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

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Executive Summary

The overall objectives of this project were to (i) develop winterhardy barley germplasm that would increase profitability in LDC and U.S. environments, (ii) contribute to an understanding of the genetic mechanisms controlling winterhardiness, and (iii) develop the intellectual and technical infrastructure for barley genetics research in Turkey. The focus of the project was on the doubled haploid technique and its application to barley germplasm enhancement. During the course of this project, there has been an explosive growth in barley genetics research. This project grew in line with technological developments, and we have exceed our initial objectives. We have developed germplasm that has found application in environments as diverse as Turkey, Russia, and the Pacific Northwest of the USA. In fact, four doubled haploids developed in the course of this project are now in regional evaluation as potential varieties. We have located genes controlling components of winterhardiness and have extended these efforts in a new PSTC project (12.091) to focus on molecular marker-based approaches. We have trained three Turkish scientists and provided the Eskisehir Institute with the basic equipment required for tissue culture and doubled haploid production. In summary, we have (i) developed germplasm that will lead to variety release, (ii) contributed to a fundamental understanding of barley genetics, and (iii) equipped the Turkish national program with skills and equipment. Four years ago the doubled haploid concept was greeted with skepticism in Turkey. Today, doubled haploid lines are in replicated yield trials in the Anatolian plateau and Turkish scientists are thinking how best to apply molecular marker techniques to their own breeding objectives.

Research Objectives

Winter and facultative barley in perspective

Barley plays a pivotal role in the 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.

Overall project objectives

The three overall objectives of this project were to (i) assess the effectiveness of the doubled haploid technique as applied to international germplasm enhancement, (ii) develop a fundamental understanding of the genetics of winterhardiness in order to develop more winterhardy barley germplasm, and (iii) develop the capacity for contemporary barley genetics research in Turkey.

Doubled haploid germplasm enhancement

The traditional approach to international cereal germplasm enhancement - typified by IARC programs - is to (i) centrally intermate germplasm with the idea of developing unique combinations of target traits, (ii) distribute the cross progeny via nurseries to cooperators in target environments, (iii) select progeny based on nursery performance in target environments, and (iv) repeat the cycle. In principle, this amounts to a breeding technique called recurrent selection. Unfortunately, due to the genetic structure of autogamous crops, such as barley and wheat, these cycles of recurrent selection are incredibly protracted. Released varieties are relatively homozygous genotypes, and it takes up to 6 generations of self-pollination after crossing before near-homozygosity is reached and sufficient seed is available for the replicated evaluation required to effectively measure quantitative trait phenotypes. In the case of winter barley, vernalization requirements preclude the shuttle breeding techniques used for rapid advance of spring germplasm. With doubled haploid techniques, homozygosity is achieved in one generation after crossing (Hayes and Chen, 1989), and after one cycle of increase, there is sufficient seed for multi-location, replicated evaluation. Of the various haploid techniques available, *Hordeum bulbosum* mediated chromosome elimination is the most useful for genetic studies capitalizing on sexual rather than induced variation (Kasha et al., 1990). With the *bulbosum* technique, embryo rescue leads to the direct formation of a haploid plant, bypassing the callus phase putatively responsible for the somaclonal variation characteristic of anther culture. We have shown that *H. bulbosum* - derived DH lines represent a random sample of F_2 gametes and are actually superior to F_2 lines

for linkage map construction (Schön et al., 1990), and we have developed the doubled haploid mapping populations used by the North American Barley Genome Mapping Project. **Thus, an objective was to develop a genetically broad-based array of doubled haploid germplasm and to assess this material in diverse environments. This was the first such large-scale test of the technique.**

Genetics of winterhardiness

Winterhardiness - the deceptively simple relationship between the initial stand of a winter barley crop and the number of healthy plants surviving to produce yield - is the product of highly complex interactions of factors ranging from low temperature tolerance to biotic stress resistance. Long-term field survival data is the final measure of winterhardiness, but the expense and time required limit selection response. Temporally and spatially limited field-based genetic studies and selection efforts are fraught with genotype x environment interaction and the infrequent occurrence of a test winter that allows for effective discrimination among genotypes (McIntyre et al., 1988). The alternative - breaking winterhardiness into its component parts - will obviously not do justice to the full complexity of trait expression. However, by determining the genetic basis of various components, we may be able to proceed, in a stepwise fashion, to build components of winterhardiness into successively released varieties.

Several factors contribute to the phenotype of "winterhardiness". Foremost among these are low temperature tolerance, vernalization requirement, photoperiod response, protein metabolism, carbohydrate status, and membrane lipids. The precise role and relative importance of these factors in winter survival is not clear. Single factor studies tend to attach great importance to the target character and often ignore the maxim that correlation is not causation. Furthermore, many of these factors can, in turn, be further subdivided and their genetic basis is either unknown, unclear, or complicated. Moreover, low temperature tolerance is not a constitutive character: cold hardiness is achieved only after acclimation, or hardening. We have also found that there is substantial genotype x environment interaction for low temperature tolerance, due in large part to genotypic differences in rate and degree of acclimation and de-acclimation (Kolar et al., 1991).

Thomashow (1990), in reviewing the molecular genetics of cold acclimation in higher plants, cites over 20 reports that, cumulatively, assign cold tolerance genes to every chromosome in each of the three genomes of wheat. As described in the "Methods/Results" section, we have evidence that genes controlling winter survival and allied traits form a multi-locus cluster on barley chromosome 7.

The apparent complexity of cold tolerance has led to the widespread use of biometrical approaches for studying quantitatively inherited traits. The results are inconclusive. The work of Sutka (1981), typifies the diallel analysis approach, in which genetic parameters are estimated based on the phenotypes of segregating generation progeny. As Allard (1988) has observed, such procedures are notoriously unreliable and are often based on

untenable assumptions. This may account for the fact that there has been little or no selection response for cold tolerance, although estimates of additive genetic variance are frequently high. When we proposed to approach the genetic analysis of cold tolerance via variance component estimation, we anticipated that evaluations based on doubled haploid line performance would be more robust than evaluations based on individual plants in segregating populations. From a statistical standpoint, our assumption was correct. **In view of developments in molecular marker analysis and quantitative trait locus estimation procedures, we recognized that knowing where genes were located would be infinitely more useful to plant breeding than knowing the relative importance of additive and additive x additive genetic variance in a genetic reference population. Thus, although we initially planned to base our genetic analysis of cold tolerance on variance component procedures, it became obvious that a gene mapping strategy would be more appropriate for this dimension of the project.**

For over 50 years geneticists have sought methods by which simply inherited markers could be used to select for linked qualitative and quantitative trait loci (QTL). From an applied plant breeding standpoint, the impetus for building a complete linkage map is to locate genes underlying economically important traits and to use this information for marker-assisted selection. The intent is to reduce the complexity of quantitative characters to the simplicity of Mendelian analysis. Ideally, marker genes should be distributed throughout the genome, should be individually identifiable, codominant in expression, and should not affect phenotype. Molecular markers, principally restriction fragment length polymorphisms (RFLPs), have allowed for explosive growth in genome mapping and QTL analysis. Medium density genome maps have been constructed in a number of species, including barley (Hayes et al., 1993; Kleinhofs et al., 1993).

