Genetic studies of cold tolerance in barley
by Somvong Tragoonrung

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Crop and Soil Science
Montana State University

Abstract:
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One hundred doubled haploid lines derived from F1 plants from a cross between Dicktoo (winter) x Morex (spring) were used to study the genetics of field survival, LT50, fructan content, flowering date and prostrate growth habit. A medium density linkage map was constructed from these doubled haploid lines using DNA, protein isoenzyme, and morphological markers. This map was then utilized to identify the location of quantitative trait loci (QTL) associated with these traits. Sequence-tagged-sites (STS) were developed for polymerase chain reaction as DNA (PCR) markers.

Significant correlations between crown fructan content and winter hardiness parameters were detected. These results suggested that fructan content may be one factor contributing to cold tolerance. However, selection based on high fructan content did not positively improve other winterhardiness associated traits.

The STS-PCR system facilitated map construction in this cross. A set of 77 DNA markers were resolved into eight linkage groups and distributed to seven chromosomes. Large QTL effects for fructan content and all winter hardiness parameters except prostrate growth habit were detected in a region of approximately 20 centiMorgans on the long arm of chromosome 7. Careful analysis of lines carrying recombination breakpoint within this 20cM interval suggested that at least 2 genes affecting measured parameters were located within this interval. This interval analysis suggested that the association among at least some of these traits is due to linkage rather than pleiotropy. Smaller QTL effects modifying characters measured in this study were detected on chromosome 2 and chromosome 5.

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APPROVAL

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Somvong Tragoonrung

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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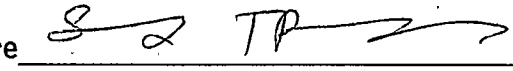
Signature 

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ABSTRACT

The poor efficiency of selection for improved cold tolerance in winter barley has been attributed to a combination of complex inheritance and difficulties in trait assessment. Several physiological parameters have been used as selection criteria for cold tolerance. However, definitive analysis of the Mendelian factors controlling these parameters has not yet been performed. The objectives of this research were to genetically investigate the relationships among characters associated with winter survival and to identify the chromosomal locations of genes controlling those traits.

One hundred doubled haploid lines derived from F_1 plants from a cross between Dicktoo (winter) x Morex (spring) were used to study the genetics of field survival, LT_{50}, fructan content, flowering date and prostrate growth habit. A medium density linkage map was constructed from these doubled haploid lines using DNA, protein isoenzyme, and morphological markers. This map was then utilized to identify the location of quantitative trait loci (QTL) associated with these traits. Sequence-tagged-sites (STS) were developed for polymerase chain reaction as DNA (PCR) markers.

Significant correlations between crown fructan content and winter hardiness parameters were detected. These results suggested that fructan content may be one factor contributing to cold tolerance. However, selection based on high fructan content did not positively improve other winterhardiness associated traits.

The STS-PCR system facilitated map construction in this cross. A set of 77 DNA markers were resolved into eight linkage groups and distributed to seven chromosomes. Large QTL effects for fructan content and all winter hardiness parameters except prostrate growth habit were detected in a region of approximately 20 centiMorgans on the long arm of chromosome 7. Careful analysis of lines carrying recombination breakpoint within this 20cM interval suggested that at least 2 genes affecting measured parameters were located within this interval. This interval analysis suggested that the association among at least some of these traits is due to linkage rather than pleiotropy. Smaller QTL effects modifying characters measured in this study were detected on chromosome 2 and chromosome 5.

CHAPTER 1

INTRODUCTION

Its limited ability to survive cold stress limits the production of winter barley, a crop with tremendous potential for diversifying the prevailing winter wheat monoculture of North America. Several attempts have been made to understand the genetic and physiological control of winter hardiness in cereals. The association of fructan accumulation with cold tolerance is one of the physiological parameters that has been identified. However, accurate estimates of the genetic dependence of winter hardiness on fructan content have not been reported.

The complex genetic basis of cold tolerance has traditionally been studied through techniques of quantitative analysis. The development of molecular marker technologies permitted the rapid construction of genetic maps and evaluation of the genetic control of quantitative traits. In barley, isozymes, seed storage proteins, and DNA markers have been used in linkage analysis experiments. The resulting linkage maps have permitted the description of the genetic control of quantitative characters.

Traditionally, DNA markers were identified using Southern blotting to detect restriction fragment length polymorphisms (RFLPs). A rapid, non-radioactive, and practical technique, polymerase chain reaction (PCR) has been used to detect RFLPs in many genome mapping projects. The technique requires short sequence primers which promote the selective amplification of target DNA fragments. The Sequence-Tagged-site (STS) approach helped span the gap between RFLP-based mapping projects and PCR technology.

Doubled haploid techniques can provide immortal genetic reference populations free of 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. A doubled haploid population from a spring x winter barley was used in this study. Our objectives were i) to genetically study the relationship between fructan accumulation and winter hardiness; ii) to develop the STS-PCR map technology; iii) to generate the genetic map of the cross and locate the QTL controlling winter hardiness and its associated characters.

CHAPTER 2

GENETICS OF FRUCTAN CONTENTS AND WINTERHARDINESS IN A WINTER X SPRING BARLEY

Introduction

Several studies on the relationship between fructan accumulation and winter hardiness have been reported in many plant species including barley (Pontis and del Campillo, 1985; Chatterton et al, 1988; Chatterton et al, 1989; Pontis, 1989; Suzuki and Nass, 1987; Livingston et al, 1989). Chatterton et al (1989) demonstrated that temperature had a profound effect upon the accumulation of total nonstructural carbohydrates in many grass species. Exposure of many cereals and grasses to low temperatures induced large accumulation of fructan and sucrose (Pontis and Del Campillo, 1985). In barley, Livingston et al (1989) demonstrated that fructan content was increased in cold acclimated plants and that stored fructans were hydrolyzed when the plants reinitiated growth at normal temperatures. Long chain fructans (high DP fructans) were found to be more closely associated with cold hardiness than low DP fructans (Livingston 1991, Suzuki and Nass, 1987). Although the presence of fructans in plant appeared to be advantageous for winter hardiness (Pointis and Del Campillo, 1985), no direct evidence of cryoprotection by fructans has been presented (Pollock, 1986).

The genetic and physiological bases of winterhardiness are poorly understood. Physiological measurements which have been commonly used to estimate cold hardiness in cereals include long-term field survival (Olien, 1978) and controlled freeze tests to determine LT_{50} (temperature at which 50% of the plant population is killed) (Pomeroy and Fowler,

1973).

While the genetic control of winterhardiness has not yet been elucidated, winter and spring growth habit in barley were found to be controlled by the action of alternative alleles at three unlinked loci. These genes strongly affected vernalization requirement, photoperiod response, and maturity (Takashashi and Yasuda, 1969). Epistasis at these three loci made it difficult to study gene action directly. Takahashi and Yasuda (1969) found that the genetic control of growth habit could more easily be studied if plants were grown in controlled environments under 24 hour lighting. Doll et al. (1989) reported that vernalization requirement is associated with cold tolerance.

In this experiment we studied the genetic control of crown fructan accumulation in a set of doubled haploid lines which derived from a simple cross between a cold-susceptible spring barley and a hardy winter barley. Our objectives in this study were i) to provide the first genetic investigation of fructan accumulation, winter hardiness and related traits in a population of lines derived from a winter x spring barley cross ii) to evaluate the relationships between physiological characters associated with winter survival in this population of lines and iii) to determine how much of the apparent association between characters was due to pleiotropy.

Materials and Methods

Plant Material

One hundred doubled haploid (DH) lines were developed using the *Hordeum bulbosum* technique, as described by Chen and Hayes (1989), from F_1 plants of the cross of Dicktoo x Morex. Dicktoo (CI 5529) is a six-rowed

winter feed barley of poorly characterized ancestry which was released by the Nebraska Agricultural Experiment Station in 1952 . Morex (CI15773) is a six-rowed spring malting barley released by the Minnesota Agricultural Experiment Station in 1978 (Rasmussen, 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 randomized complete block experiment containing three replications. Mean survival (MeanSUR) was the mean of the field survival estimates at both locations. 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 was 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).

Heading date was estimated using the heading date of unvernalized plant material under greenhouse conditions of 18°C day/night and 24 h light.

The content of fructans with a degree of polymerization (DP) $\geq$ 5 was determined using crowns from field hardened plants. Five plants of each genotype were dug from field plots at Corvallis, OR in January, 1992. In

1991, only the fifteen least and most hardy based on field survival measurement at Corvallis, OR in 1991 were sampled for crown fructan analysis. 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. Five 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 the eluate volume adjusted to 5 ml with 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 through a 0.45 micron filter prior to injection into the HPLC, which was equipped with a BioRad carbo-C guard column and a BioRad HPX-42 C carbohydrate column. Fructan content (≥ DP 5) was expressed as mg g^{-1} on a dry weight basis (mg/gdw).

RFLP and Correlation Analysis

Seventy-seven markers were used in this experiment. Genomic and cDNA clones were supplied by Cornell University, the North American Barley Genome Project or were among those described in Shin et al.(1991). PCR markers were developed as described by Tragoonrung et al (in press, Chapter 3 in this thesis). Briefly, for RFLP analysis, plant DNA was prepared from fresh tissue using the technique of Dellaporta et al.(1984). DNA samples (10 μg) were digested with appropriate restriction endonucleases, separated on 0.8% agarose gels in Tris-Borate buffer and transferred to charged nylon membranes using the alkaline transfer 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. 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 using a stereomicroscope or with a hand lens.

Results and Discussion

Fructan chromatograms of Dicktoo and Morex cultivars harvested under both hardening and non-hardening conditions are shown in Figure 1. When non-hardened crown tissue was used, fructan was not detected in either Dicktoo or Morex. After hardening in the field in 1992, Dicktoo crowns contained 137 mg fructan/gdw, while Morex crowns contained 66.2 mg fructan/gdw. In 1991, barley plants grown in the greenhouse were transferred to a growth chamber and hardened at 2°C for five weeks. Fructan contents of Dicktoo and Morex were 90.9 and 15.7 mg fructan/gdw, respectively. When compared to fructan standards, the major fructan species which differed in concentration between Dicktoo and Morex were DP5 or greater. Lower DP (DP3-DP5) fructans were detected in plants which were acclimated in the growth chamber (1991). Field hardened plants (1992) contained less DP3-DP5 fructan than growth chamber acclimated plants. Similar resulted were reported by Chatterton et al. (1989) and Pollock (1986).

The range and mean of crown fructan content, LT_{50}, field survival, and heading date are shown in Table 1. Specifics on the distribution of

these traits were reported in our companion paper (Hayes et al, submitted). Fructan content of tested doubled haploid lines in 1991 was less than that of 1992 (Table 1). This may be due to differences in growth stages of the plants at harvest, or to environmental variation. Plant tissues collected from the field were larger and had been dormant longer than the plants grown in the greenhouse and acclimated in growth chambers. Levitt (1980) reported that the degree of winter hardiness of plants depended upon developmental stage and environmental conditions. Average field survival at Corvallis, OR (ORSUR) was higher than that at Bozeman, MT (MTSUR). Half of the doubled haploid lines screened at Bozeman, MT suffered complete winterkill. Field survival of Morex were 10%, and 0% at Corvallis, OR and Bozeman, MT, respectively. Dicktoo showed 82% survival at Corvallis and 62% survival at Bozeman. LT_{50} and heading date for Dicktoo and Morex were -5.5°C and 62 days, and -3.9°C and 45 days, respectively.

Table 1. Mean, minimum, and maximum values of physiological traits for winter hardiness.

Traits	Min	Mean	Max
Fructan91	0.00	36.00	103.00
Fructan92	58.60	97.80	237.40
LT_{50}	-11.00	-6.07	-2.70
Heading date	27.00	47.18	97.00
ORSUR	1.00	53.73	100.00
MTSUR	0.00	25.08	85.00

Fructan91 = fructan contents measured in 1991; Fructan92 = fructan contents measured in 1992; ORSUR = field survival at Corvallis, OR in 1991; MTSUR = field survival at Bozeman, MT in 1992.

