



The identification of new genes through the use of molecular marker technology in barley (*Hordeum vulgare* L.)
by Deven Robert See

A thesis submitted in partial fulfilment of the requirements for the degree of Masters of Science in
Agronomy
Montana State University
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Abstract:

Molecular marker technology has permitted many advancements in crop breeding. Three applications of this marker technology are discussed within this paper.

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The application of SNP markers in mapping led to the discovery of a multigene family in barley (*Hordeum vulgare* L). The puroindoline *pinA* and *pinB* genes are important factors in the end use milling and halting quality in wheat (*Triticum aestivum*). Wheat has a single copy of the *pinB* gene on each of its three chromosome, however only the *pinB* gene on 5D is expressed. This report demonstrates that barley has multiple, expressed copies of the *pinB* gene on barley chromosome 7 (5H).

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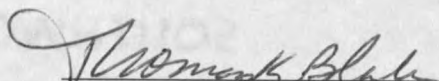
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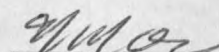
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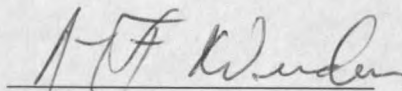
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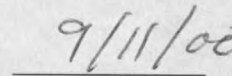

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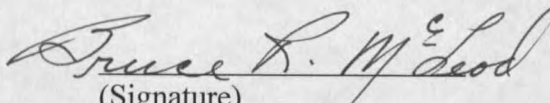
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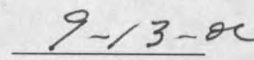

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The application of SNP markers in mapping led to the discovery of a multigene family in barley (*Hordeum vulgare L.*). The puroindoline *pinA* and *pinB* genes are important factors in the end use milling and baking quality in wheat (*Triticum aestivum*). Wheat has a single copy of the *pinB* gene on each of its three chromosome, however only the *pinB* gene on 5D is expressed. This report demonstrates that barley has multiple, expressed copies of the *pinB* gene on barley chromosome 7 (5H).

CHAPTER 1

INTRODUCTION

Nitrogen and Malting

Barley (*Hordeum vulgare* L.) is primarily utilized for livestock feed and malting. In beer production one of the most important factors affecting malt quality is grain protein content (Bezant et al., 1996). The American Malting Barley Association AMBA requires six-rowed and two-rowed malt barleys to maintain grain protein lower than 135 and 130g kg⁻¹ respectively (Weston et al., 1993). Many studies have shown that while high total grain protein content is good for monogastric feed, it is negatively correlated with malt extract yield (Garcia del Moral et al., 1998, Ulmer et al., 1985).

Trait improvement depends upon the availability of heritable variance, and upon effective selection. While most barley varieties are highly responsive to both fertilizer treatment and moisture stress with respect to grain protein content, one variety, 'Karl' has been shown to be relatively non-responsive. 'Karl' produces grain with low grain protein content under a wide array of environments (Weston et al., 1993). Karl has been used as a parent in many barley improvement programs, and to date no accepted 6-rowed barley variety has been developed which combines low, stable grain protein percentage with acceptable agronomic performance and malting quality. Since variation for grain protein percentage is both reasonably heritable and easily measured, this is either the result of poor application of selection or of undesirable repulsion linkages. In this project we

mapped the chromosomal locations of genes responsible for variation in grain protein content, plant height, flowering date and grain yield. This project was undertaken with the expectation that a better understanding of the genetics underlying variation in grain protein content would help us understand how best to select for desired protein content.

Marker Technology

Genetic markers can be made into useful tools for plant breeding. In plants like barley, morphological markers like rachilla length, awn texture or head type, are easy to identify making them quick determinants of the source of inheritance of specific traits. The limited number of good morphological markers emphasizes a need for more common and informative classes of markers. The characterization of DNA sequence diversity has brought about new genetic markers. The first class of genetic markers based upon sequence differences were restriction fragment length polymorphisms (RFLP) (Botstein et al., 1980). RFLPs rely upon digestion of genomic DNA using restriction endonucleases, blotting (Southern et al., 1975), and hybridization with a radioactively labeled probe (Rigby et al., 1975). Restriction endonucleases cleave DNA at specific recognition sequences producing polymorphic products between alleles of different genotypes.

With the invention of the polymerase chain reaction (PCR) by Mullis and Faloona, (1987), more rapid methods for sequence polymorphism detection became available. PCR permits the exponential amplification of specific DNA sequences. PCR uses primers, short lengths of DNA, in conjunction with buffers and DNA Polymerase II derived from bacteria living in extreme thermal environments. PCR provided the machinery to be able to produce billions of identical copies of DNA from a single copy of sequence present in the genomic DNA. The discovery of PCR technologies by Mullis

opened up molecular biology to new and faster methods for research. These new methods include the use of sequence tagged site (STS) markers (Tragoonrung et al., 1992), simple sequence repeat (SSR) markers (Liu 1996), and amplified fragment length polymorphisms (AFLP) (Vos et al., 1995). STS markers are usually derived from known sequences from RFLP probes. STS markers are amplified using short primer pairs ~ 20bp in length that are used to prime the amplification of DNA. This DNA can derive from two sources, genomic DNA or cDNA which is complementary DNA produced by reverse transcriptase from a mRNA template. SSRs, are simple sequence repeat motifs which mutate rapidly due to DNA polymerase slippage during synthesis and repair. Primer pairs which direct amplification of SSRs often identify multiple alleles within a population. SSRs are excellent markers in mapping. (Liu et al., 1996). The AFLP technique (Vos et al., 1995) is a combination of RFLP and PCR. The AFLP technique uses four and six base restriction enzymes to digest genomic DNA. PCR amplification of the digested fragments using selectable primer combinations produces multiple restriction fragments per gel. While RFLPs were able to identify one polymorphism at a time, AFLPs are able to identify multiple polymorphisms at once thus increasing the efficiency of the technique and research productivity. In a comparison between various marker types and their ability to detect polymorphisms, Russell et al., (1997) determined that AFLPs produce more polymorphisms than RFLPs. This high rate of polymorphism detection makes AFLPs an ideal tool for detecting differences between individuals within a population (Powell et al., 1997). They are also ideal for constructing a genetic map

(Becker et al., 1995, Waugh et al., 1997) since they produce multiple scored markers per primer combination.

A recent type of molecular markers are single nucleotide polymorphisms (SNPs). SNPs are the most abundant type of discriminating DNA sequence differences that can be found in any genome. Marker technologies based upon point mutations take advantage of single base differences between alleles of individuals. These single base differences permit determination of allele states. Recently Cho et al., (1999) developed a genetic map in (*Arabidopsis thaliana*) using SNPs. Since SNPs are the most abundant type of mutations within populations this technology will become more prevalent as more cost effective and accurate development and detection approaches are developed.

Mapping & Marker Assisted Selection

Linkage maps and quantitative trait analysis can help breeders determine whether or not a trait with marginal heritability may be suitable for improvement through selection. Genetic maps locate genes contributing to variation for a trait of interest, and as a consequence identify genetic markers tightly linked to valuable alleles. While most traits are determined by the complex interactions of many gene products with the environment, a few gene differences generally control most of the heritable variation for many traits of interest, rendering marker-assisted selection often practically useful.

Quantitative traits by definition are traits that are controlled by more than one gene. Genetic maps are ideal for isolation of the numerous loci regulating quantitative traits especially in crop plants where genotype by environment interactions are high. Genetic maps also assist in the cloning of genes of interest. Wing et al., (1994) used map based cloning to identify the position and sequence of the jointless gene in tomato.

In barley, as in many crop plants genetic maps have been developed to not only identify genes regulating traits, but to use these maps to identify markers that can be used in future breeding programs.

Marker assisted selection depends upon the development of genetic maps to identify linked markers to valuable traits. Marker assisted selection applies genetic markers to population development to increase the likelihood of favorable alleles in the outcome of crosses. Disease resistance is a good example of simple marker assisted

selection, where marker assisted selection is used to report the successful incorporation of favorable alleles (Toojinda et al., 1998). 'Valier' (PI 610264) a feed barley released in Montana in 1999, was developed with the aid of molecular markers. (Marker assisted selection should be effective for tightly linked markers to favorable alleles under the conditions that there is little or no epistatic interactions between genes. One concern in marker assisted selection is the resolution of the QTL with respect to linked markers. Selection using markers that are weakly associated with QTL may actually reduce trait heritability in selected populations if the alleles of interest are inadvertently lost through recombination (Lande and Thompson, 1990). Sufficiently high resolution mapping is the key to successful identification of informative markers that are tightly linked to QTLs of interest. Good genotype by environment statistical analysis is essential in determining the importance of gene by environmental effects, and therefore the reliability of heritability estimates for specific QTL.) Applying stringent criteria to mapping and analysis of QTLs helps alleviate problems before they arise.

Technology development, technique application and serendipity are each important components of a research project. In this project, we developed a practically useful approach to SNP detection. We then used SNP and other marker technologies to develop a genome-wide linkage map in a population of recombinant inbred lines derived from the cross 'Lewis' x 'Karl'. Using three years' replicated yield trial data, we identified the major genes contributing to variation for grain protein content which were observed to segregate in this population. We demonstrated that two of these genes were

associated with genes modifying flowering date, a result which perhaps explains the slow progress made to date in grain protein content improvement. A serendipitous observation in SNP analysis led to a series of experiments in which we discovered that a gene (*PinB*) which is single-copy in wheat is multi-copy in barley.

CHAPTER 2

SINGLE NUCLEOTIDE POLYMORPHISM DETECTION

Introduction

Several approaches have been described which permit the assay of DNA sequence polymorphisms within individual targeted loci (Chen et al., 1997, Haff et al., 1997, Landegren et al., 1998, Liu et al., 1997, Shumaker et al., 1996). The value of massively multiplexed linkage analysis has been reported for the yeast genome utilizing microarrays (Winzeler et al., 1998). A study using human volunteers utilized a similar detection platform and reported discrimination among alleles at over 100 loci (Wang et al., 1998). The human genome (3×10^9 base pairs per haploid genome) is roughly 300 times larger than the yeast genome, resulting in a 300-fold dilution of target sequence per nanogram of DNA. Preamplified specific genomic sequences containing characterized allelic variants prior to allele detection are needed in order to make allele detection successful (Wang et al., 1998). Products of amplification were then annealed to arrays carrying short oligonucleotides which spanned regions containing previously characterized mutations. Differential duplex stability due to internal sequence mismatch helped to differentiate among alleles within samples. Recently, a chip-based platform was described which provides the possibility of electrophoretic concentration of amplification products at hybridization sites (Gilles et al., 1999). Like that of Wang (Wang et al., 1998),

this detection approach depends upon preamplification, then utilizing two differently-labeled allele-specific primers per locus, each of which matches one of two possible allele states at the 3' nucleotide. Following initial amplification labeled internal primers were added to the preamplification mix along with fresh Taq polymerase, unlabeled opposite strand primer, dNTPs and reaction buffer. Fluorescent products were amplified then resolved using either polyacrylamide gels for detection on the FMBIO II (Hitachi) gel scanning system, or denaturing polyacrylamide gels for detection on the ABI377 automated DNA sequencer. These approaches permit characterization of products both by size and by fluorescent label, which permits efficient multilocus analysis.

Materials and Methods

SNP Identification

Total barley DNA was isolated from foliar tissue of the barley cultivars Steptoe (CI15229), Morex (CI15773), Lewis (CI15856), Baronesse (PI568246) and Karl (CI15487) using the technique of Dellaporta et al.,(1983). Amplification was done according to Tragoonrung et al., (1992), PCR products were cleaned up to remove excess salts and primers on Quiagen columns. Sequencing was done on an ABI377 automated DNA sequencer and sequence was read in both the forward and reverse direction. Data analysis was done with DNASIS. A total of 20.86kb of DNA was analyzed per cultivar, of that 13.12kb was from genomic DNA and 7.74kb was from cDNA. From the DNA analyzed a few representative SNP's were chosen for fluorescent analysis.