The medium density map is used to locate QTL by identifying non-random associations of phenotypes and marker loci. We have used QTL analysis procedures to locate QTLs for winterhardiness traits (Hayes et al., 1993), agronomic traits (Hayes et al., in press) and disease resistance traits (Chen et al., in press). Once located, markers flanking QTL should be useful for maximizing transgressive segregation and selecting for or against alleles in segregating populations derived from crosses between inbred lines, minimizing linkage drag during backcrossing, and increasing selection gains per unit of time. **We thus focussed on a QTL strategy for locating winterhardiness genes, with the objective of identifying molecular markers that could be used for germplasm introgression and enhancement. This work is summarized in the Methods and Results section and described in detail in the attached "Genome" reprint by Hayes et al. (1993).**

Research Infrastructure

A cornerstone of international agricultural development has been training, both in short courses and in post-graduate study. Indeed, this effort has been so successful that in many countries, Turkey being a prime example, each institute and research station boasts a complement of US-trained M.S. and Ph.D. scientists. In most cases, these scientists are

well-trained in conventional breeding techniques and classical genetics, but they often lack the intellectual upgrading and facilities required to implement newer techniques. **Thus, a sabbatical leave for Dr. Fahri Altay, Director of the Eskisehir Institute, was directly funded by this project; Nusret Zencirci received his M.S. degree with CIMMYT funding; and Ergun Ozdemir is currently completing his M.S. degree with FAO funding. Essential equipment for the establishment of a doubled haploid production facility at the Eskisehir Institute was purchased with funds from this project.**

Methods and Results

Germplasm development

A population of 300 doubled haploid lines was developed by the *Hordeum bulbosum* technique, as described by Chen and Hayes (1989), from the circulant partial diallel mating of 10 sources of barley germplasm listed in Appendix Table 1. Parental accessions represent a broad array of 6-row and 2-row winter and facultative barley of diverse geographic origin and growth habit. Several authors have suggested using recurrent selection in doubled haploid populations. Kasha and Reinbergs (1981) discussed the applications of haploidy for sampling male sterile facilitated recurrent selection populations, and Choo et al. (1979) presented a cyclic selection program in which a number of parental selections were intermated in a complete diallel fashion and doubled haploids were produced from the resulting F1s. Our choice of the circulant partial diallel was based on the fact that it allows for sampling relatively large germplasm arrays with a minimum of crosses. The principal drawback of the mating design, a sacrifice in the estimation of SCA (Bray, 1970), is immaterial in this case, since there is no dominance variance in a doubled haploid population.

Over 700 DH lines were produced from the 15 crosses used to develop this population. Three hundred (20 per cross) were chosen at random for formal assessment. The remaining lines, together with those from the population itself, were simultaneously evaluated in the mainstream OSU breeding program. This germplasm was also sent to Krasnodar, Russia and multiple locations in Turkey for evaluation in mainstream breeding efforts. Four of these lines (ORW6, ORW7, ORW8, ORW9) have been submitted to the 1993-1994 Western Regional Winter Barley Nursery for evaluation at over 20 locations in the Western U.S. Quality and agronomic data for these lines are presented in Appendix Tables 2 and 3. These are the first winter barley lines produced in the U.S. that approach malting quality standards. Malting barley commands a significant premium over feed barley, in both the US and LDCs, so release of such varieties could significantly increase the profitability of barley production.

For more formal evaluation, the entire population of three hundred lines, plus the 10 parents, was grown in row and hill plots at Corvallis, Oregon and at three locations in Turkey (Eskisehir, Bursa, and Hamidye). Mr. Ergun Ozdemir is currently completing the

analyses of these data. Preliminary results are presented in Appendix Figures 1, 2, and 3. These figures show population frequency distributions for yield, heading date, and plant height and are typical of the additional data (not shown) for kernel weight, spike length, and kernel size. Additional data were taken on micronutrient toxicity response, which, given the doubled haploid structure of the population, may allow for a straightforward determination of the genetic basis of trait expression. Several important generalizations can be made from these frequency distributions. Despite the significant differences between environments - i.e. position of the distributions on the horizontal axes - trait expression was essentially normal in all environments, and there were significant numbers of positive transgressive segregants. That is, there were a number of lines that consistently exceed parental performance in specific environments and an additional subset of lines that exceeded parental performance across environments. The merits of broad vs. environment-specific adaptation are endlessly argued in IARC circles, with CIMMYT generally arguing for the latter and ICARDA for the former. In this population, it is apparent that germplasm of both types can be recovered, and the top performing line in the US and Turkey came from a cross of Russian and French varieties! If conventional breeding procedures had been used, we would be, as of this writing, distributing our first samples of near-homozygous seed.

The full analyses of these data is in progress. Within the context of the OSU breeding effort we are already into our third cycle of intermating selected doubled haploids. That is, the ORW lines numbers 6 - 9, which originated from this population and are candidates for variety release, have already been used as parents in two cycles of crossing and their doubled haploid progeny are going into replicated yield trials this season. Within the context of the Eskisehir program, selected lines from this population have been crossed with local accessions and Mr. Ozdemir is currently producing DH lines from these crosses. A list of pedigrees of these germplasm introgression stocks is presented in Appendix Table 7.

Genetics of cold tolerance

The results of this work are described in the attached reprint of the Genome article published by Hayes et al. (1993). The implications of this work are far reaching. We provide the first molecular marker evidence for the chromosome location of genes controlling winter hardiness traits. Since the publication of the Genome report, we have expanded our map to cover all 7 chromosomes and we have generated complete data sets on vernalization and photoperiod reaction. These analyses are in progress and will be completed in the next few months, as I have been invited to present these data in the context of QTL mapping of metabolic pathways at the Plant Genome II conference in January, 1994. This work will continue on several fronts.

Within the context of the USAID/PSTC program, we have established a new project (12.091) in cooperation with the Martonvasar Research Institute in Hungary. The focus of this project is on expanding our understanding of cold tolerance genetics into other

germplasm bases. I will also be submitting a proposal to the National Research Initiative Competitive Grants Program in Dec. 1993, which will focus on finer structure mapping of cold tolerance and growth habit effects on chromosome 7.

Development of research capacity

During his sabbatical leave at OSU, Dr. Altay availed himself of opportunities for formal classroom instruction, group discussion and participation in all phases of the OSU breeding and genetics efforts. As Director of the Eskisehir Institute, Dr. Altay is in a position to encourage the development of doubled haploid techniques and genetic analyses in the context of the Turkish national program. As described in the first section, Mr. Ergun Ozdemir is completing his M.S. thesis research at OSU. He has primary responsibility for analysis of the DH population data, but the focus of his M.S. thesis will be on statistical tools for quantitative trait locus analysis.

Impact, Relevance, and Technology Transfer

The most direct impact of this project will be on the Eskisehir Research Institute, which is intended to serve as a regional model for the application of doubled haploid techniques to barley germplasm enhancement. The basic equipment for a doubled haploid lab has been supplied to the ERI and it will ultimately be housed in an optimum facility, once construction of a World Bank facilities improvement project is completed.

The results of this project will certainly be used. As shown in Appendix Table 7, selected DH lines have already been extensively used in the Eskisehir crossing program. The population has also been evaluated in Konya and Ankara, Turkey, and I understand from ICARDA researchers that they have also selected lines for expanded regional evaluation. There is also an attitude parameter that is difficult to quantify: if breeders throughout the region can see that completely homozygous, agronomically relevant germplasm can be developed within a short time frame and that their colleagues at Eskisehir are actively using and generating such material, there is a much greater likelihood of technology adoption. Larger scale trials of the material are warranted, but not within the context of this project: lines with agronomic potential will make their way into variety testing trials. The identification of chromosome regions associated with winterhardiness traits has certainly had a significant impact on the world scientific community, as is evidenced from the following section detailing project activities and outputs. More importantly, we are now to the point of applying these findings to the development of more winterhardy barley varieties.

