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

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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:

We are completing a second round of field evaluation of the doubled haploid recurrent selection population. The Oregon trials will be harvested the first week of July, 1992 and the population will be harvested at approximately the same time in Turkey. A primary objective of this project is to test the hypothesis that diverse arrays of doubled haploids could prove a more effective vehicle for international germplasm enhancement than segregating or conventionally advanced homozygous lines. **From the U.S. perspective, the results are very encouraging: four DH lines from this population are in accelerated release as varieties.**

Hayes visited the Eskisehir Institute 6/11 - 6/22. This project has had a significant impact in several arenas. 1.) DH lines have been used extensively in the Eskisehir breeding program to expand the germplasm base. Selected lines have shown excellent cold tolerance and yield potential. 2.) Certain crosses will fortuitously provide data on the genetics of resistance to elevated levels of boron. Much of the Transitional Region is plagued by elevated levels of boron; progeny from three crosses are segregating for boron resistance, under field conditions at Hamidye. Altay is summarizing these data and will transmit to Hayes for analysis. 3.) Researchers at the Eskisehir Institute are seriously questioning their breeding strategies and acknowledging the importance of integrating genetic analyses and variety development. 4.) Hayes presented seminars on gene mapping and DH techniques at Anadolu and Uludag Universities. The reception was enthusiastic, and the way may be paved for the removal of Institutional barriers

limiting University/Institute collaboration in Turkey. **Specifically, there is an exciting opportunity to establish a model program uniting the strengths of the Eskisehir Institute and Uludag University. A draft of a collaborative proposal is attached.**

The results of the cold tolerance gene mapping effort have been submitted to Genome. A draft of the manuscript is attached. Please do not circulate, as this manuscript is still in review.

C.) Scientific Impact of Collaboration:

The Eskisehir Institute typifies the quandary of "second world" agricultural research. There is a strong professional group with U.S. and European graduate training. To a large extent, these researchers are frustrated by their inability to apply the techniques they have learned abroad. Turkey has moved well beyond the "Green Revolution" scenario of releasing high yielding varieties as a stop-gap measure. There is now the luxury to be concerned with grain quality and to develop varieties that will allow for more sustainable production. Doubled haploid and molecular marker strategies are proven breeding tools that can carry a country such as Turkey into the 21st century of plant breeding.

An FAO-sponsored M.S. student from the Eskisehir Institute - Ergun Ozdemir - joined my lab in Fall, 1991. He has completed English training and will be working with the doubled haploid recurrent selection population for his M.S. research.

D.) Description of Project Impact:

The project has developed both the intellectual and technical capability for doubled haploid breeding at the Eskisehir Institute. As noted in Section "B", the project has made significant progress on several fonts: germplasm, genetic analysis, and research approaches.

E.) Strengthening of Developing Country Institutions:

Equipment and reagents for doubled haploid production have been received and installed by Dr. Altay. This project has the potential to serve as a model for integrating University/Institute research. A draft of such a project is attached.

F.) Future Work:

The project is on schedule. The C0 doubled haploid recurrent selection population has been evaluated under field conditions in Oregon and Turkey. Samples of this germplasm have been distributed to the USSR, Hungary, and ICARDA. Selections were intermated in Spring, 1992.

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 copy of a manuscript submitted to Genome is attached.

Ergun Ozdemir, the FAO-sponsored student from the Eskisehir Institute, will be working in the area of cold tolerance genetics and doubled haploid germplasm enhancement for his M.S. thesis research. He has completed an intensive English course and will begin coursework in Fall, 1992.

Section II

A.) Managerial Issues:

All is well.

B.) Budget:

No changes.

C.) Special Concerns:

No changes.

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

Hayes travelled to Turkey in May, 1992. The exciting project impacts are detailed elsewhere in this report.

Publications Relevant to This Project:

Chen, F., and P.M. Hayes. 1991. 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 26:179-184.

Schön, C., and P.M. Hayes. 1991. Gametophytic selection in a winter x spring barley cross. Genome 34:918-922.

Tragoonrung, S., V. Kanazin, P.M. Hayes, and T.K. Blake. 1992. STS facilitated PCR for barley genome mapping. Theoretical and Applied Genetics (in press).

Chen, F., and P.M. Hayes. 1992. The genetic basis of seed set varying in compatibility with *Hordeum bulbosum*. Genome (In press).

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.) in press. Advances in plant cold hardiness. CRC Press, Boca Raton, Florida.

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

This project is rewarding from the standpoint of establishing collaborative linkages and achieving concrete progress in applied plant breeding. However, after reviewing research at the Eskisehir Institute and presenting seminars at nearby Universities, I am appalled at the lack of collaboration between Turkish institutions. The problem is not peculiar to these institutions: it is symptomatic of much of the developing world, and particularly "second world" countries that have developed intellectual and technical capability. The Dean of Agricultural College of Uludag University, the Director of the Eskisehir Institute and I have prepared a draft proposal that would strengthen collaboration. Such a project is not within the mandate of the PSTC program, but we would appreciate assistance in identifying appropriate donors. I see the Turkish situation of just requiring a "jump start" . With modest outside support, I have every confidence that the Turkish Ministry of Agriculture would provide significant matching funds.