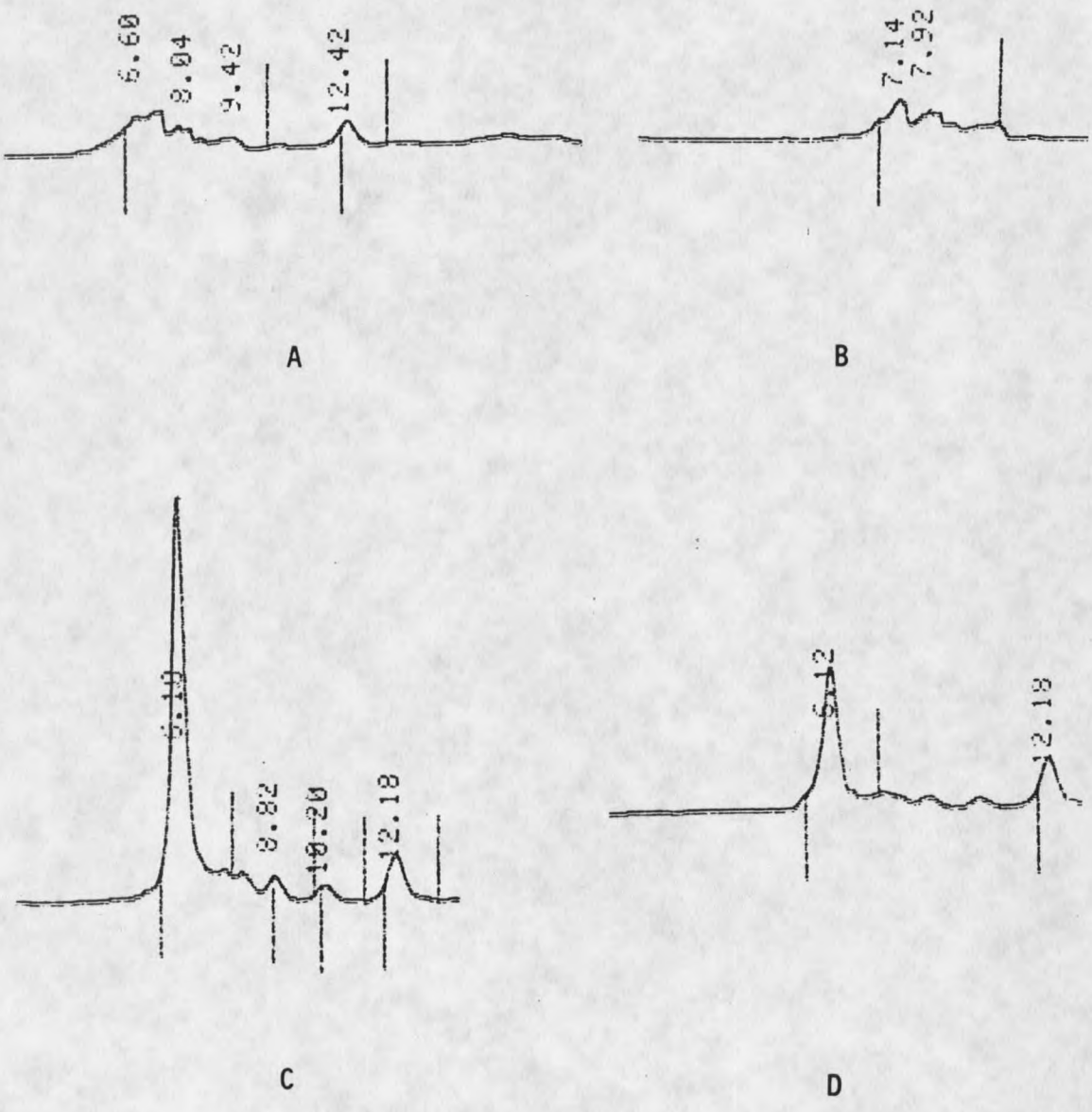

Figure 1. HPLC chromatograms of fructan contents for Dicktoo and Morex cultivars. A) Fructan chromatogram of non-hardened Dicktoo tissue. B) Fructan chromatogram of non-hardened Morex tissue. C) Fructan chromatogram of hardened Dicktoo. D) Fructan chromatogram of hardened Morex tissue.

Correlations among crown fructan content and physiological parameters associated with winter hardiness are shown in Table 2. Significant correlations were observed between crown fructan content and each of the other measured parameters in the 1992 experiment. In 1991, only the fifteen most and least hardy lines were sampled for crown fructan content. As would be expected, the reduced sample size relative to the 1992 experiment resulted in reduced experimental sensitivity.

Table 2. Correlation coefficients between fructan content in 1991 and 1992 and physiological parameters.

Traits	Fructan91	Fructan92
LT_{50}	0.29^{ns}	0.27^{*}
Heading date	0.24^{ns}	0.47^{**}
ORSUR	0.63^{**}	0.47^{**}
MTSUR	0.57^{**}	0.41^{**}
MeanSUR	0.60^{**}	0.50^{**}

** Significant level at 1%
* Significant level at 5%
ns Non-significant.
ORSUR = field survival at Corvallis, OR in 1991; MTSUR = field survival at Bozeman, MT in 1992; MeanSUR = mean field survival from both locations.

Field survival is among the most error-prone measurements commonly made by plant scientists. We compared the similarity of tails of distributions had selection of tails been performed at either Bozeman, Corvallis, or using their overall mean value (Table 3). The simple correlation value between crown fructan content and field survival was higher using data from Corvallis than that at Bozeman. The correlation between fructan content in 1991 and 1992 was 0.46. Obviously, if one is going to perform a tailed-distribution analysis (Lander and Botstein, 1989) one must be careful in obtaining the distribution upon which the

tails are selected.

The correlation between fructan content and LT_{50} was much lower than that reported by Livingston at al. (1989). This may result from the difference in sample size or reduced dependence of LT_{50} on crown fructan content in progeny from this cross. Significant positive correlations were found between LT_{50} and heading date, LT_{50} and MEANSUR, and heading date and MEANSUR (0.43, 0.45, 0.5, respectively).

Table 3. Percentage of lines in common among physiological traits at 15 and 25% selection intensities.

Traits	LT_{50}	Heading date	Fructan92
15% Dicktoo type			
Oregon survival	40	33	47
Bozeman survival	33	33	40
Heading date	56	100	60
LT_{50}	100	56	36
15% Morex Type			
Oregon survival	20	33	13
Bozeman survival	ND	ND	ND
Heading date	20	100	33
LT_{50}	100	20	26
25% Dicktoo type			
Oregon survival	44	60	40
Bozeman survival	52	52	48
Heading date	56	100	48
LT_{50}	100	56	36
25% Morex type			
Oregon survival	40	32	20
Bozeman survival	ND	ND	ND
Heading date	48	100	36
LT_{50}	100	48	28

ND= Data not determined.
Fructan92 = fructan contents measured in 1992.

To determine how effectively selection for any of these characters would result in correlated selection for another of these characters, we calculated the percentage of common lines among distribution tails (Tragoonrung et al., 1990). The percentage of line in common for pairs of characters using tails comprising either 15% and 25% of each distribution are shown in Table 3. If selection were performed solely on the basis of high fructan content, approximately 50% of the lines selected would show good survival and delayed heading date. Thirty-six percent of lines with high fructan content showed low LT_{50}.

It has been suggested that fructans may act as tissue cryoprotectant by modifying ice crystal formation (Olien and Lester, 1985). It has also been suggested that delayed spring heading and low LT_{50} values have independent functions in attaining the ultimate phenotype needed for successful overwintering. To determine the association of winterhardiness traits and genetic markers, a set of markers was used for correlation analysis with the four physiological characters. Four markers showed significant correlations for all the traits (Table 4). These four markers map to the long arm of barley chromosome 7 and comprise a fairly tightly linked set of loci. Six other markers showed significant correlations with fructan content and a combination of significant correlations with the other measured characters. This suggests that one gene or gene cluster on the long arm of barley chromosome 7 coordinately modifies crown fructan content, LT_{50}, winter survival, and flowering date. This could result from either linkage or pleiotropy. On chromosome 5 there is another locus in the vicinity of the gene encoding D hordein which has significant impact on both crown fructan content and survival at Corvallis

Oregon, but which showed little interaction with other characters. On the satellite of chromosome 7 (KSU32) there appears to be a gene which has an effect on crown fructan content and heading date.

These results suggest that a gene (or perhaps a tightly linked cluster of genes) with major impact on development resides on the long arm of barley chromosome 7. This gene or gene cluster, when derived from Dicktoo, results in a barley line with many of the characteristics associated with good winter survival. The Dicktoo allele results in delayed heading, high crown fructan content, low LT_{50} and relatively good field survival at either Bozeman or Corvallis. Two other loci, one on chromosome 5 and the other on the satellite of the short arm of chromosome 7, have an impact on crown fructan content. The chromosome 5 locus is also implicated in winter survival while the locus on the satellite of chromosome 7 is implicated in delayed flowering.

Some of the difficulty experienced in developing barley germplasm with improved cold tolerance can be explained by these results. The largest factor resulting in the excellent field survival potential of Dicktoo appears to be inherited as though it were a single Mendelian gene on the long arm of chromosome 7. Unfortunately, this one Mendelian gene appears to result in delayed flowering in addition to improved survival, lowered LT_{50} and increased crown fructan content. Plant breeders have for decades sought earlier flowering, hardy barley genotypes. Since it is both easier and more effective to select for flowering date than for winter survival, in wide crosses using Dicktoo as a parent the selected progeny would likely not carry the Dicktoo allele for winter survival at this locus due to its association with delayed heading.

Two of the three loci found to modify crown fructan content were also shown to affect flowering date. It would seem reasonable to hypothesize a physiological relationship between fructan deposition (or reduced turnover) and delayed inflorescence. Using the simple chromosome 7 marker, rough awns, and the flanking marker BCD265b, it should be straightforward to develop isogenic lines which vary for this region. Once available, physiological analysis of differences among lines would appear reasonable. Line development is in progress, and isogenic pairs can be accessed from Dr. Patrick M Hayes when they are available.

Table 4. Correlation coefficeints of RFLP markers that showed significant interactions with fructan content (Fructan92).

Markers	Loc[1]	Fructan92	LT50	Heading date	MTSUR	ORSUR	MeanSUR
AWN	7L	0.45^{**}	0.45^{**}	0.54^{**}	0.72^{**}	0.57^{**}	0.72^{**}
pKSU24	7L	0.36^{**}	0.34^{**}	0.42^{**}	0.62^{**}	0.60^{**}	0.65^{**}
BCD265b	7L	0.38^{**}	0.44^{**}	0.61^{**}	0.75^{**}	0.44^{**}	0.66^{**}
C3a	7L	0.50^{**}	0.43^{*}	0.65^{**}	0.77^{**}	0.78^{**}	0.80^{**}
RACH	7L	0.23^{**}	0.03^{ns}	0.23^{*}	0.28^{**}	0.29^{**}	0.32^{**}
HorD	5L	0.25^{*}	0.05^{ns}	0.02^{ns}	0.07^{ns}	0.20^{*}	0.16^{ns}
ap340a	5L	0.26^{*}	0.01^{ns}	0.01^{ns}	0.04^{ns}	0.19^{*}	0.14^{ns}
pKSU32	7S	0.44^{**}	0.32^{ns}	0.59^{**}	0.32^{ns}	0.37^{ns}	0.36^{ns}
C8	?	0.39^{*}	0.23^{ns}	0.07^{ns}	0.02^{ns}	0.03^{ns}	0.01^{ns}
F4	?	0.48^{*}	0.05^{ns}	0.05^{ns}	0.21^{ns}	0.23^{ns}	0.22^{ns}

[1] Chromosomal location of locus
** = Statistically significant level at 99%
* = Statistically significant level at 95%
ns = Statistically non significant
MTSUR = field survival at Bozeman, MT in 1992; ORSUR = field survival at Corvallis, OR in 1991; MeanSUR = mean field survival of both locations.

CHAPTER 3

STS FACILITATED PCR FOR BARLEY GENOME MAPPING.

Introduction

Current genomic linkage maps are generally based on restriction fragment length polymorphisms (RFLPs) (Botstein et al., 1980). Conventional detection of RFLPs by Southern blot analysis (Southern, 1975) is laborious and costly (Beckmann and Soller, 1983; Beckmann, 1988). The polymerase chain reaction (PCR) (Saiki et al., 1985; Mullis and Faloona, 1987) provides a rapid, safe and efficient method for screening large populations. Unlike Southern blot analysis, PCR can directly distinguish between insertion/deletion or point mutation events. Point mutation polymorphisms can be detected by hybridizing PCR products with allele-specific oligonucleotides (Higuchi et al., 1988; Li et al., 1988; Kurth et al., 1991), DNA sequencing of PCR products (Wong et al., 1987), cleavage of PCR products with a restriction endonuclease (Saiki et al., 1985), and denaturing gradient gel electrophoresis (Myers et al., 1987; Riedel, et al., 1990). Insertion/deletion polymorphisms can be analyzed by sizing of PCR products via gel electrophoresis (Higuchi et al., 1988; Shin et al., 1990). A major limitation for PCR analysis is the need for extensive sequence information in order to synthesize the appropriate primers (Williams et al., 1991). Williams et al.(1990) proposed using arbitrary nucleotide sequences as single primers for amplification of random DNA fragments (RAPD).

In the human genome mapping project, an attempt to convert the genetic map to a physical map has been greatly simplified by using

sequence-tagged sites (STS). An STS is a short, unique sequence, amplified by PCR, which identifies a known location on a chromosome (Olson et al., 1989). To date, all STSs that have been used in mapping projects have been derived from well-characterized DNA probes or sequences (Cole et al., 1991). D'Ovidio et al.(1990) and Weining and Langridge (1991) showed that PCR can be used to detect genetic polymorphisms in cereals with primer sequences derived from the sequence of a γ-gliadin gene and a α-amylase gene, respectively.

