barley in the Czech Republic, used for production of top quality malt for domestic use as well as for export.

Also Bonus (1984-1994) was grown in great extend during the 80's and the 90's. It had good malting quality, high yields and powdery mildew resistance *Mla13*.

Zenit (1985-1991) was the first feed barley variety registered in then Czechoslovakia with combination of *Mla13* and *Rph3* genes.

Variety Rubín was replaced by new cultivar Akcent in 1992. Akcent is variety with good malting quality and excellent lodging resistance. During the 90's it was the leading variety on the Czech market, preferred by most of malthouses besides other features because of its short duration of dormancy.

Forum was the first variety with *mlo* powdery mildew resistance gene, registered in 1993, and followed by Atribut (1996), which combined *mlo* resistance and good tolerance to BYDV.

There are two special varieties, suitable for production of the Czech-type premium lager beer, viz. Amulet (1995) and Tolar (1997). Amulet's distinction is excellent sieving (even if dry conditions during the grain filling period occur), top lodging resistance, and good tolerance to the low soil pH. Tolar is high yielding variety with good field powdery mildew resistance despite no major gene was detected in its genome.

Variety Heris was released in 1998. It is feed barley with unique combination of *mlo* powdery mildew resistance gene and *Rph7* brown rust resistance gene.

Recently registered varieties Malz (2002) and Respekt (2003) as well as new varieties-candidates tested in the official trials still have to prove their potential under constantly increasing competitive pressure from abroad.

Winter Barley Breeding and Varieties

Concerning winter barley breeding, its history is quite different. For long time winter barley growing was not too important in Bohemia and Moravia, breeding programs were rather limited and only few varieties were released, most of them being six-row forms. In 1974 the winter barley breeding was even entirely closed up and no breeding activity occurred here till 1980. After re-establishing of the winter barley breeding several successful six-row

feed varieties were registered. They distinguish by high yield and good winterhardiness, which is important under semi-continental climate of the Czech Republic. The most important varieties are Lunet (1990), Kromoz (1992), Okal (1992), Kamil (1993), Kromir (1995), Luxor (1996), and Luran (1998).

References

Annual reports of the Central Institute for Supervising and Testing in Agriculture, Brno (1965-2002).

Annual reports of the Research Institute of Brewing and Malting, Malting Institute, Brno (1965-2002).

BOUMA J. (1966): New variety of spring barley "Diamant" in Czechoslovakia. In: Induzierte Mutationen und ihre Nutzung. Erwin Baur Gedächnisvorlesungen IV., 1966. Abh. Dt. Akad. Wiss. Berlin, Akademie-Verlag.

BRÜCKNER F. (1982): Vyšlechtění jarního ječmene odrůdy Karát. Genetika a šlechtění, **18**: 265-270.

BRÜCKNER F. (1993): Vyšlechtění odrůdy sladovnického ječmene nového morfotypu Forum. Genetika a šlechtění, **29**: 199-203.

DREISEITL A., JORGENSEN J.H. (2000): Powdery mildew resistance in Czech and Slovak barley cultivars. Plant Breeding, **119**: 203-209.

DREISEITL A. (2003): Adaptation of Blumeria graminis f. sp. hordei to barley resistance genes in the Czech Republic in 1971-2000. Plant Soil Environ., **49**: 241-248.

DREISEITL A., SVAČINA P. (2001): Registration of Heris barley. Crop Sci., **41**: 1992-1993.

LANGER I., LANGROVÁ M. (1997): Spring Barley Atribut. Genetika a šlechtění, **33**: 77-78.

LEKEŠ J. (1994): E. Proskowetz světovému ječmenářství. Úroda, **10**: 30

LEKEŠ J. (1997): Šlechtění obilovin na území Československa. Brázda Praha, 279 pp.

MAŘÍK P. (1997): Winter barley Luxor. Genetika a šlechtění, **33**: 75-76.

MAŘÍK P. (1999): Winter barley Luran. Czech J. Genet. Plant Breed., **35**: 62-64.

SVAČINA P. (1998): Spring barley Tolar. Czech J. Genet. Plant Breed., **34**: 77-78.

SVAČINA P. (1998): Spring barley Heris. Czech J. Genet. Plant Breed., **34**: 78-79.

SVAČINA P. (2002): Spring barley Malz. Czech J. Genet. Plant Breed., **38**: 139-140.

ŠPUNAROVÁ M. (2003): Spring barley Respekt. Czech J. Genet. Plant Breed., **39**: 27-28.

Organizing Committee

Agricultural Research Institute Kromeriz, Ltd.
Jaroslav Spunar
Katerina Vaculova
Ludvik Tvaruzek
Antonin Dreiseitl
Jarmila Milotova
Mendel University of Agriculture and Forestry Brno
Oldrich Chloupek
Jaroslava Ehrenbergerova
Research Institute of Crop Production Prague
Jaroslava Ovesna
Vaclav Sip
Research Institute of Brewing and Malting, PLC Prague
Karel Kosar
Vratislav Psota
Institute of Experimental Botany Olomouc
Jaroslav Dolezel
Ludmila Ohnoutkova
SELGEN,Co.,Prague – Stupice
Ivan Langer
Czech Breeder and Seed Association Prague
Ludek Krehlik
Sladovny Soufflet CR,Member of Groupe Soufflet
Jean Francois Fevre
Marek Schmiedt

.

International Committee

Professor Bryan Harvey – Canada
Dr. Jaroslav Spunar – Czech Republic
Dr. Sylvia German - Uruguay
Dr. Victor Shevtsov – Russia
Professor Jerome Franckowiak – USA
Professor Wayne Powell – United Kingdom
Professor Roland von Bothmer – Sweden
Professor Wolfgang Friedt – Germany
Dr. Liu Xui – People´s Republic of China
Professor Kazuyoshi Takeda - Japan

Authors of CD ROM

Antonin Pospisil, Antonin Soucek, Jarmila Janikova

Agricultural Research Institute Kromeriz, Ltd.

http://www.vukrom.cz
Phone: +420 573 317 193
Fax: +420 573 339 725
Email: ibgs@vukrom.cz