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Progress Report
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Application of doubled haploid and tissue culture techniques to winter barley germplasm enhancement

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Eskisehir, Turkey

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Executive Summary:

Barley plays a pivotal role in subsistence agroecosystems of high elevation and cold stress regions of the developing world. In sub-optimal environments characterized by low fertility, winter rainfall, low temperature, and a short growing season, winter and/or facultative barley has a comparative advantage over other cereal crops. ICARDA estimates that over 6 million ha in West Asia and North Africa are, or could be, producers of winter-sown barley. The crop also has tremendous potential in Eastern Europe and the Andean region of South America. However, greater winterhardiness is needed to realize the full potential of barley in these agroecosystems.

Higher incomes as a consequence of economic development lead to greater demands for livestock and poultry products. The LDC small farmer stands to gain from the increased demand for feed grain, if we can capitalize on the crop's innate capacity for stress resistance, particularly cold tolerance.

Despite considerable progress made in understanding the physiological mechanisms of cold damage and tolerance, there has been little progress over the past 50 years in actually improving the cold tolerance of barley. Some authors have argued that genetic variation for cold tolerance is exhausted.

We argue that genetic variability is not exhausted and that substantial improvements can be made in selection for cold tolerance through a creative synthesis of classical and contemporary plant breeding and genetics techniques. Specifically, we propose a two-pronged strategy for the genetic analysis and improvement of barley cold tolerance. We will use doubled haploid technology to generate a rapid-cycling recurrent selection population that will be screened in both field and laboratory tests. We will also use molecular markers to map cold tolerance genes in a doubled haploid population and relate the expression of cold tolerance to such physiological parameters as plant growth, vernalization, photoperiod response, and cell wall constituents.

Section I

A.) Research Objectives:

Our goal is to use classical plant breeding, tissue culture strategies, and molecular approaches to 1) understand the genetics of cold tolerance and 2) use this information to develop germplasm that will be of use in the developing world.

Our objectives are to:

1) develop a doubled haploid recurrent selection population based on the intermating of geographically and genetically diverse sources of germplasm and evaluate the recurrent selection population in field and controlled environment tests.

2) estimate classical quantitative genetic parameters (variances and heritability) in the recurrent selection population and initiate recurrent selection for cold tolerance.

3) develop a medium resolution RFLP map in a doubled haploid population with the objective of mapping cold tolerance genes.

4) reconcile classical quantitative parameters with a molecular marker analysis of cold tolerance.

B.) Research Accomplishments:

The doubled haploid population has performed well in field trials. The Oregon trial will be harvested the first to second week of July. The trial in Turkey will be harvested at the same time. Some differential winterkill was observed at both locations, allowing selection for cold tolerance based on field performance.

We are selectively genotyping the doubled haploid progeny of the cross of Dicktoo X Morex and by July 18 we will have 30 RFLp markers mapped. We will also have the first evidence on the genetic basis of fructans and their relationship to cold tolerance.

We have initiated DNA extractions on the 10 parents of the CPD and we will screen them with polymorphic probes identified by the North American Barley Genome Mapping Project.

Dr. Altay and I will present a joint paper on this project at the Sixth International Barley Genetics Symposium. Helsingborg, Sweden, July 20 - 27. The abstract is attached in Appendix 1.

I am presenting a paper on the cold tolerance gene mapping work. A copy of the abstract is attached in Appendix 2.

Our survey of cold tolerance in winter and facultative barley germplasm has been accepted for publication in Crop Science. A copy of the manuscript is enclosed.

Our gametophytic selection for cold tolerance paper has been accepted for publication in Genome. A copy of the manuscript is enclosed.

C.) Scientific Impact of Collaboration:

Dr. Altay has identified some outstanding germplasm in his trial and plans for extended evaluation in the 1991/1992 season (see Appendix 3).

As indicated above, Dr. Altay and I will be jointly presenting a paper at the IBGS.

This project has stimulated interest and infrastructure for applied biotechnology. An FAO-sponsored M.S. student from the Eskisehir Institute will join my project in fall, 1991.

D.) Description of Project Impact:

Dr. Altay has returned to his Institute and is directing the development of a new and improved research infrastructure. As a direct result of his period as a visiting scientist at OSU, Dr. Altay can personally oversee the transfer of technology to Turkey. Turkey, then, can assume regional leadership in technology application and transfer. Germplasm has been distributed to Dr. Altay and other interested cooperators.

E.) Strengthening of Developing Country Institutions:

Equipment for doubled haploid production has been received by Dr. Altay. Tissue culture reagents will be shipped this week.

F.) Future Work:

The project is on schedule. The C0 doubled haploid recurrent selection population is under field evaluation in Oregon and Turkey. Samples of this germplasm have also been distributed to the USSR, Hungary, and ICARDA. Preliminary analyses of quantitative genetic parameters will begin after the 1991 harvest.

The gene mapping dimension, while primarily supported by other sources of funding, promises to considerably expand the application of this project. Indeed, we have a rare opportunity to reconcile classical quantitative genetics and molecular biology - all with the objective of more rapidly developing cold tolerant barley germplasm. A population of 100 DH lines has been developed and increased. A partial map of this material is under construction and will be supplied in the next report.

Section II

A.) Managerial Issues:

All is well.

B.) Budget:

No changes.

C.) Special Concerns:

No changes.

D.) Collaboration, Travel, Training, and Publication:

Hayes will travel to Sweden to present a joint paper reporting on progress in the project at the IBGS. Travel and per diem will be not be paid from this grant. 1992.

Publications Relevant to This Project:

Blake, T. Lybeck, N., and P. Hayes. 1991. Good, bad, and untested ideas in RFLP and QTL analysis. Plant Breeding Abstracts 61:1-7.

Chen, F., and P.M. Hayes. In press. Effect of exogenous plant growth regulators on *in vitro* seed set, embryo development, and haploid production in a *H. vulgare* X *H. bulbosum* cross. Plant Cell, Tissue, and Organ Culture.

Hayes, P.M., and T. Blake. 1991. Genetic analysis of cold tolerance in winter barley. 1991. Sixth International Barley Genetics Symposium. Helsingborg, Sweden, 22 - 27 July, 1991. In press.

Hayes, P.M. 1991. Production of recombinant inbred lines: the biology and the technology. In Biotic Stress of Barley in Arid and Semi-arid Environments. 31 July - 2 August, 1990. Big Sky, Montana. MSU/ICARDA

Kolar, S., P.M. Hayes, and T. Chen. In press. Genotypic variation for cold tolerance in barley. Crop Science.

Kolar, S., P.M. Hayes, and R. Linderman. In review. Effect of inoculation with VAM fungi on the expression of cold tolerance in winter barley. Canadian Journal of Plant Science.

Schön, C., and P.M. Hayes. In press. Gametophytic selection in a winter x spring barley cross. Genome.

E.) Request for A.I.D. or BOSTID Actions:

No specific requests. I urge you to do all you can to maintain funding for the USAID/PSTC program. This is an outstanding program for facilitating good, peer-reviewed science in the international arena.

Appendix 1

DOUBLED HAPLOIDS AS A TOOL FOR GERMPLASM ENHANCEMENT

Abstract presented at the

Sixth International Barley Genetics Symposium

Helsingborg, Sweden
July 20-27, 1991

DOUBLED HAPLOIDS AS A TOOL FOR GERMPLASM ENHANCEMENT

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International germplasm enhancement efforts have focussed on screening nurseries consisting of fixed lines, Male Sterile Facilitated Recurrent Selection (MSFRS) populations, and segregating generation populations. The improvement of many LDC plant breeding infrastructures and the need to enhance germplasm for defined target traits calls for a new approach. The use of doubled haploid (DH) breeding techniques can considerably increase the efficiency of international germplasm enhancement. Completely inbred recombinant DH lines allow for more efficient selection, providing cooperators with both parental material and lines for subsequent increase and release. DH lines can be generated via mating designs or from MSFRS populations. After several cycles of selection in diverse environments, such materials provide data for monitoring changes in allele frequency through molecular marker analysis.

We are using recurrent selection, a technique designed to increase the frequency of favorable alleles in individuals in a population, for improving winter barley germplasm resources. Recurrent selection with intermating can be more effective than selfing and subsequent selection in self-pollinated crops (Ramage, 1977; Reinhold, 1990). Kasha and Reinbergs (1981) pointed out the applications of haploidy in sampling male sterile-facilitated recurrent selection populations. Choo et al. (1979) presented a cyclic selection program in which a number of parental cultivars are crossed in a partial diallel, and doubled haploids are obtained from the resulting F1s. Hayes and Stucker (1989) presented statistical considerations in implementing doubled haploid recurrent selection based on a circulant partial diallel mating.

After cycles of intermating, effective selection for quantitatively inherited traits requires near-homozygosity in lines to be selected for the next cycle of intermating. Conventional accelerated approaches to homozygosity require relatively long cycle times and are not practical in winter barley. The use of doubled haploids reduces cycle time and offers advantages for both applied breeding and molecular marker analysis.