Production of Fluorescent Product

Following amplification, the alternative alleles of ABC255, ABG65, and ABC305 for these five barley cultivars were sequenced using an ABI377 automated DNA sequencer and the Perkin Elmer Big Dye sequencing reaction kit. Sequencing was performed in both directions, and final sequences analyzed using DNASIS (Hitachi corp.) see (Table 1) for specific sequence and primers. Allele-specific fluorescently labeled primers were synthesized by IDT using either 6-carboxyfluorescein,(Fam) or 4,7,2',4',5',7'-Hexachloro-6-carboxyfluorescein (Hex). ABC255 and ABC305 map to barley chromosome 1, while ABG65 maps to barley chromosome 6 (Kleinhofs et al., 1993). Since the ABC255 polymorphism lies within an *Fnu4HI* restriction site, we were

able to validate it using restriction analysis. Primary amplification products from ABC 255 were combined with 1 unit of *Fnu4HI* and one microliter of 10x reaction buffer (New England Biolabs), then incubated for 1 hour at 37°C prior to electrophoretic analysis. In the parallel fluorescent SNP detection reactions one microliter of primary PCR product was mixed with 1 unit Taq polymerase and the solution brought to a final concentration of 1mM of each dNTP, 1.5mM MgCl₂, 10mM Tris-Cl pH 8.0 and 50 ng each of both fluorescent internal primers and unlabeled reverse strand primers.

Following one minute of denaturation at 92°C, samples were amplified through thirteen cycles (30 sec 92°C, 30 sec 45°C, 45 sec 72°C), with five minutes at 72°C prior to reaction termination at 4°C when using the FMBIOII. Only seven cycles of amplification were required for detection using the ABI377. A simulated heterozygote was produced by mixing equal quantities of DNA from both Lewis and Karl. This sample was treated as previously described.

Detection Platforms

The Hitachi FMBIO II is a flatbed fluorescence detection platform which can be utilized as a gel scanner. One was generously loaned to our project for the purposes of conducting this experiment by Hitachi-USA (San Jose, CA).

Ten microliters of secondary reaction mix or restriction digest were mixed with 2 microliters of 50% glycerol containing a small amount of bromphenol blue, then loaded onto a 7% acrylamide gel running in a continuous 0.5X TBE (Maniatis 1989) buffer system at 200V (20V/cm). HaeIII-digested PhiX174 was utilized as molecular weight

marker in the restriction digestion. Gels were run until the bromphenol blue migrated to the edge of the gel. Following electrophoresis, the restriction digest gel was stained with ethidium bromide. Both gels were transferred onto a borosilicate glass plate, placed in the FMBIO II and scanned. The restriction digest gel used a 605 nm cutoff filter which permits detection of ethidium bromide fluorescence (figure 1). The SNP detection gel was scanned twice, using filter sets which differentiate among FAM at 505nm and HEX at 585 nm. In figure 2 the FAM-labeled products are green and the HEX-labeled products are red. The ABI 377 is primarily used in our laboratory for sequencing and amplified fragment length polymorphism (AFLP) detection (Vos 1995). This platform also works well to characterize SNPs . One microliter of secondary amplification product was diluted 1:30 in 24ul ddH₂O and 5ul Promega loading dye. Samples were denatured at 94°C for 3 min. One microliter of diluted product was loaded on a 48 well membrane comb CAM48-750 obtained from The Gel Company. The samples were run in a 6% polyacrylamide denaturing gel in a 1X TBE buffer. The gel was prerun for 15 min. then loaded and run for 3 hrs. at 750V and 51°C.

Results

To determine the validity of identifying point mutations two experiments were performed. The first experiment tested whether the ABC 255 transition mutation at position 330 would be faithfully detected by the fluor-tagged primers. This was done by comparing a restriction digestion of the point mutation in an array of segregants using *Fnu4HI* and then evaluating the products of secondary amplification with fluor-tagged primers. The samples tested included the cultivars Steptoe and Morex, two cultivars which differ at position 330 by a C/T transition mutation, and 13 samples from a double haploid line (DHL) population derived from a Steptoe by Morex cross (figure 1). The *Fnu4HI* digestion produced a 410bp fragment in the Steptoe allele and a 316bp fragment in the Morex allele. Figure 2 shows Steptoe and Morex and the 13 DHL segregants with the transition mutation C (red, Morex) or T (green, Steptoe) alleles. The second experiment involved multiple allele and multiple cultivar analysis using ABC 255, ABG 65, and ABC305 with the cultivars Lewis, Karl, Morex, Steptoe, and Baronesse. This was a two part assay evaluating both multiplexing and heterozygosity analysis. The first part of this test involved multiplexing the primer sets ABC 255, ABG 65, and ABC305 through both the initial PCR and secondary SNP amplification. All of the samples in figure 4 were run under the same amplifying conditions. The first panel of figure 3 shows the results obtained when all three primer sets were used to detect polymorphisms simultaneously. The next three panels show the results obtained using the primer sets individually. Panels are separated by molecular weight ladders.

Figure 1

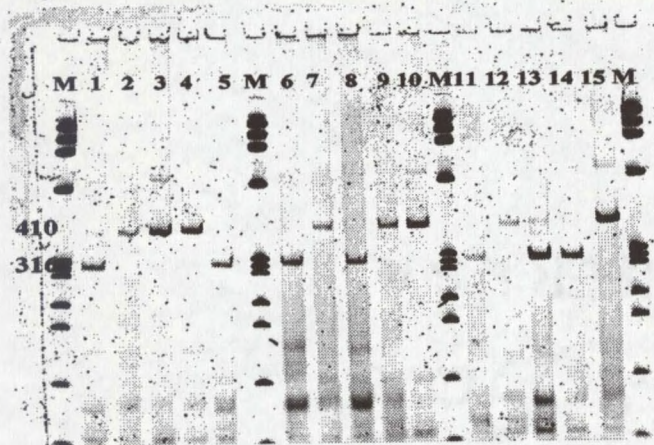


Figure 1. FMBIOII, ethidium stained *Fm4H1* restriction of ABC255 amplification products of Morex (lane 1), Steptoe (lane 2) and a subset of doubled haploid lines from their F_1 . Molecular weight standards Phi X lambda are in lanes labeled 'm'.

Figure 2

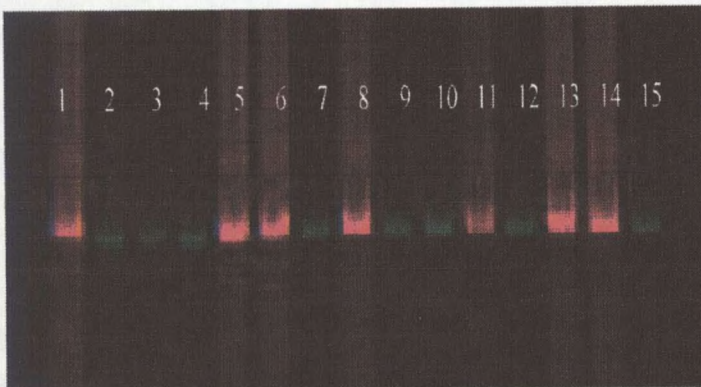


Figure 2. FMBIOII, Fluorescent primer detection of the ABC255 C/T transition mutation using the same DNA samples used in figure 2.

FIGURE 3

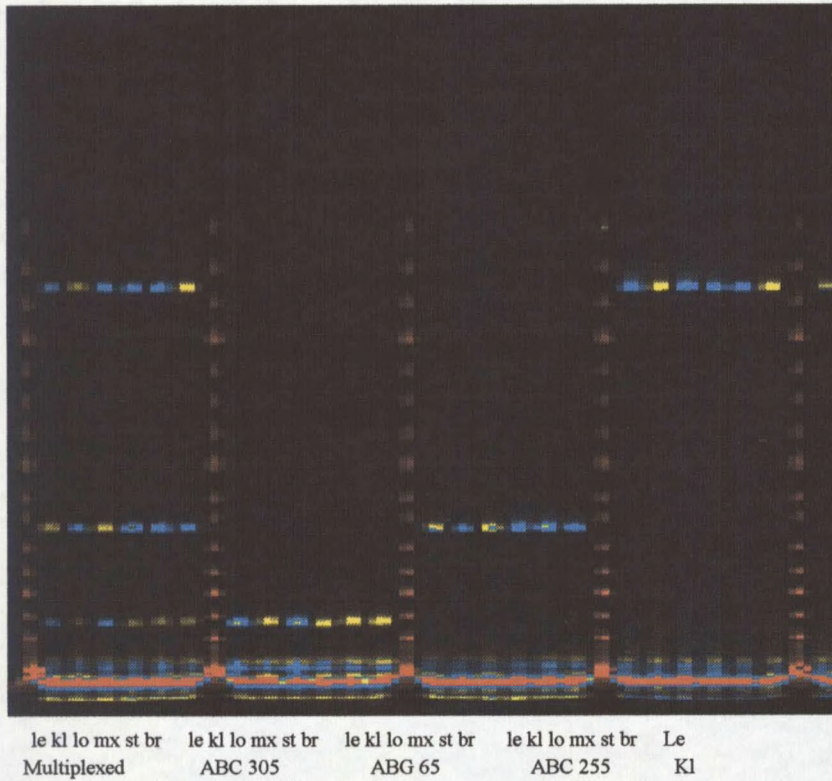


Figure 3: ABI 377

fluorescent primer detection of multiplexed SNP reaction using the cultivars Lewis, Karl, Logan, Steptoe, Morex, and Baronesse respectively. The first six samples are the result of multiplexing ABC255, ABG 65, and ABC 305 thru both the primary PCR and secondary SNP annealing PCR. The next three sets of samples are used as controls to show proper annealing of SNP primers at transition sites with ABC 305, ABG 65, and ABC 255 respectively. The sample in the last lane shows the heterozygote of Lewis and Karl. Fluorescent ladder (CXR) 60-400 bases from Promega.

Figure 4

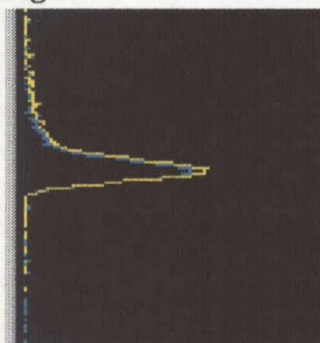


Figure 4. ABI 377 fluorescent primer detection of heterozygosity between Lewis and Karl at the ABC255 C/T transition mutation. Image on left side is the electropherogram peak intensity showing near equal emission of both the Fam and Hex labeled primers.

In the last panel, DNA from Lewis and Karl were mixed together to simulate a heterozygous sample. This simulated heterozygote was amplified with ABC 255 primers. Migration of the heterozygous product is shown in the last lane of figure 4. Peak intensity and color of the sample was evaluated, showing approximately equal incorporation of both the C and T primers in figure 4.