Here, we propose two approaches to obtain STSs for barley genome mapping. One approach is to utilize sequence data from Genebank to construct primers. Another is to sequence anonymous DNA clones used in genome mapping projects. Point mutation polymorphisms within amplified fragments were detected on polyacrylamide gels of digested PCR products with four-base cutters. Insertion/deletion events were observed directly by gel electrophoresis.

Material and Methods

Genetic Stocks

Two doubled haploid populations were generated from barley cv. Dicktoo x Morex (DxM), a winter x spring cross, and from Steptoe x cv. Morex (SxM), a spring x spring cross by the North American Barley Genome Mapping Project (NABGMP). For the DxM cross, 30 doubled haploid lines were used for segregation analysis. One hundred and fifty doubled haploid progenies were used for segregation analysis in the SxM cross. A set of wheat-barley (cv. Chinese Spring-cv. Betzes cultivars) chromosomal addition lines described by Islam et al. (1981) was utilized for chromosomal assignment of polymorphisms.

DNA Isolation

Genomic DNA was prepared using the procedure of Ausubel et al. (1987), modified as follows: 10 g fresh weight of young leaf tissue was ground to a fine powder under liquid nitrogen and incubated at 55 C in 20 ml of extraction buffer (100 mM Tris-HCl pH 8.5, 100 mM EDTA, 250 mM NaCl, 0.5% SDS, and 100 ug/ml proteinase K) for 2 hrs. The lysate was extracted with 20 ml phenol:chloroform solution (50% phenol, 49% chloroform, 1% isoamyl alcohol), followed by chloroform extraction, and ethanol precipitation. DNA was centrifuged at 5000 rpm for 15 min., suspended in 2 ml of sterile water, and treated with 20 ug DNase free RNase for 2 hr. DNA was extracted with phenol:chloroform, and chloroform, adjusted to a concentration of 0.3 M sodium acetate, and ethanol precipitated. The purified DNA was dissolved in sterile water and stored at -20 C until use.

DNA Sequencing

Four DNA clones, Pstl-319, Pstl-327, Pstl-337, and Pstl-340, were randomly selected for sequencing from a PstI barley genomic library, provided by Dr. Nora Lapitan (Colorado State Univ.). Single-stranded DNA (ssDNA) templates were generated using PCR as described by Higuchi and Ochman (1989). PCR primers, 5′ TACGACTCACTATAGGGC 3′ and 5′ CGCCAAGCTATTTAGGTG 3′, were used to amplify the inserted DNA fragments in the pGEM vector. One of the primers was phosphorylated prior to PCR with T4 DNA kinase. After PCR amplification, the double-stranded DNA (dsDNA) was treated with lambda exonuclease, a 5′ to 3′ nuclease which specifically digests only the DNA strand synthesized from the phosphorylated primer leaving the complementary strand (ssDNA). The ssDNA was purified using Prep-A-Gene (BIO-RAD), and sequenced by the technique

of Sanger et al., 1977 using Sequenase following the manufacturer's protocol (USB).

Primer Synthesis

Oligonucleotide primers were synthesized by standard phosphoramidite chemistry on a Applied Biosystems 391 DNA synthesizer. After hydrolysis from the solid support and removal of protecting groups in 30% ammonium hydroxide at 55 C for 15 hr., the DNAs were dried under vacuum, and dissolved in 200 ul of sterile water. DNA yield was approximately 800 ug per synthesis.

PCR Amplification

PCR amplifications were performed in a 100 ul reaction containing 2.5 units Taq polymerase (Perkin Elmer Cetus), 1x PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, and 1.5 mM $MgCl_2$), 200 uM of each dNTP, 0.3 uM primers, and 25 ng of genomic DNA templates. The reaction was overlaid with mineral oil and subjected to one cycle of 95 C for 5 min., followed by 32 cycles of 94 C for 1 min., 55 C for 1 min., and 72 C for 90 sec.

Detection of Polymorphisms

PCR products were run on 1% agarose gels to detect insertion/deletion polymorphisms. To detect base substitution polymorphisms, 20 ul of amplified fragments were digested with four base cutters: AluI, HaeIII, HhaI, HinfI, MspI, RsaI, and TaqI. Two units of the restriction endonucleases, except RsaI, were directly added to 20 ul of the PCR reaction after amplification. For RsaI, 20 ul of the PCR product were precipitated with three volumes of 95% ethanol and the pellet was washed with 70% ethanol prior to digest. Digested fragments were electrophoresed

on 7% polyacrylamide gels at 250 Volt for 100 min.

Results

STS Production

Eight PCR primer sets and their sources are shown in Table 5. Four primer sets were designed from published sequences of α-hordothionin, alcohol dehydrogenase 2 (ADH-2), B1-hordein, and the BamHI-SstI fragment of pMSU21. The other four, Pstl-319, Pstl-327, Pstl-337, and Pstl-340, were obtained from sequencing short portions of DNA clones from a Pstl genomic library. Figure 2 shows the DNA sequence of short portions at both ends of the genomic clones obtained by the method described. From these sequences, PCR primers were designed to be about 20 bases long, contain 50% GC and harbor no inverted repeat sequences.

PCR amplification and polymorphism detection

Each primer set was used to amplify sequences from total DNA of Dicktoo and Morex barley (Figure 3). Two primer sets differentiated between the two cultivars based on agarose gel electrophoresis. Amplified DNA fragments from pMSU21 primers identified an insertion/deletion polymorphism. A 670 bp fragment is amplified from Dicktoo DNA, and a 450 bp fragment is amplified from Morex DNA when these primers are used. Parental and progeny banding patterns are shown in Figure 5A. This polymorphism was previously determined to be allelic to that identified by Southern blot analysis with the probe pMSU21 (Shin et al., 1990). B1-hordein primers amplified two fragments in Dicktoo (approx. 960 and 860 bp) and one fragment in Morex (approx. 860 bp). The PCR and protein polymorphisms (determined by SDS-PAGE) demonstrated perfect cosegregation (Table 6).

PCR products amplified with the other six primer sets did not show size polymorphisms. These products were digested with seven four-base cutters and electrophoresed on 7% polyacrylamide gels. Restriction site polymorphisms were detected in PCR products amplified from α-hordothionin, alcohol dehydrogenase 2, Pstl-327, Pstl-337, and Pstl-340 primer sets (Figure 4). No polymorphism was detected with the Pstl-319 primers. Segregation based on PCR amplification from Pstl-327 and Pstl-337 primers agreed perfectly with that determined by Southern blot analysis of doubled haploid lines from the DxM cross (Table 6). Parental and progeny banding patterns for the analysis based on the Pst-337 primers are shown in Figure 5B. The product of ADH-2 primer amplification, however, did not show allelism with any of five RFLP polymorphisms identified through the use of the ADH-2 clone in Southern blot analysis of a series of doubled haploid lines (pers. comm, A. Kleinhofs, NABGMP, 1991).

Chromosomal Location

Chromosomal locations of STSs were identified by linkage analysis with previously mapped markers in doubled haploid progenies and by PCR amplification of wheat-barley addition lines (Table 5 and Figure 6). The recombination analyses were performed using MAPMAKER (Lander and Botstein, 1989). The Pstl-327 and α- hordothionin amplification products were evaluated in both the SxM (population size = 150) and DxM (population size = 30) crosses. They were located on chromosome 2 and 5 respectively in both crosses. Identical chromosomes were located using recombination analysis and PCR amplification of DNA from the wheat-barley addition lines.

The polymorphism identified by the ADH-2 based primers mapped to

barley chromosome 7 by both recombination analysis and PCR amplification of wheat-barley chromosome addition lines. Curiously, although the probe ADH-2 hybridizes to several polymorphic bands from genomic digests, none of the RFLP polymorphisms cosegregated with the PCR polymorphism.

Efficiency of PCR Analysis

Four previously unmapped clones were selected from a Pst-1 library preselected to exclude repeat sequences and organellar sequences. Sequence data was obtained from the terminal 200 bp from each end of each clone and primers were synthesized. Seven restriction endonucleases with four base pair restriction sites were utilized with the four PCR products from the barley cultivars Morex and Dicktoo. A total of 79 restriction sites were surveyed, and a total of 16 were absent in one of the two genotypes. This provides a conservative estimator of sequence divergence between these two genotypes of 16/316 bases, about 5%. Since the clones were not preselected on the basis of polymorphisms, we have no reason to expect bias in this estimate of base pair differentiation between homologous single copy sequences of Dicktoo and Morex barley.

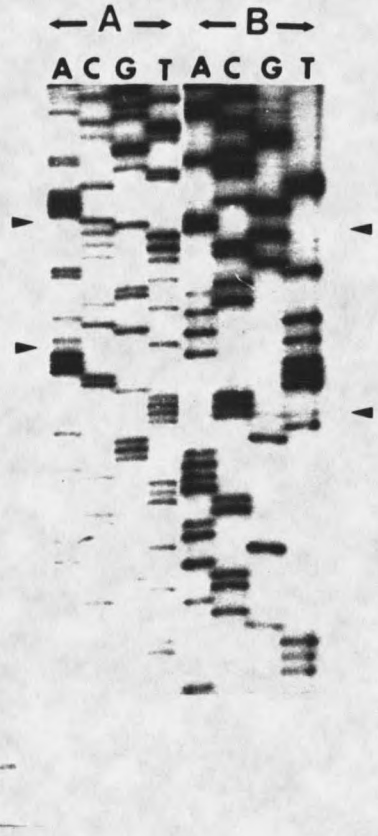

Figure 2. DNA sequence of PstI-340 clone. Short portions of both ends (A and B) of the clones were used to design PCR primers. The arrows indicate the portions of sequence from which primers were designed.

Table 5 Primer sequences, sources, chromosomal locations and allelism analyses.

Primer set	Primer sequences	Primer source	Chromosomal location(Allelism)
aMSU21	5'GGTCTTTCATGTACCTACC 3' 5'CGAGCTCCTGTCGAGG 3'	Shin et al., 1991	2[1](100/100)
B1-Hordein	5'CCACCATGAAGACCTTCCTC 3' 5'TCGCAGGATCCTGTACAACG 3'	Forde et al., 1985	5 (70/70)
α-Hordothionin	5'CTGGGGTTGGTTCTGG 3' 5'GGCAGCAACATGGCATTC 3'	Rodriguez-Palenzuela et al., 1988	5 (ND)
Alcohol dehydrogenase2	5'GGGGAGATATCGACCAAAGT 3' 5'CACGCCCTCGCCAACGCTCTCCA 3'	Trick et al., 1988	7 Unlinked[2]
PstI-319	5'AGCTGAGCAAGCTTCTTTGG 3' 5'AACATGCTGGGCAACTCCCA 3'	Genomic library	-
PstI-327	5'GGTACGAACATGGAGGTACT 3' 5'ATCCAGTTCTTGTGCACCTG 3'	Genomic library	2 (30/30)
PstI-337	5'ATCCAGTTCTTGTGCACCTG 3' 5'AGCTACGTGGATCACACCAC 3'	Genomic library	7 (30/30)
PstI-340	5'TAGCATCGGTAATCTCTCGC 3' 5'CCCTTTATATACACTGCCGA 3'	Genomic library	5 (ND)

[1]Data previously reported (Shin et al., 1990)
[2]Southern blot data communicated by Dr. A. Kleinhofs
-No polymorphisms were identified either by Southern blot analysis or PCR
ND = data not determined

Table 6. Restriction Site Variation in PCR Products.

Primer sets	DNA source	PCR amplified fragment sizes	Restriction sites						
			AluI	HaeIII	HhaI	HinfI	MspI	RsaI	TaqI
PstI-319	Dicktoo	1100 bp	4	3	1	1	1	1	3
	Morex	1100 bp	4	3	1	1	1	1	3
PstI-327	Dicktoo	1000 bp	1	2	1	3	1	ND	1
	Morex	1000 bp	2	3	1	2	0	ND	1
PstI-337	Dicktoo	1200 bp	2	3	1	2	3	ND	4
	Morex	1200 bp	4	3	3	4	4	ND	5
PstI-340	Dicktoo	1100 bp	5	0	0	5	1	0	1
	Morex	1100 bp	5	1	0	5	1	0	1
α-hordothionin	Dicktoo	1050 bp	1	2	1	1	0	ND	3
	Morex	1050 bp	1	2	1	1	0	ND	2
Alcohol dehydrogenase2	Dicktoo	630 bp	ND	3	2	0	2	0	1
	Morex	630 bp	ND	3	3	0	2	1	1

ND = data not determined.