We have developed a winter barley doubled haploid recurrent selection population based on a circulant partial diallel (CPD) mating design (Kempthorne and Curnow, 1961). This offers a straightforward approach to using doubled haploids to 1) improve a population of inbred lines, 2) develop inbreds for evaluation as potential varieties, and 3) estimate pertinent genetic parameters. As any given set of adapted parents cannot be construed as a random sample of winter barley germplasm, a fixed effects model is most appropriate. However, should one wish to use this procedure for estimation of genetic variances, the population of doubled haploids, representing a random sample of F2 gametes (Schön et al., 1990), may be identified as a base population in which to define a set of genetic parameters.

A CPD mating design allows sampling of a large germplasm array with a reduced number of crosses via a balanced design in which each parent is involved in the same number of crosses. Crosses are allocated as described by Kempthorne and Curnow (1961). We have synthesized a C0 winter barley DHRS population derived from a CPD mating. The population is currently being evaluated at Eskisehir, Turkey and Corvallis, Oregon. The population is also being characterized for cold tolerance via controlled freeze tests.

MSFRS populations offer an alternative approach to doubled haploid recurrent selection, one that could prove useful in developing "synthetic landraces". This is essentially a modified ear-to-row system that is designed to simultaneously 1) maximize gain from selection in the MSFRS populations and 2) derive inbred lines for parental stocks, release as varieties, or components of "synthetic landraces".

In dry and marginal areas, landraces are more stable and often higher yielding than pure-line cultivars. In such environments, consistent performance is more critical than occasional outstanding performance. The advantage of landraces over pure line genotypes is attributed to a "buffering effect" (Allard and Bradshaw, 1964) that may be attributed to both heterogeneity and heterozygosity. The heterogeneity of landraces for a range of qualitative and quantitative traits has been elegantly documented (Weltzien, 1989; van Leur et al. 1989).

Successful exploitation of the genetic resources found in landrace populations and the development of synthetic landraces capable of providing consistently more stable and higher returns to producers in marginal, stress environments requires the development of a rapid and efficient breeding system for exploiting crosses of landrace genotypes inter se and with exotic germplasm sources, as well as the development of a systematic approach to development of synthetic landraces. DH lines meet these criteria, providing an ideal vehicle for germplasm introgression, enhancement, and development of multi-component mixtures.

Population buffering (Allard and Bradshaw, 1964) and heterogeneity are documented to increase and stabilize yields of small grains in landraces (Cecarelli and Grando, 1989) and multi-component mixtures (Nitzche and Hesselbach, 1983). The key in capitalizing upon the advantages conferred by mixing lies in identifying optimum mixture components. Federer (1979) and Gizlice et al. (1989) present models for identifying genotypes that perform well in various mixture combinations.

We are currently researching the synthesis of synthetic landraces. In principle, cooperators at targeted locations would select male fertile plants from the MSFRS population. Selections would be assigned identifiers and reserve seed maintained. DH lines would be extracted from each selection. DH lines would be evaluated at each targeted location. The MSFRS population would be re-synthesized from reserve parental seed of superior DH lines, while cooperators at each location would select DH lines for immediate increase and further evaluation.

This research is supported by a grant from the U.S. Agency for International Development Program in Science and Technology Cooperation.

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Appendix 2

GENETIC ANALYSIS OF COLD TOLERANCE IN WINTER BARLEY

Abstract presented at the

Sixth International Barley Genetics Symposium

Helsingborg, Sweden
July 20-27, 1991

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GENETIC ANALYSIS OF COLD TOLERANCE IN WINTER BARLEY

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INTRODUCTION

Extensive study of cold tolerance physiology and genetics in winter cereals has not led to selection responses, leading some investigators to argue that genetic variation for cold tolerance has been exhausted. We hypothesize that there is genetic variation for cold tolerance in the winter barley germplasm pool and that this variation can be identified and subsequently manipulated. Our goal is to identify and map molecular markers - RFLPs, isozymes, and storage proteins - that are linked to quantitative trait loci (QTL) conferring tolerance to cold stresses. Marker-QTL associations will be based on the extensive, replicated measurement of trait expression in 100 doubled haploid lines evaluated in field and laboratory experiments.

Considerable progress has been made in understanding mechanisms of both cold-related injury and protection in barley (Olien and Smith, 1981). The complex genetic basis of cold tolerances (Gusta and Chen, 1987) has traditionally been studied through techniques of quantitative analysis. This approach is supported by cytogenetic evidence. Monosomic analysis and substitution lines have been used in wheat to locate cold tolerance genes to a total of 15 out of 21 chromosomes (Cahalan and Law, 1979; Sutka, 1981).

Long-term field performance is the final measure of cold tolerance (Olien, 1977), but short-term field genetic studies and selection efforts are fraught with genotype x environment interaction (Eunus et al., 1962) and plagued by the infrequent occurrence of a "test winter". Controlled freeze testing to determine the temperature lethal to 50% of a test population (LT_{50}) has proven to be a reliable laboratory measure of cold tolerance (Marshall et al., 1981). Genetic studies of cold tolerance as measured by regrowth after controlled freeze tests indicate a preponderance of additive genetic variation. These analyses have, without exception, been based on segregating generation progeny.

The lack of progress in direct selection for winter hardiness in barley, and other winter cereals, may be attributed to causes other than fixation at all major loci and may include 1) problems with the genetic models and level of homozygosity in test materials, 2) inappropriate environmental reference populations, and 3) lack of effective selection criteria.

Many, if not most, important characteristics of agricultural species are inherited quantitatively. Of necessity, genetic analysis of such traits has been approached with

biometrical procedures. While progress in the improvement and understanding of quantitative traits through conventional biometrical procedures is impressive, even greater efficiencies may be realized by using Mendelian markers to map and select for quantitative traits. Whether the linkage of quantitative trait effects be with major genes, isozymes, or restriction fragment polymorphisms, the intent is to reduce the complexity of quantitative characters to the simplicity of Mendelian analysis.

RFLPs provide a ready means of relating DNA-level variation to phenotypic variation. RFLPs have been used in constructing genetic linkage maps in a number of crop species, including barley (Shin et al., 1990). Molecular-marker assisted selection requires a high level of linkage disequilibrium between marker loci and QTL. As reviewed by Stuber et al. (1987), the theory behind the technique is based on using a marker locus to identify sections of the chromosome that can be followed in inheritance studies.

PROCEDURES

Cold tolerance is being assayed in controlled environment and field evaluations of a doubled haploid population from the cross of Dicktoo X Morex. As measured by long-term field survival data, Dicktoo is one of the most cold tolerant barleys available in the US. Morex, a spring barley, is the U.S. industry six-row malting barley standard. One hundred doubled haploid lines were developed by the Hordeum bulbosum technique, using the in vitro floret culture protocol of Chen and Hayes (1989). The LT_{50} of each doubled haploid line will be determined in controlled freeze tests, following a modification of the protocol of Marshall et al. (1981).

Morphological, isozyme and storage protein markers provide the best known reference points on the current barley map. Isozymes are evaluated in horizontal starch gel systems as described by Nielsen and Johansen (1986). Storage proteins are separated in 12% SDS-polyacrylamide gels as described by Blake et al. (1982). For RFLP analysis, plant DNA is prepared from freeze-dried tissue using a modified proteinase K procedure. We have identified a number of informative clones, and we are continuing to screen cDNA and genomic libraries. DNA samples (10 μg) are digested with appropriate restriction endonucleases, separated on agarose gels and transferred to charged nylon membranes using the alkaline protocol (Reed and Mann, 1985). Cloned DNA inserts are cut out from low melting point agarose and labeled by random priming (Feinberg and Vogelstein, 1984). Filters are prehybridized, hybridized overnight, washed with a final stringency of 0.2X SSC, 1% SDS, 65C, and exposed to film as needed (3-7 days). For reuse, filters are washed in 0.1X SSC, 0.1% SDS at 95C.

Map construction using MAPMAKER (Lander et al., 1987) and QTL mapping, using the flanking marker model of Knapp et al. (1990), are in progress.

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Appendix 3

DATA ON DH GERMPLASM EVALUATED AT ESKISEHIR, TURKEY

MINISTRY OF AGRICULTURE, FORESTRY AND RURAL AFFAIRS
TRANSITIONAL REGION AGRICULTURAL RESEARCH INSTITUTE
ESKISEHIR, P.O.BOX 17, TURKEY 26001

TIK-308

TEL: 22 101741
FAX: 22 101743

01.05.1991

Dr. Patrick Hayes
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Crop and Soil Science Department
Crop Science Building
Corvallis, OR 97331-3002

Dear Pat,

Enclosed is the winter observation notes on the double haploids planted this fall. Evaluations are given as percentages of plants surviving winter. Some lines have notes stating that they have not been planted. This is because of damages occuring during transportation resulting in loss of material. If you could send us 20 gr of each of these lines, it will be possible to plant full set of trials in 5 locations next fall.