Discussion

SNP detection using 3' primer selection has thus far proven reliable under standard PCR conditions across two different detection platforms. The use of the 3' primer base selection provides a specificity that permits direct scoring of polymorphisms and determination of heterozygotes within a population. We used individuals from a doubled haploid line (DHL) population as a model system to validate SNP segregation. In figure 1 DNAs from a subset of the Steptoe x Morex DHL population were used as a source of templates for lanes 3-15 (Hayes et al., 1994). *Fnu4HI* digestion produces a 410bp fragment from the Steptoe allele and a 316bp fragment from Morex. The proper annealing of the SNP primers to these same DHL samples is demonstrated in Figure 2. We can clearly differentiate among alleles carrying either C (red, Morex) or T (green, Steptoe) at position 330 within ABC255. The SNP detection results perfectly match restriction site analysis (figure 1). Two further assays were carried out to demonstrate the possibility of multiplexing and the use of other detection platforms. Figure 3 shows the fluorescent detection of ABC 255, ABC 305, and ABG 65 after multiplexing. In the multiplexing reaction the three primer sets were used to simultaneously direct product amplification. The products from this multiplexed primary PCR were used in the secondary PCR amplification with the corresponding SNP primers. In the next three sets of samples ABC 305, ABG 65 and ABC 255 were amplified individually. The last sample in figure 3 is a simulated Lewis/ Karl heterozygote amplified with ABC 255

primers. Figure 4 shows the electropherogram peak intensity of the simulated heterozygote between Lewis and Karl at the ABC 255 loci. Both multiplexing and heterozygote analysis provided accurate genotype designation. Experiments in which large numbers of individuals must be surveyed for informative meioses in specific genomic regions demand experimental designs which permit cost-effective analysis of large numbers of individuals. Parallel systems (like gels) provide the opportunity to obtain moderately dense datasets for large numbers of individuals. Amplification products containing different fluorophores may be separated by size, permitting potentially many loci to be assayed per gel lane.

Multiplexing of any DNA analysis system has limitations. Reassociation kinetics determines the specificity of the priming step of DNA synthesis, and the error rate of reassociation (i.e. the relative frequency of inappropriate priming events) is dependent upon the complexity of the DNA in solution, and the ratio of potential appropriate versus inappropriate priming sites. Inappropriate priming events occur more often when a large number of potential priming sites are available. The simplest approach to lessen this problem is to preamplify the sites of interest, and in doing so reduce the pool of available sites for detection to a relatively small number. As a rule of thumb, sequences found in genomes larger than 10^9 base pairs per haploid complement will require preamplification prior to detection (Syvanen et al., 1999). This strategy has been used and an effective limit in multiplexing has been found to exist at ~ 20 target loci per primary amplification reaction (Wang 1998). In a gel based assay 20 target loci, and 20

In a gel based assay 20 target loci, and 20 fluorescent bands of DNA per gel lane should be simple to resolve if primers are well selected and amplification product sizes are sufficiently distinct.

Table 1: SNP Sequence and Primers**ABC255**

ACAGCGACAA	GGCCGCAGAT	ATCTTGGTCA	ACTTCTTTGA	GAAGAGCACG
GCGGATCCAA	GCTACTGGGA	CAAAATCTCC	CAGGGAGGCC	TGAAGAGAAT
TTATGAGAAG	TATGTGTATT	CTCTTCTTGT	TGTGCCAGGA	TAGTTCCAAG
TCATGTCCCT	GAACAAATGT	TGATCCTATA	ATTTCTGAAT	GCTCAGGTAC
ACCTGGAAGC	TCTACTCAGA	GAGGCTGATG	ACCC'TGACCG	GTGTGTATGG
GTTCTGGAAG	TACGTGAGCA	ACCTGGAGAG	GCGCGAGACT	CGCCGTTACC
TGGAGGATGT	<u>TCTACGCTCT</u>	<u>CAAGTACCGY</u>	<u>AGCTGGTAGG</u>	TTATCTGACC
AACAGAGAGG	AACATTATCA	GTGTCCACTT	CAGTATGCTT	ACTTAGGGTT
TGTATGTTTT	GTTGAATTCA	GGCTGCTGCA	GTTCCATTGG	CGGTGCACGG
CGAGAGCAGC	GGCAACTAGC	GCGGATTCGG	GGCATGAAGA	GGGCGCATTC
ATCGAGCAGG	AGGGTGAAGT	GTCGGCTGCG	CTATGATTTG	TCTACCGAGT

ABG65

TGGCGACTCT	GATGCTACGA	TTGGATCAGC	AGAGCCCACA	AGTTGGCCCC
<u>CGCCTGGGAA</u>	<u>GGACCGTTCA</u>	<u>TCYTCTCTAA</u>	GGTGCAGAAC	AACGGAGCAT
ATCGACTTTA	CAACCTCGAC	AGGGAAACGG	ACGAGCCGCG	AGCATGGAAT
GGAGATCTAC	TGAAGCGCTT	CTACACATAA	CCGCCGATAG	ATCCTCA

ABC305

TGAACACTTC	CCTTGTTCAGA	TTAGACTGGC	GTTTATTTAT	CGTTTTTATAT
TGTGCCCGTG	ACTGGCGTGT	GTTGTCATAT	TCTGTTGTGT	CGTGCAATGC
GAGCCTGCC	TCAATTGCAG	CCTCTTTGAA	TTTTGCTTGT	TGGTATGCTA
TGTGATTGGT	<u>TCTTTTGAAC</u>	<u>ACTGCTGAAC</u>	<u>CTTTTTTYTT</u>	TTTTGAGTGT
AAACACTGCT	GAACCTGATG	AGTAAATTTG	TTGGCG	

INTERNAL SNP PRIMERS:

ABC255:

HEX TCT ACG CTC TCA AGT ACC GC
 FAM-- TCT ACG CTC TCA AGT ACC GT

ABG65:

HEX - CTG GGA AGG ACC GTT CAT CG
 FAM- -CTG GGA AGG ACC GTT CAT CA

ABC305:

HEX - ACA CTG CTG AAC CTT TTT TT
 FAM- -ACA CTG CTG AAC CTT TTT TC

RESTRICTION ENZYME:

*Fnu*4H I restriction sequence = **GC[^]NGC**

CHAPTER 3

MAPPING THE GENES UNDERLYING PROTEIN CONTENT IN A LEWIS BY KARL CROSS

Introduction

A 146 member recombinant inbred population was produced from a cross between a normal grain protein percentage line 'Lewis' CI 15856. (Hockett et al.1985) and a low protein line 'Karl' CI 15487 (Wesenberg et al. 1976). This population was developed with the intent of mapping the genes responsible for the 'Karl' low protein phenotype through quantitative trait locus (QTL) analysis (Paterson et al., 1988).

Karl, is a unique six-rowed malt barley which produces grain of consistently lower protein content and higher malt extract than most other barleys (Wesenberg et al., 1976). Karl was derived from CI 7147 which was developed in Idaho in 1936. CI 7147 is the product of a cross between Good Delta and Everest, however neither 'Good Delta' or Everest exhibit this stable low protein phenotype,(Wesenberg et al., 1976). The other parent in this cross is 'Lewis'. 'Lewis' is a two-rowed spring feed barley variety derived from 'Hector' and 'Klages', The primary reason for studying a cross of these two diverse barleys is to develop a genetic map to identify the QTL controlling the low protein content property inherent in 'Karl'. Previous studies focusing on Karl's low protein content discovered that 'Karl' differs from both its reported parents with respect to grain protein content (Burger et al., 1979). Weston et al., (1993) found that Karl maintained its

low protein characteristic under varying N fertilizer rates. This data supports the contention that Karl's low protein content is less effected by the environment then that of other varieties tested. This low genotype by environment interaction should permit reliable location of the 'Karl' low protein QTL within a genetic map.

Many studies have determined the location of the QTL regulating grain nitrogen protein. In the development of a QTL map for malting quality traits for winter-habit barleys, Oziel et al., (1996) found grain protein QTL on chromosomes 1, 4, 5, 6, and 7. This study evaluated both winter and spring-habit barley populations. In the winter barley experiments map locations of grain protein QTL were on chromosomes 4, and 7. In the spring trials QTL were reported on chromosomes 5, 6, and 7. In a study done with AFLPs on twelve quantitative traits in barley by Powell et al., (1997) QTL for grain nitrogen were found on chromosomes 2, 3, 4, and 7. Bezant et al., (1997) reported eight QTL for grain nitrogen, these QTL were found on every chromosome except chromosome 3. Statistically significant, ($P < 0.001$), QTL were reported on chromosomes 2, 6, and 7. These data show that multiple QTL can be found for grain protein on any chromosome depending on the varieties tested and the conditions under which these tests were conducted.

Materials and Methods

Population Development

'Karl' was crossed with the two-rowed variety 'Lewis'. One hundred forty six six-rowed F_5 derived recombinant inbred lines were isolated. The progeny of the cross were grown in the plant growth center in the winter of 1995. F_5 derived plants were developed through single seed descent, with the F_2 generation grown at the A. H. Post Research Farm, near Bozeman, MT. Plots were harvested in the summer of 1996 and to increase progeny turnover rates, the F_3 and F_4 plants were grown again in the plant growth center over the winter and spring of 1996 and 1997. The F_5 plants were grown at the post research farm. The F_6 plots were planted at the post farm, in two row plots. In 1999 and 2000 the F_7 and F_8 plots were planted at the post research farm in four row plots to help facilitate yield studies.

Previously mapped morphological, storage protein, STS, SSR, and SNP markers, (Kleinhofs et al., 1993, Liu et al., 1996, Kunzel et al., 2000) were used to anchor the map while AFLP markers (Vos et al., 1995 as modified by Blake et al., 1998) were used to fill the map.

Protein and Morphological Markers

Three morphological markers were visually scored. These included: Rachilla hair length, (long for Karl and short for Lewis), and the *v* and *i* genes which control spike morphology. The B and C hordein banding patterns were identified with SDS-PAGE.

electrophoresis from ground seed (Blake et al., 1982).

STS, SNP, and SSR Markers

DNA was extracted from F₅ derived leaf tissue using the protocol of Dellaporta et al., (1983). Amplification of DNA fragments was done by PCR following the protocol of (Tragoonrung et al., 1992). PCR reaction volumes were decreased to 25ul in 1X PCR buffer (Promega, Madison, WC) (50mM KCL, 10mM Tris-HCL, 0.1% Triton X-100,) 2.5mM MgCl₂, 0.1mM of each dNTP 2ng of both the forward and reverse primer, and 0.5 units of taq polymerase (Promega). The thermocycler protocol times were decreased for all applications except for fragments > 1000bp. The program included 94°C 3 min. followed by 35 cycles of 94°C 20sec., 50°C 20 sec., and 72°C 20sec. with a final extension time of 72°C for 2 min. For STS and SSR markers with size polymorphisms (table 2), marker segregation was determined on polyacrylamide gels, (7% acrylamide: 19:1 acrylamide: bisacrylamide), in 0.5X TBE electrophoresis was run at 200V for 3 hours. Segregation of SNP markers was determined by 4% denaturing poly acrylamide gel in 8M urea and 1X TBE (See et al., 2000).

AFLP Marker Detection

The AFLP protocol follows that of Vos et al., (1995) with modifications for detection of fluorescently labeled products for the ABI 377 automated DNA sequencer. Genomic DNA was digested in a 15ul reaction with ~ 100ng DNA digested for 2 hours at 37°C with 3 units of *EcoR* I and 3 units of *Hpa* II. After digestion the restriction enzymes were deactivated at 65°C for 10 min. To the digested DNA a 15ul mix of ligating adapters

Hpa II adapter 5' GACGATGAGTCCTGAG 3' at 150ng
3' TACTCAGGACTCGC 5' at 132ng
EcoR I adapter 5' CTCGTAGACTGCGTACC 3' at 16.8ng
3' CATCTGACGCATGGTTAA 5' at 17.4ng
1X ligation buffer, and 1 unit of T1 Ligase (New England Biolabs). This reaction was run at 37°C for 2 hours. The preamplification reaction consisted of 1ul of ligation reaction product in 30 ul of 0.1mM each dNTP, 1X buffer (Promega) 25nM MgCl₂, 0.5 units Taq polymerase and 30ng of both *EcoR* I primer and *Hpa* II primer. Thermocycler conditions were run for 20 cycles at 94°C 1 min., 56°C 1 min. 72°C 1 min. the secondary reaction included 1ul of preamplification mix product in 20 ul of 0.1mM each dNTP, 1X buffer (Promega) 25nM MgCl₂, 0.5 units Taq and 5ng of *EcoR* I fluorescently tagged primer and 30ng of *Hpa* II primer. Thermocycler conditions followed (Vos et al., 1995). The three selectable base combinations used in the AFLP analysis were *EcoR* I AGA, *Hpa* II CTA, G, C, T, CAA, G, C, T and CGG, C, T. AFLP products were resolved on an ABI 377 automated DNA sequencer in a 4% denaturing poly acrylamide gel in 8M urea and 1X TBE at 250V for 2.5 hours. Internal molecular weight standard Genescan-500 ROX from (PE Applied Biosystems), loaded with each sample, allowed for equilibration of markers within lanes. Segregating AFLP data was analyzed with Genographer (Benham et al., 1999).