Strengthening Inter-Institutional Linkages for Biotechnology in Barley Research: A Cooperative Project Involving The Transitional Agricultural Research Institute, Uludag University and Oregon State University

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Justification:

Applied agricultural research and training infrastructure is well established in the Transitional Region of Turkey's Anatolian Plateau. The barley breeding program at the Transitional Agricultural Research Institute is productive. The agricultural faculty at Uludag University is strong. The pieces are in place to move agricultural research into the 21st Century, given a modest infusion of capital, research equipment, training, and international collaboration. Without such an infusion, however, the synergistic growth that is possible with University/Institute collaboration will not occur. Rather, the former will focus on theory while the latter will struggle to re-allocate scarce resources from mission oriented research.

There are agricultural biotechnologies ranging from the applied to the basic that, when focussed on a model crop system, can set an example for University/Institute collaboration throughout Turkey. Barley is the ideal model crop system: tissue culture and doubled haploid techniques are now routinely used in variety development in the U.S. and Europe; molecular marker analysis is coming into its own as a breeding tool; transformation has real potential; and barley is a critically important crop in the Transitional Region.

Objective:

This project is intended to serve as a model system for integrating basic and applied research in a fashion that will have tangible short and intermediate term benefits for Turkish farmers and Turkish scientists. The project will develop the basic research

capacity for molecular marker and transformation research at Uludag University and ensure that these technologies are transferred to the doubled haploid lab and the fields of the Transitional Region Agricultural Research Institute. The Oregon State University Barley Project has developed a collaborative program in doubled haploid technology with the Transitional Region Agricultural Research Institute. At Oregon State University, the Project is active in molecular marker analysis and transformation technology.

Projected Benefits:

A strong agricultural sector is vital for the stability of the Turkish rural economy, and barley is a cornerstone of Transitional Region agriculture. A cadre of U.S. and European-trained scientists is present at both Uludag University and the Transitional Region Agricultural Research Institute. Much of this talent will be lost to the private sector, or wasted, due to perceived inter-institutional barriers and the lack of start-up funding.

Turkey is a critical center of genetic diversity for barley and other cereals. If Turkish scientists are equipped with the tools of contemporary genetics, they will be in a position to exploit this diversity and therefore take a pro-active role in its conservation. This project is intended to serve as a model for University/Institute collaboration in other crops throughout the country.

Workplan:

This project is envisioned to run for a three year period. Scientists and laboratory facilities will be equipped for doubled haploid and tissue culture work, molecular marker analysis, and transformation.

Training:

With FAO support, a scientist from the Transitional Region Agricultural Research Institute is currently training at OSU in doubled haploid technology. This project would fund 2 scientists, each for one year at OSU. In year one, a scientist will focus on molecular marker mapping strategies and in year 2 the second scientist will focus on transformation technology.

Training budget:

Post-doctoral salary and benefits at OSU:	$35,000 X 2 = $70,000
Travel	$ 2,000 X 2 = $ 4,000
Total training:	$74,000

Equipment:

A doubled haploid laboratory has been established at the Transitional Research Institute with USAID and World Bank support. This project would equip a laboratory at Uludag for non-radioactive molecular marker analysis and ancillary supporting

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equipment. Lease of a particle acceleration device may be negotiated.

Equipment budget:	
DNA extraction, purification, storage:	$75,000
DNA separation, visualization	$75,000
Lab support and computer base:	$50,000
Supplies:	$15,000
Total equipment:	$215,000

Cooperating Institution costs:	
Technical salary support:	$35,000 X 3 = $70,000
Supplies:	$10,000 X 3 = $30,000
Travel (annual to Turkey)	$2,000 x 3 = $ 6,000
Total cooperating institution:	$106,000

Total Project: $395,000

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Mapping of traits associated with winterhardiness in a winter x spring barley cross

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Abstract

The locations of quantitative trait loci (QTL) determining traits associated with winterhardiness – field survival, LT_{50}, growth habit, and crown fructan content – were determined using a set of 33 markers in a population of 100 F_1-derived doubled haploid lines from a winter x spring cross. There was significant transgressive segregation for all traits, indicating that either both parents contributed favorable alleles or that epistasis is an important determinant of trait expression. The 33 markers were resolved into seven linkage groups. The most complete coverage is provided for chromosomes 7, 5, and 4. Large QTL effects for all traits were found on chromosome 7. Lesser effects were detected on chromosome 5, and an interval, putatively on chromosome 2, is also an important determinant of trait expression. No QTL controlling target traits were found on chromosome 4. The largest QTL effects for all traits were detected in a region of approximately 21 recombination units on chromosome 7. Several lines of evidence support the hypothesis that trait association is due to linkage rather than pleiotropy. High fructan content and winter growth habit, although positively associated with cold tolerance, are not required for trait expression.

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

INTRODUCTION

The deceptively simple relationship between the initial stand of a winter barley crop and the number of healthy plants surviving to produce yield - winterhardiness - is the ultimate expression of interacting factors ranging from low temperature tolerance to biotic stress resistance. Foremost among these contributing factors are low temperature tolerance, vernalization requirement, photoperiod response, protein metabolism, carbohydrate status, and membrane lipid composition (Thomashow 1990). The genetic and physiological bases of most of these factors are only partially understood. We are addressing the genetic basis of winterhardiness by systematically measuring its components in a doubled haploid population of barley and relating these phenotypic data sets to molecular marker genotypes to identify quantitative trait loci (QTL).