Best regards,

Fahri

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Germplasm Sent from Oregon State University - List Generated: 09/11/90 Page 1

DOUBLED HAPLOID AMBA INCREASE

CORVALLIS

Packet Number	Permanent Oregon ID	Variety Name or State Number	Pedigree	Source Nursery		Winter Survival (%)
1	2863150	PLAISANT	Ager/Nymphe	GSQP	1	Not Planted
2	1861112		IGRI/B68-1285//PULL12222/3/FB73 -194/NY6005-19	GSQP	2	Not Planted
3	1600518	WINTERMALT	TRIALL/HUDSON	GSQP	3	Not Planted
5	2863136	NOVATOR		GSQP	5	90
6	1730003	ROBUR		GSQP	6	90
7	1700173	SCIO		GSQP	7	90
8	1861009		IGRI/MOB2639,F1//F1,P13161/IGRI	GSQP	8	95 ⊕⊕
9	2863151	FLAMENCO		GSQP	9	80
10	1860012		72Ab83/WINTERMALT	GSQP	10	90
11	1907652		PLAISANT/NOVATOR	DHF-E	466	98 ⊕⊕⊕
12	1907654		PLAISANT/NOVATOR	DHF-E	468	98 ⊕⊕⊕
13	1907655		PLAISANT/NOVATOR	DHF-E	469	95 ⊕⊕
14	1907656		PLAISANT/NOVATOR	DHF-E	470	90
15	1907657		PLAISANT/NOVATOR	DHF-E	471	95 ⊕⊕
16	1907658		PLAISANT/NOVATOR	DHF-E	472	95 ⊕⊕
17	1907660		PLAISANT/NOVATOR	DHF-E	474	90
18	1907661		PLAISANT/NOVATOR	DHF-E	475	90 ⊕
19	1907662		PLAISANT/NOVATOR	DHF-E	476	90
20	1907663		PLAISANT/NOVATOR	DHF-E	477	90
21	1907664		PLAISANT/NOVATOR	DHF-E	478	98 ⊕⊕⊕
22	1907665		PLAISANT/NOVATOR	DHF-E	479	90
23	1907666		PLAISANT/NOVATOR	DHF-E	480	90
24	1907667		PLAISANT/NOVATOR	DHF-E	481	90 ⊕
25	1907668		PLAISANT/NOVATOR	DHF-E	482	90
26	1907669		PLAISANT/NOVATOR	DHF-E	483	90
27	1907674		PLAISANT/NOVATOR	DHF-E	488	90

Germplasm Sent from Oregon State University - List Generated: 09/11/90 Page 2
DOUBLED HAPLOID AMBA INCREASE
CORVALLIS

Packet Number	Permanent Oregon ID	Variety Name or State Number	Pedigree	Source Nursery	Winter Survival (%)
28	1907675		PLAISANT/NOVATOR	DHF-E 489	90
29	1907676		PLAISANT/NOVATOR	DHF-E 490	90
30	1907679		PLAISANT/NOVATOR	DHF-E 493	90
31	1907687		PLAISANT/ROBUR	DHF-E 499	90
32	1907688		PLAISANT/ROBUR	DHF-E 500	95 ⊕⊕
33	1907689		PLAISANT/ROBUR	DHF-E 501	90
34	1907690		ROBUR/PLAISANT	DHF-E 502	85
35	1907691		ROBUR/PLAISANT	DHF-E 503	90
36	1907692		ROBUR/PLAISANT	DHF-E 504	90
37	1907693		ROBUR/PLAISANT	DHF-E 505	90
38	1907694		ROBUR/PLAISANT	DHF-E 506	90
39	1907695		ROBUR/PLAISANT	DHF-E 507	90
40	1907696		1861112/ROBUR	DHF-E 508	85
41	1907698		1861112/ROBUR	DHF-E 510	85
42	1907699		1861112/ROBUR	DHF-E 511	60
43	1907701		1861112/ROBUR	DHF-E 513	85
44	1907702		1861112/ROBUR	DHF-E 514	90
45	1907703		1861112/ROBUR	DHF-E 515	85
46	1907704		1861112/ROBUR	DHF-E 516	90
47	1907705		1861112/ROBUR	DHF-E 517	85
48	1907706		1861112/ROBUR	DHF-E 518	90
49	1907707		1861112/ROBUR	DHF-E 519	Not planted
50	1907710		1861112/ROBUR	DHF-E 522	90
51	1907711		1861112/ROBUR	DHF-E 523	85
52	1907712		1861112/ROBUR	DHF-E 524	85
53	1907703		1861112/ROBUR	DHF-E 525	90

DOUBLED HAPLOID AMBA INCREASE
CORVALLIS

Packet Number	Permanent Oregon ID	Variety Name or State Number	Pedigree	Source Nursery	Winter Survival (%)
54	1907714		1861112/ROBUR	DHF-E 526	70
55	1907716		1861112/ROBUR	DHF-E 528	80
56	1907717		1861112/ROBUR	DHF-E 529	80
57	1907718		1861112/ROBUR	DHF-E 530	90 ⊕
58	1907720		1861112/ROBUR-1A	DHF-E 531	85
59	1907721		1861112/ROBUR-1B	DHF-E 532	50
60	1907722		SCIO/1861112	DHF-E 533	95 ⊕⊕
61	1907724		SCIO/1861112	DHF-E 535	90
62	1907725		SCIO/1861112	DHF-E 536	80
63	1907726		SCIO/1861112	DHF-E 537	90
64	1907727		SCIO/1861112	DHF-E 538	90
65	1907728		SCIO/1861112	DHF-E 539	90
66	1907729		SCIO/1861112	DHF-E 540	90
67	1907730		SCIO/1861112	DHF-E 541	90
68	1907732		SCIO/1861112	DHF-E 543	90
69	1907733		SCIO/1861112	DHF-E 544	85
70	1907734		SCIO/1861112	DHF-E 545	90
71	1907735		SCIO/1861112	DHF-E 546	Not Planted
72	1907736		SCIO/1861112	DHF-E 547	90 ⊕
73	1907738		SCIO/1861112	DHF-E 549	90
74	1907739		SCIO/1861112	DHF-E 550	90
75	1907742		SCIO/1861112	DHF-E 553	90
76	1907743		SCIO/1861112	DHF-E 554	85
77	1907744		SCIO/1861112	DHF-E 555	90
78	1907746		SCIO/1861112	DHF-E 557	90
79	1907747		SCIO/1861112	DHF-E 558	90

DOUBLED HAPLOID AMBA INCREASE
CORVALLIS

Packet Number	Permanent Oregon ID	Variety Name or State Number	Pedigree	Source Nursery	Winter Survival (%)
80	1907776		SCIO/WM	DHF-E 586	90
81	1907778		SCIO/WM	DHF-E 588	85
82	1907779		SCIO/WM	DHF-E 589	85
83	1907780		SCIO/WM	DHF-E 590	90
84	1907781		SCIO/WM	DHF-E 591	90
85	1907782		SCIO/WM	DHF-E 592	90
86	1908190		FLAMENCO/NOVATOR	DHF-E 953	90
87	1907784		SCIO/WM	DHF-E 594	85
88	1907788		SCIO/WM	DHF-E 598	90
89	1907790		SCIO/WM	DHF-E 600	90
90	1907791		SCIO/WM	DHF-E 601	90
91	1907792		SCIO/WM	DHF-E 602	Not Planted
92	1907793		SCIO/WM	DHF-E 603	Not Planted
93	1907795		SCIO/WM	DHF-E 605	90
94	1907796		SCIO/WM	DHF-E 606	90
95	1907797		SCIO/WM	DHF-E 607	85
96	1907798		SCIO/WM	DHF-E 608	90
97	1907799		SCIO/WM	DHF-E 609	90
98	1907801		SCIO/WM-1A	DHF-E 611	90
99	1907802		SCIO/WM-1B	DHF-E 612	95 (+)(+)
100	1861009		IGRI/MOB2639,F1//F1,P13161/IGRI	GSQP 8	95 (+) (+)
101	1907803		1861009/WM	DHF-E 613	90 (+)
102	1907804		1861009/WM	DHF-E 614	98 (+)(+)(+)
103	1907806		1861009/WM	DHF-E 616	98 (+)(+)(+)
104	1907807		1861009/WM	DHF-E 617	98 (+)(+)(+)
105	1907808		1861009/WM	DHF-E 618	98 (+)(+)(+)