Map Construction

Mapping construction of the 146 progeny in the Lewis by Karl six row population was done using Mapmaker 3.0 (Lander et al., 1987). Using a minimum $l_o d$ score of 4.0 as criterion for linkage. Progeny were scored such that Lewis alleles were designated A and

Karl alleles B, all heterozygous scores were treated as missing data. Preliminary chromosome designations were assigned to STS, SSR, and SNP markers based upon previous marker assignments by (Kleinhofs et al., 1993, Liu et al., 1996, Kunzel et al., 2000).

Statistical Analysis

QTL analysis was performed using mapmaker QTL. The F₅ derived plants were grown at the post farm in replicated randomized block designs. Data was collected for each replication, averaged and the averaged data was used in the QTL analysis.

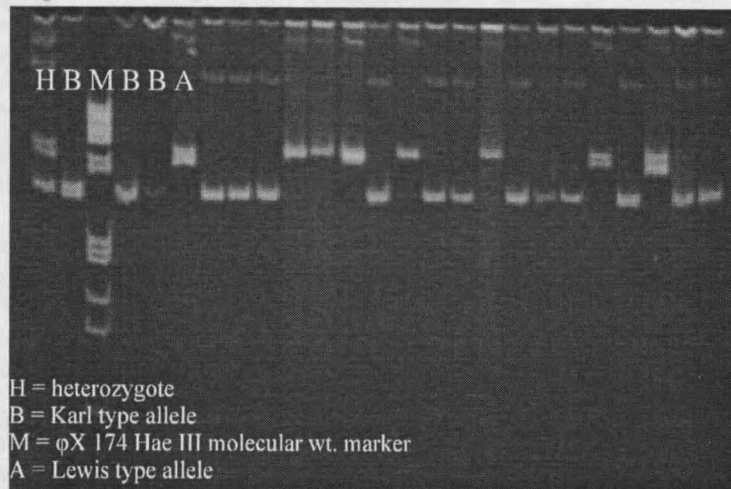
Statistical analysis, ANOVA, was conducted on protein content, heading date, plant height, and yield using a statistical analysis software, (SAS 1988). Phenotypic and QTL marker heritability was determined using regression analysis. Once major QTL were identified, these were evaluated to determine whether they showed additive or epistatic interactions.

Results

STS, SNP, and SSR Markers

While the bulk of the genetic map was made with AFLP markers. A total of twelve STS and SNP eight SSR markers, the hordeins and the three morphological characters were used to anchor the seven chromosomes. STS markers with size polymorphisms like TB 33,34 (figure 5), which has an insertion of ~ 100bp permitted direct scoring of segregation among the recombinant inbred lines.

Figure 5



Five of the twelve STS markers showed no size polymorphisms and were scored with the aid of restriction enzymes. Only one unexpected map location occurred using previously mapped RFLP based STS markers. MWG 502 mapped to the short arm of chromosome 6 instead of the short arm of chromosome 7 according to previous maps. Seven SSR markers were also incorporated into the map as anchor markers. Like STS markers SSR markers are also able to identify a heterozygote within the population. Chapter two

within the population. Chapter two discussed in detail SNP techniques and the ability to identify allele states between individuals. In the Lewis by Karl map a few SNP markers were utilized to validate their ability as viable markers in mapping populations. ABC255, and ABC305 previously discussed in chapter two were scored see in the 146 individuals of the Lewis by Karl six-rowed population. Figure 6 is an example of SNP markers segregating in the mapping population using ABC305. Clearly the SNP detection method works well in recombinant inbred populations. Distinguishing Lewis (A) from Karl (B).

Figure 6
MAABAABH

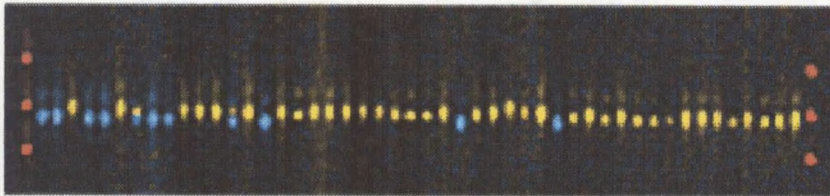


Figure 6: SNP detection of segregation at the ABC305 locus between the progeny of Lewis and Karl. Lewis allele state = A, Karl = B. fluorescent ladder (Genescan-500 ROX PE Applied Biosystems). Fragment size ~148bp. H = heterozygous allele.

The SNP detection method also identified heterozygotes as indicated by the (H) in figure 6. The marker for the puroindoline gene, *pinB* was tested as well, however ambiguous sequence interpretation lead to the amplification of non specific priming events. Due to the ambiguous SNP results, the *pinB* marker was used as an anchor marker on chromosome 7 with the aid of restriction enzymes. Another candidate SNP marker MWG502 was digested with restriction enzymes instead due to the large size of the fragment.

Protein and QTL

Two protein markers (figure 7) on chromosome 5, the B and C hordeins were scored with SDS PAGE. They map to within 6.5cM of each other with 12.5% recombinant individuals within the population. The morphological markers used were rachilla hair length.

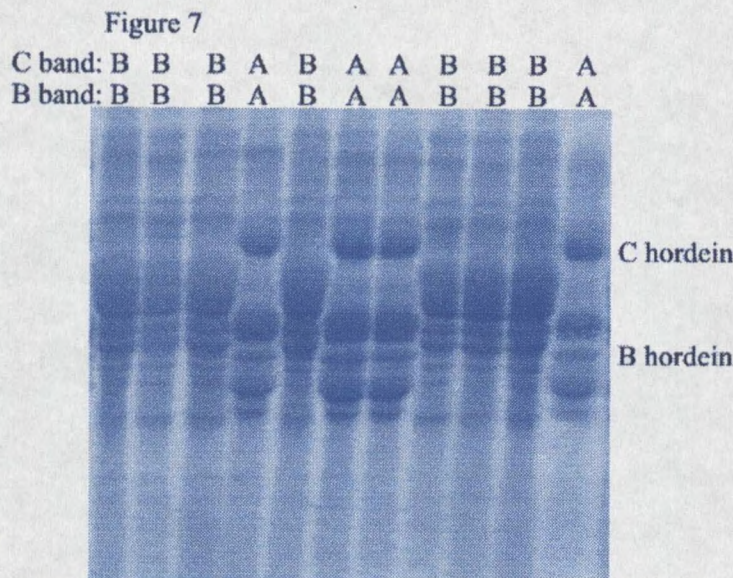


figure 7; SDS-PAGE; B and C hordein segregation between a subset of the Lewis by Karl population. A = Lewis allele. B = Karl allele.

AFLP Markers

AFLP markers comprised the bulk of the map with 89 markers total. Table 8 list the markers used, the size and the chromosome locations. Of the 89 markers scored, only 9 were not used in the map construction due either to low LOD scores or lack of linkage to any known markers. Twelve primer combinations were used in developing the map. *Eco R I* was used as the six base restriction enzyme. *Hpa II* was used as the four base restriction enzyme due to the fact that *Hpa II* is methylation sensitive. Due to the large heavily methylated, recombinationally inactive heterochromatic component of the barley

genome, traditional AFLP markers cluster around the centromeric and other heterochromatic genomic regions. Using a restriction enzyme that is methylation sensitive results in AFLP markers that are better dispersed throughout the linkage map.

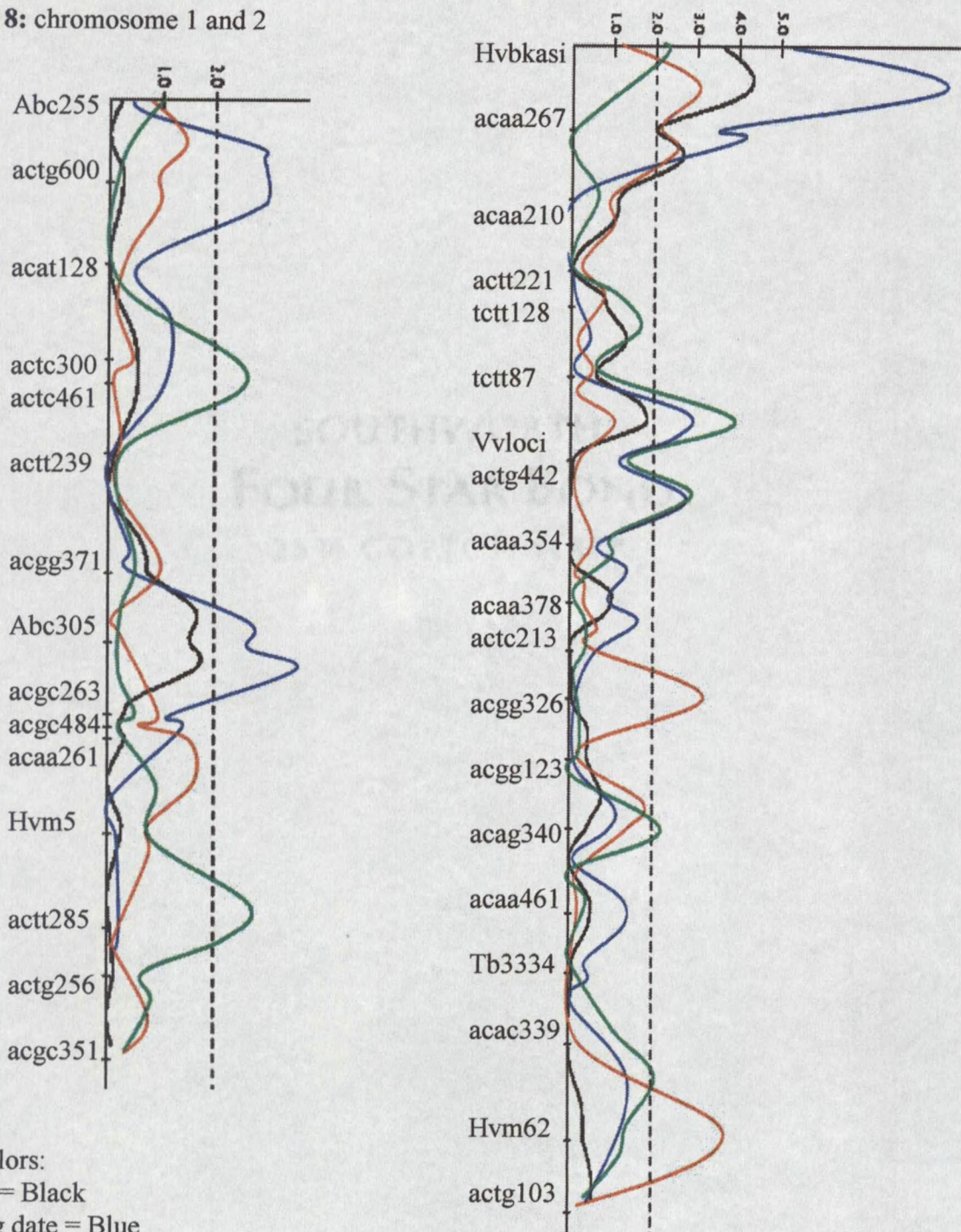
Map Construction and QTL Analysis

The genetic map of the 146 individuals in the Lewis by Karl six-row cross was developed with 110 markers producing a total genome map length of 2393cM. Anchor markers were interspersed in both the long and short arm of all chromosomes. No markers exceeded a cM value of fifty except in the case of previous mapped markers like HVM40 which was mapped to the short arm of chromosome 4 by Liu et al.,(1996), and on chromosome 3 due to the lack of anchor markers, the middle of the chromosome has a cM distance greater than fifty due to the lack of a more refined definitive location. Previously mapped markers linked to their reported locations with the exception of, HVM62 which linked to the distal end of the long arm in chromosome 2 instead of its reported location on the end of the long arm of chromosome 3. MWG502 an RFLP probe reported to locate on the tip of the short arm of chromosome 7 instead segregated with AFLP markers on the microsatellite region of chromosome 6. Table 2 shows the location of the markers on the seven chromosome and there distances in centimorgans. Highlighted markers are previously linked markers from known maps.