In terms of trait assessment, cold tolerance poses a tremendous challenge. Long-term field survival is the final measure of winterhardiness (Olien 1978), but the expense and time required limit selec-

tion response. Temporally and spatially limited field-based genetic studies are fraught with genotype x environment interaction. Furthermore, the infrequent occurrence of a test winter that allows for effective discrimination among genotypes makes field-based selection impractical (McIntyre et al. 1988). Of the many biochemical and physiological tests available, controlled freeze tests to determine LT_{50} (the temperature at which 50% of the population is killed) most satisfactorily meet the criteria of simplicity, efficiency, and correlation with field performance (Stushnoff et al. 1984). Thomashow (1990), in reviewing the molecular basis of cold tolerance in higher plants, cites over 20 reports that, cumulatively, assign cold tolerance genes to every chromosome in each of the three genomes of wheat. Chromosomes 5A and 5D are most often implicated; Sutka and Snape (1989) and Roberts (1990) provide compelling evidence for the importance of chromosome 5A.

Vernalization and cold tolerance are associated, but trait association in barley (Doll et al. 1989) and wheat (Roberts 1990) is attributed to linkage rather than pleiotropy. Separation of growth habit into its components of vernalization requirement, photoperiod sensitivity, and maturity is challenging. Three unlinked loci have been identified which behave epistatically to determine winter vs. spring habit in barley

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(Takahashi and Yasuda 1971). The winter habit phenotype can be determined by both vernalization and photoperiod, and separation of these effects can be further complicated by the action of maturity genes (Roberts et al. 1988). Barnham and Rasmusson (1981) reported that photoperiod response, as distinct from maturity, is quantitatively inherited.

Carbohydrates, and particularly fructans, are reported to play a cryoprotective role in cereals, and a number of mechanisms have been proposed (Olien and Lester 1985). Livingston et al. (1989) found a marked effect of carbohydrate level on the freezing tolerance and sugar composition of barley crown tissue, including that of our winter parent (Dicktoo). However, there was no direct relationship between specific compositional makeup of total carbohydrate and freezing tolerance. Thus, while carbohydrate content and makeup may contribute to cryoprotection, their use as criteria for indirect selection for cold tolerance could be ineffective or could even lead to negative effects on partitioning that would have adverse effects on other agronomic traits.

Doubled haploids provide an immortal genetic reference population free of the non-additive types of gene action that complicate segregating generation analyses in autogamous species. They are also

ideally suited for linkage map construction and QTL mapping. The components of barley winterhardiness, which have posed challenges for conventional genetic analyses and thwarted selection efforts, are excellent targets for QTL mapping in doubled haploid populations.

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 x Morex. 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

We used three measures of cold tolerance: field survival at Corvallis, Oregon, field survival at Bozeman, Montana, and LT_{50}. Field survival at Corvallis 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. Field survival at Bozeman was measured as the difference between initial (October, 1991) and post-cold stress (March, 1992) plant stands in a three replicate experiment. 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 ten 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 measured using the heading date of unvernalized plant material under greenhouse conditions of 18°C day/night and 24 h light.

The crown content of fructans with a degree of polymerization (DP) $\geq$ 5 was determined using field hardened plant material. Five plants of each genotype were dug from field plots at Corvallis, OR 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 50 mg per sample were used for fructan extraction. 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 to 8 h. Dried samples were dissolved in 1 ml of 60°C water and centrifuged at 12,000 rpm for 10 min. The supernatant was filtered using a 0.45 micron filter prior to injection into the HPLC, which was equipped with BioRad carbo-C guard and BioRad HPX-42 C carbohydrate columns. Fructan content (≥ DP 5) is expressed as mg g^{-1} on a dry weight basis.

Map construction and QTL analysis

Markers, descriptions, and chromosome assignments are listed in Table 1. The following conventions were used. The prefixes "m" and "i" designate morphological and isozyme markers, respectively. Clones supplied by Cornell University were named following the nomenclature of Heun et al. (1990), where the prefixes WG, BCD, and CDO designate wheat genomic, barley cDNA, and oat cDNA clones, respectively. Requests for these clones should be directed to: Dr. Mark Sorrells, 252 Emerson Hall, Cornell University, Ithaca, NY 14853-1902. The ABC

prefix designates a cDNA clone developed by the North American Barley Genome Project. Requests for these clones should be directed to: Dr. Andy Kleinhofs, 271 Johnson Hall, Washington State University, Pullman, WA 99164-6420. The "ap" prefix designates known DNA sequence polymorphism visualized by polymerase chain reaction. Requests for primer sequences should be directed to: Dr. Tom Blake, 320 Leon Johnson Hall, Montana State University, Bozeman, MT 59715.

Detailed mapping protocols, including restriction enzyme choice and polymorphic fragment size data, are available upon request from Dr. F. Chen, Crop Science Building 248, Oregon State University, Corvallis, OR 97331-3002. Briefly, for RFLP analysis, plant DNA was prepared from fresh tissue using the proteinase k procedure. DNA samples (10 μg) were digested with appropriate restriction endonucleases, separated on agarose gels and transferred to charged nylon membranes using the alkaline protocol of Reed and Mann (1985). Cloned DNA inserts were cut from low melting point agarose and labeled by random priming (Feinberg and Vogelstein 1984). Filters were prehybridized, hybridized overnight, washed with a final stringency of 0.1 XSSC, 0.1% SDS, 65 C, and exposed to film as needed. PCR markers were

based on published sequences or on partial sequencing of the termini of informative RFLP clones. Sequencing and oligonucleotide synthesis procedures were described by Tragoonrung et al. (in press). Isozymes were assayed as described by Nielsen and Johansen (1986) and storage proteins were assayed as described by Blake et al. (1982). The morphological markers were scored under a stereomicroscope or with a hand lens.