This project will leave in Turkey a willingness to try new breeding systems, the basic equipment for a doubled haploid lab, and a core of germplasm that will be used in future cycles of selection. Likewise, the scientific capabilities of collaborating scientists have been upgraded: doubled haploid production will no longer be perceived as an arcane luxury available only to breeders in developed countries. From doubled haploid

production, it is a logical step to genome mapping and QTL analysis. This productive fusion of techniques is one that the Turkish Research Institutes and universities will hopefully develop.

Project Activities/Outputs

Relevant Presentations at Professional Meetings

ICARDA Review of Cereals Research. "Winter and facultative barley germplasm enhancement". Aleppo, Syria. 10/89.

American Society of Agronomy. "Doubled haploid recurrent selection in barley based on a circulant partial diallel mating". Denver, CO. 11/89.

Krasnodar Institute for Agricultural Research. "Genetic analysis of cold tolerance: applications of doubled haploid and gene mapping techniques". Krasnodar, Russia 6/90.

International Symposium on breeding barley for stress environments. "Production of recombinant inbred lines: the biology and the technology". Bozeman, MT. 7/90.

International Barley Genetics Symposium. "Doubled haploids as a tool for germplasm enhancement". Helsingborg, Sweden. 7/91.

Anatolian University. "Doubled haploid-based gene mapping of abiotic stress resistance traits in barley". Eskisehir, Turkey. 5/92.

Uludag University. "Doubled haploid techniques in barley". Bursa, Turkey. 5/92.

Gordon Conference. "Genetics of winterhardiness in barley" Oxnard, CA. 2/93.

Relevant Publications

Chen, F., and P.M. Hayes. 1989. A comparison of *Hordeum bulbosum* - mediated haploid production efficiency in barley using *in vitro* floret and tiller culture. Theoretical and Applied Genetics 77:701-704.

Hayes, P.M., and F. Chen. 1989. Genotypic variation for *Hordeum bulbosum* - mediated haploid production in winter and facultative barley. Crop Science 29:1184-1188.

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Book Chapters

Hayes, P.M., T. H.H. Chen, and T.K. Blake. 1992. Marker-assisted genetic analysis of cold tolerance in winter barley. In P.H. Li and L. Christersson (ed.). Advances in plant Cold Hardiness. CRC Press, Boca Raton, USA

Patents

All technology and germplasm developed in the course of this project is in the public sector and freely available.

Project Productivity

As described in the preceding sections, this project has been productive. We have developed doubled haploid germplasm that promises to lead to variety release in the US, Russia, and Turkey. These stocks will likely appear in the pedigrees of new varieties over the next 10 years. Broad-based doubled haploid populations should be an excellent adjunct to the conventional material developed by IARCs. There is no question that when genetic factors, such as vernalization requirement, preclude conventional rapid generation advance, and when quantitatively inherited traits are selection targets, doubled haploid approaches are warranted. The technique is cost-effective. The OSU barley program is based entirely on DH production, and we currently produce at least 1,000 lines per year. We have presented the first molecular marker data on the location of winterhardiness traits, and we are in the process of finer structure mapping in diverse germplasm accessions that will allow us to systematically manipulate cold tolerance, vernalization response, and photoperiod reaction. We have provided three Turkish scientists with the techniques required for DH production, and we have stimulated an interest in innovative breeding strategies.

Future Work

In an immediate sense, this project is terminated, as Turkey is apparently not eligible for continued USAID funding. However, this work will continue on several fronts. We will continue to exchange germplasm with Turkish and other regional national programs, as funding allows. Turkish scientists will always be welcome at OSU. The genetic analysis of cold tolerance will continue, both in the context of the USAID/PSTC-supported effort with the Martonvasar Research Institute and in other competitive grant programs. We are grateful to the USAID/PSTC program for supporting Dr. Hayes in his first competitive grant project, for supporting Dr. Altay's efforts at development of laboratory capability, and for affording the opportunity to develop lasting professional relationships based on respect, friendship, and common goals.

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Appendix Table 1. Parental stocks and F_1 combinations generated from a circulant partial dialle mating.

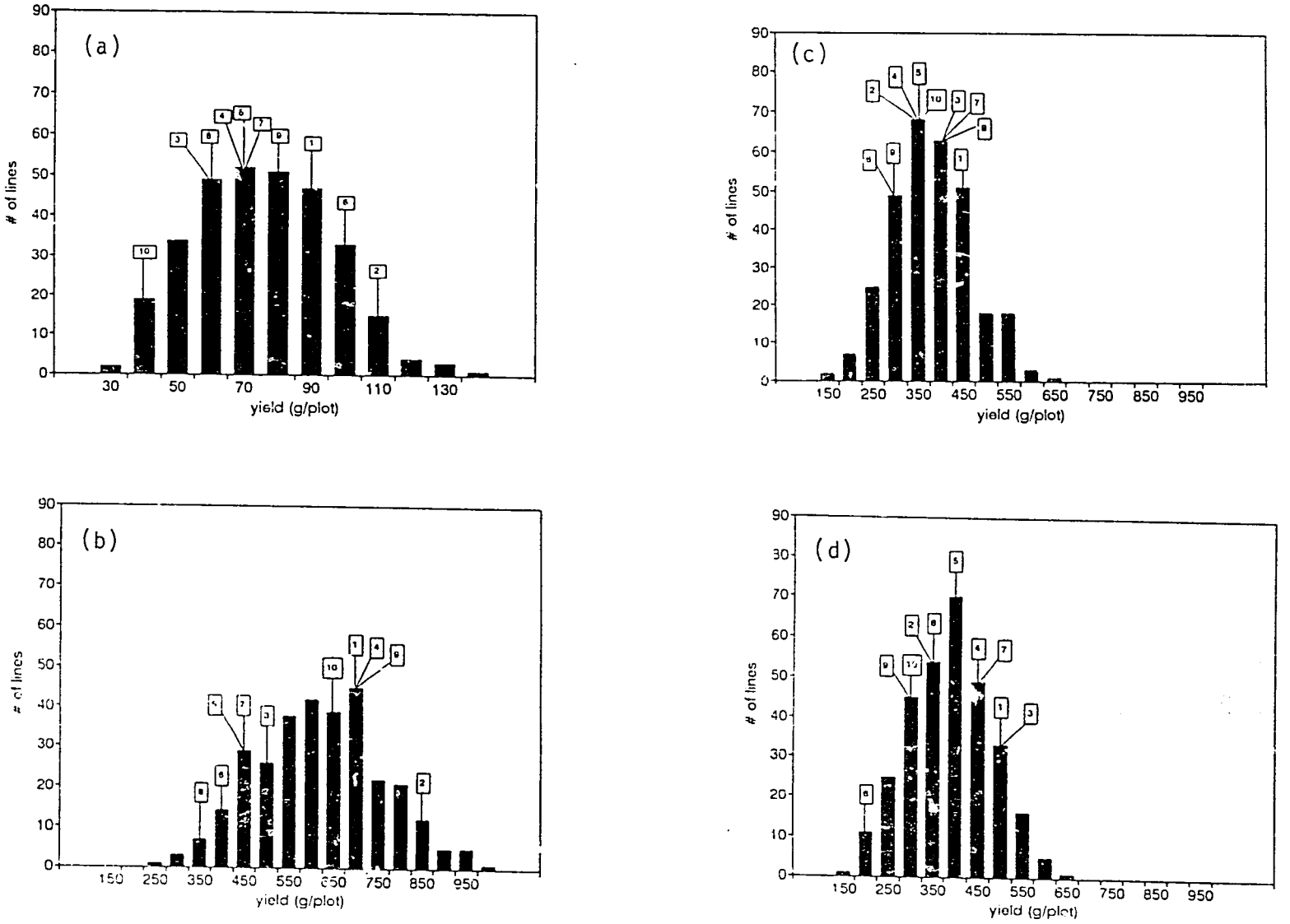
	Origin	Type	Comments
1.) Plaisant	France	6-row	Malt quality
2.) OR186112	Oregon	6-row	Net blotch resistance
3.) Wintemalt	Oklahoma	6-row	Cold and draught tolerance
4.) Kold	Oregon	6-row	Barley stripe rust resistance
5.) Novator	Russia	6-row	Cold tolerance
6.) Robur	France	6-row	Earliness
7.) Scio	Oregon	6-row	Yield
8.) OR1861009	Oregon	2-row	BYDV resistance
9.) Flamenco	France	2-row	Malt quality
10.) AB812	Idaho	6-row	Yield/reduced height
F_1 combinations	DH lines produced		
PLAI NOV	62		
PLAI ROB	57		
PLAI SCI	49		
1861112/ROB	65		
1861112/SCI	49		
1861112/1861009	36		
WIN/SCI	51		
WIN/1861009	43		
WIN/FLA	31		
KOLD/1861009	49		
KOLD/FLA	73		
KOLD/1861002	65		
NOV/FLA	54		
NOV/1861002	41		
ROB/1861002	32		