Table 2 continued:

CH5	CH6	CH7
6.5- Chord		
- Bhord		
18.9-	37.4- -tctt298	17.5- -acgt63
- abc156	- tctt273	9.3- -actc296
21.7-	24.1-	- actc55
- abg53	- acgg515	13.3-
22.7-	20.3-	- acgc140
- acgc424	- acgc132	11.3-
27.5-	38.0-	5.7- -acaa389
- acgt424	- mwg502	0.0- pinB1
	31.1-	- pinB2
	9.2- -acgt517	20.8-
	1.2- mwg2218	- acgg174
66.8-	- hvm34	
- actc410		44.2-
19.1-	42.3-	- acat333
- tb2122	- acgc311	
17.6-	17.8-	
- actt149	- actt166	58.8-
32.1-	19.2-	- rachilla
- tctt109	8.5- -actt298	21.7-
	9.0- abg458	- acat428
	- hvm74	33.1-
	14.1-	- acaa270
	- mwg2029	10.0-
	10.3-	- acaa327
	- mwg820	
	31.4-	
	- acta180	
	15.5-	
	- acgc419	
	11.3-	
	- acat472	
	35.7-	
	- actg177	

Figure 8: chromosome 1 and 2



Next page: chromosomes 3, 4, and 5

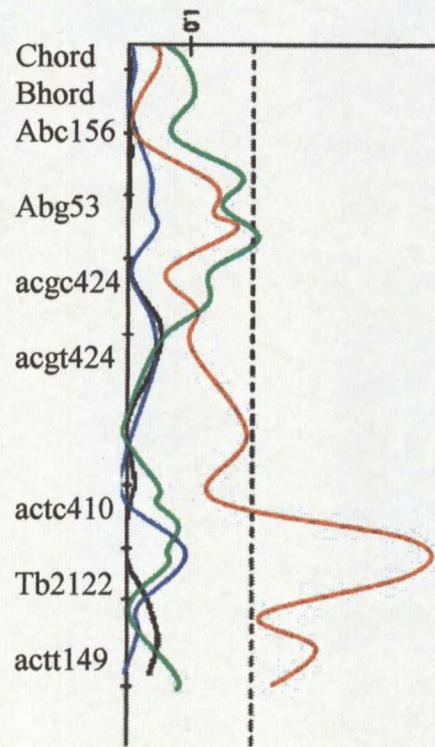
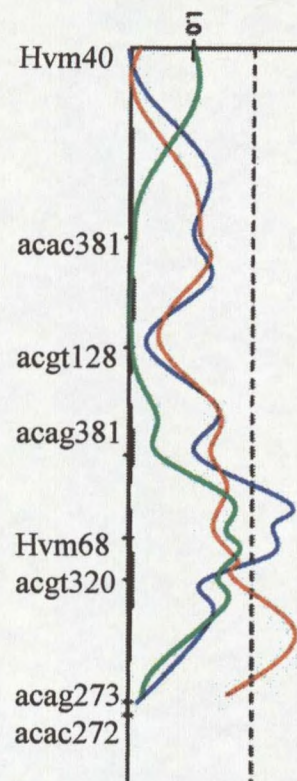
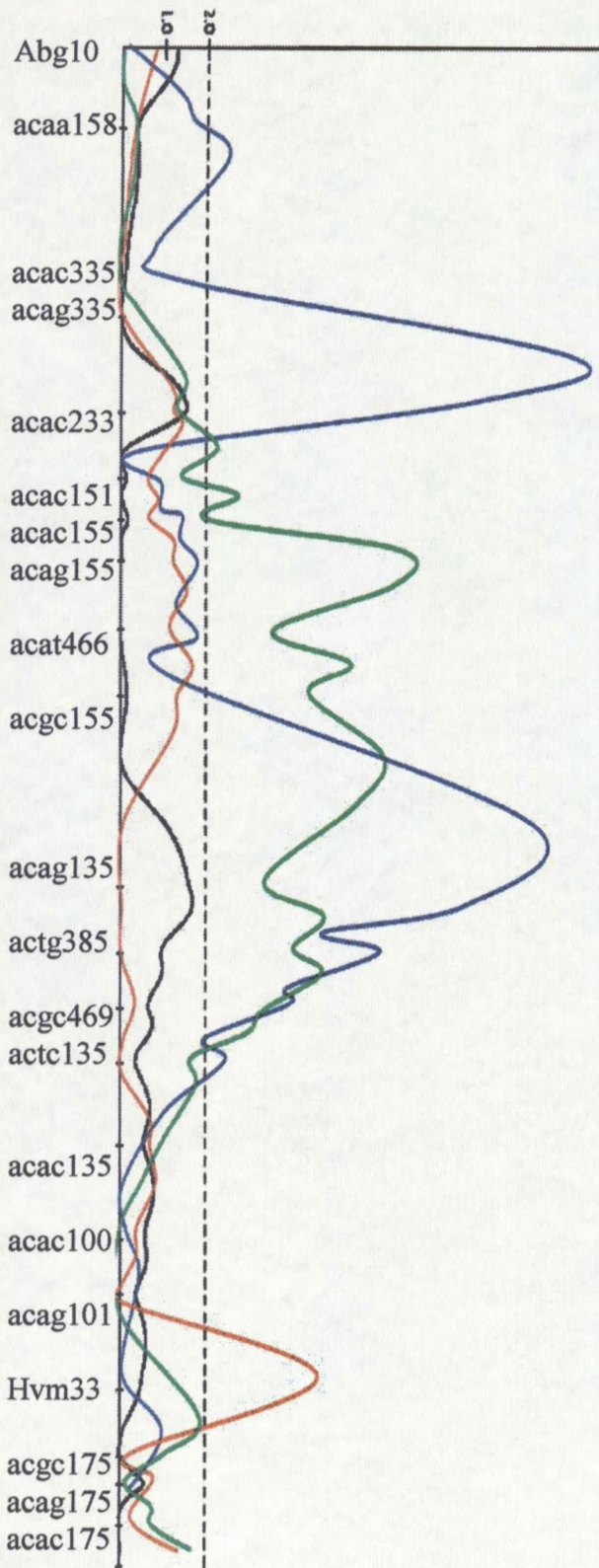
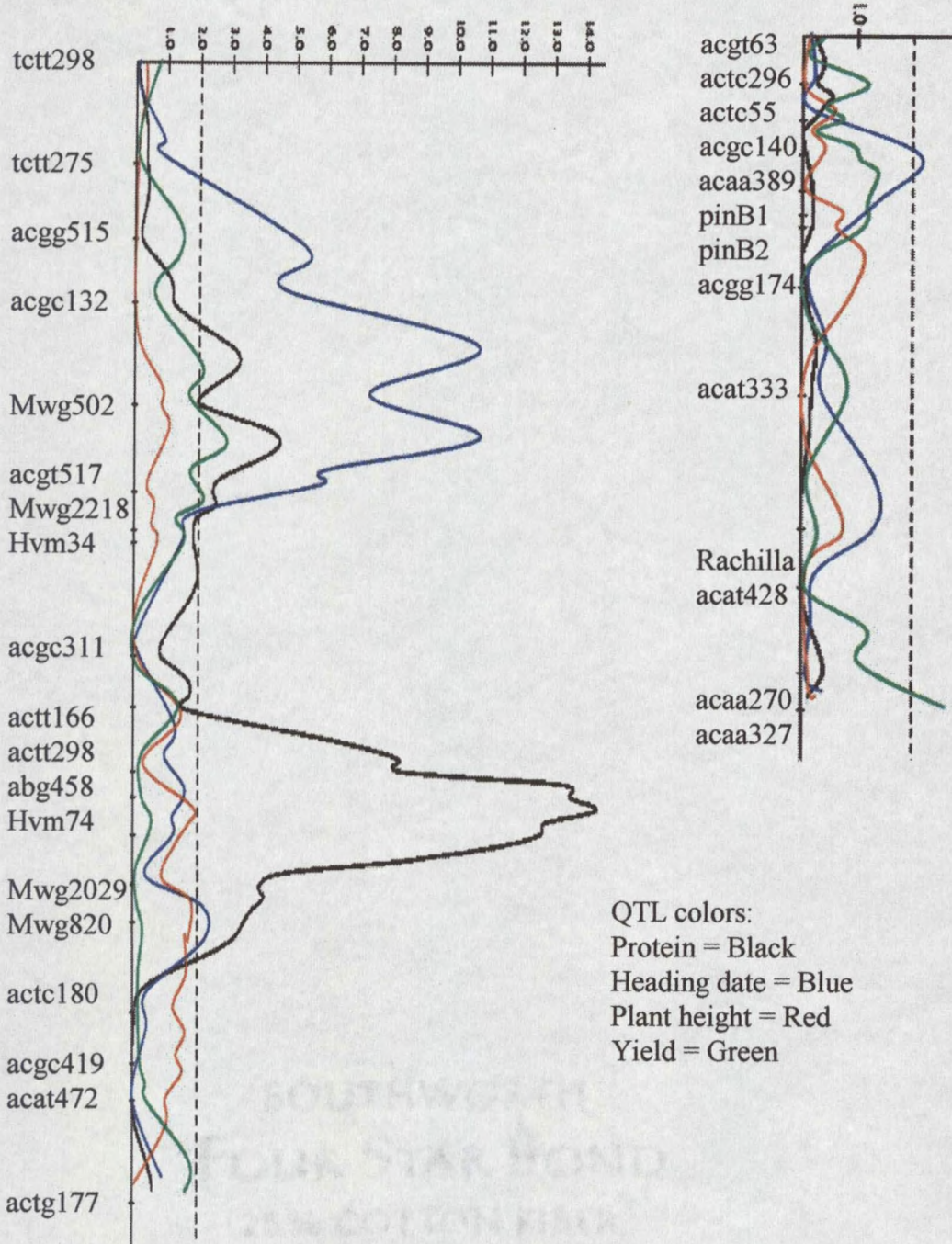


Figure 8: chromosomes 6 and 7



The intent of this map was to permit the chromosomal location of the protein QTL. QTL for heading date, plant height, and yield were analyzed to determine if they impacted upon grain protein content. Figure 9 shows the QTL for heading date, plant height, and yield superimposed over the QTL for protein. Chromosome 1 shows QTL for heading date around AFLP marker actg600 and near ABC 305. Yield has two QTL on chromosome 1 near actc461 and on the long arm near actg256. Chromosome 2 shows multiple alleles for all four traits of specific interest is the overlap of QTL for plant height, heading date and protein near the Hvbkasi. At the V locus QTL overlap for heading date and yield. Chromosome 3 has large QTL for heading date between acac233 and acag335, and another near acag135. Overlapping the QTL for heading date near acag135 is a broad QTL peak for yield. Plant height shows a small peak over the SSR marker HVM33. On chromosome 4 there were no significant QTL for the four phenotypes observed. Chromosome 5 shows one QTL for yield near the STS marker TB 21,22. Chromosome 6 is the most interesting in regards to protein QTL, between markers acgc132 and MWG2218 is a broad peak, overlapping this QTL is a peak for heading date. HVM74 a SSR marker, is positioned just above the nuclear organizer region on the short arm of chromosome 6, and it is the nearest marker to the most significant QTL for grain protein. Chromosome 7 shows no significant QTL.