DOUBLED HAPLOID AMBA INCREASE
CORVALLIS

Packet Number	Permanent Oregon ID	Variety Name or State Number	Pedigree	Source Nursery	Winter Survival (%)
106	1907812		1861009/WM	DHF-E 622	100
107	1907813		1861009/WM	DHF-E 623	90
108	1907814		1861009/WM	DHF-E 624	100
109	1907819		1861009/WM	DHF-E 629	100
110	1907821		1861009/WM	DHF-E 631	100
111	1907822		1861009/WM	DHF-E 632	90
112	1907826		1861009/WM	DHF-E 636	70
113	1907827		1861009/WM	DHF-E 637	90
114	1907830		1861009/WM	DHF-E 640	95
115	1907834		1861009/WM	DHF-E 644	90
116	1907835		1861009/WM	DHF-E 645	80
117	1907837		1861009/WM	DHF-E 647	90
118	1907838		1861009/WM	DHF-E 648	90
119	1907848		1861009/WM	DHF-E 658	60
120	1907849		1861009/WM	DHF-E 659	85
121	1907879		FLAMENCO/WM	DHF-E 686	85
122	1907898		FLAMENCO/WM	DHF-E 705	80
123	1907902		FLAMENCO/WM-2A	DHF-E 708	90
124	1907882		FLAMENCO/WM	DHF-E 689	90
125	1907883		FLAMENCO/WM	DHF-E 690	85
126	1907884		FLAMENCO/WM	DHF-E 691	90
127	1907886		FLAMENCO/WM	DHF-E 693	95
128	1907887		FLAMENCO/WM	DHF-E 694	90
129	1907888		FLAMENCO/WM	DHF-E 695	85
130	1907889		FLAMENCO/WM	DHF-E 696	90
131	1907890		FLAMENCO/WM	DHF-E 697	85

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DOUBLED HAPLOID AMBA INCREASE
CORVALLIS

Packet Number	Permanent Oregon ID	Variety Name or State Number	Pedigree	Source Nursery		Winter Survival (%)
132	1907894		FLAMENCO/WM	DHF-E	701	85
133	1907895		FLAMENCO/WM	DHF-E	702	85
134	1907896		FLAMENCO/WM	DHF-E	703	80
135	1907900		FLAMENCO/WM-1A	DHF-E	706	90
136	1907901		FLAMENCO/WM-1B	DHF-E	707	85
137	1907903		FLAMENCO/WM-2B	DHF-E	709	85
138	1907904		FLAMENCO/WM-3A	DHF-E	710	90
139	1907905		FLAMENCO/WM-3B - SAVE FOR AMBA TRIAL	DHF-E	711	80
140	1907906		FLAMENCO/WM-CALLUS - SAVE FOR AMBA TRIAL	DHF-E	712	85
141	1907910		1841007/1861009	DHF-E	716	90
142	1907911		1841007/1861009	DHF-E	717	85
143	1907912		1841007/1861009	DHF-E	718	85
144	1907914		1841007/1861009	DHF-E	720	80
145	1907915		1841007/1861009	DHF-E	721	90
146	1907916		1841007/1861009	DHF-E	722	90
147	1907917		1841007/1861009	DHF-E	723	80
148	1907918		1841007/1861009	DHF-E	724	85
149	1907919		1841007/1861009	DHF-E	725	90
150	1907920		1841007/1861009	DHF-E	726	85
151	1907921		1841007/1861009	DHF-E	727	85
152	1907923		1841007/1861009	DHF-E	729	90
153	1907924		1841007/1861009	DHF-E	730	90
154	1907926		1841007/1861009	DHF-E	732	75
155	1907930		1841007/1861009	DHF-E	736	80
156	1907931		1841007/1861009	DHF-E	737	90
157	1907934		1841007/1861009	DHF-E	740	90

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DOUBLED HAPLOID AMBA INCREASE
CORVALLIS

Packet Number	Permanent Oregon ID	Variety Name or State Number	Pedigree	Source Nursery	Winter Survival (%)
158	1907935		1841007/1861009	DHF-E 741	85
159	1907937		1841007/1861009	DHF-E 743	95 (+)(+)
160	1907938		1841007/1861009	DHF-E 744	85
161	1908036		1841007/1860012	DHF-E 841	80
162	1908038		1841007/1860012	DHF-E 843	75
163	1908040		1841007/1860012	DHF-E 845	80
164	1908041		1841007/1860012	DHF-E 846	80
165	1908043		1841007/1860012	DHF-E 848	90
166	1908044		1841007/1860012	DHF-E 849	85
167	1908045		1841007/1860012	DHF-E 850	80
168	1908046		1841007/1860012	DHF-E 851	95
169	1908047		1841007/1860012	DHF-E 852	85
170	1908048		1841007/1860012	DHF-E 853	90 (+)
171	1908049		1841007/1860012	DHF-E 854	80
172	1908050		1841007/1860012	DHF-E 855	70
173	1908051		1841007/1860012	DHF-E 856	Not Planted
174	1908053		1841007/1860012	DHF-E 858	60
175	1908054		1841007/1860012	DHF-E 859	40
176	1908055		1841007/1860012	DHF-E 860	75
177	1908056		1841007/1860012	DHF-E 861	50
178	1908057		1841007/1860012	DHF-E 862	80
179	1908058		1841007/1860012	DHF-E 863	80
180	1908060		1841007/1860012	DHF-E 865	90
181	1908091		NOVATOR/FLAMENCO	DHF-E 894	90 (+)
182	1908092		NOVATOR/FLAMENCO	DHF-E 895	30
183	1908094		NOVATOR/FLAMENCO	DHF-E 897	85

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DOUBLED HAPLOID AMBA INCREASE
CORVALLIS

Packet Number	Permanent Oregon ID	Variety Name or State Number	Pedigree	Source Nursery	Winter (%) Survival
184	1908095		NOVATOR/FLAMENCO	DHF-E 898	85
185	1908096		NOVATOR/FLAMENCO	DHF-E 899	85
186	1908188		NOVATOR/FLAMENCO	DHF-E 900	85
187	1908110		FLAMENCO/NOVATOR	DHF-E 914	95 (+) (+)
188	1908112		FLAMENCO/NOVATOR	DHF-E 916	90
189	1908115		FLAMENCO/NOVATOR	DHF-E 919	85
190	1908116		FLAMENCO/NOVATOR	DHF-E 920	Not Planted
191	1908120		FLAMENCO/NOVATOR	DHF-E 924	Not Planted
192	1908121		FLAMENCO/NOVATOR	DHF-E 925	90
193	1908122		FLAMENCO/NOVATOR	DHF-E 926	90
194	1908123		FLAMENCO/NOVATOR	DHF-E 927	75
195	1908124		FLAMENCO/NOVATOR	DHF-E 928	98 (+) (+)(+)
196	1908133		FLAMENCO/NOVATOR	DHF-E 937	85
197	1908135		FLAMENCO/NOVATOR	DHF-E 939	95 (+) (+)
198	1908141		FLAMENCO/NOVATOR	DHF-E 945	98 (+)(+)(+)
199	1908151		FLAMENCO/NOVATOR-2B	DHF-E 957	90
200	1908153		FLAMENCO/NOVATOR-3B	DHF-E 959	98 (+)(+)(+)

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Genome

Gametophytic selection in a winter x spring barley cross

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Abstract

Segregation distortion and the consequences of gametophytic selection were assessed in a winter x spring barley cross by comparing segregation of enzyme, storage protein, DNA, and morphological markers in three populations derived from the same cross: a control F_2 (F_{2C}), a doubled haploid (DH) population, and an F_2 derived from F_1 plants self pollinated at 10°C (F_{2T}). Segregation distortion was present in the F_{2T} and the DH population. Based on a comparison of the F_{2C} and the F_{2T}, gametophytic selection as a consequence of self-pollination at 10°C was operative on chromosome 7 in regions linked to Rrn2. Segregation distortion in favor of the winter parent was found in the DH population. There were significant deviations from expected segregation ratios at two loci, but only at one of the loci was the gene number significantly different from the F_{2C}. Despite segregation distortion, the DH population should be suitable for linkage analyses, as estimates of recombination based on F_2 and DH data were in close agreement.

Key words: *Hordeum vulgare* - segregation distortion - doubled haploids - gametophytic selection - cold tolerance

Introduction

Genetic and environmental factors responsible for selection at the gametophytic level can significantly affect the sporophytic generation (Rick 1966, Zamir et al. 1982).

Selection at the gametophytic level could potentially enhance efficiencies in breeding. Substantial overlap between sporophytic and gametophytic gene expression has been demonstrated for a number of species, including barley (reviewed by Mascarenhas 1989). Zamir et al. (1982) reported preferential transmission of *Lycopersicum hirsutum* alleles in backcrosses of *L. esculentum* x (*L. esculentum* x *L. hirsutum*) under low temperature and concluded that, due to gametophytic gene expression, pollen grains carrying alleles from the cold tolerant *L. hirsutum* parent were more successful in fertilization. Such a relationship of sporophytic and gametophytic gene expression could be exploited for the production of F_2 progenies with increased frequencies of desired alleles.

Zamir et al. (1981) demonstrated differential *in vitro* pollen germination and pollen tube growth under low temperature for two *Lycopersicum* species (*L. hirsutum* and *L. esculentum*). Kison (1979) reported that barley pollen is viable for only five to ten minutes after it is shed. *In vitro* studies of barley

pollen germination and pollen tube growth under cold stress were, therefore, precluded.