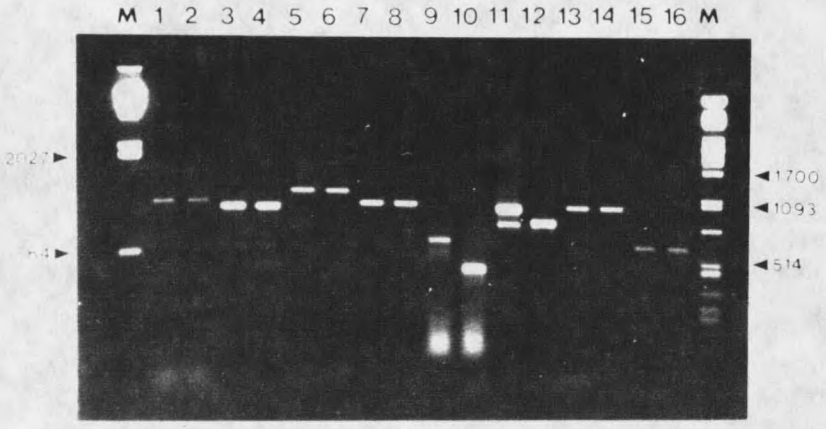

Figure 3. Amplification of barley DNA from Dicktoo and Morex with eight primer sets on a 1% agarose gel. DNA templates in the odd lanes are Dicktoo, in the even lanes are Morex. Amplified products with PstI-319 primers are in lanes 1 and 2, those with PstI-327 primers are in lane 3 and 4, those with PstI-337 primers are in lane 5 and 6, those with PstI-340 primers are in lane 7 and 8, those with pMSU21 primers are in lane 9 and 10, those with B1-hordein primers are in lane 11 and 12, those with α-hordothionin primers are in lane 13 and 14, those with alcohol dehydrogenase 2 primers are in lane 15 and 16. DNA size markers are shown in lane M. The sizes of the marker bands are given in base pairs.

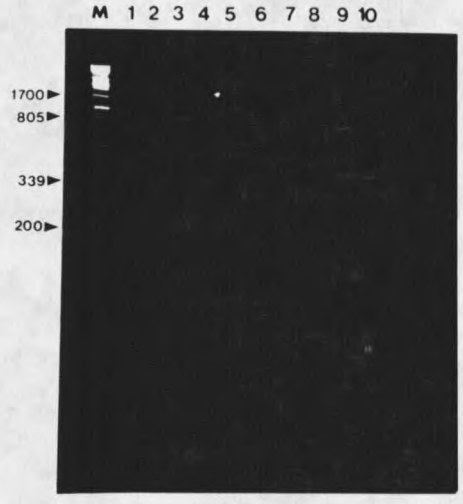

Figure 4. Digestion of PCR products with restriction endonucleases with four-base recognition sequences were electrophoresed on a 7% polyacrylamide gel. HinfI digestion of amplified products with PstI-327 primers are in lane 1 (Dicktoo) and lane 2 (Morex). HinfI digestion of amplified products with PstI-337 primers are in lane 3 (Dicktoo) and lane 4 (Morex). HaeIII digestion of amplified products with PstI-340 primers are in lane 5 (Dicktoo) and lane 6 (Morex). TaqI digestion of amplified products with α-hordothionin primers are in lane 7 (Dicktoo) and lane 8 (Morex). RsaI digestion of amplified products with alcohol dehydrogenase 2 primers are in lane 9 (Dicktoo) and lane 10 (Morex). DNA size markers are shown in lane M with the size of the marker bands given in base pairs.

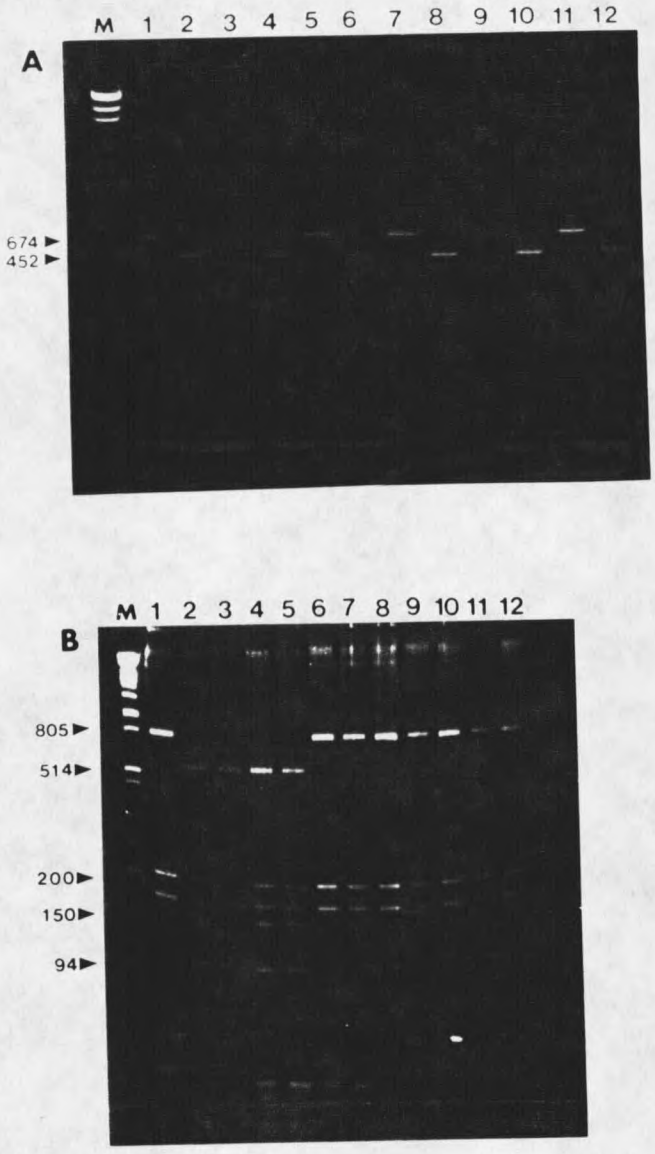

Figure 5A, B. Segregation of DxM doubled haploid lines. DNA size markers are shown in lane M with the size of marker bands given in base pairs. The template DNA was from Dicktoo in lane 1, Morex in lane 2 and doubled haploids in lane 3 to lane 12. **A** Amplified products with pMSU21 primers showed insertion/deletion polymorphisms on a 1% agarose gel. **B** HinfI digestion of amplified products with PstI-337 primers were electrophoresed on a 7% polyacrylamide gel.

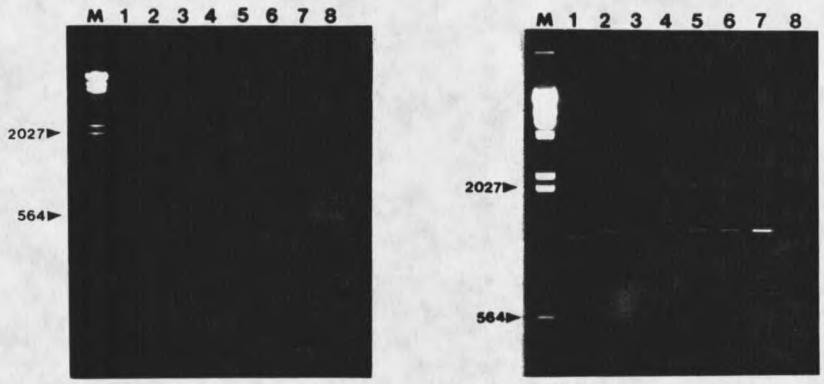

Figure 6A, B. Amplification of wheat-barley chromosome addition lines was from barley cv Betzes in lane 1, Chinese spring wheat in lane 2, barley chromosome 1, 2, 3, 4, 6, 7 addition lines are in lanes 3 to 8, respectively. DNA size markers are shown in lane M with a given size of marker bands in base pairs. **A** Amplification of wheat-barley chromosome addition lines with PstI-327 primers electrophoresed on a 1% agarose gel. **B** Amplification of wheat-barley chromosome addition lines with PstI-337 primers electrophoresed on a 1% agarose gel.

Discussion

Three of the four randomly selected single copy clones from which we obtained primer sequences identified allelic variation between Morex and Dicktoo barley. Further, it appears as though about one base in twenty varies between Morex and Dicktoo. Approximately 37% of the randomly isolated single copy genomic clones tested against Morex and Dicktoo identified polymorphisms when six six-base restriction endonucleases were utilized. When individual restriction sites were evaluated, we actually observed a frequency of 1 site in 5 lost by mutation (39 mutations/1188 bases evaluated), or 3.3% (data not shown). These frequencies (5% vs. 3.3%) seem fairly similar and suggest that polymorphisms between winter and spring barley cultivars are frequent enough to permit effective mapping using either RFLP or PCR based approaches.

Five of the six PCR-based evaluation systems in which allelism was determined for Southern blot based polymorphisms provided expected linkages to previously mapped markers. The sixth, ADH-2, provided a clear polymorphism but no linkage to polymorphisms identified by Southern blot analysis. On one hand, it is clear that the ADH-2 primers used in this study provide a useful genetic marker. On the other hand, it is likely that the locus amplified by these primers has no relationship to alcohol dehydrogenase. Further study could help explain this anomaly.

This study demonstrates an alternative method to Southern blot analysis for genome mapping. However, the need of primer sequences can be a limiting factor in PCR-based genome mapping. To overcome this problem, the RAPD approach was introduced (Williams et al., 1990). D'Ovidio et al. (1990) and Weining and Langridge (1991) derived PCR primers from published

DNA sequences. Comparable to STS proposed in the human genome project (Olson et al.,1989), I extended the STS idea by evaluating four randomly selected DNA clones from a genomic library. PCR products of three of these four clones identified variation between Morex and Dicktoo. PCR-based sequencing methods (Gyllensten and Erlich, 1988; Innis et al., 1988; Higuchi and Ochman, 1989; Kusukawa et al., 1990) make production of STS from selected DNA clones practical.

The production of medium density genomic maps using RFLPs has proven to be feasible in many crop species. Using these maps for efficient QTL manipulation demands the use of technology simpler than Southern blot analysis. In this experiment we found that seven of the eight marker sequences tested could be easily manipulated to permit evaluation of segregation by PCR. As significant QTL loci are identified in crops, converting mapped RFLP markers which flank agronomically important loci to PCR based detection systems will provide a user-friendly technology to the ultimate users of genome maps, the plant breeders.

Although the variation outside the STS region cannot be detected as in Southern blot analysis, it has been demonstrated that there is sufficient exists variation within amplified fragments in mammalian systems (Li et al., 1988; Litt and Luty, 1989; Tautz, 1989; Weber and May, 1989). In plant systems, our study supports a similar conclusion. In contrast to Skolnick and Wallace (1988), this study demonstrates that PCR polymorphisms are mainly due to the absence or presence of restriction endonuclease sites rather than the fragment length differences.

Chromosomal locations were identified using both recombination analysis and use of the wheat-barley addition lines. Chromosomal

locations identified in progeny from two crosses (Dicktoo x Morex and Steptoe x Morex) corresponded well and were confirmed by PCR analysis of DNA from the wheat-barley chromosome addition lines.

Although relative costs might be debated, the ease of PCR analysis, the clarity of restriction pattern of PCR products and the ease of distribution of primer sequence data (as opposed to maintenance of clones) all argue in favor of PCR as a tool in genome analysis. Clearly, the chromosomal location of a polymorphism must be well-characterized and sTable over crosses if it is to be generally useful. All seven polymorphisms evaluated in this study appear sTable and appear to map to single loci. One of the primer sets (ADH-2) amplified a product from an unexpected locus. The clone from which our sequence data derived clearly hybridized to sequences at several dispersed loci. We would suggest that loci of this sort, dispersed, multigene families, may be unsuitable candidates for PCR based genetic analysis.

CHAPTER 4

MAPPING OF TRAITS ASSOCIATED WITH WINTERHARDINESS IN A WINTER x SPRING BARLEY CROSS

Introduction

The proportion of winter barley plants surviving to produce grain - winterhardiness - is determined by many interacting factors. These are thought to range from low temperature tolerance to biotic stress resistance. Included 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 poorly understood. In this report we addressed the genetic basis of winterhardiness by systematically measuring its components in a doubled haploid population of barley and regressing these phenotypic data sets on molecular marker genotypes to identify quantitative trait loci (QTL).

Long-term field survival is the best measure of winterhardiness (Olien 1978), but obtaining long term performance data are expensive and difficult. High levels of genotype x environment interaction and enormous measurement error increase the difficulty of correct data interpretation. Furthermore, the infrequent occurrence of a test winter that permits effective discrimination among genotypes makes field-based selection generally ineffective (McIntyre et al. 1988).

Crown fructan content and controlled freeze tests to determine LT_{50} (the temperature at which 50% of the population is killed) are reasonably simple measures which show significant correlations with field survival

(Stushnoff et al. 1984). Thomashow (1990), in reviewing the molecular basis of cold tolerance in higher plants, cited 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) provided compelling evidence for the importance of chromosome 5A.