Statistical Analysis

Statistical analysis using SAS (1988) was done to determine if correlations were present between the observed quantitative traits: Table 3 shows the means of the observed traits. Table 4 shows the correlations between each observed quantitative trait.

Table 3: Statistical Analysis of Quantitative Traits.

<u>Variable</u>	<u>N. entry</u>	<u>Mean</u>	<u>Std. Dev</u>	<u>Minimum</u>	<u>Lewis</u>	<u>Karl</u>	<u>Maximum</u>
yield	146	64.46073	10.99157	23.44476	71.500	68.000	101.91237
heading date	146	185.67123	1.69221	182.00000	186.00	186.50	192.00000
plant height	146	84.57911	6.08890	63.00000	88.00	92.00	101.00000
protein	146	13.74430	1.08712	11.20950	14.93	13.39	16.92000

Table 4: Correlations Between Quantitative Traits.

<u>Yield</u>	<u>yield</u>	<u>heading date</u>	<u>Plant height</u>
Heading date	-0.48407*		
Plant height	-0.02573	0.08095	
Protein	-0.27892*	0.46503*	0.19180*

Note: * = P value < 0.05

The correlation data in table 4 shows a significant negative correlation between heading date and yield. In looking at the protein data, there is a significant correlations with all of the observed traits. Protein has a positive correlation with heading date and plant height indicating that shorter plants that head earlier will produce less protein. Yield has a negative correlation with protein indicating higher yielding plants produce less protein.

Protein Quantitative Traits

To determine whether epistatic or additive effects were taking place between the protein QTL the effects that nearest markers had upon each significant QTL and each other were examined. For protein there were three significant QTL peaks, on the distal end of the short arm of chromosome 2 the nearest marker for this peak was acaa267. On

chromosome 6 there were two markers with close proximity to QTL peaks MWG502 and HVM74. Table 5 shows the mean values for the different allele states for each marker. In the case of all three markers, the Karl allele carries the low protein values.

Table 5: Marker Means.

<u>Marker</u>	<u>Allele</u>	<u>N. obs</u>	<u>Protein mean</u>	<u>Std. Dev</u>	<u>Treatment p value</u>
HVM74	missing*	3	13.7620000	0.934	
	A	81	14.2958827	0.975	
	B	62	13.0228306	0.774	0.0001
ACAA267	missing	2	14.4210000	2.735	
	A	30	14.2276667	1.321	
	B	114	13.6052281	0.955	0.0128
MWG502	missing*	8	13.3090625	0.698	
	A	54	14.1166852	1.234	
	B	84	13.5463631	0.946	0.0049

Note: * indicates missing or heterozygous data. Lewis allele = A; Karl allele = B.

Table 6: Marker Interaction.

<u>HVM74</u>	<u>mwg502</u>	<u>acaa267</u>	<u>observations</u>	<u>protein</u>	<u>deviation</u>
A	A		36	14.6135972	1.01656131
A	B		42	14.0541429	0.89334550
B	A		15	12.9950333	1.04305390
B	B		42	13.0385833	0.69824467
A		A	16	14.9639063	1.11686551
A		B	63	14.1222540	0.81158434
B		A	11	13.2837727	1.05070256
B		B	51	12.9665490	0.70207702
	A	A	18	14.2184444	1.36515485
	A	B	35	14.0003857	1.13672284
	B	A	12	14.2415000	1.31206600
	B	B	71	13.4438028	0.82603410

Table 6a:	<u>Source</u>	<u>DF</u>	<u>Type III SS</u>	<u>Mean Square</u>	<u>F Value</u>	<u>Pr > F</u>
	hvm74*acaa267	2	1.45600827	1.45600827	1.95	0.1647
	mwg502*hvm74	2	2.74522577	1.37261289	1.77	0.1741
	mwg502*acaa267	2	7.70166120	3.85083060	3.67	0.0279*

HVM74 is the marker defining the greatest difference in allelic state for grain protein content. Lines carrying the Karl allele at this locus were on average one percent lower in grain protein content than those carrying the Lewis allele. Table 6 shows the relationship

of markers upon each other and their combined effect upon protein. Epistasis was observed between the markers MWG502 and AFLP marker acaa267. The lowest protein mean was in a combination of the Karl allele in HVM74 and acaa267, however there were no significant epistatic effects between these two genes.

Heading Date QTL

Heading date had four main QTL peaks, accounting for 88 percent of the heritable variance for heading date (table 6). Two peaks at acaa267 and MWG502 on chromosome 6 overlap the QTL peaks for grain protein. The two other peaks are on chromosome 3 around acaa233 and acag135. There were no major epistatic interactions between the four markers, however there was a slight epistatic interaction between the marker MWG502 and acac233.

Plant Height QTL

Analysis of the three major QTL for plant height accounted for 62 percent of the heritable variance for this trait. One peak was located on chromosome 2 near the marker HVM62. On chromosome 3 HVM33 was centered under a peak, and on chromosome 5 TB 21,22 had the most significant QTL peak. The Lewis allele contributed to low plant height at TB21,22 and HVM33 . The Karl allele contributed to the low plant height allele at HVM62. Epistasis was observed between the markers HVM33 and HVM62, with the Lewis allele at HVM33 and the Karl allele at HVM62 producing the lowest mean plant height of 81cm. There was an additive effect between the markers HVM33 and TB 21,22 when both Karl alleles are present, causing a significant increase in plant height to 89cm.

Yield QTL

Four major QTL controlled 29 percent of the heritable yield in this population. The *V* gene on chromosome 2 controlled 7 percent of the yield variance and also impacted plant height. On chromosome 1 there are two QTL peaks, one at AFLP marker *actc300* and another at AFLP marker *actt285*. The broadest QTL peak is on chromosome 3, the marker centered under this peak is *acag155*. The chromosome 3 QTL is overlapped by the two large QTL for heading date. Analysis of the QTL for epistatic or additive effects showed no significant marker to marker interactions. Three of the markers, *acag155*, *actt285*, and *actc300* affected yield greatest when expressed by the Lewis allele. At the *V* gene, yield was effected by the Karl allele. Regression analysis of the markers associated with the QTL was done in order to calculate the heritable percentage each QTL attributed to the phenotype. The nearest markers for all observed QTL traits were analyzed. Table 7 shows the results of heritability of each of the QTL. Of the results obtained the protein QTL is the most reliable due to the data used in the statistical analysis. Regression analysis for plant height, heading date, and yield, were calculated using data from 1999 field trials obtained in two locations with two reps each. Protein regression analysis was calculated using field trial data from 1997, 1998, and 1999, for one location with two reps each year. In analyzing the data from table 7 for protein QTL, a total of 56% of the genetic variance is accounted for by the three QTL. The marker HVM74 accounts for 40% of the total protein heritability. Interactions of heading date, yield and plant height QTL at the other

Table 7: Heritability

<u>phenotype</u>	<u>Marker</u>	<u>Heritability h²</u>	<u>QTL % = R²</u>	<u>Calculated QTL heritability (R²/h²)</u>
Protein		82.65 %		
	HVM74		33.21%	40.18%
	acaa267		5.91%	7.15%
Height	MWG502		7.17%	8.68%
		46.60%		Total = 56.00%
	HVM33		10.15%	21.78%
	TB21,22		8.37%	17.96%
Heading date	HVM62		7.29%	15.64%
	acgg326		5.54%	11.89%
		80.69%		Total = 62.27%
	Mwg502		16.47%	20.41%
Yield	acaa267		8.99%	11.14%
	acag135		21.97%	31.84%
	acac233		20.07%	24.87%
		86.22%		Total = 88.26%
	acag155		12.25%	14.21%
V gene			5.77%	6.69%
	actc300		3.22%	3.74%
	actt285		4.40%	5.10%
				Total = 29.74%

two QTL for protein down play the significance of their heritability for protein alone.

There were four QTL for plant height accounting for 62 percent of the total genetic variance. HVM33 had the largest effect on plant height with 22 percent of the total heritability. Heading date was highly heritable with four QTL accounting for 88 percent of the genetic variance. The two AFLP markers, acag135, and acac233 on chromosome 3 accounted for 57 percent of the total. Yield was highly heritable with an h² value of 86%, however the four QTL for yield only accounted for 30 percent of the total heritability.

Discussion

Markers and Mapping

Two types of markers were used in the development of this genetic map, the first class of markers was AFLP markers, STS, SSR, and SNP markers consisted of the second class. AFLP markers were very useful in bulking up the map, however this type of marker comes with its own problems. The first problem that is apparent with AFLP markers is the fact the heterozygotes can not be identified with this technology. Secondly, AFLP markers are only reproducible within a specific population. The problems that arose within this population were a few instances where different primer combinations appeared to amplify the same products, most notably on chromosome 3 where the markers acac155, acag155, and acgc155 segregated together. This problem arose most likely because of nonspecific priming events that occurred during PCR, and would perhaps be remedied by more stringent thermocycler annealing conditions.

The STS and SSR markers worked well to anchor the AFLP markers to the map, however in a couple of instances these markers did not map to their previous reported location according to previous maps done by (Kleinhofs et al., 1993, Liu et al., 1996, Kunzel et al., 2000). Specifically marker MWG502 which should have anchored the short arm of chromosome 7 and linked to pinB did not, instead this marker fit best on the microsatellite region of chromosome 6. The most likely explanation for this discrepancy is due to the fact that this marker is derived from an RFLP marker and perhaps the RFLP marker location does not segregate with the STS marker location.

Map construction in this population was aided by the high number of AFLP markers that were scored. Using the methylation sensitive restriction enzyme *Hpa* II prevented clustering of AFLP markers in methylated regions of the genome allowing for a more random placement of markers along the chromosomes. Powell et al., (1997) observed in barley that the methylated regions while physically large, are recombinationally very small. Most chromosomes were well represented, however chromosomes 3, 4, 5, and 7 could have used more anchored markers at the distal regions of the arms and near the centromeres. Due to the sparse density of this map recombination frequency were not looked at, however the parental allele distribution per chromosome was looked at. Two chromosomes had abnormal percentages of one allele type over the other. On chromosome 2, only 24 percent of the allele distribution was accounted for by Lewis. The most obvious explanation for this is the fact that the vast majority of this population is six-row derived with only eight of the one hundred forty six progeny being two-row. The other discrepancy arose on the short arm of chromosome 7, from the tip to below pinB, this short arm is eighty seven percent Lewis. No specific reason for this skewed result is known.

Quantitative Trait Loci

The development of the recombinant inbred population between Lewis and Karl for the purpose of identifying the novel gene from Karl that regulates protein content worked well. Three major QTL were identified. Two of the QTL, *aca267* and *MWG502* are not likely candidates for protein specific QTL, due to the interaction at

these loci with QTL for heading date, plant height and yield. Most likely early heading date is causing less production of protein giving false positives at these locations within the map. The QTL above HVM74 is a novel QTL for protein in barley. In a Dicktoo by Morex cross Oziel et al., (1996) reported finding a QTL for grain protein in the region between ABG387 and WG223b on the short arm of chromosome 6. The placement of this QTL is in the region of the minor QTL above acaa267, distal to HVM74. In the Steptoe by Morex cross Kleinhofs et al., (1993) reported three QTL for grain protein on the long arm of chromosome 6, the nearest was 5cm below MWG2029. This new QTL for low grain protein should prove transferable into other agronomically acceptable malting varieties. The three QTL accounted for 56 percent of the total heritable protein phenotype inferred by the three nearest markers, HVM74 accounts for 40 percent.