Appendix Table 2. Quality of doubled haploid selections compared to Plaisant.

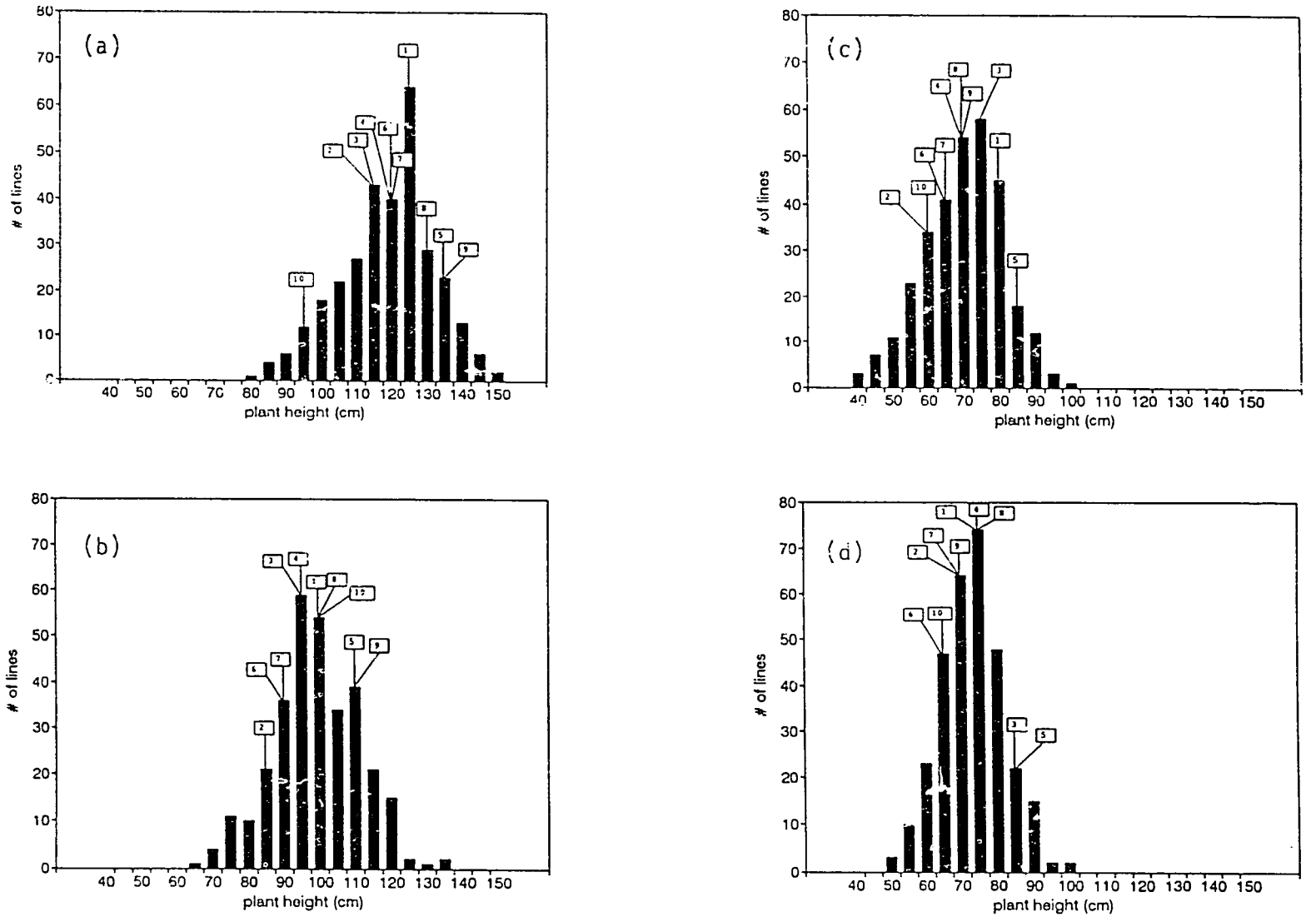
Line	Grain protein %	S/T ratio	DP Deg	α amylase 20 Deg units	Extract %	Viscosity
Pendleton, OR						
ORW-6	8.0	38.3	61	37.4	80.4	1.45
ORW-7	8.5	33.5	81	25.8	78.9	1.76
ORW-8	8.9	40.5	105	47.4	79.7	1.46
ORW-9	8.4	45.2	132	40.7	81.1	1.38
Plaisant	7.6	43.5	89	41.1	81.3	1.40
Pullman, WA						
ORW-6	11.3	36.9	51	37.6	78.5	1.57
ORW-7	12.8	38.8	90	31.8	79.0	1.58
ORW-8	13.9	34.4	94	41.4	79.2	1.47
ORW-9	13.9	36.9	89	36.5	77.4	1.55
Plaisant	12.6	36.5	87	30.6	80.1	1.48
Aberdeen, ID						
ORW-6	10.2	37.4	88	34.5	77.6	1.53
ORW-7	12.4	25.8	153	31.5	75.5	1.43
ORW-8	10.9	33.1	127	40.1	78.2	1.51
ORW-9	11.2	33.9	142	39.7	78.3	1.49
Plaisant	10.8	31.3	137	36.2	77.6	1.50
Average						
ORW-6	9.8	37.5	66.7	36.5	78.8	1.51
ORW-7	11.2	32.7	108	29.7	77.8	1.59
ORW-8	11.2	36.0	109	42.9	79.0	1.48
ORW-9	11.2	38.6	121	38.9	78.9	1.47
Plaisant	10.3	37.1	104	35.9	79.6	1.46

Appendix Table 3. Grain yield of doubled haploid selections compared to checks.