Preferential transmission of alleles from one parent can occur, especially in the progeny of wide crosses (Zamir and Tadmor 1986). Such shifts in gene frequency are undesirable when progeny are used for breeding or linkage analyses. Preferential transmission of alleles from one parent is also a concern when populations, such as Hordeum bulbosum-derived DH lines in barley, are obtained through tissue culture.

Based on these considerations, this study had two objectives: (i) to test the effectiveness of gametophytic selection for cold tolerance, and (ii) to determine if there was segregation distortion in F_2 and Hordeum bulbosum-derived DH progeny of a winter x spring barley cross.

Materials and Methods

Crosses were made between cultivars Morex, a six-row spring malting barley, and Dicktoo, a winter habit six-row feed cultivar that has consistently ranked among the most cold tolerant entries in repeated tests throughout the United States. Twenty F_1 plants were grown in the greenhouse at 20/16°C (day/night) with a 16 h photoperiod. Three days before anthesis of the main culm, the F_1 plants were divided into three groups. Five plants received a low temperature treatment at anthesis by placing them in a growth chamber maintained at a constant 10°C, with a 16 h photoperiod. Below 10°C pollen tube growth is in general inhibited (Richards 1986). Spikes were checked daily for protrusion of anthers and pollen shed. After an average of 14 days the first and second tiller inflorescences had gone through fertilization and plants were returned to the greenhouse. The F2 progeny of these plants are subsequently referred to as the F_{2T}. Ten plants were used for the extraction of 130 *Hordeum bulbosum*-mediated DH lines using *in vitro* floret culture as described by Chen and Hayes (1989). All DHs were vernalized for six weeks at 8°C with an 8 h photoperiod. Five plants constituted the parents of the control F_2 population (F_{2C}).

Seed was harvested from the first and second tiller of each cold-treated and control F_1 plant. Twenty six seeds were ran-

domly chosen from each plant and planted in the greenhouse at two planting dates. After three weeks plants were transferred to a vernalization chamber at 8°C with a 8 h photoperiod for six weeks. Plants were transplanted to soil after vernalization and grown to maturity in the greenhouse.

Segregation of each of ten Mendelian markers: five enzymes, three hordeins, and two morphological markers, awn roughness (R/r) and rachilla hair length (S/s), was followed in 130 F_2 control plants (F_{2C}), 130 F_2 plants derived from cold treated F_1s (F_{2T}), and 130 DH lines. In both F2 populations, but not in the DH lines, segregation also was monitored for one polymerase chain reaction (PCR) marker and for alleles at the Rrn2 locus. The chromosomal locations of all markers used in this study are shown in Table 1.

Aconitate hydratase 1 (Aco1), esterases 1 and 4 (Est1, Est4), glucosephosphate isomerase 1 (Gpi1), and phosphogluconate dehydrogenase 2 (Pgd2) were evaluated in horizontal starch gel systems as described by Nielsen and Johansen (1986). Hordeins B,C, and D (Hor2, Hor1, Hor3) were separated in 12% SDS-polyacrylamide gels as described by Blake et al. (1982). When plants were four weeks old, DNA was extracted from 1 g of fresh leaf tissue (Dellaporta et al. 1983) for RFLP and PCR analysis. 15 μg aliquots were digested with Bam H1, electrophoresed in

0.8% agarose gels and transferred to Zeta-probe nylon membranes (Reed and Mann 1985). Filters were hybridized according to Sambrook et al. (1989) with 5x Denhardt's reagent, 6x SSC, 0.5% SDS and 100 μg/ml herring sperm DNA. A genomic DNA clone, pTA71, containing the entire wheat rDNA repeat unit (Gerlach and Bedrook 1979), was labeled using primer extension (Feinberg and Vogelstein 1984). Primers for the PCR reaction were generated based on the sequence of clone pMSU21 (Shin 1988). The amplification reaction consisted of 30 cycles with a cycling protocol of 1 minute at 94°C, 2 minutes at 37°C and 4 minutes at 72°C. Total reaction volumes were 30 μl. Reaction products were electrophoresed in 1.4% agarose gels. Awn roughness and rachilla hair length were evaluated under a stereomicroscope. Phenotypes were classified into smooth or rough and short or long, respectively.

All markers were assumed to follow monohybrid patterns. Goodness-of-fit to hypothesized ratios was tested in all three populations with the log likelihood ratio test (G-test). In the F_2 populations, fit to a 3:1 ratio was tested for markers with dominant inheritance. For codominant markers, a fit to expected gene ratios (1:1) was tested in the F_2 populations to allow for a comparison between the F_{2c} and the DH population. Heterogeneity was tested for single loci between populations and for

pooled data between populations. When testing pooled segregation within populations, markers with dominant inheritance were tested separately from codominant markers. The Rrn2 locus was excluded from the pooled tests within populations, because it was the only marker with a homozygous recessive Dicktoo phenotype. Other dominant markers were recessive for the Morex phenotype.

The multipoint linkage map for chromosome 5 and two-point recombination frequencies for the remaining markers were calculated using Mapmaker (Lander et al. 1987). The standard error of the recombination frequency estimates from the F_2 and the DH populations was calculated as described by Allard (1956).

Results and Discussion

Effectiveness of gametophytic selection for cold tolerance

The evaluation of cold tolerance in the sporophytic generation of barley is complicated, whether the criteria be field survival (Fowler and Carles 1979) or survival in controlled freeze tests (Kolar et al., 1991). Therefore, it would be desirable to increase the frequency of cold tolerance alleles in breeding populations through gametophytic selection. There is evidence for gametophytic expression of cold tolerance in tomato (Zamir et al. 1982). Qian et al. (1986) found differential response in wheat genotypes exposed to low temperatures during anthesis. In barley, Pedersen et al. (1987) demonstrated an overlap of gene expression in the sporophyte and the gametophyte. Clegg et al. (1978) showed that gametophytic selection was operative in barley Composite Cross V.

We tested the consequences of gametophytic selection for cold tolerance by comparing the F_{2C} and F_{2T} populations. Segregation at Rrn2 deviated significantly from the expected 3:1 ratio in the F_{2T} ($p < 0.01$), in favor of the homozygous recessive Dicktoo phenotype. The heterogeneity test showed a highly significant difference in phenotype numbers between the F_{2C} and the F_{2T} (Table 2). There was a significant reduction of homozy-

gous recessive Morex phenotypes at Est1 in the F_{2C}. At the same locus the F_{2T} segregated according to expectations. A heterogeneity test indicated a significant difference between the two F_2 populations at the Est1 locus, but this was not reflected at the tightly linked Est4 locus. The two remaining dominant markers S/s and R/r did not deviate from expectations in either F_2 population.

Goodness-of-fit to the hypothesized gene ratio (1:1) was tested at the eight codominant marker loci (Table 3). No deviations were detected in the F_{2C}. There was a significant excess of Dicktoo genes at the Aco1 locus in the F_{2T}. However, the heterogeneity test identified the excess of Dicktoo types at locus Aco1 to be not significantly different from the F_{2C} (Table 3). Goodness-of-fit tests were not significant for the remaining codominant markers.

An effect of the cold treatment was detected in a portion of the genome linked to Rrn2, which raises the question whether Rrn2 and/or linked genes are involved in the genetic control of cold tolerance in barley. In a study on the geographical distribution of different alleles at this marker locus, Saghai-Maroof et. al. (1990) found that allele 107 was predominant in Hordeum spontaneum, behaving as the wild-type allele, but that, on a worldwide basis, allele 104 was predominant in Hordeum

vulgare. After 53 generations of natural selection in an experimental population of barley, allelic frequencies at this locus had changed dramatically in favor of the 107 allele. Alleles at the Rrn2 locus were shown to be associated with environmental factors, including temperature.

The segregation data presented for Rrn2 are based on digestion with Bam H1. Subsequently, digestion with Sst I showed that Dicktoo carries the 107 allele at Rrn2 and Morex the 104 allele (Saghai Maroof, personal communication).

In wheat, a close correlation between the intensity of rRNA synthesis and frost resistance has been observed (Paldi and Devay 1977). Rrn2 is located on barley chromosome 7, which is homoeologous to the group 5 chromosomes in wheat (Islam and Shepherd 1981). The distorted segregation in favor of the Dicktoo allele observed at the Rrn2 locus may have been due to a gene homoeologous to the cold tolerance gene mapped to chromosome 5A of wheat by Sutka and Snape (1989).

To test if deviations from normal segregation favored the alleles of one parent, segregation data were pooled over all codominant markers in both F_2 populations (Table 3). The goodness-of-fit test to a hypothesized 1:1 gene ratio was not significant in the F_{2C}. Although Dicktoo alleles were significantly over-represented in the F_{2T}, no significant overall

change in gene numbers as a consequence of the cold treatment was by the heterogeneity test of pooled codominant marker data.

It remains to be seen if the segregation distortion in favor of the Dicktoo phenotype at the Rrn2 locus is correlated with an increase of sporophytic cold tolerance. We are currently constructing a medium density map based on this cross and we are evaluating the DH population for both field survival and survival in controlled freeze tests.