Chromosome 6 while defined well in the areas of importance to grain protein QTL, could have been refined better specifically between the centromere and the nuclear organizer region containing the most important protein QTL. The Kunzel map, (Kunzel et al., 2000) shows both the physical and genetic distances of the short arm of chromosome 6. The distance between the centromere and the nuclear organizer while physically large is recombinationally quite small. HVM74 which is the closest marker to the protein QTL, lies just above the nuclear organizer region. Markers between these two regions were analyzed for polymorphisms within this population, however none were found that segregated between Lewis and Karl, leaving the nearest marker, MWG2029, beneath HVM74 around the middle of the long arm.

Yield had many QTL, besides the four reported, there were many smaller peaks throughout the seven chromosomes. Due to the large quantity of QTL for yield the difficulty lie in estimating the genetic variance. One specific problem with estimating heritability in this population, was the high R^2 value for yield, at 86 percent this value seems higher than a trait as dependent upon the environment as yield is. Even with this high heritability, the four main QTL only accounted for 30 percent of the total genetic variance. A second problem in determining the proper QTL for yield arose on chromosome 3 where there was a very broad QTL for yield that spanned a large portion of the center of the chromosome Larson et al., (1996) reported that chromosome 3 had two of the largest QTL effecting yield in a Steptoe by Morex cross. This problem could be solved with a higher density map. As predicted by the higher yield in Lewis compared to Karl, most of the significant markers associated with major QTL derived there high yield from Lewis alleles.

Heading date was covered well in this population with four main QTL that accounted for 88 percent of the genetic variance of this trait. There was only one statistically significant interaction between markers, however the markers MWG502 and acaa267 which showed an epistatic interaction in the protein QTL also shows up in the heading data analysis, while not statistically significant at the 0.05 level there is as obvious decrease in heading date when the Karl allele is present at both markers. The heading date data is a good example of transgressive segregation. Lewis and Karl both flower at approximately 186 days, however when these two parents are crossed the

progeny segregate away from the parental means, ranging from 182 days to 192 days

Plant height had four significant QTL that accounted for 62 percent of the total genetic variance. While the R^2 values for the four QTL were similar, HVM33 was the highest with 22%. The Lewis allele regulated shortened plant height at the markers HVM33 and TB21,22, and Karl produces more effects at HVM62. There was one interaction between HVM33 and HVM62. This possible additive effect was regulated with HVM33 producing a more significant contribution than HVM62.

Marker Assisted Selection

In summary of the data presented, marker assisted selection could be applied to this population or future experiments using these parents. In the case of grain protein content, it is a reasonable highly heritable trait. HVM74 accounting for the majority of the genetic variance could be used as a marker for pre selection of low protein lines; this would assure that Karl's low protein gene would be present. Heading date and plant height could both be manipulated with marker assisted selection. Plant height selection, due to interactions between HVM33 and HVM62 would require positive selection at both of these markers in order to insure gains in plant height. Heading date has one STS marker, MWG502, this marker would work well in selection for two reasons. First because of its high R^2 and secondly due to the overlapping of heading date and protein at this marker. the other markers for heading date selection are AFLP markers. In this population these markers would be beneficial due to their reproducibility, however in other populations this reproducibility is questionable. Yield while having a high R^2 , does

not have any markers than can account for significant genetic variation. Yield would be best selected for by phenotypic measurements instead of molecular markers.

The data presented has shown that new and novel genes can be identified that regulate grain protein content, marker assisted selection could be applied using these genes, and agronomically advanced malting lines would benefit from the results presented .

Table 8: AFLP Markers.

Marker	Primer combination	Ch	Marker	Primer combination	Ch
acaa158	EcoRI aga/ HpaIII caa	3	actc296	EcoRI aga/ HpaIII ctc	7
acaa210	EcoRI aga/ HpaIII caa	2	actc300	EcoRI aga/ HpaIII ctc	1
acaa261	EcoRI aga/ HpaIII caa	1	actc410	EcoRI aga/ HpaIII ctc	5
acaa267	EcoRI aga/ HpaIII caa	2	actc461	EcoRI aga/ HpaIII ctc	1
acaa270	EcoRI aga/ HpaIII caa	7	actc55	EcoRI aga/ HpaIII ctc	7
acaa327	EcoRI aga/ HpaIII caa	7	actg103	EcoRI aga/ HpaIII ctg	2
acaa354	EcoRI aga/ HpaIII caa	2	actg177	EcoRI aga/ HpaIII ctg	6
acaa378	EcoRI aga/ HpaIII caa	2	actg245	EcoRI aga/ HpaIII ctg	un
acaa389	EcoRI aga/ HpaIII caa	7	actg256	EcoRI aga/ HpaIII ctg	1
acaa401	EcoRI aga/ HpaIII caa	un	actg385	EcoRI aga/ HpaIII ctg	3
acaa461	EcoRI aga/ HpaIII caa	2	actg442	EcoRI aga/ HpaIII ctg	2
acac100	EcoRI aga/ HpaIII cac	3	actg600	EcoRI aga/ HpaIII ctg	1
acac135	EcoRI aga/ HpaIII cac	3	actt149	EcoRI aga/ HpaIII ctt	5
acac151	EcoRI aga/ HpaIII cac	3	actt166	EcoRI aga/ HpaIII ctt	6
acac155	EcoRI aga/ HpaIII cac	3	actt196	EcoRI aga/ HpaIII ctt	un
acac175	EcoRI aga/ HpaIII cac	3	actt221	EcoRI aga/ HpaIII ctt	2
acac233	EcoRI aga/ HpaIII cac	3	actt239	EcoRI aga/ HpaIII ctt	1
acac272	EcoRI aga/ HpaIII cac	4	actt285	EcoRI aga/ HpaIII ctt	1
acac335	EcoRI aga/ HpaIII cac	3	actt298	EcoRI aga/ HpaIII ctt	6
acac339	EcoRI aga/ HpaIII cac	2	tctt109	EcoRI att/ HpaIII ctt	5
acac381	EcoRI aga/ HpaIII cac	4	tctt128	EcoRI att/ HpaIII ctt	2
acag101	EcoRI aga/ HpaIII cag	3	tctt273	EcoRI att/ HpaIII ctt	4
acag135	EcoRI aga/ HpaIII cag	3	tctt298	EcoRI att/ HpaIII ctt	6
acag155	EcoRI aga/ HpaIII cag	3	tctt87	EcoRI att/ HpaIII ctt	2
acag175	EcoRI aga/ HpaIII cag	3	actc135	EcoRI aga/ HpaIII ctc	3
acag273	EcoRI aga/ HpaIII cag	4	actc213	EcoRI aga/ HpaIII ctc	2
acag335	EcoRI aga/ HpaIII cag	3	acat344	EcoRI aga/ HpaIII cat	un
acgg281	EcoRI aga/ HpaIII cgg	un	acat428	EcoRI aga/ HpaIII cat	7
acgg326	EcoRI aga/ HpaIII cgg	2	acat466	EcoRI aga/ HpaIII cat	3
acgg371	EcoRI aga/ HpaIII cgg	1	acat472	EcoRI aga/ HpaIII cat	6
acgg412	EcoRI aga/ HpaIII cgg	un	acgc132	EcoRI aga/ HpaIII cgc	6
acgg515	EcoRI aga/ HpaIII cgg	6	acgc140	EcoRI aga/ HpaIII cgc	7
acgg71	EcoRI aga/ HpaIII cgg	un	acgc155	EcoRI aga/ HpaIII cgc	3
acgt128	EcoRI aga/ HpaIII cgt	4	acgc175	EcoRI aga/ HpaIII cgc	3
acgt320	EcoRI aga/ HpaIII cgt	4	acgc263	EcoRI aga/ HpaIII cgc	1
acgt424	EcoRI aga/ HpaIII cgt	5	acgc311	EcoRI aga/ HpaIII cgc	6
acgt517	EcoRI aga/ HpaIII cgt	6	acgc347	EcoRI aga/ HpaIII cgc	un
acgt63	EcoRI aga/ HpaIII cgt	7	acgc351	EcoRI aga/ HpaIII cgc	un
acta180	EcoRI aga/ HpaIII cta	6	acgc419	EcoRI aga/ HpaIII cgc	6
acat128	EcoRI aga/ HpaIII cat	1	acgc424	EcoRI aga/ HpaIII cgc	5
acat295	EcoRI aga/ HpaIII cat	3	acgc469	EcoRI aga/ HpaIII cgc	4

CHAPTER 4

***PINB* A MULTIGENE FAMILY IN
BARLEY (*HORDEUM VULGARE* L.)****Introduction**

Endosperm hardness is an important trait for milling and baking in wheat (*Triticum aestivum*) (Morris and Rose 1996), and has been implicated as a quality factor in malting barley (Powell et al., 1995,1996). In wheat, endosperm hardness is controlled by the Hardness (Ha) locus on the short arm of chromosome 5D (Giroux, Morris 1997, Nelson et al., 1995). The Puroindoline genes *pinA* and *pinB* may act as an integral part of the starch granules in the starchy endosperm of mature wheat seeds (Gautier et al., 2000). In wheat the puroindoline genes are assumed to be single copy genes on chromosomes 5A, 5B, and 5D genomes, however only the 5D genome homologues are expressed (Giroux, Morris 1998)..The puroindoline genes are well studied in wheat, however little attention has been paid to their homologues in barley (*Hordeum vulgare* L.). In barley *pinB* maps to chromosome 7 which is homoeologous to wheat chromosome 5 (Rouves et al., 1996). To date it has been assumed that all genomes in the *Triticeae* carried single copy puroindoline genes. We present evidence showing that in barley there are multiple copies of the *pinB* gene, and that at least two copies are expressed. Two types of sequence will be described in this report. The previously reported sequence type for *pinB* (Beecher et al., *in press*) is named the primary sequence type. The new copy of *pinB* secondary.

Materials and Methods

In analyzing *pinB* to determine if it is multiple copy in barley, three main areas of research were conducted. Cloning of the *pinB* sequence types was done with genomic DNA samples extracted from foliar tissue using the protocol of Delaporta et al., (1983). DNA was extracted from six barley varieties, Karl (CI15487), Lewis (CI15856), Steptoe (CI15229), Morex (CI15773), Baronesse (PI1568246), and Logan (PI592784). *PinB* was amplified using the primers PB5 (5'ATGAAGACCTTATTCCTCCTA3') and PB3 (5'TCACCAGTAATAGC CACTAGGGAA3'). Thermocycler conditions were 94°C 2min. followed by 35 cycles of 94°C 30sec, 55°C 20sec, 72°C 20sec, followed by a 2 min extension at 72°C. PCR products were purified using Quiagen columns to remove excess primers and salts followed by precipitation with 5M NaAc and 95% ethanol to concentrate products. Cloning was accomplished with the T Vector System I by Promega (Madison, WI). Colonies were picked and amplified again with the PB5 and PB3 primers, the products were sequenced in the forward and reverse direction using the ABI377 automated DNA sequencer and the BigDye™ sequencing reaction kit (PE Biosystems). Sequence was analyzed using DNASIS (Hitachi).