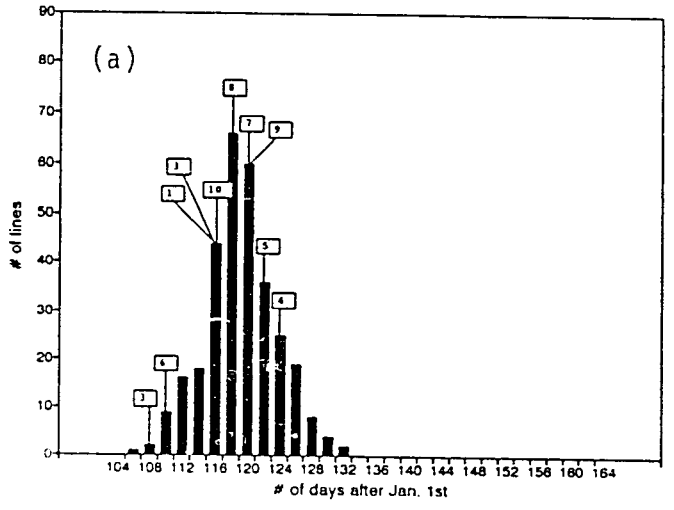
	Yield, kg/ha			
Line	Pendleton	Corvallis	Aberdeen	$\bar{x}$
Scio	6190	4993	7488	6223
Plaisant	7805	6316	6326	6815
Hundred	5877	6840	7440	6719
Eight-twelve	7012	3483	7200	5898
ORW-6	8347	4760	8520	6209
Scio	6434	4720	8496	6550
Plaisant	7489	6521	7152	6843
ORW-7	6858	7043	8112	7337
ORW-8	6997	5074	6700	6257
Scio	5900	4497	7776	6250
Plaisant	6968	6366	6096	6466
ORW-9	6620	6048	7432	6700

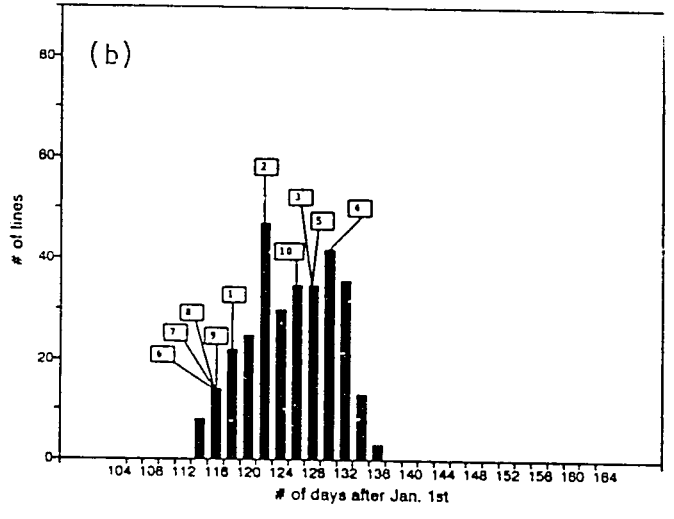


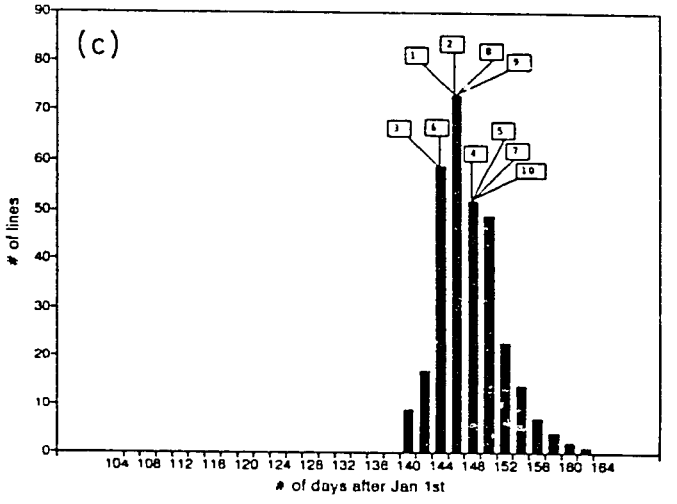
Appendix Figure 1. Grain yield frequency distributions for a barley doubled haploid population evaluated at Corvallis, OR (a), and Bursa (b), Eskisehir (c), and Hamidiye (d), Turkey. Numbers indicate parental lines, per Appendix Table 1. Plot sizes were identical in Turkey. Corvallis data are from hill plots.



Appendix Figure 2. Plant height frequency distributions for a barley doubled haploid population evaluated at Corvallis, OR (a), and Bursa (b), Eskisehir (c), and Hamidiye (d), Turkey.







Appendix Figure 3. Heading date frequency distributions for a barley doubled haploid population evaluated at Corvallis, OR (a), and Bursa (b), and Eskisehir (c), Turkey.

Appendix Table 7. Pedigrees of doubled haploid parental sources including OSU/Eskisehir germplasm.

THE PEDIGREES OF THE F_1's

1= YEA235//Plaisant/Novator

2= YEA2936//Plaisant/Novator

3= YEA2954//Plaisant/Novator

4= YEA2955//Plaisant/Novator

5= YEA2960//Robur/Plaisant

6= YEA2961//Robur/Plaisant

7= YEA2965//Robur/Plaisant

8= YEA2967//1861112/Robur

9= YEA3003//Scio/1861112

10= YEA3009//Scio/1861112

11= Novator//72AB83/Winter Malt/3/Tokak

12= 1861112/1861009//Tokak

13= NS313 188010 Winter Hardy/4/Igri/B68-1285//Pul12222/3/3/FB73194/ NY60005-19/5/Tokak

14= Kamiak/Belts67-875//WA1094-67/3/OACWB142-6/4/NS313 188070 Winter Hardy/5/Tokak

15= Cyclone/4/Igri/B68-1285//Pull112222/3/FB73-194/NY6005-19/5/Tokak

16= 72 AB83/Winter Malt//Cyclone/3/Tokak

17= Cyclone/4/Kamiak/Belts67-875//WA1094-67/3/OACWB142-6/5/Tokak

18= Plaisant//Wbelt32/3/Tokak

19= Cyclone/3/Mal/OWB753328-51-1 P//Perga/Boyer/4/Tokak

Quantitative trait loci on barley (*Hordeum vulgare* L.) chromosome 7 associated with components of winterhardiness[1]

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Quantitative trait loci (QTL) controlling traits associated with winterhardiness in barley (field survival, LT_{50}, growth habit, and crown fructan content) were mapped to chromosome 7 in a population of 100 F_1-derived doubled haploid lines. The largest QTL effects for all traits were detected in a 21% recombination interval on the long arm of chromosome 7. QTL in this region accounted for 37–68% of the variation for three measures of cold tolerance, 47% of the variation for growth habit, and 28% of the variation in crown fructan content. Trait association may be due to linkage rather than pleiotropy.

Key words: *Hordeum vulgare*, genome mapping, quantitative trait loci, winterhardiness, cold tolerance, fructan, growth habit.

HAYES, P. M., BLAKE, T., CHEN, T. H. H., TRAGOONRUNG, S., CHEN, F., PAN, A., et LIU, B. 1993. Quantitative trait loci on barley (*Hordeum vulgare* L.) chromosome 7 associated with components of winterhardiness. Genome, **36** : 66–71.

Les locus de traits quantitatifs (QTL) qui contrôlent les traits associés à la résistance au froid chez l'orge : survivance au champ, LT_{50}, mode de croissance et teneur en fructanes de la couronne, ont été cartographiés sur le chromosome 7 chez une population de 100 lignées F_1 dérivées de plantules haploïdes doublées. Les effets des QTL les plus marqués pour tous les traits ont été décelés dans un intervalle de recombinaison de 21% sur le bras long du chromosome 7. Dans cette région, les QTL ont été à l'origine de 37 à 68% de la variation pour des mesures de résistance au froid, de 47% de la variation pour le mode de croissance et de 28% pour la variation de la teneur en fructanes de la couronne. L'association des traits peut être reliée au linkage plutôt qu'à la la pléiotropie.

Mots clés : *Hordeum vulgare*, cartographie des génomes, locus des traits quantitatifs, résistance au froid, fructanes, mode de croissance.