Segregation distortion in unselected populations

The parents used in this cross - Dicktoo and Morex - were chosen in order to maximize genetic variation for cold tolerance in the progeny and polymorphism at marker loci. This resulted in a relatively wide (winter x spring) cross, and we were concerned that alleles from one parent might be preferentially transmitted to the progeny and that estimates of recombination frequencies would be biased by aberrant segregation at more than one locus. The F_{2c} and DH populations were used to test for segregation distortion.

In the F_{2c}, Est1 showed a significant deviation from the expected 3:1 ratio (Table 2). Est1 and Est4 are tightly linked on chromosome 3 (Kahler and Allard 1970). In the F_{2c}, Est4 exhibited the same deficiency of the homozygous recessive Morex

genotype as Est1. However, the deviation from the expected gene ratio (1:1) were not significant at the Est4 locus (Table 3). Because no recombinant genotype between Est1 and Est4 was found in the F_{2c}, we assumed that the gene frequency at Est1 would not differ significantly from estimates obtained for Est4. Therefore, there was no evidence for segregation distortion in the F2C.

For all ten markers in the DH population goodness-of-fit to a 1:1 ratio was tested (Table 4). Significant deviations from expected numbers were in favor of the Dicktoo genotypes at two unlinked loci (Pgd2 and Gpi1). Genotype numbers summed over all loci in the DH population were significantly different from the expected 1:1 ratio, with 54 percent Dicktoo genotypes and 46 percent Morex genotypes.

Codominantly expressed markers were compared in the DH and the F_{2c} populations (Table 5). Single locus heterogeneity tests comparing the DH and the F_{2c} populations were significant for the Gpi1 locus. When data were pooled over the seven loci used to compare the two populations, the heterogeneity test was significant.

This preferential transmission of alleles from one parent - Dicktoo - contrasts with reports by Powell et al. (1986a,b) and Schön et al. (1990), who demonstrated, based on segregation of

Mendelian markers and distributions of quantitative traits, that Hordeum bulbosum-derived DH lines were derived from a random sample of gametes. An ongoing analysis of marker segregation at additional RFLP loci is underway and will show the extent of segregation distortion in this winter x spring cross.

Success rates in DH production are known to be genotype dependent (Hayes and Chen 1989). Alleles present in Dicktoo that confer a selective advantage during culture may account for aberrant segregation. Megaspore competition based on partial incompatibility with Hordeum bulbosum pollen must also be considered as a cause for deviations from expected ratios. Alternatively, the significant excess of Dicktoo alleles in the DH population may be attributable to differential survival rates during the colchicine treatment required to double the genome of haploid plants generated by Hordeum bulbosum-mediated chromosome elimination. Plants with more tillers, in general plants with winter growth habit, have a greater chance to survive the colchicine treatment. However, the overall doubling efficiency in our program is greater than 90% (unpublished data) and excessive mortality during the colchicine doubling phase was not observed with the Dicktoo x Morex population. In addition, neither genes for growth habit nor genes for tillering have been

mapped to barley chromosome 5, where distortion was most pronounced.

If DH lines are to be used for breeding and mapping, F_1-derived DH lines should represent a random sample of gametes. Since genotype numbers in the DH population did not meet expectations at two loci, we tested the possible consequences of these results on linkage by comparing linkage data obtained from the F_2 and DH population. For all markers, chromosome locations and estimates of recombination frequencies are available in the literature. Gpi1, Pgd2, Hor1, Hor2, and Hor3 have all been mapped to chromosome 5 and Aco1 to chromosome 6 (Brown et al. 1989). The PCR marker on chromosome 2 was mapped by Shin (1988). Est1 and Est4 are known to be tightly linked on chromosome 3 (Kahler and Allard 1970). The two morphological markers form a linkage group on chromosome 7 (Nilan 1964). Polymorphism can be detected with pTA71 at the Rrn1 and Rrn2 loci located on chromosomes 6 and 7 (Saghai-Maroof et al. 1984).

The non-significant heterogeneity tests between F_2 populations for loci included in the linkage analysis allowed data to be pooled. Therefore, for the comparison of estimated recombination fractions between DHs and F_2s, calculations were based on all 260 F_2 individuals. All estimates of recombination values were in close agreement with values previously reported

in the literature. Estimates of recombination frequencies based on DH data matched the F_2 data very well (Table 6). Thus, linkage analysis in the DH population was not significantly affected by the shift in gene frequency in favor of the Dicktoo parent.

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Table 1. Phenotype, expected F_2 phenotypic ratios, and chromosome location of twelve marker loci used to follow segregation in progeny of a Dicktoo x Morex cross.

Marker	Description	Expected ratio	Chromosome
PCR	PCR polymorphism	1:2:1	2
Est1	esterase 1	3:1	3
Est4	esterase 4	1:2:1	3
Gpi1	glucosephosphate isomerase 1	1:2:1	5
Pgd2	phosphogluconate dehydrogenase 2	1:2:1	5
Hor2	hordein B	1:2:1	5
Hor1	hordein C	1:2:1	5
Hor3	hordein D	1:2:1	5
Aco1	aconitate hydratase 1	1:2:1	6
Rrn2	RFLP (rDNA)	3:1	7
S/s	rachilla hair length	3:1	7
R/r	awn roughness	3:1	7

Table 2. Dominant markers, their single-locus and pooled phenotype numbers, G-statistics testing fit to a 3:1 ratio, and heterogeneity tests (HET) between the control F_2 (F_{2C}) and the treated F_2 (F_{2T}) populations.

Marker	F_{2C}		F_{2T}		HET
	D_:M_†	G-statistic	D_:M_†	G-statistic	G-statistic
Rrn2	13:52	0.19	23:29	9.06**	7.99**
Est1	92:19	3.99*	84:36	1.54	5.36*
S/s	77:26	0.00	65:20	0.09	0.074
R/r	72:31	1.37	62:23	0.19	0.210
Pooled+	241:76	0.179	211:79	0.762	0.849

+ Rrn2 not included

† D_ = number of Dicktoo phenotypes, M_ = number of Morex phenotypes

*,** significant at the 0.05 and 0.01 level, respectively

Table 3. Codominant markers, their single-locus and pooled gene numbers, G-statistics testing fit to a 1:1 ratio, and heterogeneity tests (HET) between the control F_2 (F_{2C}) and the treated F_2 (F_{2T}) populations.

Marker	F_{2C}		F_{2T}		HET
	M:D†	G-statistic	M:D	G-statistic	G-statistic
PCR	52: 62	0.88	65: 61	0.13	0.86
Est4	100:122	2.18	122:120	0.02	1.34
Gpi1	112:110	0.02	112:130	1.34	0.81
Pgd2	108:118	0.44	100:106	0.18	0.03
Hor2	93:103	0.51	104:120	1.14	0.04
Hor1	129:123	0.14	112:136	2.33	1.82
Hor3	94:100	0.19	98:122	2.62	0.63
Aco1	89: 83	0.21	62: 88	4.53*	3.49
Pooled	777:821	1.21	775:883	7.04**	1.15

† M = number of Morex genes, D = number of Dicktoo genes

*,** significant at the 0.05 and 0.01 level

Table 4. Single-locus and pooled genotype numbers and G-statistics testing fit to a 1:1 ratio in the doubled haploid population.

Marker	MM : DD[†]	G-statistic
Est4	35 : 53	3.71
Est1	24 : 35	2.06
Gpi1	49 : 75	5.49*
Pgd2	49 : 73	4.75*
Hor2	44 : 48	0.17
Hor1	42 : 50	0.69
Hor3	38 : 54	2.79
Aco1	52 : 48	0.16
S/s	56 : 46	0.98
R/r	60 : 45	2.15
Pooled	449 : 527	6.24*

[†]MM = number of Morex genotypes, DD = number of Dicktoo genotypes

*significant at the 0.05 level

Table 5. Goodness-of-fit tests to hypothesized gene ratios (1:1) and heterogeneity tests (HET) between the control F_2 (F_{2c}) and the doubled haploid (DH) population for seven codominant markers.

Marker	G-statistic		
	F_{2c}	DH	HET
Est4	2.18	3.71	0.72
Gpi1	0.02	5.49*	3.84*
Pgd2	0.44	4.75*	1.87
Hor2	0.51	0.17	0.04
Hor1	0.14	0.69	0.83
Hor3	0.19	2.79*	1.29
Aco1	0.21	0.16	<0.01
Pooled	0.78	11.96**	5.49*

*,** significant at the 0.05 and 0.01 level

Table 6. Comparison of recombination values (r) and associated standard errors (SE) between the F_2 and the doubled haploid (DH) populations.