G-Mendel 2.0, an unreleased version of G-Mendel (Liu and Knapp 1990) was used for linkage map construction. Locus order was determined by simulated annealing, and multi-point recombination frequencies were estimated using the maximum likelihood algorithm of Lander and Green (1991). Quantitative trait loci were mapped using QTL-STAT, an interval mapping program that uses linear models to estimate QTL parameters (Liu and Knapp, unpublished). This software expresses QTL in terms of a single and multilocus ANOVAs and by plotting LODs ($\log_{10}$ of the maximum likelihood estimate ratios) against specific map segments. To separate QTL effects in adjacent intervals, we used the non-linear model procedure of Knapp (1991).

RESULTS AND DISCUSSION

Map construction

The 33 markers were resolved into seven linkage groups (Table 1 and Figure 1). One group (3) is anchored by the *mHs* morphological marker on the long arm of chromosome 4. The log likelihood for this map is -141.6, and it spans a region of 56.9 recombination units. Two groups (4 and 5) are anchored by known-location isozyme markers on chromosome 5. The log likelihood for the group 4 map is -68.1 and the map is 30.18 recombination units long. The group 5 map spans a region of 31.7 recombination units; the log likelihood -97.2. We have yet to identify markers that will join the two groups and incorporate *HOR3*. No recombination was detected between the *iEST1*, *iEST4* and *ABC173c* markers on chromosome 3 (group 2). The group 6 map is anchored by known location morphological (*mR* and *mS*) and DNA (*Rm2*) 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. More markers are needed to unequivocally determine the chromosome location of the group 1 markers. However, *CDO64* and *BCD175* were reported to be 43 cM apart on chromosome 2 (Heun et al. 1991). We found 30.9% recombination between these loci (log likelihood -61.9). The chromosome

locations of *HOR3* and *iACO-1* are known, but they were not linked to any of the markers we used. The chromosome location of four markers remains undetermined. Linkage maps for chromosomes 4, 5, 7, and two segments without unequivocal chromosome assignments are presented in Figure 1.

Cold tolerance

Each measure of cold tolerance gave a distinct distribution (Figures 2a, 2b, and 2c). 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. At Corvallis, eight lines had 100% survival and only two showed complete mortality. At Bozeman, 50 lines suffered complete mortality and the highest survival was 85%. The hardening and freezing stresses used to determine LT_{50} led to a more normal distribution of trait performance: LT_{50} values ranged from - 2.7 to - 11.0 °C. The transgressive segregants for cold tolerance indicate that the spring parent, Morex, may have contributed favorable alleles and/or that epistasis is an important determinant of trait expression. When the top fifteen survivors were tabulated for each of the three measures of cold

tolerance, there were only four lines in common: DH 16, DH 33, DH 50, and DH 67 (Figure 4); for the two-way comparisons, an average of seven lines were in common. These modest associations of trait performance measured in distinct environments typify the genotype x environment interaction that has confounded prior genetic analyses.

The QTL analysis approach provides a clearer picture of trait relationship, and it confirms that the three measures have a common genetic basis (Figures 3a, 3b and 3c). As Knapp and Bridges (1990) have observed, 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.

Large QTL effects for all measures of cold tolerance were found on chromosome 7 (Figures 3a, 3b, and 3c). For field survival in Oregon, QTL effects exceeding the LOD $\geq$ 3.0 threshold were detected in every interval except *mS - WG364b*. The r^2 for the *mR - BCD265b* interval is 0.40. In the multi-locus 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 exceeding the LOD $\geq$ 3.0 threshold were detected in three

intervals: *BCD298 - mS*, *WG364b - mR*, and *mR - BCD265b*. Only the latter interval was identified in the multi-locus ANOVA. The r^2 was 0.68. For LT_{50}, LODs ≥ 3.0 were detected in the following intervals: *Rm2 - apADH*, *WG364b - mR* and *mR - BCD265b*. Only the *mR - BCD265b* interval was identified in the multilocus model, with an r^2 of 0.37.

A modest QTL effect for field survival in Oregon was detected in the *apHRTH - BCD265c* interval on chromosome 5, with a maximum LOD of 3.2. The r^2 for this interval was 0.13 and it was identified in the multi-locus ANOVA. The same distribution of LODs was coincident for Montana field survival and LT_{50}. However, the ≥ 3.0 threshold was not exceeded.

Because this represents a first attempt to map QTL associated with cold tolerance in barley, it is difficult to put our findings in the perspective of previous reports. We reported significant segregation distortion, in favor of the winter parent, in the vicinity of *Rm2* in the F_2 progeny of Dicktoo X Morex F_1 plants self-pollinated under low temperature (Schön et al. 1991). No segregation distortion was detected in the vicinity of *mR*. In the case of Oregon field survival and LT_{50}, modest QTL effects were detected in the vicinity of *Rm2*. In the face of popula-

tion frequency distributions such as those shown in Figures 2a, 2b, and 2c, previous investigators have resorted to classical quantitative procedures to estimate genetic variances and heritability. The results have not been particularly satisfying. For example, Rhode and Pulham (1960) reported heritabilities for field survival ranging from -8% to 85%. The availability of appropriate cytogenetic stocks in wheat has allowed for the measurement of chromosome effects, and both 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 and Shepard 1981).