To identify sequence types for RNA expression analysis, total RNA was extracted from Lewis, Karl, Steptoe, and Morex seeds at 20 days post anthesis using LiCl (McCarty 1986). RNA was treated with RQ1 RNase-free DNase (Promega) to remove contaminating DNA. Reverse transcriptase PCR was done using an RT-PCR kit

(Promega, cat.# M6101) following the manufacturer's suggested protocol. First strand cDNA products were amplified using the *pinB* primers PB5 and PB3. Amplified products were cloned using the T Vector System (Clontech cat# A3600). Sequence types were determined by restriction digestion using *Dra* III (New England Biolabs Beverly, MA); to digest the secondary *pinB* sequence type within the cultivars Lewis, Morex, Baronesse, and Logan. *Fok* I (NEB), digested the secondary *pinB* type within the cultivars Karl and Steptoe. Digested products were visualized in a 7% acrylamide gel running in a 0.5 x TBE buffer system (Maniatis 1989). The molecular weight marker was *Hae* III-digested ϕ X 174 DNA (Promega).

The restriction enzyme *Bst*NI (NEB) was used for determination of the sequence types in the six varieties used in this study. To map the primary and secondary sequence types, *pinB* was amplified from genomic DNA in one hundred forty six F₅ derived recombinant inbred lines derived from a cross of Lewis and Karl. Separation of fragment types was done using *Dra* III (NEB) which digested the Lewis secondary sequence type and *Scr*FI (NEB), instead of *Bst*NI was used to produce three bands in Lewis and four bands in Karl for the primary sequence type.

Results

Sequence Analysis

Fourteen independent *PinB* clones from genomic amplifications of *PinB* from the varieties 'Lewis' and 'Karl' were sequenced in both forward and reverse directions. Sequence analysis confirmed that at least two different sequence were present in both Lewis and Karl (Table 9). The primary sequence is the same as that reported by Beecher et al (*in press*) with one discrepancy at 282bp. (table 9). The secondary sequence type is 95.5% identical to the primary 'Lewis' sequence, differing at eighteen points within the region amplified by the *pinB* primers. Karl's secondary sequence is 96% identical to the primary Karl sequence, differing at sixteen points. The Lewis and Karl secondary sequences are 98.5% identical. Table 10 shows the deduced amino acid sequence and variations between the four amino acid sequences. The presence of the two genes was confirmed in the other cultivars used in this study by restriction enzyme analysis. Figure 9 identifies the primary and secondary *pinB* fragments with restriction digestion. The primary sequence digestion was done with *Bst*NI cutting *pinB* at two sites in Lewis, Morex, Baronesse, and Logan. Three fragments are seen in Karl and Steptoe. In the second gel *Dra* III was used to digest the secondary gene, this was done to validate the presence of both the primary and secondary sequence types. These digestions were done using genomic DNA amplification products. Due to this the presence of both genes in each cultivar, the original 446bp fragment can be seen undigested depending upon the restriction enzyme used.

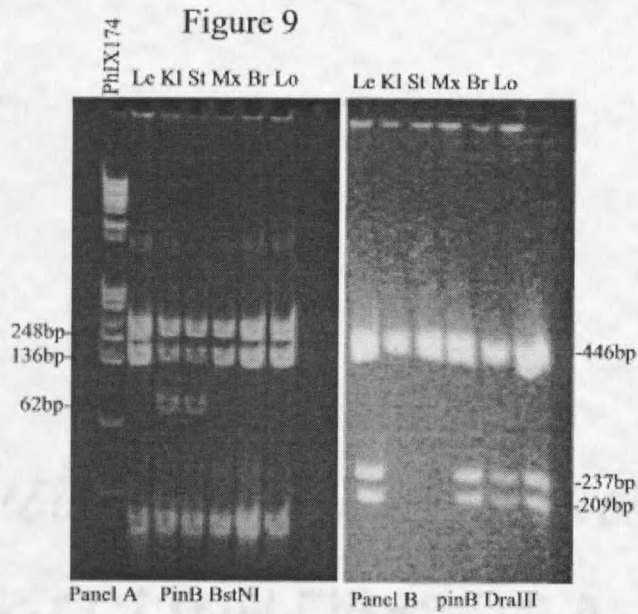


Figure 9: Panel A, *Bst*NI digested genomic amplification fragments indicating the presence of the primary *pinB* sequence.
 Panel B, *Dra*III digestion producing two fragments in the secondary *pinB* sequence, while leaving the primary *pinB* sequence undigested.

Table 9: PinB Sequences.

Restriction Enzymes:

Fok I

Le1 1 ATGAAGACCTTATTCCTCCTAGCTCTCCTTGCTCTTGTAGCAAGCACAAACCTTCGCGCAA
 Kl1 *****T*****
 Le2 *****C*****
 Kl2 *****A*C*****

Le1 61 TACTCAGTTGGCGGTGGTTACAATGACGTTGGCGGAGGAGGCGGTTCTCAACAATGCCCA
 Kl1 *****
 Le2 *****T*****T*****T*****
 Kl2 *****T*****T*****T*****

*BstN I ScrF I**Fok I*

Le1 121 CAGGAGCGGCCGAACCTAGGCTCTTGCAAGGATTACGTGATGGAGCGGTGTTTCACGATG
 Kl1 *****G*****G*****
 Le2 **A*****A*****T*****
 Kl2 **A*****A*****T*****

BstN I ScrF I Dra III

Le1 181 AAGGATTTTCCACTTACCTGGCCACAAAATGGTGGGAAGGGAGGCTGTGAACAAGAGGTT
 Kl1 *****G*****
 Le2 *****G*****G**G*****G**T*****
 Kl2 *****G*****G*****G**T*****

Le1 241 CGGGAGAAGTGTGCCAGCAACTGAGCCAGATAGCACCACAATGTCGCTGTGATGCTATC
 Kl1 *****T*****C*****
 Le2 *****G*****T*****C*****
 Kl2 *****G*****C*****

ScrF I

Le1 301 CGGGGAGTGATCCAAGGCAAGCTCGGTGGTATCTTTGGCATTGGGGGAGGTGATGTATTC
 Kl1 *****G*****
 Le2 *****C*****
 Kl2 *****

Le1 361 AAACAAATTCAGAGGGCCCAAATCCTCCCCTCAAAGTGCAACATGGGCGCCGACTGTAAG
 Kl1 *****G*****
 Le2 *****G*****T*****
 Kl2 *****G*****

Le1 421 TTCCCTAGTGGGCTATTACTGGTGA
 Kl1 *****
 Le2 *****
 Kl2 *****

Notes: Le1 and Kl1 are primary sequence type;

Le2 and Kl2 are secondary sequence type;

Restriction Enzymes: *Fok I* GGATG (9/13)*BstN I* CC/WGG*ScrF I* CC/NGG*Dra III* CACNNN/GTG

Table 10: Protein Sequences

```

Le1  ALLALVASTTFAQYSVGGGYNDVGGGGGSQQCPQERPNLGSCKDYVMERCFTMK
K11  *****I*****R**
Le2  *****S*****
K12  *****S*****

Le1  DFPLTWP TKWVKGGCEQEVREKCCQQLSQIAPQCRCDAIRGVIQGKLG GIG
K11  *****R*****
Le2  **V*****H*****H*****
K12  **V*****H*****

Le1  GGDVFKQIQRAQILPSKCNMGADCK
K11  *****
Le2  **A*****V***
K12  *****

```

Notes: inferred amino acid sequence

Le1 and K11 are primary amino acid sequences

Le2 and K12 are secondary amino acid sequences

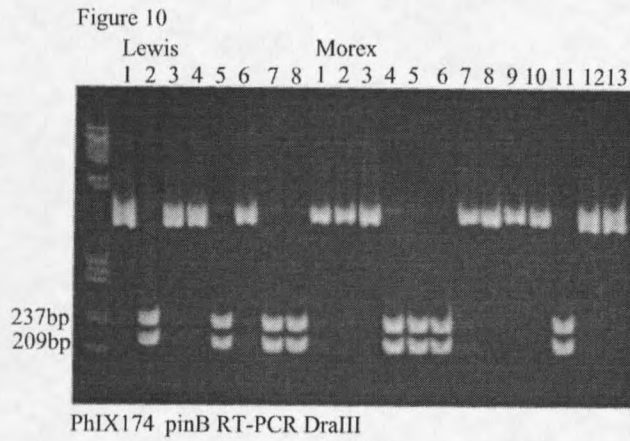


Figure 10a; First strand cDNA clones of Lewis and Morex, digested with *Dra* III indicating samples Le2, 5, 7, 8 and Mx 4, 5, 6 and 11 have the secondary *pinB* sequence.

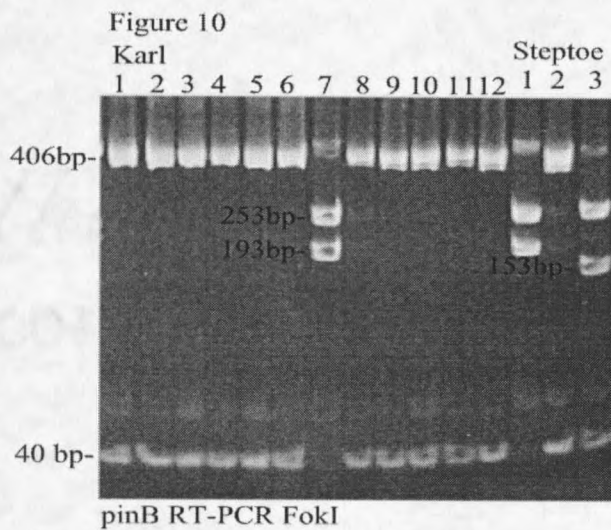


Figure 10b; Karl and Steptoe digested with *Fok* I showing the presence of the secondary sequence in all samples except Karl7 and Steptoe 1 and 3.

RT-PCR Analysis of *PinB* Genes

Sequence analysis of genomic clones confirmed the presence of multiple copies of the *pinB* gene in each of the six tested cultivars. Reverse Transcriptase PCR was done with RNA from the four of the six cultivars to determine whether both *pinB* genes were expressed. The RT-PCR products were cloned and digested to determine the presence or absence of the predicted different *pinB* variants. Figure 10a shows the digestion of the cloned RT-PCR products of Lewis and Morex. This gel clearly shows two sequence types, one without a *Dra* III site and one with a *Dra* III site in the middle. Figure 10b confirms the presence of the primary and secondary *pinB* in the cultivars Karl and Steptoe. Lane 3 from Steptoe suggests the presence of a third sequence variant, carrying both *Fok*1 sites. The two bands at 406 and 40bp indicate the presence of the secondary *pinB*, while the two fragments at 253 and 193bp indicate the digestion of the primary *pinB* sequence type. One discrepancy in this gel is the presence of the fragment at 153bp in the Steptoe sample 3. These restriction fragments show the presence of both sequence types in one expressed genotype. While over amplification or mispriming during PCR could produce these results. The most logical explanation is the presence of a third sequence type.

Mapping of *PinB*

Mapping of the two fragments was done using the one hundred forty six recombinant inbred lines used in the Lewis by Karl map discussed in chapter 3. *PinB* has already been mapped to the short arm of chromosome 7 as seen in table 4 in chapter 3.

Mapping was done using *ScrFI*, figure not shown, to look for segregants among the primary *pinB* gene. *Dra* III was used to digest the secondary *pinB* allele type. Of the one hundred forty six progeny digested, no recombinants were identified. Within the limitations of this experiment, the primary and secondary *PinB* structural genes were completely linked, suggesting that they are members of a multigene family.

Discussion

Genomic sequence analysis, restriction assay and RT-PCR, all clearly indicate the presence of multiple expressed copies of *pinB* within the six cultivars analyzed. The sequence analysis of the cloned *pinB* fragments from genomic DNA indicates the presence of a third sequence type.

Linkage analysis of the *pinB* primary and secondary structural genes found no intergene recombination, suggesting that these genes lie less than a centimorgan apart, in a region of the genome which is characterized as a 'high frequency recombination' region (Kunzel et al., 2000). The other *Triticeae* members for which *PinB* has been characterized have been found to contain one *PinB* sequence per genome, suggesting that the barley *PinB* multigene family may represent a recent gene duplication event.

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