Vernalization and cold tolerance are considered to be associated, but trait association in barley (Doll et al. 1989) and wheat (Roberts 1990) has been 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 (Takahashi and Yasuda 1971). The winter habit phenotype can be modified 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.

Prostrate or rosette growth habit has been shown to be correlated with cold hardiness in wheat (Salmon 1971; Taylor 1983; Chaudhry 1986). Low temperatures induced reduction in rosette growth habit (Roberts and MacDonald 1984). In our barley parents, the winter cultivar (Dicktoo) showed prostrate growth while the spring cultivar (Morex) showed a much more erect growth pattern.

Carbohydrates, and particularly fructans, are reported to play a cryoprotective role in cereals. Several mechanisms have been proposed

(Olien and Lester 1985) to explain this association. Livingston et al. (1989) found a marked effect of carbohydrate level on the freezing tolerance and sugar composition of barley crown tissue in a set of barley genotypes which included our winter parent (Dicktoo). However, there was no direct relationship between specific compositional makeup of total carbohydrate and freezing tolerance.

Substantial interest has developed in the cloning and characterization of genes regulated or induced by specific stresses. To date, three genes regulated by cold in barley have been reported (Cattivelli and Bartels, 1990; Dunn et al., 1991). Two clones were kindly provided by Dr. Cattivelli. One of the two clones (A086) was found to identify a polymorphism between Morex and Dicktoo. Although one clone evaluated in these experiments was not readily obtained, published sequence data permitted us to recover homologous sequences from several of these using PCR. No polymorphism was detected from PCR using this marker in the cross. The clone A086 was examined to determine whether it mapped to loci associated with cold tolerance or its components.

Doubled haploids provide genetic reference populations 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 F_1 plants of the cross of Dicktoo x Morex. Dicktoo is a six-rowed winter feed barley of unknown ancestry and mixed description released by the Nebraska Agricultural Experiment Station in 1952. Morex is a six-rowed spring malting barley released by the Minnesota Agricultural Experiment Station in 1978.

Trait Assessment

We measured three traits which are direct 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 thrice replicated 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).

Two estimates of heading date were made. Heading date was measured

using unvernalized plant material in the greenhouse at a constant temperature of 18°C and 24 h light. The heading date under normal green house conditions was also measured at the same time as the heading date under continuous light. Normal green house conditions at Corvallis were 16 h light, with temperatures of 20°C during the day, and 18°C during the night.

Growth habit data were taken in the field at Corvallis, OR 1992. A scale from one to eight was used. One indicated extreme prostrate habit and eight indicated erect growth. These data were taken before digging plants for fructan analysis.

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. Five 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 ($\geq$ DP 5) is expressed as mg g^{-1} on

a dry weight basis (mg/gdw).

Map Construction and QTL Analysis

Markers, descriptions, and chromosome assignments are listed in Table 7. 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.

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. Map construction and QTL analysis were performed using MAPMAKER and MAPMAKER/QTL programs (Lander and Botstein, 1989).

The Sequence-Tagged-Site approach (Olsen et al., 1991) was used to generate primers homologous to the sequences published for the cold regulated genes A086, T59 and BLT4 (Cattivelli and Bartels, 1990; Dunn et al., 1991). From the published sequence data primer sequences were selected. Amplification regimes were selected to promote amplification of appropriately sized products which were then evaluated for restriction fragment length polymorphisms or size polymorphisms using template DNA from the cultivars Dicktoo and Morex. These were then utilized as molecular markers.

Results and Discussion

Map Construction

The 77 markers were resolved into eight linkage groups (Table 7 and Figure 7). Two unlinked groups were mapped to barley chromosome 1. One of these was anchored to chromosome 1 by the presence of His3 and the other by a Ubiquitin locus, both of which were previously mapped to chromosome 1 by the North American Barley Genome Mapping Project (Kleinhofs and Killian, in press). The chromosome 2 map was anchored by aWG110S and aPST240B and aMSU21, spaning a region of 213.1 cM. Six markers were found on chromosome 3 linked with the known marker iEST4.

These markers spanned a distance of 110.6 cM. Chromosome 4 was anchored by marker aGlu2 and aHrth, both of which had previously been mapped to barley chromosome 4. These markers span a region of 152.3 cM long. Known location seed storage protein markers on chromosome 5 were detected in a linkage group of 13 markers. The map of chromosome 5 covered a distance of 180.3 cM. Chromosome 7 spanned a region 189.8 cM long. Six of the 13 markers mapped to chromosome 7 are known-location markers either from previous mapping projects (Shin et al., 1990; Kleinhofs and Killian, in press) or by PCR of wheat-barley chromosome addition lines. One linkage group consisting of 7 markers is ambiguous for chromosome location. No unambiguously previously mapped markers mapped within this group. One of the markers mapped within this group is a product of amplification directed by primers with homology to the barley nitrate reductase (Nar1) locus, known to reside on barley chromosome 6. Unfortunately, three amplification products which upon digestion provided restriction fragment length polymorphisms were produced under the direction of these primers. Two of them were linked with markers on chromosome 2. The third is assumed to reside on chromosome 6, but we suggest that this may be a weak assertion.

Table 7. Descriptions, chromosome location, and numbers of mapped progenies 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	Numbers of lines mapped
aNar1a	STS-PCR	1	100
aHIS3	STS-PCR	1	100
WG118S	Wheat genomic RFLP	1	30
D2A	Barley cDNA RFLP	1	30
C8	Barley cDNA RFLP	1	30

Table 7. (continued)

Marker	Description	Chromosome	Numbers of lines mapped
aUbiq	STS-PCR	1	100
apT59b	STS-PCR	1	30
C3b	Barley cDNA RFLP	1	30
aWG110S	STS-PCR	2	100
WG645	Wheat genomic RFLP	2	100
aPST327	STS-PCR	2	100
aMSU21	STS-PCR	2	100
PST59	Barley genomic RFLP	2	30
A086	Barley cDNA RFLP	2	30
CDO588	Oat cDNA RFLP	2	100
aNar7HinF	STS-PCR	2	100
aWG178	STS-PCR	2	100
aNAR7S	STS-PCR	2	100
aPST340b	STS-PCR	2	30
A10A	Barley cDNA RFLP	3	30
A12	Barley cDNA RFLP	3	30
D7A	Barley cDNA RFLP	3	30
iEST4	Esterase 4	3	100
F8	Barley cDNA RFLP	3	30
F4	Barley cDNA RFLP	3	30
aGlu2	STS-PCR	4	100
WSEP	H_2O-soluble endosperm protein	4	100
TB19-20	STS-PCR	4	100
WG1026b	Wheat genomic RFLP	4	100
BCD265a	Barley cDNA RFLP	4	100
PST316	Barley genomic RFLP	4	100
D7B	Barley cDNA RFLP	4	30
HorB	B-hordein	5	100
HorC	C-hordein	5	100
aGlu1	STS-PCR	5	100
iGPI1	Glucosephosphate isomerase 1	5	100
WG789	Wheat genomic RFLP	5	30
WG118L	Wheat genomic RFLP	5	30
A10B	Barley cDNA RFLP	5	30
aWG110L	STS-PCR	5	100
HorD	D-hordein	5	100
aPST340a	STS-PCR	5	100
iPGD	Phosphogluconate dehydrogenase 2	5	100
BCD265C	Barley cDNA RFLP	5	100
aHRTH	STS-PCR	5	100
D2B	Barley cDNA RFLP	6	30
aWG669	STS-PCR	6	100
pKSU44	Barley cDNA RFLP	6	30
aNar7L	STS-PCR	6	100
LMWP	Low molecular weight protein	6	100
pKSU24S	Barley cDNA RFLP	6	100
OPA11	Barley cDNA RFLP	6	30

Table 7. (continued)

Marker	Description	Chromosome	Numbers of lines mapped
pKSU32	Barley cDNA RFLP	7	30
pTA71	Barley genomic RFLP	7	100
apT59a	STS-PCR	7	30
aADH	STS-PCR	7	100
BCD298	Barley cDNA RFLP	7	100
mS	Rachilla hair length	7	100
WG364b	Wheat genomic RFLP	7	100
PST321	Barley genomic RFLP	7	30
pKSU24	Barley cDNA RFLP	7	100
C3a	Barley cDNA RFLP	7	30
mR	Awn roughness	7	100
BCD265b	Barley cDNA RFLP	7	100
aPST337	STS-PCR	7	100

Cold Tolerance and QTL Analysis

Each measure of cold tolerance gave a distinct distribution of phenotypes (Figures 8, 9, and 10). Field survival is thought to be 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 or that epistasis may be an important determinant of trait expression.

Large QTL effects for all measures of cold tolerance were found on chromosome 7. For field survival in Oregon, the QTL effect was detected in the interval of mR-BCD265b with the log-likelihood of 8.56 and account

for 37.8% of the variation. For field survival in Montana, QTL effects exceeding the log-likelihood of 2.0 threshold were detected in two intervals: WG364b-PST321, and pKSU24-C3a. The later interval was covered up to mR-BCD265b interval. Analysis of the QTL for mean of the field survival in the two locations detected the QTL effects at two intervals: WG364b-PST321, mR-BCD265b. Log-likelihood of the two intervals were 6.07 for WG364b-PST321, and 19.23 for mR-BCD265b. For LT_{50}, LODs $\geq$ 3.0 were detected in the following intervals: apT59a-aADH, and mR-BCD265b.

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 Rrn2 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 Rrn2.

In the face of population 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 et al, 1981).

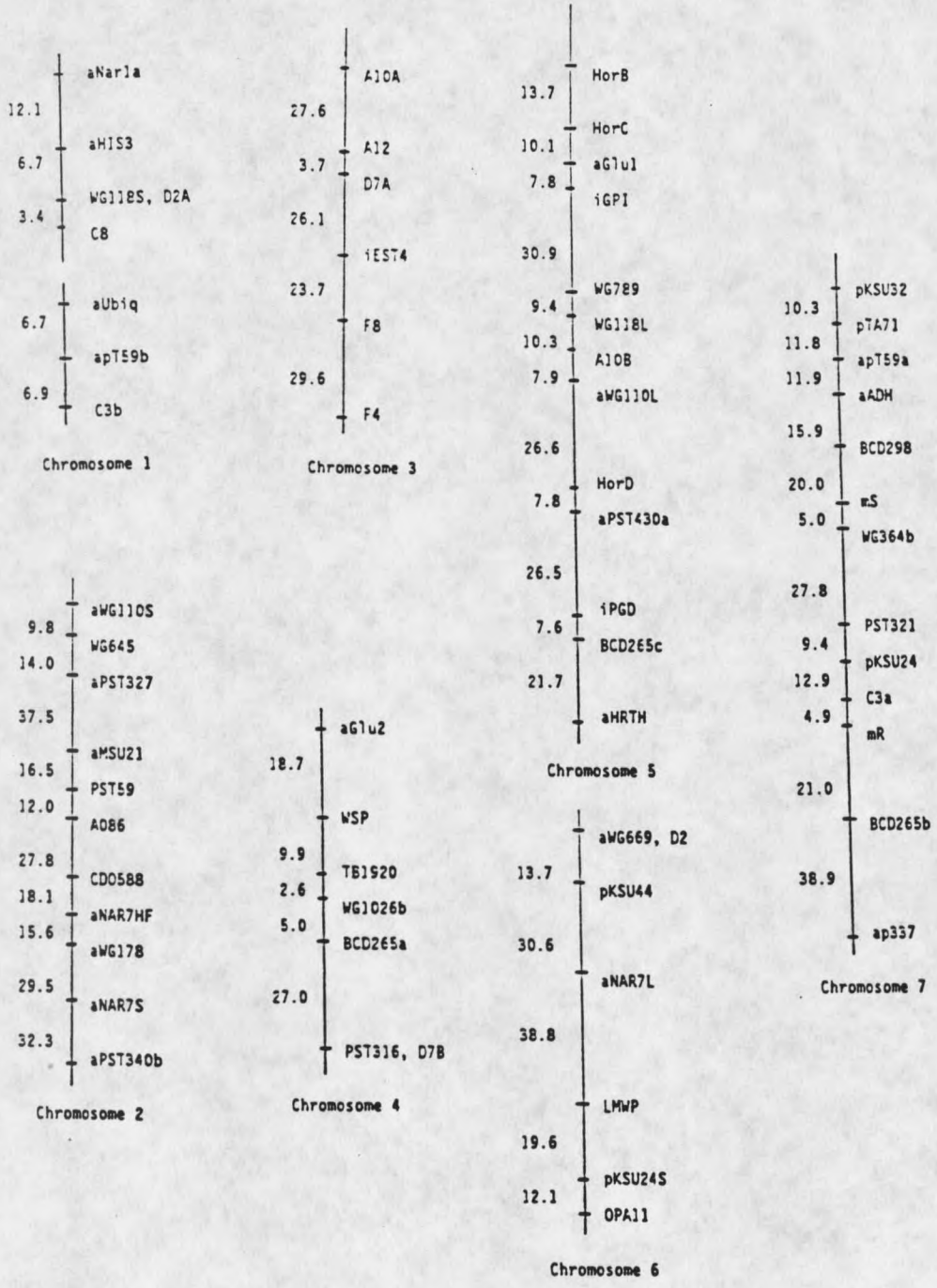

Figure 7. Map construction of Dicktoo x Morex cross.