Growth habit

Vernalization response, maturity, and photoperiod response genes interact to determine winter vs. 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. The significant transgressive segregation and discontinuous expression of heading date under 24 light, referred to subsequently as growth habit, can be attributed to multigenic control. As with the measures of cold tolerance, both parents may have contributed winter growth habit alleles, or trait expression is the result

of epistasis (Figure 2d). 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. It is already clear from a series of ongoing experiments involving various permutations of vernalization, photoperiod, and ambient temperature, that the winter phenotype observed under field conditions is attributable to photoperiod sensitivity rather than to a vernalization requirement. An examination of several 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.

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 vs. spring growth habit is determined by the epistatic interaction of 3 loci: *Sh* on chromosome 4; *sh2* on chromosome 7; and *sh3* on chromosome 5. The allelic constitution *ShShsh2sh2sh3sh3* is required for expression of winter habit and all other allelic combinations give spring growth habit. While there is reasonable fit to a two-locus, 3:1

model (Chi square = 2.61; $p > .100$) based on the grouping of all phenotypes earlier maturing than Dicktoo vs. those later than the winter parent, such a grouping ignores the presence of transgressive segregants on both sides of the distribution.

Based on the level of genome saturation that we have achieved to date, it would appear that Dicktoo and Morex may carry contrasting alleles at only one of the *Sh* loci. The *Sh* locus is reported to be linked to the *mHs* (Takahashi and Yasuda 1971), but LODs for growth habit were $\leq$ 1.0 throughout the entire length of chromosome 4. Significant QTL effects for growth habit were not detected on chromosome 5 (site of the *Sh3* locus), although, as with Montana field survival and LT_{50}, there was a reduced mirror image of the Oregon field survival QTL. Large QTL effects (LOD $\geq$ 14) were detected on chromosome 7, again in the *WG364b* - *mR* and *mR* - *BCD265b* intervals (Figure 3d). Takahashi and Yasuda (1971) reported 13.1% recombination between *mR* and the *Sh2* locus. The region between *mR* and *BCD265b* is the region showing the largest QTL effect and, as described in the section "Evidence for a multilocus cluster", this region was identified as most likely to carry the QTL by the non-linear model analysis. For this interval, the Dicktoo and Morex QTL genotype means are 62.3 days and 37.0 days,

respectively. In the *BCD175* to *CDO64* interval the Dicktoo and Morex QTL genotype means are 37.7 and 60.3 days, respectively, indicating that Morex is contributing an allele that led to the expression of transgressive winter growth habit in the progeny. LODs in this interval ranged from 3.47 to 10.47. The multilocus model accounted for 65% of the variation in trait expression with the following two intervals: *mR* - *BCD265b* and *BCD175* - *CDO64*. Extension of the map will test the validity of the preliminary assignment of this interval to chromosome 2 and thus allow for relating the presence of QTL with known-location maturity loci. Completion of ongoing experiments characterizing photoperiod, vernalization, and temperature responses will hopefully clarify the inheritance of growth habit in this population.

Fructan content of crown tissue

The parental polymorphism for fructan content, together with the wide range of crown fructan content in their progeny (Figure 2e) provides a unique opportunity to simultaneously assess the role of fructan content and cold tolerance. The population distribution is approximately normal and skewed toward the Morex phenotype. Morex had a fructan content of 63.8 mg g^{-1}. The transgressive segregants with fructan

substantially higher than Dicktoo (137 mg g^{-1}), indicates that both parents may have contributed alleles for fructan accumulation.

The QTL analysis revealed substantial effects on chromosome 7 (Figure 3e). The LOD plots follow approximately the same pattern as those for field survival, LT_{50}, and growth habit. Lesser QTL effects (maximum LOD = 2.2) were detected in both intervals of the group 4 markers on chromosome 5. For both chromosomes 5 and 7, the Dicktoo QTL genotype had the highest fructan. In the putative chromosome 2 interval(*CDO64* - *BCD175*) Morex contributed the high content allele (maximum LOD = 2.7). Two intervals - *mR* to *BCD265b* and *BCD175* to *CDO64* were identified in the multilocus model; together they account for 65% of the variation in trait expression.

Evidence for a multi-locus cluster

The QTL data raise intriguing issues regarding numbers of QTL, types of gene 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). The following tests were performed using a non-linear model procedure (Knapp 1991) in an attempt to determine if there are indeed two QTL or really one QTL

with an overwhelming effect: L1 = Q_1 vs Q_1 + Q_2; L2 = Q_2 vs Q_1 + Q_2; L3 = Q_1 vs. no Q; L4 = Q_2 vs. no Q; and L5 = Q_1 + Q_2 vs. no Q. For all traits the likelihood ratios and p value for L4 support the hypothesis that there is a single QTL at Q_2. For example, for LT_{50} L4 = 29.4 and p = 1.0×10^{-10}. Likelihood ratios of 6.6, 0.03, 15.8, and 11.2 and p values of 4.0×10^{-4}, 9.9×10^{-1} , 15.8×10^{-6} and 1.3×10^{-8} were obtained for L1, L2, L3, and L5, respectively. There is, therefore, strong evidence to support the hypothesis that there are loci controlling cold tolerance, growth habit, and crown fructan content in the *mR - BCD2-65b* region. The adjacent interval may contain genes associated with trait expression, or the apparently large QTL effects may be attributable to a "spill-over" effect. Further map saturation should more precisely reveal QTL locations.