[Traduit par la rédaction]

Introduction

Winterhardiness in cereals is the final expression of a number of interacting component traits, including low temperature tolerance (Olien 1978), growth habit (Takahashi and Yasuda 1971), and crown fructan content (Livingston et al. 1989). Responses to selection for winterhardiness have been limited, leading Fowler and Gusta (1979) to conclude that genetic variation may be exhausted. Thomashow (1990) summarized over 20 reports that, cumulatively, assign cold tolerance genes to every chromosome in each of the three genomes of wheat. Chromosomes 5A and 5D were most often implicated. Sutka and Snape (1989) and Roberts (1990) provide compelling evidence for the importance of chromosome 5A.

Tanksley et al. (1989) reviewed the developments in molecular marker technology and statistical analysis that provide tools for mapping quantitative trait loci (QTL). When genetic markers flanking target QTL are mapped, the basis of selection shifts from phenotype to genotype and selection response should be maximized. Considering the negligible response to selection for winterhardiness, these traits are logical candidates for QTL mapping. Barley (*Hordeum vulgare*) provides a model system for testing the utility of QTL mapping for the components of winterhardiness. The crop is amenable to doubled haploid production (Chen and Hayes 1989), and these immortal genetic reference populations facilitate both molecular marker map construction (Heun et al. 1991) and repeated phenotype assessments. Our objectives are to locate QTL associated with field survival, the temperature lethal to 50% of a test population (LT_{50}), crown fructan content, and growth

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habit in barley. Here we present preliminary evidence that a multilocus cluster on the long arm of chromosome 7 is associated with trait expression.

Materials and methods

Germplasm

One hundred doubled haploid (DH) lines were developed by the *Hordeum bulbosum* technique, as described by Chen and Hayes (1989), from the F_1 of the cross of 'Dicktoo' × 'Morex'. F_1 plants were generated by crossing single plants of each parental accession. 'Dicktoo' is a six-row winter feed barley of unknown ancestry and mixed description released by the Nebraska Agricultural Experiment station in 1952. 'Morex' is a six-row spring malting barley released by the Minnesota Agricultural Experiment Station in 1978.

Trait assessment

Cold tolerance was measured as (*i*) field survival in Oregon, (*ii*) field survival in Montana, and (*iii*) LT_{50}. Field survival in Oregon was measured by visually assessing percent survival following the winter of 1990–1991. Each DH line was represented by an unreplicated plot consisting of two 1.5-m rows at the Hyslop Agronomy Farm, near Corvallis, Oreg. Field survival in Montana was measured as the difference between initial and final plant stand, following the winter of 1991–1992, in a three-replicate experiment grown at the Post Agronomy Farm near Bozeman, Mont. The LT_{50} of each DH line was determined using plant material hardened at 2°C for 5 weeks with a 10-h light - 14-h dark photoperiod regime. Four temperatures (0, −4, −8, −12°C) with 10 plants at each temperature were used to determine the LT_{50} of each DH line. A total of three replicates were run from October 1990 to February 1991. Plant material was prepared for freezing and LT_{50} values were computed as described by Kolar et al. (1991).

The growth habit of each DH line was expressed as the mean heading date of three unvernalized plants of each DH line under greenhouse conditions of 18°C day/night and 24-h light.

Crown fructan content (≥DP 5) was determined using field hardened plant material. Five plants of each genotype were taken from field plots at Corvallis. Oreg., in January 1992. Plants were washed, trimmed, and the crowns were immediately frozen in liquid nitrogen. Tissue was lyophilized for 15 days. Crown tissue was ground in liquid nitrogen and 5 mL of distilled water were added to each 50-mg sample. Samples were shaken and then incubated for 15 min. in a 90°C water bath. The supernatant was poured through an Amberlite MB-3A ion exchange resin column and remixed in 5 mL water; this step was repeated twice. The column was then washed with 5 mL of 90°C water, and the water extracts were dried at 60°C for 6–8 h. Dried samples were dissolved in 1 mL of 60°C water and centrifuged at 120 000 rpm for 10 min. The supernatant was filtered using a 0.45 μm filter prior to injection into the HPLC, which was equipped with Bio-Rad carbo-C guard and Bio-Rad HPX-42 C carbohydrate columns. Fructan content (≥DP 5) is expressed as milligrams per gram on a dry weight basis. The fructan content of each DH line was determined once; the parents were run in triplicate.

Map construction and QTL analysis

Thirty-three markers have been mapped in this population. This report focusses on the eight markers located on chromosome 7, which are shown on the horizontal axis of Fig. 2. The prefix *m* designates the morphological markers rachilla hair length (*mS*) and awn texture (*m.R*). The WG and BCD prefixes designate wheat genomic and barley cDNA clones, respectively (Heun et al. 1991).

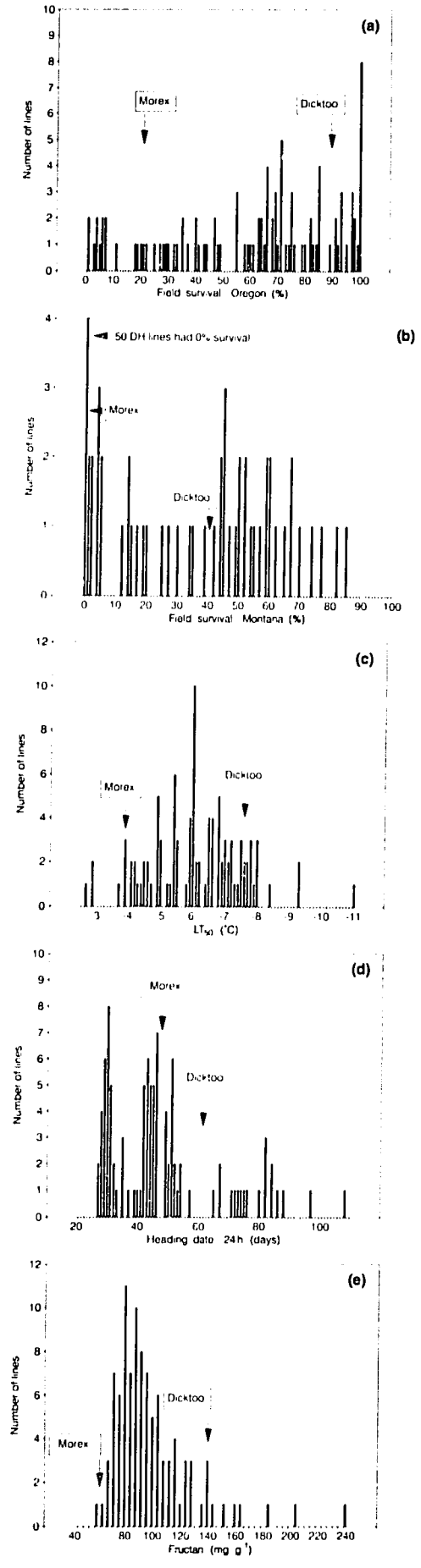
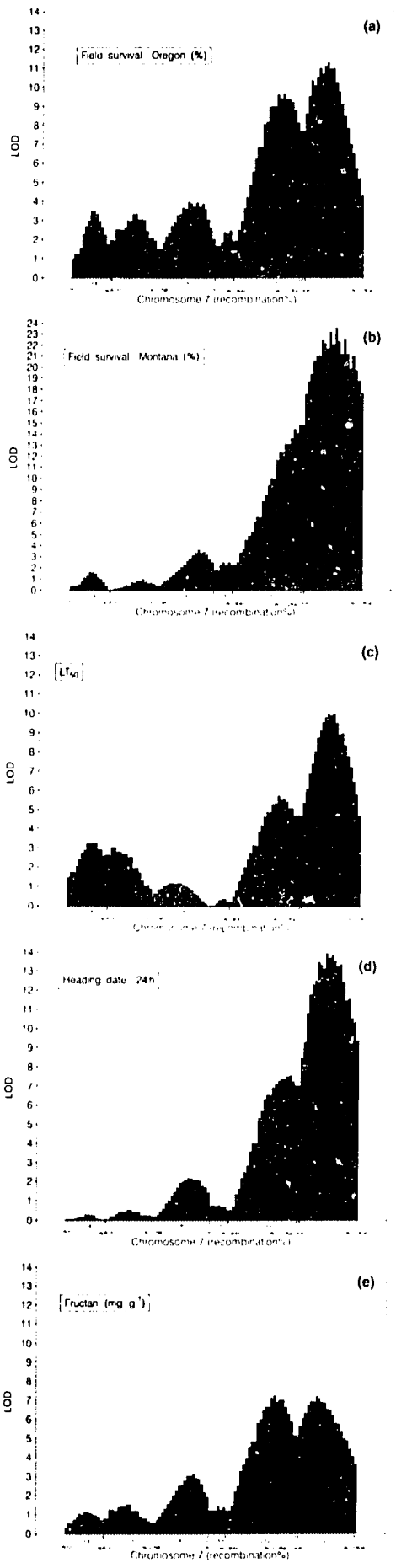