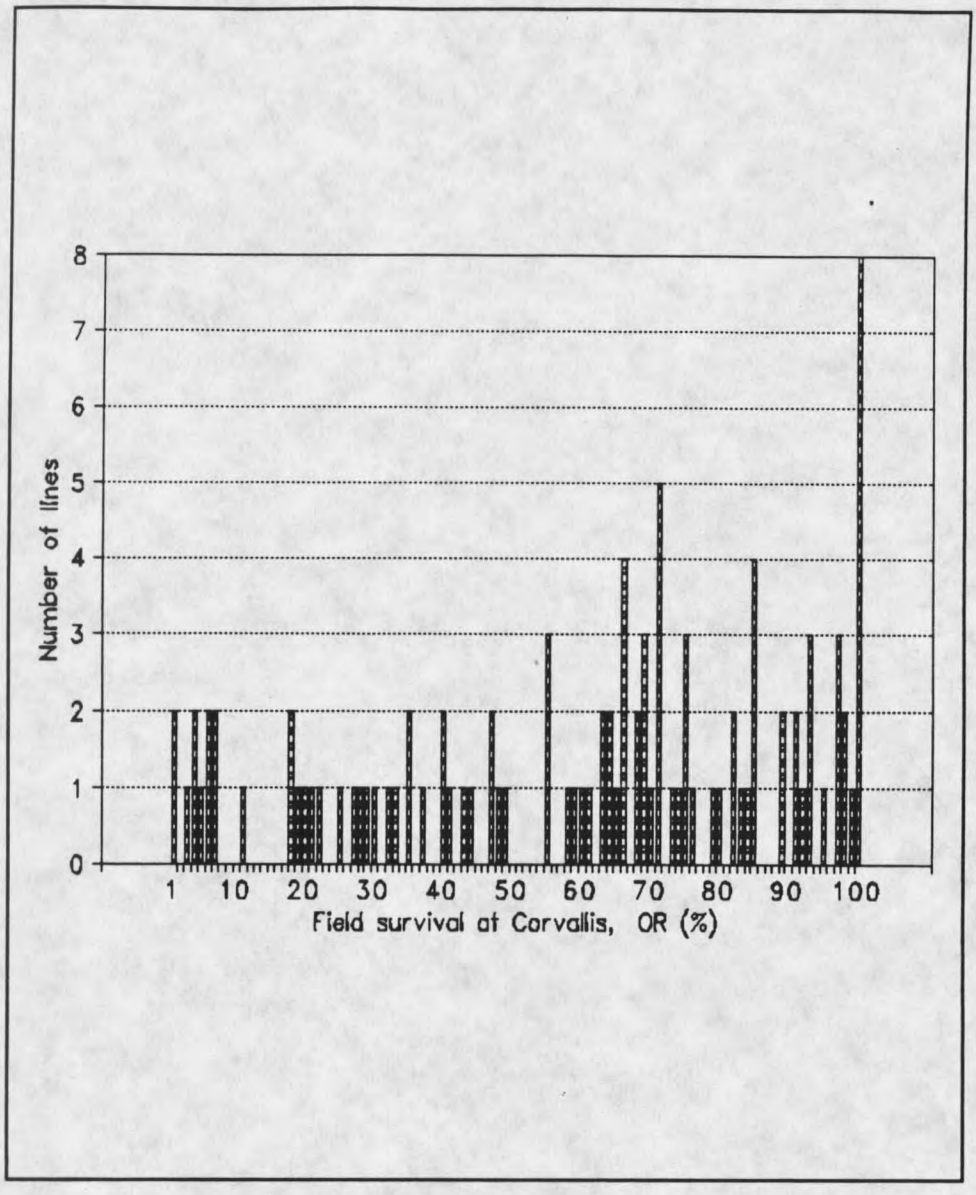

Figure 8. The distribution of field survival measured at Corvallis, OR in 1991 for Dicktoo x Morex cross.

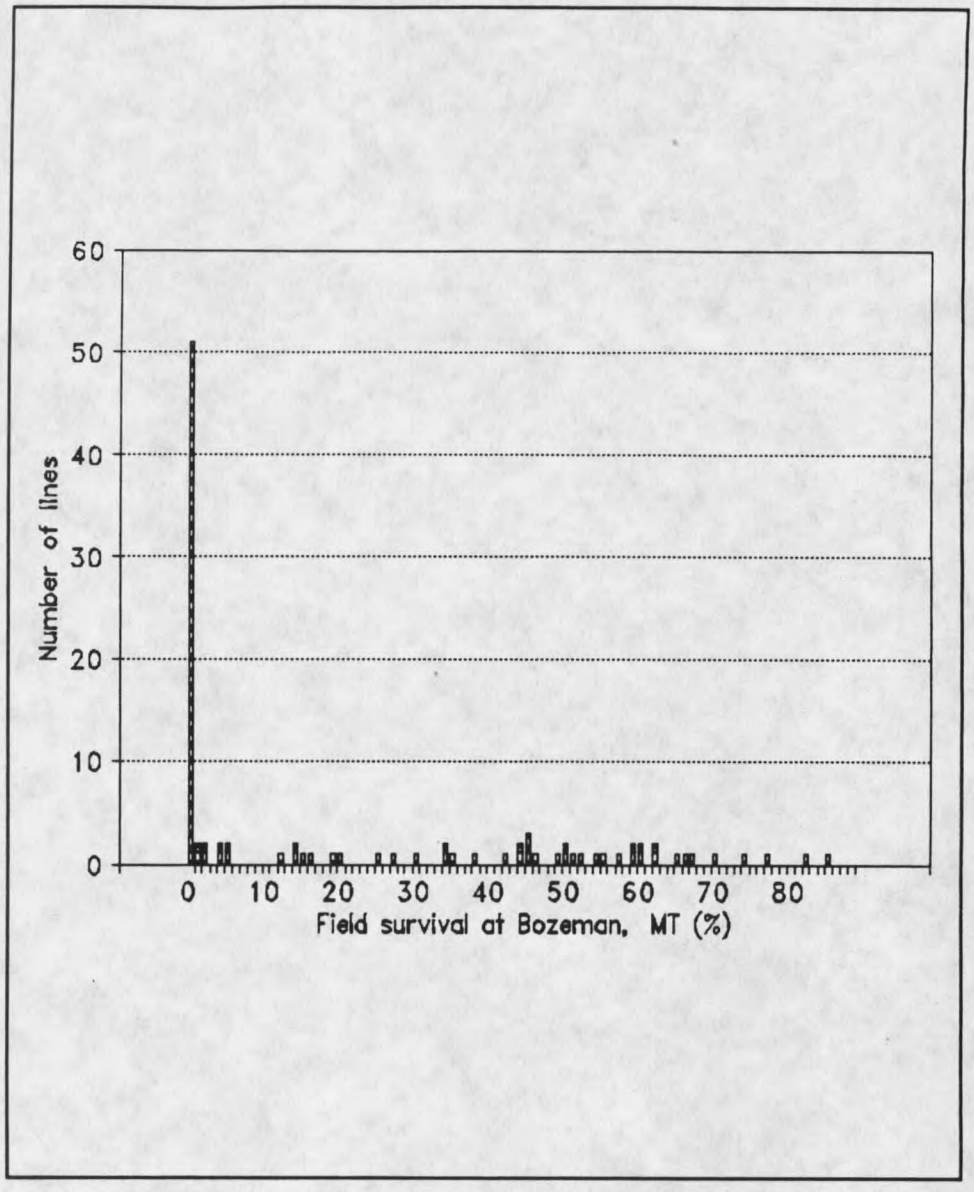

Figure 9. The distribution of field survival measured at Bozeman, MT in 1992 for Dicktoo x Morex lines.

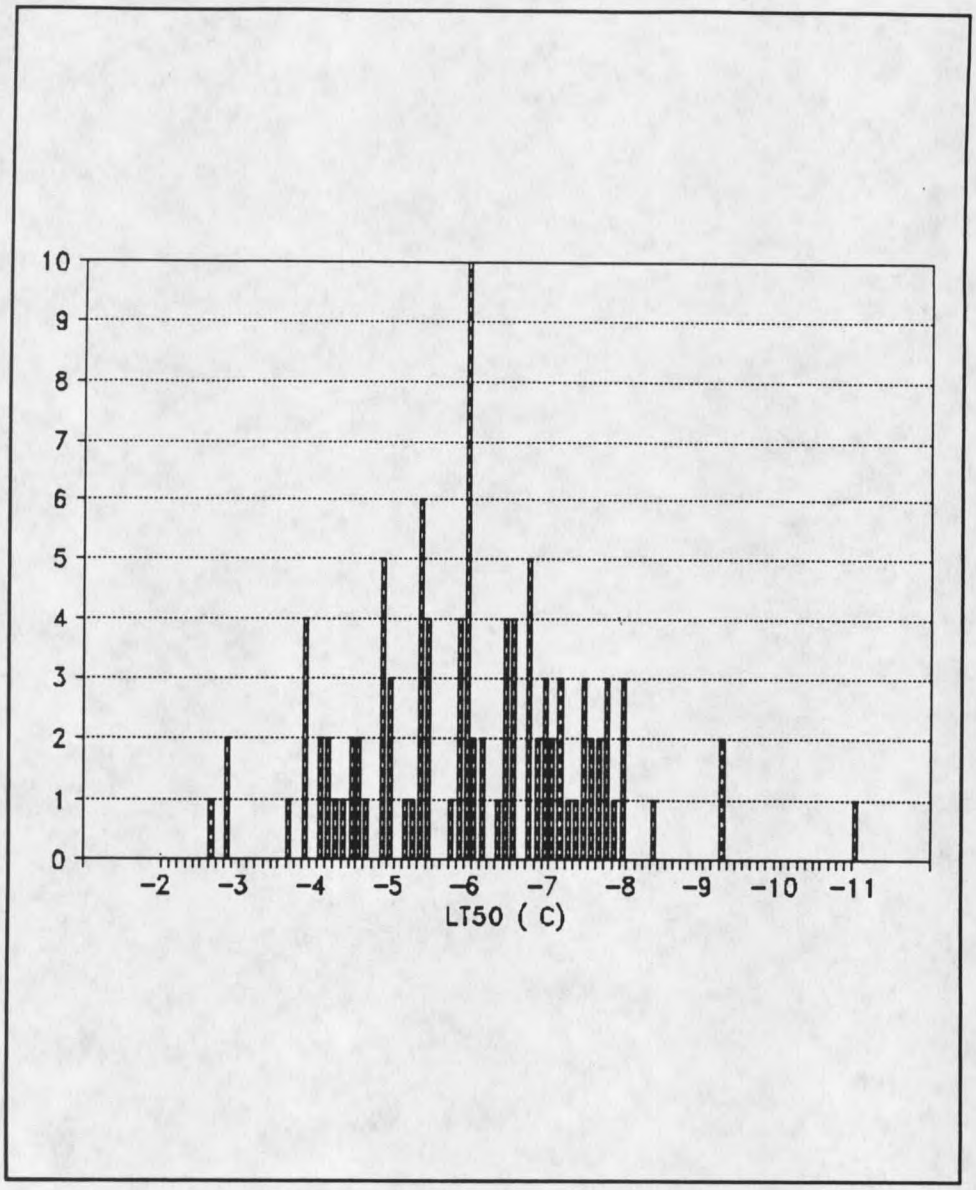

Figure 10. The distribution of LT_{50} for Dicktoo x Morex lines.