We provide evidence that there are QTL controlling multiple characteristics associated with the winterhardy phenotype on the long arm of chromosome 7. Are there 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 Figures 4a to 4f. 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 to 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 days - 108 days). Three 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 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-67 may, therefore, have resulted from a crossover between the fructan locus and the survival and growth habit loci.

A second line of evidence favoring linkage over pleiotropy is provided by Roberts (1990), who proposed that two and probably more loci on chromosome 5A of wheat determine cold hardiness and vernalization. One of these loci is, or is tightly linked to, *Vrn1*, and *Vrn1* can be considered homoeologous to the *Sh2* locus of barley. Although

Roberts did not recover any recombinants between his putative cold hardiness locus and *Vrn1*, and could therefore not rule out pleiotropy, he computed a maximum opportunity for crossover of less than 3%. Finally, 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 vs. spring - is intuitively appealing and supported by Allard's (1988) finding that natural selection favors the development of multi-locus clusters conferring adaptation.

These data have important implications for cold tolerance breeding efforts. Some improvement in cold tolerance may be possible, even in a winter x spring cross. Fructan content and growth habit QTL appear to be closely linked to cold tolerance QTL on chromosome 7, but elevated fructan content and winter growth habit are not required for a genotype to achieve a high level of cold tolerance. While the precise relationship of fructan accumulation with cold tolerance remains to be elucidated, growth habit can have a profound effect on agronomic competitiveness. Spring habit, cold tolerant genotypes may be able to withstand a mid-

winter stress, but their early heading date puts them at risk from frost damage at anthesis.

Completion of a medium density genome map in this cross, and development of a comparable map in a winter x winter cross, will hopefully allow for a more complete integration of QTL data with known location genes and narrow the intervals in which QTL effects are measured. This, in turn, should facilitate marker-assisted selection. With somewhat narrower intervals flanking cold tolerance loci mapped in the progeny of Dicktoo X Morex, we should be able to introgress cold tolerance and growth habit loci into spring barley backgrounds via marker assisted backcrossing. However, the end product would likely be somewhat less cold tolerant than the donor parent. Fowler and Gusta (1979) stated that genetic variability for cold tolerance may be exhausted and that hardy cultivars may possess all available major genes. We have shown that transgressive segregants can be recovered, even in the progeny of a winter x spring cross, and we are hopeful that extension of our mapping efforts into winter germplasm will reveal polymorphism that can be manipulated to achieve significant improvement in cold tolerance.

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Table 1. Descriptions, chromosome location, and linkage group assignments for markers used to locate quantitative trait loci in the doubled haploid progeny of Dicktoo X Morex. Definitions of marker prefixes are provided in the text.

Marker	Description	Chromosome	Linkage Group
BCD175	Barley cDNA RFLP	2	1
CDO64	Oat cDNA RFLP	2	1
iEST1	Esterase 1	3	2
iEST4	Esterase 4	3	2
ABC173c	Barley cDNA RFLP	3	2
WSEP	H_20-soluble endosperm protein	4	3
BCD265a	Barley cDNA RFLP	4	3
WG1026b	Wheat genomic RFLP	4	3
apGLU2	ß-glucanase sequence primer (Glu-2)	4	3
mHs	Hairy leaf sheath	4	3
apAHRTH	Hordothionin sequence primer (Hrth)	5	4
BCD265c	Barley cDNA RFLP	5	4

(Table 1 cont.)

Marker	Description	Chromosome	Linkage Group
iPGD2	Phosphogluconate dehydrogenase 2	5	4
iGPI1	Glucosephosphate isomerase 1	5	5
HOR1	Hordein C	5	5
HOR2	Hordein B	5	5
ABC173a	Barley cDNA RFLP	5	5
pTA71	Rrn-2 RFLP	7	6
apADH	Alcohol dehydrogenase- 2 sequence primer	7	6
BCD 298			
mS	Rachilla hair length	7	6
WG364b	Wheat genomic RFLP	7	6
mR	Awn roughness	7	6
WG1026a	Wheat genomic RFLP	7	6
BCD265b	Barley cDNA RFLP	7	6
BCD410	Barley cDNA RFLP	?	7

(Table 1 cont.)