Locus pair	F_2		DH	
	r	SE	r	SE
Est1 - Est4	0.01	0.01	0.02	0.02
Hor2 - Hor1	0.12	0.02	0.15	0.04
Hor1 - Gpi1	0.08	0.01	0.07	0.03
Gpi1 - Hor3	0.35	0.04	0.34	0.05
Hor3 - Pqd2	0.28	0.04	0.27	0.05
S/s - R/r	0.24	0.03	0.24	0.04

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Genotypic Variation for Cold Tolerance in Winter and Facultative Barley

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ABSTRACT

Improved winter survival of barley may be achieved by developing genotypes which maintain an acceptable level of cold tolerance despite fluctuations in air and soil temperature. The cold tolerance of eight winter and facultative barley cultivars, a spring barley and a winter wheat was measured in controlled freeze tests of field-grown material. The temperature lethal to 50% of each genotype population (LT_{50}) was calculated from survival data. LT_{50}s for each genotype were determined at multiple sampling dates at two locations over a two year period. Sampling dates were considered environments, and these data were used to identify genotypes showing stable, or "full-season", cold tolerance. Genotypes were grouped according to patterns of comparable LT_{50} response across the six environments. The significant genotype group x environment interaction detected in the analysis of groups and environments was due to changes in magnitude of response rather than changes of rank. Averaged over all environments, 'Norstar' winter wheat was significantly more cold tolerant than the barley germplasm, and there was little variation for cold tolerance among winter barley genotypes. Winter barley cultivars were more cold tolerant than facultative cultivars. Two joint regression stability parameters, the regression coefficient (b) and squared deviations from regression (s^2_d), revealed genotypic variation for full-season cold tolerance.

INTRODUCTION

In many production environments, winter cereals must be able to withstand relatively large fluctuations in ambient air temperature, soil temperature and snow cover. Winterhardiness is a complex character determined by a genotype's resistance to both biotic and abiotic stresses. Selection for winterhardiness is complicated not only by genotype x environment interaction, but also by the relative magnitude of stresses contributing to winter injury. A fundamental component of winterhardiness is cold tolerance (Olien, 1967).

Field survival may not be an optimum measure of cold tolerance because of the infrequent occurrence of "test" winters that allow for effective discrimination among genotypes (Fowler and Gusta, 1979). Of the various laboratory and controlled environment tests used to measure cold tolerance (Stushnoff et al., 1984), controlled freeze testing to determine the temperature lethal to 50% of a population (LT_{50}) has proven to be the most repeatable and efficient (McIntyre et al., 1988). This technique has been used extensively to characterize genotypic variation for cold tolerance in wheat and triticale (Fowler and Gusta, 1979; McIntyre et al., 1988) and to study the genetics of cold tolerance in wheat (Brule-Babel and Fowler, 1988; Lazar et al., 1988; Sutka, 1981).

Many environmental factors and their interactions determine plant cold tolerance, and winter cereal genotypes are known to vary in their level and duration of cold tolerance (Chen et al., 1983). A desirable genotype would be one that maintains an acceptable level of cold tolerance despite fluctuations in environmental conditions. Such a genotype would show "full-season", or stable, cold tolerance. The significant genotype x environment interaction found in

analyses of LT_{50} data based on field-grown material sampled at various points during the winter growing season has been attributed to the complex genetic basis of cold tolerance traits (McKersie and Hunt, 1987). The complexity of genotype x environment interaction typically remains unresolved, and cold tolerance data are often interpreted in terms of average performance across environments.

Olien (1964) used barley as the model system for his pioneering work in cold stress physiology, and Livingston et al. (1989) recently described genotypic variation for cold tolerance among three barley genotypes. There are no reports describing genotypic variation for cold tolerance in a diverse array of contemporary winter and facultative barley germplasm, nor has there been any attempt to characterize full-season cold tolerance in such material.

Our objectives were to (i) characterize genotypic variation for cold tolerance, as measured by average LT_{50}, in an array of winter and facultative barley germplasm grown in diverse field environments, (ii) determine the basis of genotype x environment interaction in the expression of cold tolerance in this germplasm, and (iii) characterize genotypic variation for full-season cold tolerance, as measured by the stability of cold tolerance expression in diverse field environments.

MATERIALS AND METHODS

Eight winter and two facultative barleys of diverse geographic origin, a spring barley 'Morex', and a winter wheat 'Norstar' (Table 1) were hand planted in field plots at the Hyslop and Sherman Experiment Stations, located at Corvallis and Moro, Oregon, respectively, in both the fall of 1987 and 1988. Planting dates, rates, and inputs were in accordance with recommended practices for each location (data available upon request).

Each two row, 3m plot was separated by a single 3m row of Norstar winter wheat. A three-replicate randomized complete block design was used at each location. Data presented in this report were generated from plants sampled at each of three dates at Corvallis in 1987 - 1988: 20 Dec., 23 Jan., and 24 Feb. In 1987 - 1988, drought conditions led to the loss of all plants at the Moro location. In 1988 - 1989, plant material was sampled on 28 Jan. and 17 Feb. at Corvallis and on 16 Dec. and 19 Jan. at Moro.

At each of the seven sampling dates, plants were washed and trimmed to 4 to 5 cm above, and to 0.2 to 0.3 cm below, the base of the crown. Crowns from each replicate were bundled into groups of ten, placed in stainless steel containers, covered with wet sand, and maintained at 0°C for 12 h. One container, the control, was maintained at 0°C. Ice crystals were then added to each the remaining containers to initiate freezing. After 24 h at -2°C, these containers were placed in a programmable freezer and subjected to a 3°C/h drop in temperature. The first season (1987-1988), containers with three replicates of the 12 genotypes (10 plants per genotype per replicate) were removed at seven temperatures: -3, -6, -9, -12, -15, -18, and -20°C. The second season (1988-89), containers with two replicates of the 12 genotypes (10 plants per genotype per replication) were removed at -4, -8, -12, and -

17°C. After freezing, samples were thawed for 15 h at 4°C.

Crowns were replanted into a 1:1 mixture of vermiculite and greenhouse soil mix and maintained in a greenhouse with a 16 h photoperiod and daily temperature varying between 16 and 18°C. Regrowth was scored four to five weeks after transplanting. Plants showing vigorous shoot and root growth were considered to have survived the freezing stress. Percent survival data were transformed by arcsin, and the LT_{50} for each genotype at each sampling date was computed as described by Pomeroy and Fowler (1973). In the subsequent discussion, LT_{50}, as determined from survival following the freeze test, is used synonymously with cold tolerance.

Data were analyzed using the Statistical Analysis System (SAS, 1987), using the General Linear Models option in cases of imbalance. Genotypes were grouped using a maximum likelihood estimation of normal distribution mixture models program (McLachlan and Basford, 1988). Subsequent analyses of variance were performed using group means. Two joint regression measures of stability (Eberhart and Russell, 1966) were employed to identify genotypes with full-season cold tolerance.

RESULTS AND DISCUSSION

Despite differences in soil temperature, air temperature, and snow cover across years, locations, and sampling dates (Table 2), the 100% survival of all winter and facultative genotypes in the field underscored the need for alternative procedures to evaluate cold tolerance. Despite the stresses associated with excavation, transport, and preparation of field-grown material, control plants (those maintained at 0°C) showed complete survival. We are thus confident that differential regrowth is a measure of stresses imposed during the freeze test.

Initial analyses of variance of the LT_{50} data were computed separately for the first and second year data. Genotypes were considered a fixed effect, while sampling dates and locations were considered random effects. In view of our objective to understand the basis of genotype x environment interaction, we used the following ANOVA-guided rationale to identify six distinct 'environments'.

In the 1987 - 1988 one-location analysis, genotype, sampling date, and genotype x sampling date effects were highly significant ($p = 0.01$). Therefore, each of the three sampling dates was considered representative of a unique combination of environmental effects causing differential expression of cold tolerance. These three sampling dates defined environments 1, 2, and 3. In the 1988 - 1989 two-location analysis, genotype and genotype x location effects were significant ($p=0.05$). Separate analyses of variance were then conducted for each location. At Corvallis, genotype, sampling date, and genotype x sampling date effects were again significant ($p=0.05$), leading to the resolution of an additional two environments (4 and 5). Finally, because there was no genotype x sampling date interaction at Moro, environment 6 was

derived from main effect means of the two sampling dates at this location. Subsequent analyses were based on genotype performance in these six environments.

There was an overall trend for genotypes to show maximum cold tolerance in environments 1,4, and 6, and the least cold tolerance in environments 2 and 5 (Table 2). Exceptions to this generalization led to significant genotype x environment interaction. McKersie and Hunt (1987) attributed significant genotype x environment interaction in the expression of three distinct cold tolerances in winter wheat to complex genetic mechanisms. While plausible, this hypothesis is of little assistance in designing cold tolerance selection criteria.

With the objective of identifying patterns of comparable LT_{50} response so that genotype x environment interaction could be studied on the basis of group responses to environments rather than the responses of individual genotypes, we used the mixture model analysis procedure of McLachlan and Basford (1988). Based on log likelihood ratio testing (McLachlan and Basford, 1988), six groups were identified (Table 1).