FIG. 1. Frequency distributions for field survival in Oregon (*a*), field survival in Montana (*b*), LT_{50} (*c*), growth habit (*d*), and crown fructan content (*e*) of 100 F_1-derived doubled haploid lines from the cross of 'Dicktoo' × 'Morex'.



apADH is an alcohol dehydrogenase-2 locus visualized by polymerase chain reaction (PCR). *Rrn2* was scored with a genomic DNA clone, pTA71, containing the entire wheat rDNA repeat unit (Gerlach and Bedrook 1979). Detailed mapping protocols are available upon request. Restriction fragment length polymorphism (RFLP) procedures were as described by Schön et al. (1991). Sequencing and oligonucleotide synthesis procedures for PCR reactions are described by Tragoonrung et al. (1992). Morphological markers were scored under a stereomicroscope.

G-Mendel 2.0, an unreleased version of G-Mendel (Liu and Knapp 1990) was used for linkage map construction. Quantitative trait loci were mapped using QTL-STAT, an interval mapping program that uses linear models to estimate QTL parameters (B.H. Liu and S.J. Knapp, unpublished). QTL were assessed in terms of a single and multilocus ANOVAs and by plotting LODs ($\log_{10}$ of the maximum likelihood ratio estimate) against specific map segments.

Results and discussion

Map construction

The chromosome 7 map is anchored by known location morphological (*mR* and *mS*) and DNA (*Rrn2*) markers on chromosome 7. *WG1026a* and *mR* showed complete cosegregation. The log likelihood for the chromosome 7 map is −245.0 and the total length is 102.3 recombination units.

Cold tolerance

Field survival is the consequence of a unique set of hardening, growth, and stress conditions and the distinct population frequency distributions for Oregon and Montana field survival reflect these differences (Figs. 1*a* and 1*b*). At Corvallis, eight lines showed 100% survival and only two showed complete mortality. At Bozeman, 50 lines suffered complete mortality and the highest survival was 85%. The standard error was ±11.1%. LT_{50} values ranged from −2.7 to −11.0°C (Fig. 1*c*). The standard error was ±1.8°C. When the top 15 survivors were tabulated for each of the three measures of cold tolerance, there were four lines in common. These modest associations of trait performance measured in distinct environments typify the difficulties associated with cold tolerance measurement and selection.

The QTL analysis approach may provide a clearer picture of trait relationship. The primary determinant of the power of tests of hypotheses about QTL genotype means is the number of replications of QTL genotypes (i.e., the number of individuals in the genetic reference population), not the number of times each individual is replicated (Knapp et al. 1990). For example, in an unreplicated phenotype evaluation of this population, each QTL genotype was actually replicated 50 times.

Large QTL effects for all measures of cold tolerance were found on chromosome 7 (Figs. 2*a*, 2*b*, and 2*c*). A threshold of LOD ≥3 is approximately equal to $p = 0.001$ (Ott 1991). For field survival in Oregon, QTL effects exceeding the LOD ≥3.0 threshold were detected in every interval except *mS–WG364b*. The r^2 for the *mR–BCD265b* interval is 0.40. In the multilocus model, the two intervals *apHRTH–BCD265c* and *mR–BCD265b* together account for 51% of the variation for field survival observed in the population. For field survival in Montana, QTL effects

FIG. 2. Chromosome 7 LOD values for field survival in Oregon (*a*), field survival in Montana (*b*), LT_{50} (*c*), growth habit (*d*), and crown fructan content (*e*) of 100 F_1-derived doubled haploid lines from the cross of 'Dicktoo' × 'Morex'.