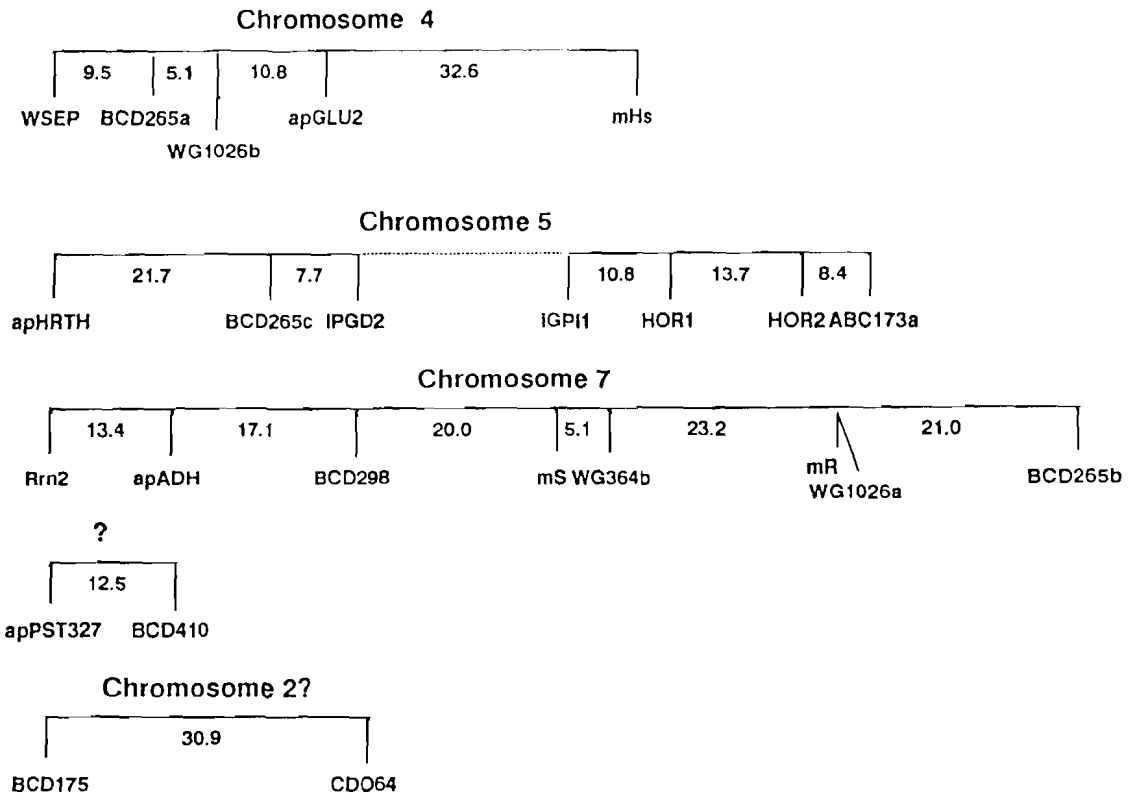
Marker	Description	Chromosome	Linkage Group
apPST327	Pst-genomic clone primer	?	7
HOR3	Hordein D	5	unlinked
ACO1	Aconitate hydratase 1	6	unlinked
LMWSP	Low molecular weight storage protein	?	unlinked
aPST337	Pst-genomic clone primer	?	unlinked
WG223	Wheat genomic RFLP	?	unlinked
WG364a	Wheat genomic RFLP	?	unlinked

Figure 1. Linkage maps based on segregation of 33 markers in 100 F_1-derived doubled haploid lines from the cross of Dicktoo X Morex. Map distances are expresses as percent recombination. Markers are defined in Table 1.

Figure 2. Frequency distributions for field survival in Oregon (a), field survival in Montana (b), LT_{50}, growth habit (d), crown fructan content (e) of 100 F_1-derived doubled haploid lines from the cross of Dicktoo X Morex.

Figure 3. Chromosome seven LOD values for field survival in Oregon (a), field survival in Montana (b), LT_{50}, growth habit (d), crown fructan content (e) of 100 F_1-derived doubled haploid lines from the cross of Dicktoo X Morex.

Figure 4. 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).



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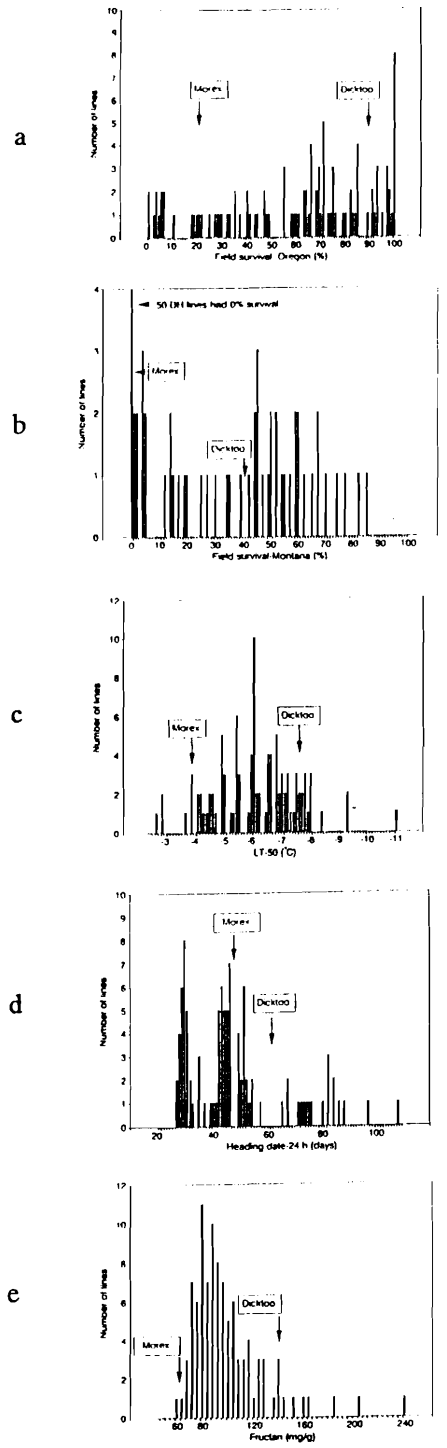