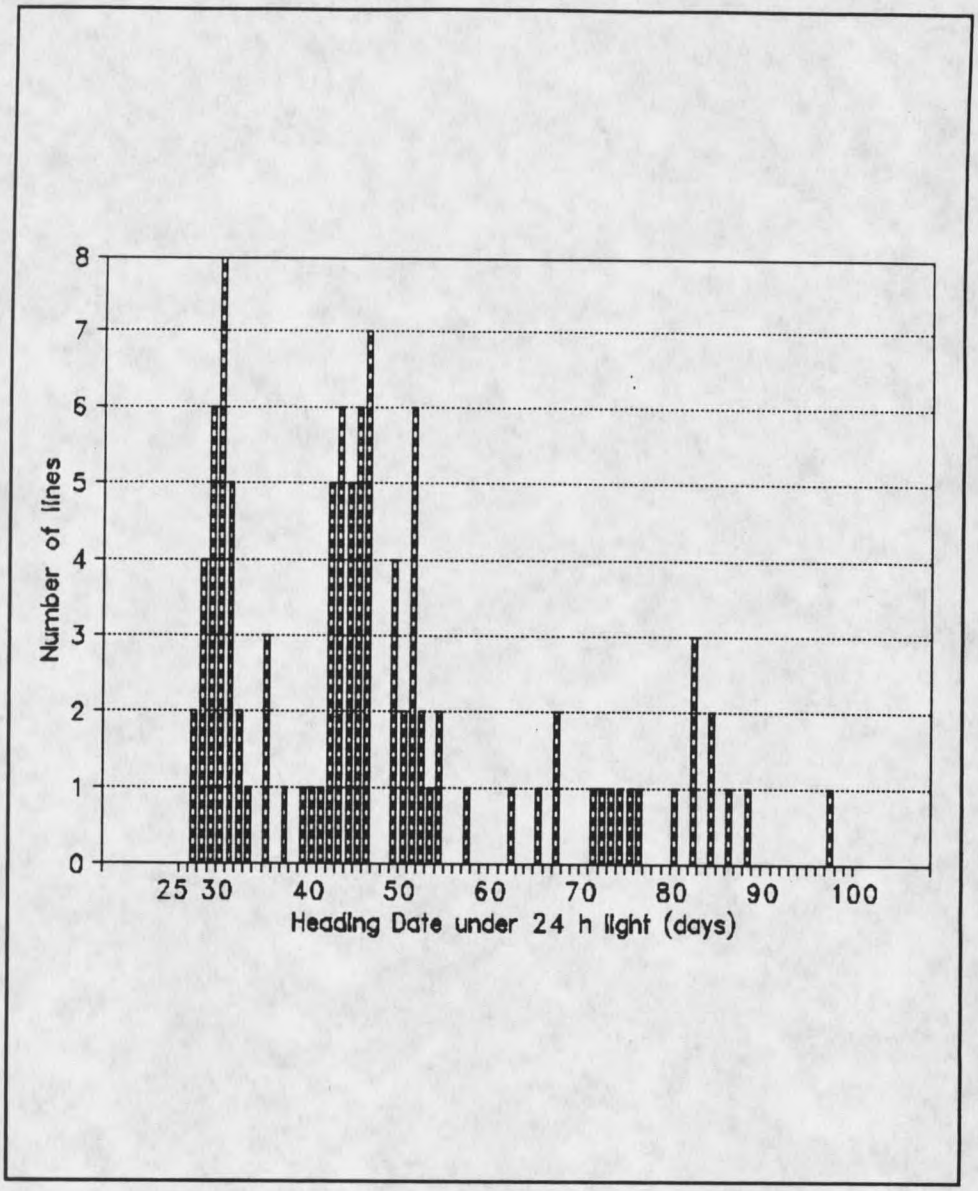

Figure 11. The distribution of heading date under 24 h light for Dicktoo x Morex lines.

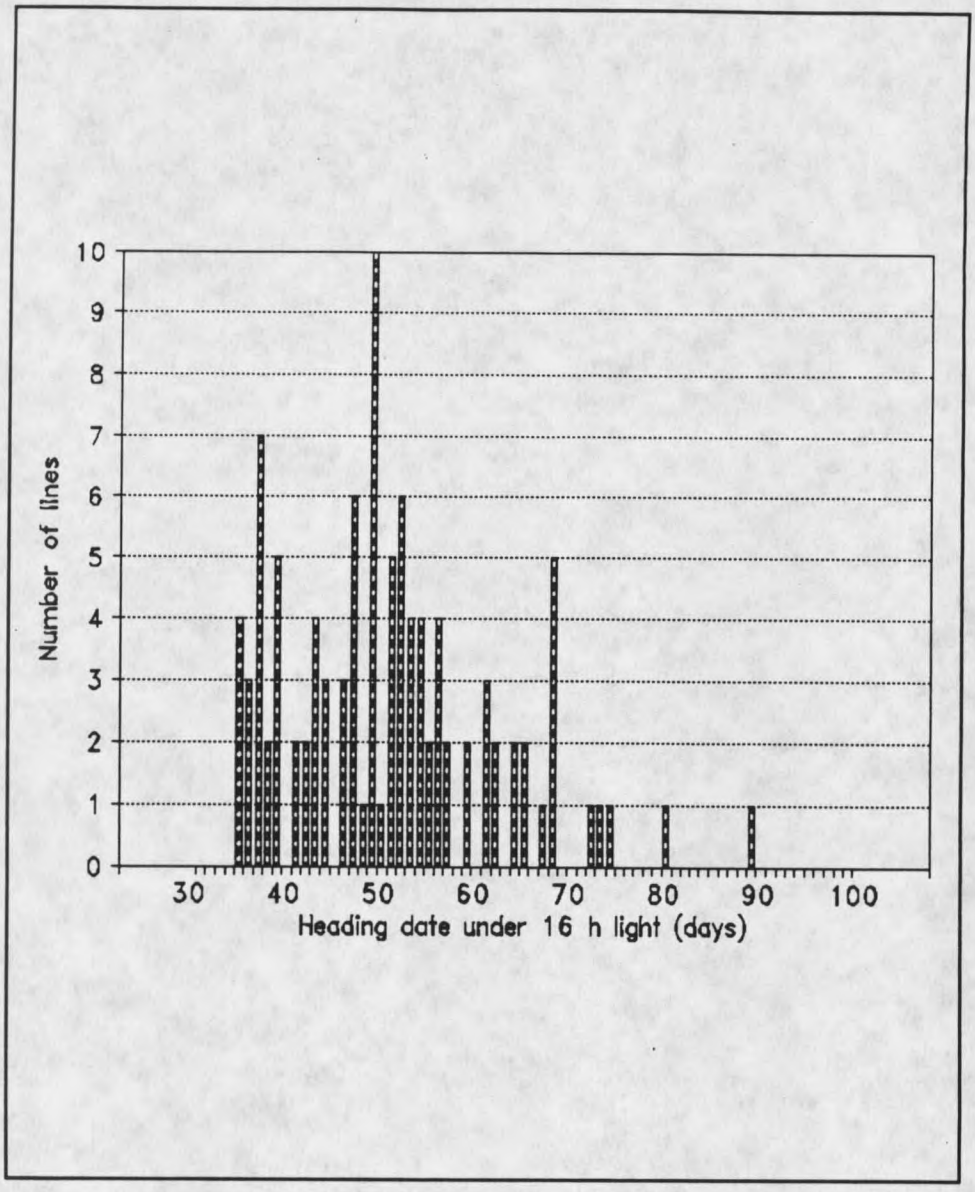

Figure 12. The distribution of heading date under greenhouse condition (16 h light) for Dicktoo x Morex lines.

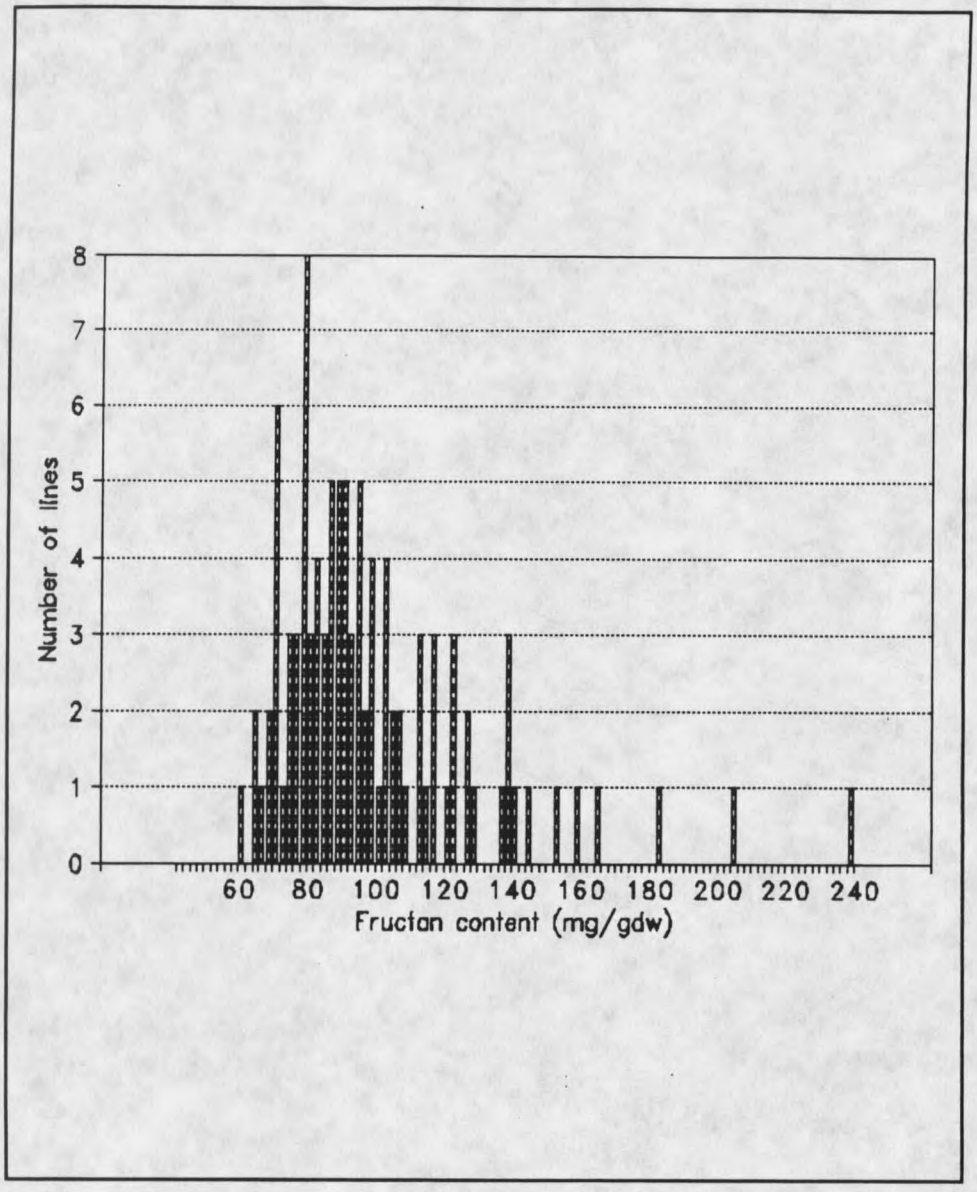

Figure 13. The distribution of fructan content in 1992 for Dicktoo x Morex lines.

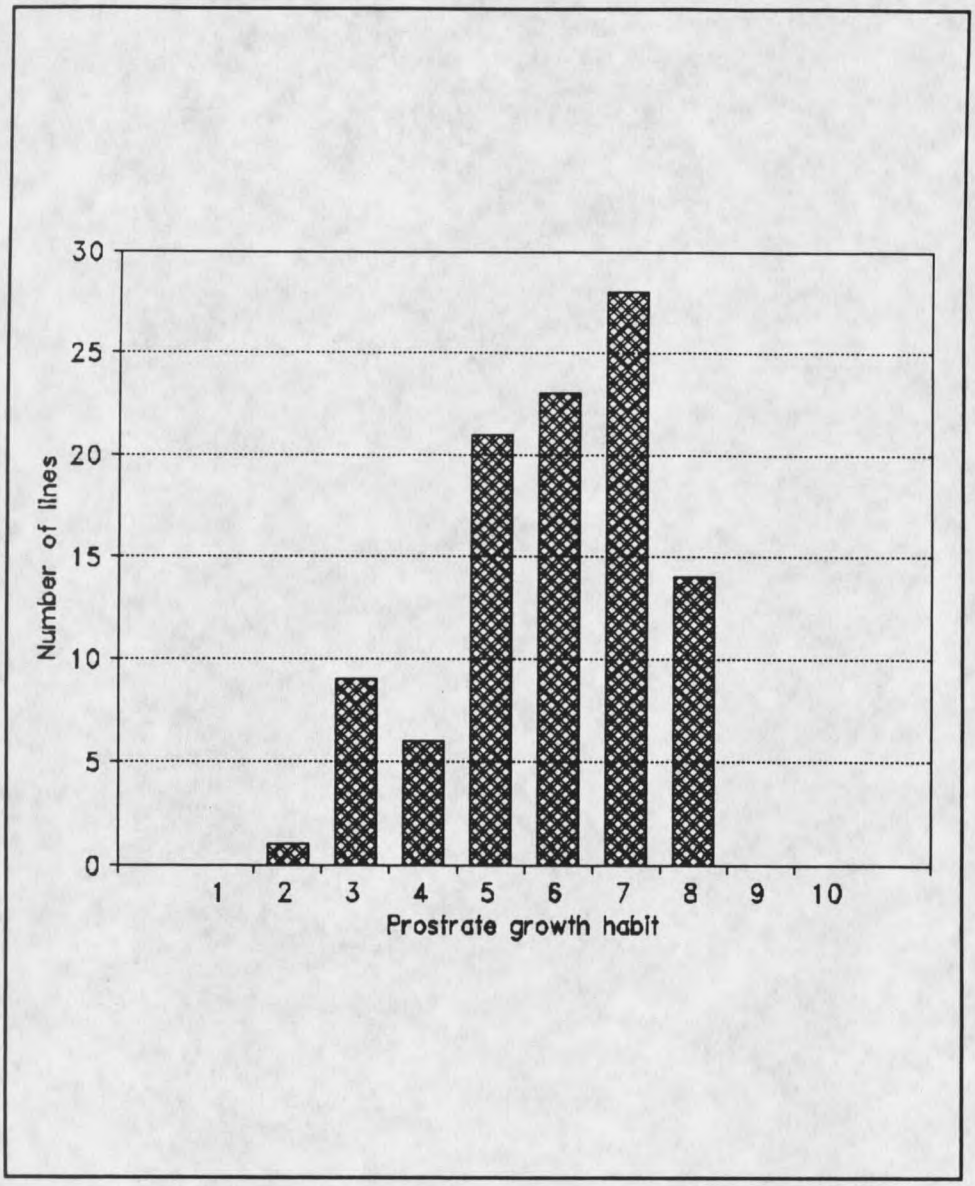

Figure 14. The distribution of prostrate growth habit for Dicktoo x Morex lines.