In the subsequent analysis of variance of groups and environments, the group, environment, and group x environment effects were significant sources of variation. Crossover interactions were not significant, according to the test of Azillini and Cox (1984). Therefore, the interaction of groups and environments was due to changes in magnitude of response rather than changes in rank. Primary sources of interaction were 1) the poor performance of group 2 genotypes relative to groups 3 to 6 in environment 3, and 2) the high level of cold tolerance of Norstar wheat in environment 6 (Table 2). This grouping approach may be a useful in breeding for cold tolerance. First, a group of genotypes is identified that has acceptable cold tolerance over an array of

environments. Subsequently, genotypes within the target group can be selected that meet other selection criteria.

Partitioning of the highly significant group sums of squares into mutually orthogonal contrasts revealed that, in terms of mean LT_{50}, the winter and facultative barley genotypes were less cold tolerant than Norstar winter wheat ($p = 0.05$). There is modest variation for cold tolerance in this diverse array of winter barley germplasm (groups 3, 4, and 5). The facultative genotypes (group 2) and the spring barley Morex were less cold tolerant than the true winter types. The exception to the last generalization was 'Robur', a winter barley, which was grouped with the facultative habit cultivars in group 2.

In view of our objective of characterizing full-season cold tolerance, we employed stability parameters in an attempt to identify genotypes that achieved and maintained a level of cold tolerance in a diverse set of environments. Stability analyses were based on genotype, not group, means. We found that weather data (Table 2) was of little assistance in explaining genotypic variation and genotype x environment interaction for cold tolerance. We therefore relied on the average LT_{50} of all genotypes in an environment as the index of that environment.

Becker and Leon (1988) have made a useful distinction between static and dynamic concepts of stability: cold tolerance is an example of a trait where an ideal genotype exhibits static stability, possessing "an unchanged performance regardless of any variation of the environmental conditions". Finlay and Wilkinson (1963) defined such genotypes as having regression coefficients (b) less than 1.0 and described them as having "above average stability". Yield is an example of a trait where an ideal genotype exhibits dynamic stability. Such genotypes follow a predictable response to environments and have "no deviation from this response to environments" (Becker and Leon, 1988).

Eberhart and Russell (1966) accordingly defined a stable genotype as having b = 1.0 and squared deviations from regression (s^2_d) of 0.

In terms of static stability, a genotype with full-season cold tolerance should have b = 0 and s^2_d = 0. The most cold tolerant barley genotypes (groups 4 and 5) came closest to meeting these criteria. The cultivars Kearney and Schuyler had the lowest slopes of the winter barleys, (0.60 and 0.65, respectively) and also had some of the lowest s^2_d values (Table 1). Kearney was released in 1952 and Schuyler in 1968 (Baum et al., 1985). Although subsequent winter barley releases exceed these cultivars in agronomic performance, Kearney and Schuyler may represent promising sources of full- season cold tolerance. 'Novator' (group 5) and 'Cyclone' (group 4) were developed at the Krasnodar Research Institute of Agriculture, Krasnodar, USSR using controlled freeze testing screening procedures and are agronomically competitive both in the Pacific Northwest US and in their area of adaptation in the Russian Republic of the USSR. Both regions are subject to irregular snow cover and fluctuating winter temperatures. Given the diverse origin and presumably diverse genetic backgrounds of the four winter barley genotypes, some progress from selection for cold tolerance among their cross progeny might be expected.

Of the group 3 winter barleys, the performance of 'Dicktoo' is of particular interest. Dicktoo, with the highest slope (1.56) of the winter barleys, and a high s^2_d compared to the groups 4 and 5 genotypes, was very unstable in its expression of cold tolerance. Dicktoo, released simultaneously with Kearney, is a cold tolerant check in the Uniform Barley Winterhardiness Nursery (UBWHN). In the 1989 UBWHN, Dicktoo had the highest survival (82.0%) of any winter barley (USDA-ARS, 1989). In our experiments, Dicktoo was grouped with 'Plaisant' and 'Scio'. Plaisant is characterized as moderately "frost resistant" in

Europe (European Brewery Convention, 1990).

Cultivars Dicktoo, Scio, and the group 3 facultative barleys Tokak and Steptoe appear to be unstable, by the static definition of stability, perhaps because they are responsive to conditions favoring cold tolerance expression. These genotypes may be sensitive to environmental fluctuations and do not achieve and maintain a consistent level of cold tolerance across a range of environmental conditions. High survival ratings in long-term barley winter-hardiness trials may be a measure of Dicktoo's expression of cold tolerance in uniformly cold environments that favor trait expression. For example, in environment 6, which had the lowest environmental index for LT_{50}, Dicktoo had the lowest LT_{50} of any barley genotype (-11.8°C).

Of the group 2 genotypes, Steptoe and Tokak are both fall and spring-planted in the Pacific Northwest US and the Anatolian Plateau of Turkey, their respective areas of adaptation, and are considered facultative genotypes. The overall LT_{50}s of these genotypes are low, but the fact that they were significantly more cold tolerant than the spring barley Morex provides further confirmation for the hypothesis that a vernalization requirement is not a prerequisite for a level of cold tolerance (Doll et al., 1989). Tokak was particularly notable for having the highest slope and the highest s^2_d of any genotype.

The inclusion of Norstar provides a baseline, allowing for comparison of our data with previous work. McKersie and Hunt (1987) reported a LT_{50} of -12.8°C for Norstar and noted that this variety was considerably less cold tolerant in their experiments in eastern Canada than in western Canada. Norstar was relatively unstable, with a slope near 1 and a s^2_d exceeded only by the two facultative barleys.

The genetic and physiological basis of full-season cold tolerance merits further study under controlled environment conditions. Improved winter

survival of barley may be achieved through the systematic intermating of genotypes with acceptable, stable levels of cold tolerance. For breeding purposes, screening of field-grown cross progeny in controlled freeze tests, followed by mixture model analysis grouping, may give a positive selection response. To this end, we have developed, and are currently characterizing, a winter barley doubled haploid recurrent selection population.

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Table 1. Genotypic differences associated with cold tolerance in a winter wheat and an array of barley germplasm evaluated in six environments.

Genotype	Origin	Growth habit	LT_{50} (°C)	LT_{50}† group	Stability parameters‡ b	s^2_d
Morex	USA	Spring	-1.3	1	0.36	1.18
Robur	France	Winter	-7.3	2	1.02	1.41
Steptoe	USA	Facultative	-6.9	2	1.17	2.50
Tokak	Turkey	Facultative	-5.5	2	1.83	2.76
(Group mean)			-6.6			
Dicktoo	USA	Winter	-9.2	3	1.56	1.25
Plaisant	France	Winter	-9.8	3	0.90	1.56
Scio	USA	Winter	-8.3	3	1.48	1.15
(Group mean)			-9.1			
Cyclone	USSR	Winter	-10.0	4	0.80	0.58
Kearney	USA	Winter	-10.1	4	0.65	0.68
(Group mean)			-10.1			
Novator	USSR	Winter	-10.5	5	0.71	0.94
Schuyler	USA	Winter	-10.3	5	0.60	0.72
(Group mean)			-10.4			
Norstar	Canada	Winter Wheat	-11.8	6	0.91	2.08

†Grouping defined in text.
‡Parameter defined in text.

Table 2. Ambient and soil temperatures at sampling and since planting at Moro and Corvallis, OR during the winter periods of 1987-1988 and 1988-1989.

Sampling date	Location	Environ-ment	Group LT_{50} (°C) 1	2	3	4	5	6
20 Dec. 87	Corvallis	1	-3.5	-10.0	-10.9	-11.2	-10.7	-13.0
23 Jan. 88	Corvallis	2	-0.8	-5.9	-7.9	-9.7	-9.1	-10.1
24 Feb. 88	Corvallis	3	-0.5	-3.4	-10.2	-9.3	-10.8	-11.2
28 Jan. 89	Corvallis	4	-2.0	-9.7	-10.7	-10.6	-11.9	-11.0
17 Feb. 89	Corvallis	5	-1.0	-4.0	-4.4	-7.9	-8.4	-9.5
16 Dec. 88	Moro	6[††]	-0.1	-6.7	-10.7	-11.7	-11.3	-16.2
19 Jan. 89	Moro	6[††]						

[†] Total number of days since planting with measurable snow on the ground.
[††]Environment six is comprised of the mean of the two sampling dates.

Table 2 - right side

Temperature (°C)								
On sampling date				Since planting				Snow†
Ambient air		Soil at 10 cm		Ambient air		Soil at 10 cm		
Max.	Max.	Max.	Min.	Max.	Min.	Max.	Min.	
1.7	-0.6	3.3	2.8	22.8	-1.1	16.1	1.7	1
6.7	1.7	5.0	4.4	22.8	-5.0	16.1	1.1	3
13.5	-1.7	8.9	3.9	22.8	-5.0	16.1	1.1	4
8.9	-1.7	6.7	4.4	23.3	-5.0	19.4	0.6	5
9.4	5.0	5.6	3.9	23.3	-13.9	19.4	0.6	5
1.7	-7.8	2.2	2.2	28.9	-8.3	22.8	2.2	0
12.8	-2.2	6.7	3.9	28.9	-10.0	22.8	0.6	5