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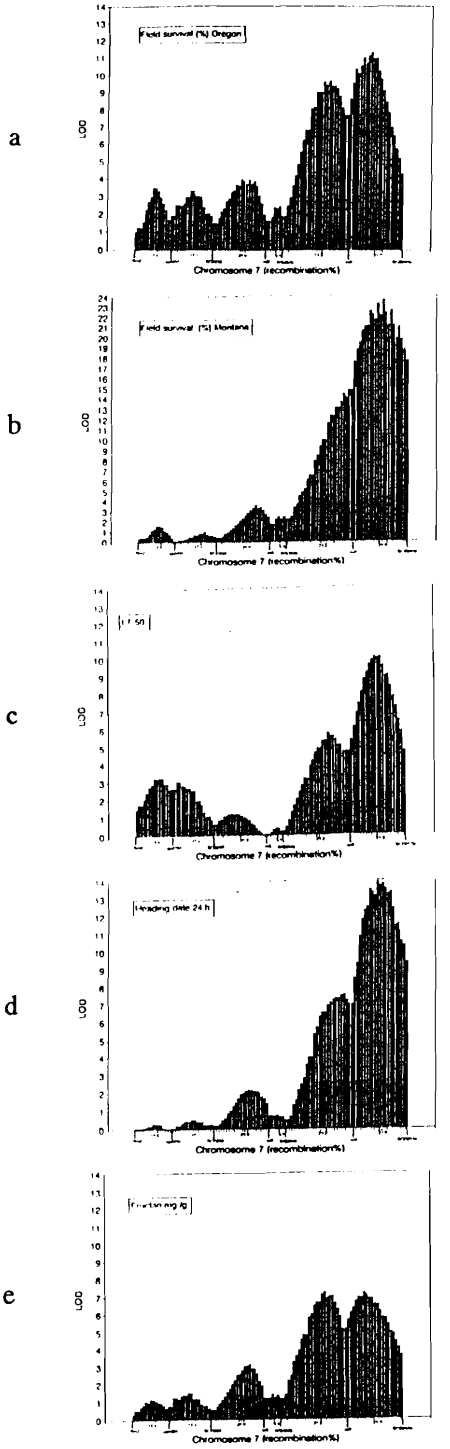

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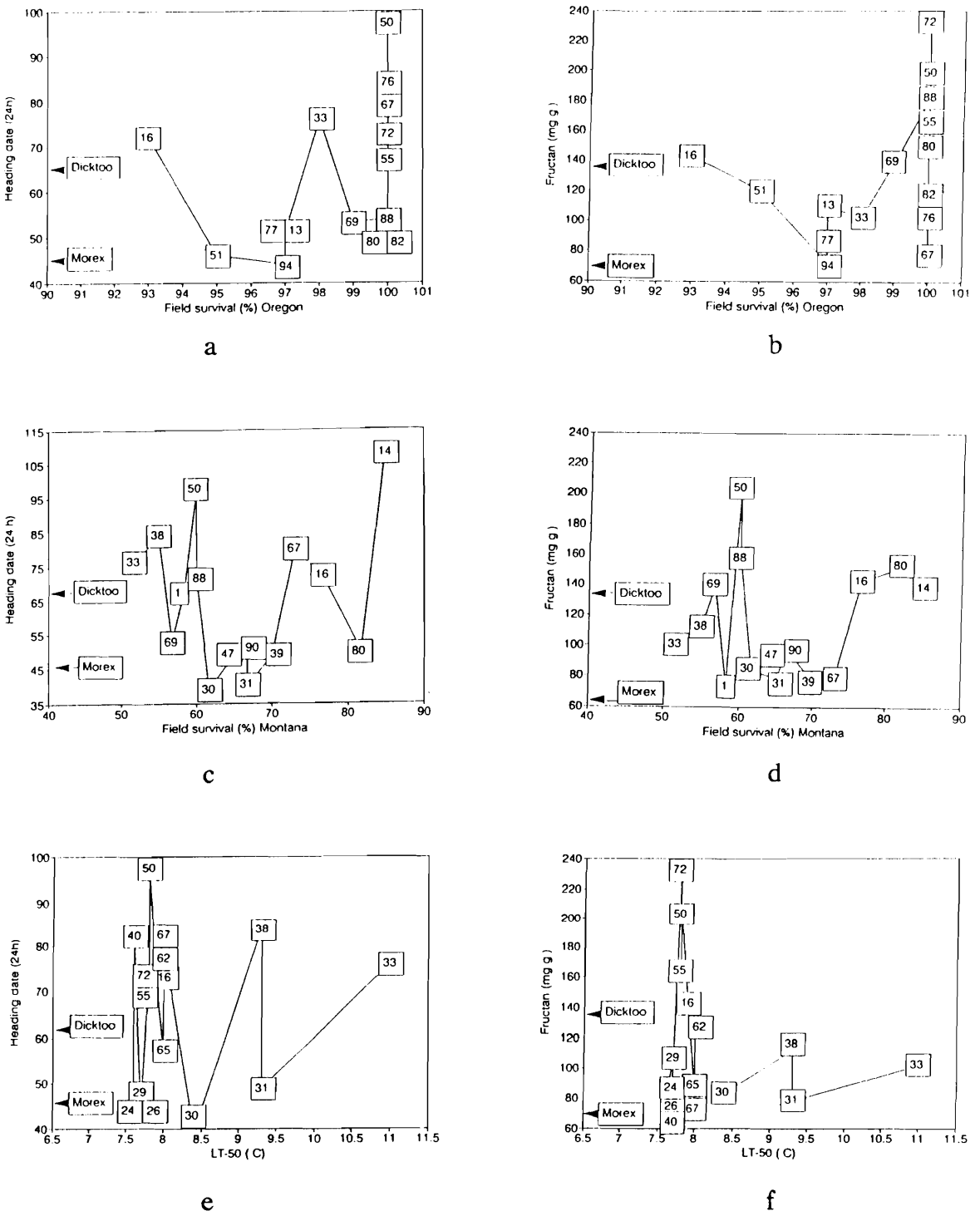

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