Heading Date

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 character associated with growth habit. The significant transgressive segregation and discontinuous expression of heading date under 24 light 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 11 and 12). 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. Significant QTL effects for growth habit were not detected on chromosome 4 and 5 (site of the Sh and Sh3 loci, respectively). Large QTL effects (LOD $\geq$ 19) were detected on chromosome 7, again in the mR-BCD265b interval. 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. Small QTL effects were detected in chromosome 2 in the aNAR7HF-aWG178 interval with the LOD value 2.48. In the greenhouse, QTL for heading date trait also fell in the same interval as heading date at 24 hr light. The log-likelihood for the heading date QTL in mR-BCD265b was 11.37.

Fructan Content of Crown Tissue

The parental polymorphism for fructan content, together with the wide range of crown fructan content in their progeny (Figure 13) 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/gdw. The transgressive segregants with fructan substantially higher than Dicktoo (137 mg/gdw), indicates that both parents may have

contributed alleles for fructan accumulation.

The QTL analysis revealed substantial effects on chromosome 7 The LOD plots follow approximately the same pattern as those for field survival, LT_{50}, and growth habit. Log-likelihood of the QTL in the mR-BCD265b was 5.05 and accounted for 20.8% of the variation.

Prostrate Growth Habit

The distribution of prostrate growth habit in this population is shown in Figure 14. Morex is erect while Dicktoo is prostrate. There was transgressive segregation toward Dicktoo, the winter barley. QTL effects of this trait fell into three intervals; ap327-aMSU21, ap340a-iPGD, and pKSU24-C3a. Log-likelihood values of the first two intervals were 2.31 and 2.36, respectively. Log-likelihood of the pKSU24-C3a (Chromosome 7) was 3.37.

From our data, the mR-BCD265b interval on the long arm of chromosome 7 contributed to QTL effects for all the traits except prostrate growth habit. Multiple regression analysis was performed in this interval and the R^2 values are shown in Table 8. As expected, the R^2 of prostrate growth habit is the smallest among the traits. Field survival at Bozeman, MT gave highest R^2. The difference of R^2 values for field survival between the two locations may result from the differences in cold severity conditions and the precision of the experiment. Three replications conducted at Bozeman may have increased the precision of field survival measurements. Obviously, the accuracy of QTL analysis is dependent upon the accuracy with which the traits can be measured. Considering the R^2 values of the two flanking markers of the interval, the mR locus has higher association with fructan content, and field survival than has

BCD265b locus does. The reverse is true for heading date at 24 h light. Since different R^2 values between the two markers were detected for heading date under 24 h light and field survival at Oregon were detected (Table 8), means of the ration of the two traits were analyzed from the two groups of the recombinant individuals in the interval. Two fold differences between the two means were observed. This suggested that the relationship between field survival at Oregon and heading date under 24 h light was due to linkage, not pleiotropy. More markers and individual carrying recombinant type in this interval are needed to resolve the question of pleiotropic effect.

Table 8. Regression coefficients of mR and BCD265b loci with winterhardiness parameters

Traits	mR	BCD265b	mR and BCD265b
Fructan	0.21	0.15	0.23
Oregon survival	0.31	0.18	0.33
Bozeman survival	0.51	0.56	0.68
LT_{50}	0.20	0.20	0.25
Heading date 16 h light	0.32	0.33	0.41
Heading date 24 h light	0.29	0.38	0.42
Prostrate growth habit	0.13	0.04	0.13

As was suggested by Lebowitz et al (1986) and Lander and Botstein (1989), the power with which we detected QTL variance effects with selective genotyping was smaller than with random sampling. Weller and Wyler (1992) found that if 2% of the individuals were selected at the middle and 8% from the tail of the distribution, then power was approximately equal to that obtained with random sampling. Since many marker loci used in this map were based on selection of phenotypes from the tails of the field survival distribution at Corvallis, OR, we

constructed a correlation matrix to estimate the mean effects of mapped markers on phenotypes. Historically, this was the most commonly utilized technique to map genes controlling quantitatively measured phenotypes (Lincoln and Lander, 1990).

Of the 77 markers mapped in this experiment, 30 markers were mapped using 30 individuals while the remainder were mapped using the entire population. Sample size can have a dramatic impact on the accuracy of a linkage map, and the precision of our recombination estimates between markers therefore is variable within the linkage map. Further, interval analysis approaches to QTL identification utilize data only when genotypic data is available on both sides of the interval. If a marker in which a complete dataset was available was adjacent to a marker containing only 30 genotype datapoints, then the QTL analysis would utilize only the 30 phenotypic values corresponding to the intersection of the two genotype datasets. As an example, this occurred in the interval on chromosome seven from KSU24-c3a. The accuracy of the QTL analysis as implemented by Mapmaker-QTL is questionable in instances of this type.

Simple correlation and regression analysis provides a simple approach to QTL analysis which utilizes all of the data available for each mapped marker. When Table 9 is contrasted with the LoD score estimates of Mapmaker-QTL, all of the significant interactions identified by Mapmaker-QTL are similarly identified by simple correlation analysis. However, Mapmaker-QTL failed to identify interactions detected through the use of correlation analysis. When datasets are less than perfect, correlation analysis may provide more information than interval analysis.

A separate question concerns the use of selective genotyping as a

cost-effective approach for the identification of QTL. The best way to address this question with this dataset is to ask whether the QTL identified using complete datasets would likewise have been identified with selective genotyping.

When correlation analysis of marker mR, KSU24, and BCD265b were conducted on the 30 lines identified by selective genotyping, significant correlations were found for all traits, and the correlations were of the same magnitude as were those identified using 100 progeny. The levels of statistical significance for LT_{50} and mR, and LT_{50} and BCD265b were reduced to 5% instead of 1%. More complete datasets are always to be preferred to less complete, but it appears in this case that a more complete dataset would result in the conclusions we obtained.

Table 9. Significant levels of associationa among markers and winterhardiness parameters.

Markers	Fructan	Oregon survival	Bozeman survival	Prostrate growth	Heading date(16)	Heading date(24)	LT_{50}
C8	*	NS	NS	NS	NS	NS	NS
aMSU21	NS	NS	NS	**	NS	NS	NS
aPST327	NS	NS	NS	NS	*	NS	NS
PST59	NS	*	NS	NS	NS	NS	NS
CDO588	NS	NS	NS	NS	NS	*	NS
aWG110S	NS	NS	NS	*	NS	NS	NS
aNAR7HnF	NS	NS	NS	NS	*	**	NS
aWG178	NS	NS	NS	NS	**	**	NS
aPST340b	NS	*	NS	NS	NS	NS	NS
F8	NS	NS	NS	**	NS	NS	NS
F4	*	NS	NS	*	NS	NS	NS
aGlu2	NS	NS	NS	**	NS	NS	NS
BCD265a	NA	NS	NS	NS	NS	*	NS
WG1026b	NS	NS	NS	*	NS	*	NS
D7B	NS	NS	NS	NS	**	**	*
WSP	NS	NS	NS	*	NS	NS	NS
HorD	*	NS	NS	*	NS	NS	NS
BCD265c	NS	*	NS	*	NS	NS	NS
aPST340a	*	NS	NS	**	NS	NS	NS
iPGD	NS	NS	NS	*	NS	NS	NS
LMWP	NS	NS	NS	**	NS	NS	NS
aNAR7L	NS	NS	NS	NS	NS	NS	*
OPA11	NS	NS	NS	NS	*	*	NS
pTA71	NS	*	NS	NS	NS	NS	**
WG364b	NS	**	**	NS	NS	NS	NS
BCD298	NS	**	NS	NS	NS	NS	NS
BCD265b	**	**	**	*	**	**	**
pKSU24	**	**	**	**	**	**	**
pKSU32	*	NS	NS	NS	**	**	NS
C3a	**	**	**	NS	**	**	*

Table 9 (continued).

Markers	Fructan	Oregon survival	Bozeman survival	Prostrate growth	Heading date(16)	Heading date(24)	LT_{50}
mS	*	**	**	NS	*	*	NS
mR	**	**	**	**	**	**	**
WG1026a	**	**	**	**	**	**	**
PST321	NS	NS	NS	NS	**	**	NS
C7	NS	*	NS	NS	NS	*	*
BCD175	NS	NS	NS	*	**	**	NS

NS = Statistically non significant.
* = Statistically significant at 95% level.
** = Statistically significant at 99% level.

CHAPTER 5

SUMMARY

This study provides the first reasonably complete dataset designed to permit evaluation of the genetic control of physiological aspects of winter hardiness in barley. The dependence of cold tolerance on crown fructan content, heading date, growth habit and LT_{50} was investigated. The location of genes modifying expression of each of these characters was identified, and the magnitude of the effects of allelic variation estimated.

Crown fructan content appeared to be associated with winter survival and cold tolerance. Growth habit and heading date appeared to be less directly associated with winter survival, although linkage among genes controlling each of these characters may have made selection for useful recombinants difficult.

Analysis of the percent of lines in tails of trait distributions demonstrated the potential difficulties which can be generated from the use of tailed distribution analysis. Long term winter survival is still the best estimator of winterhardiness, and one or two experiments' data can lead to a poor assignment of lines to distribution tails. Further, while the Bozeman winter of 1992 provided good separation among lines which showed relatively good winter survival, half of the lines in the experiment showed no survival. Multiple year and location experiments would aid in the development of accurate phenotypic description of these lines.

This project did provide support for the contention that recombinant

inbred lines, especially doubled haploids, are uniquely suited to quantitative trait analysis when the trait under analysis is difficult to measure. Only recombinant inbreds provide mapping resources which permit the development of replicated experiments at multiple locations and over multiple years.

Many RFLP maps have been constructed solely using Southern blot analysis. In this project, we used polymerase chain reaction (PCR) to facilitate map construction in this cross. The primer sequences for PCR were generated from sequence-tagged-sites of the clones from the barley literature and from the barley genome mapping project. We found that the frequency with which we detected polymorphisms using PCR was similar to Southern blot analysis.

Seventy-seven markers used in this study were resolved to eight linkage groups. Seven linkage groups were known from prior mapping of the chromosomal location of markers. One linkage group (that assigned to barley chromosome 6) may be inaccurately located. Further use of markers will clarify this problem.

QTL effects of all winter-hardiness-associated parameters (excepting prostrate growth habit) measured in this study were found to map to a single 20cM interval on the long arm of chromosome 7. The mR-BCD265b interval on chromosome 7 was the location of genes modifying these traits. When recombinants within this interval were carefully evaluated, we found that a gene modifying survival at Oregon and a gene modifying flowering date under 24 hours' illumination occupied locations at different ends of the interval. This provides to our knowledge the first test of linkage versus pleiotropy for QTL residing in a common interval. It also suggests

that recombinants can be selected which break the association between survival and late flowering.

Three loci were found to modify crown fructan content. The largest effect mapped to the interval on the long arm of chromosome 7, while two loci with less dramatic effects were mapped to the short arm of chromosome 7 and the long arm of chromosome 5. The fructan QTL on chromosome 5 appeared to cosegregate with winter survival measured at Oregon in 1991. The chromosome 7 fructan QTL showed no detectable interaction with winter survival at either location. More accurate measurement of winter survival among these lines may help determine whether crown fructan content is truly a critical factor in determining winterhardiness.

A total of three mapped intervals were found to contain QTL which modified growth habit. Intervals on chromosome 2 and chromosome 5 accounted for 15.2 and 15.7 percent of the variation for this trait. Major effects on heading date were located to the chromosome 7 interval and to an interval on chromosome 2 which accounted for approximately 13% of the variation for the character.

These data suggest that improvement in winter survival may be accompanied by earlier heading and more erect growth if appropriate selection is performed. They also accentuate the importance of appropriate selection of parents and accurate analysis of the phenotypic value of lines when performing a QTL experiment.

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