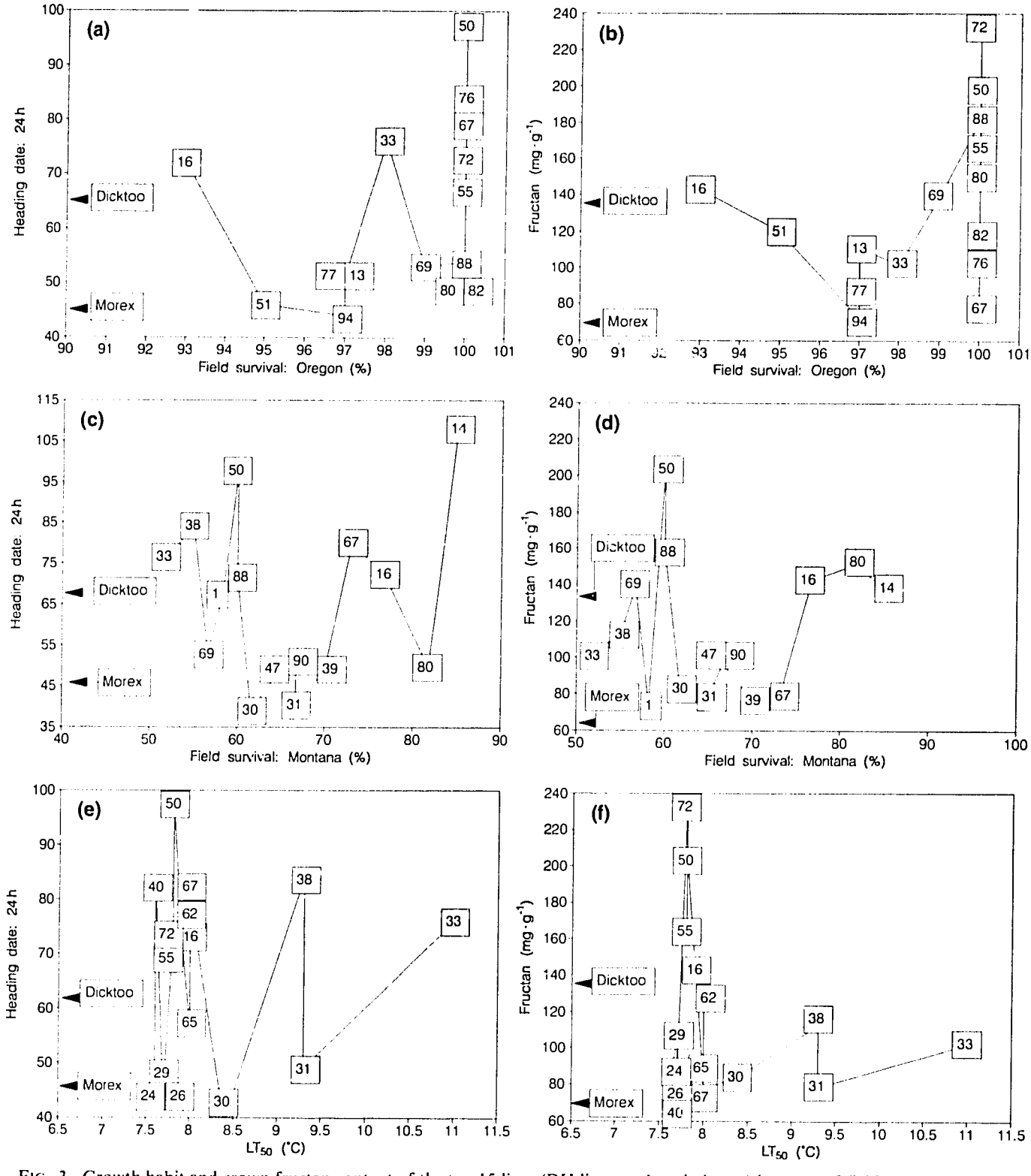


FIG. 3. Growth habit and crown fructan content of the top 15 lines (DH line numbers in boxes) in terms of field survival in Oregon (*a* and *b*), field survival in Montana, (*c* and *d*), and LT_{50} (*e* and *f*).

exceeding the LOD ≥3.0 threshold were detected in three intervals: *BCD298-mS*, *WG364b-mR*, and *mR-BCD265b*. Only the latter interval was identified in the multilocus ANOVA. The r^2 was 0.68. For LT_{50}, LODs ≥3.0 were detected in the following intervals: *Rrn2-apADH*, *WG364b-mR*, and *mR-BCD265b*. Only the *mR-BCD265b* interval was identified in the multilocus model, with an r^2 of 0.37.

Schön et al. (1991) reported that, in this same cross, gametophytic selection imposed by anthesis cold stress caused significant segregation distortion in favor of the winter parent in the vicinity of *Rrn2*. In the case of Oregon field survival and LT_{50}, modest QTL effects were detected in the vicinity of *Rrn2*. Roberts (1990) and Sutka and Snape (1989) report that loci on wheat chromosome 5 are

associated with cold tolerance. Barley chromosome 7 is homoeologous with wheat chromosome 5 (Islam et al. 1981).

Growth habit

Vernalization response, maturity, and photoperiod response genes interact to determine winter versus spring growth habit. Following the example of Takahashi and Yasuda (1971) we chose heading date under 24-h light as a measure of growth habit. Heading dates among the samples of individual DH lines varied a maximum of 2 days. The transgressive segregation and discontinuous expression of heading date under 24 h light, referred to subsequently as growth habit, can be attributed to multigenic control (Fig. 1*d*). The accession of 'Dicktoo' that we used as a parent displays typical winter growth habit under field conditions, prostrate throughout the fall and winter and mid-season maturity, but under the described test conditions it is only 16 days later to head than 'Morex'. An examination of seveal accessions of 'Dicktoo' revealed a range of vernalization responses from none to complete (data not shown). This variation may be attributable to the heterogeneity of the initial germplasm, as documented in the variety release description. Since every effort was made to ensure that the DH population was derived from pure seed, this heterogeneity should not be a factor in our analyses. However, this heterogeneity underscores the need to characterize and maintain seed of all parental stocks used in developing mapping populations.

The skewness of the growth habit frequency histogram toward the spring phenotype would lend some support to the epistatic model proposed by Takahashi and Yasuda (1971). According to this model, winter versus spring growth habit is determined by the epistatic interaction of three loci: *Sh* on chromosome 4; *sh2* on chromosome 7; and *sh3* on chromosome 5. Large QTL effects (LOD ≥ 14) were detected on chromosome 7, again in the *WG364b-mR* and *mR-BCD265b* intervals (Fig. 2*d*). Takahashi and Yasuda (1971) reported 13.1% recombination between *mR* and the *Sh2* locus. The 'Dicktoo' and 'Morex' QTL genotype means are 62.3 and 37.0 days, respectively, for the *mR-BCD265b* interval, and this interval accounted for 47% of the variation in trait expression.

Crown fructan content

The coefficient of variation (CV) among triplicate samples of the parents was 6.6%. 'Morex' had a crown fructan content of 63.8 $mg \cdot g^{-1}$. The transgressive segregants with fructan substantially higher than 'Dicktoo' (137 $mg \cdot g^{-1}$) indicate that both parents may have contributed alleles for fructan accumulation (Fig. 1*e*).

The QTL analysis revealed substantial effects on chromosome 7 (Fig. 2*e*). The LOD plots follow approximately the same pattern as those for field survival, LT_{50}, and growth habit. The *mR-BCD265b* interval accounted for 28% of the variation in trait expression.

Evidence for a multilocus cluster

The QTL data raise intriguing issues regarding numbers of QTL, types of genes action, and the contribution of each trait to the winter-hardy phenotype. For all traits principal QTL appear in the intervals *WG364b-mR* (Q_1) and *mR-BCD265b* (Q_2). We used the procedure of Knapp (1991) to test the hypotheses regarding the number of QTL in these intervals. For all traits, the greatest likelihood ratios and *p* values were found for the contrast of Q_2 versus no Q, supporting the hypothesis that there are loci controlling cold tolerance, growth habit, and crown fructan content in the *mR-BCD265b* region.

Are these linked QTL or is this a pleiotropic effect of a single locus? Several lines of evidence favor linkage over pleiotropy. The heading dates and fructan content of the top 15 lines for the two measures of field survival and LT_{50} are plotted in Figs. 3*a*–3*f*. In all cases, high surviving DH lines show a range of fructan values ranging from equivalent to 'Morex' to far in excess of 'Dicktoo' (77.8–237.4 mg g^{-1}). Likewise, these same lines show a range of heading dates from equivalent to 'Morex' to the most extreme transgressive segregant (39–108 days). Four DH lines are identified as among the top 15 by both the field survival and LT_{50} criteria. All of these lines (DH 16, DH 33, DH 50, and DH 67) exceed 'Dicktoo' in heading date, survival, and LT_{50}, and all have the 'Dicktoo' QTL genotype for these characters in the *mR-BCD265b* interval. DH67, however, has a fructan content of 83 $mg \cdot g^{-1}$ and the 'Morex' QTL genotype for this character in the R-*BCD265b* interval. The remaining DH lines have the 'Dicktoo' QTL locus genotype in this interval. The estimated recombination distances from *mR* to the fructan, survival, and growth habit QTL are 7, 9, and 10%, respectively. DH-57 may, therefore, have resulted from a crossover between the fructan locus and the survival and growth habit loci.

Roberts (1990) proposed that at least two loci on chromosome 5A of wheat determine cold hardiness and vernalization. One of these loci is, or is tightly linked to, *Vrn1*, and *Vrn1* may be homoeologous to the *Sh2* locus of barley. Doll et al. (1989) measured both field survival and vernalization requirement in doubled haploid populations of winter barley and confirmed the oft-reported association, but not cosegregation, of growth habit and cold tolerance in barley. Tight linkage among characters conferring adaptation to a given environment, in this case winter versus spring, is intuitively appealing and supported by Allard's (1988) finding that natural selection favors the development of multilocus clusters conferring adaptation.

Fructan content and growth habit QTL appear to be closely linked to cold tolerance QTL on chromosome 7, but high fructan content and winter growth habit are not required for a genotype to achieve a high level of cold tolerance. We are hopeful that extension of our mapping efforts will allow for a significant improvement in barley cold tolerance.

Acknowledgements

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