DEVELOPING A DNA FINGERPRINT FOR MIDWEST SIX-ROWED MALTING BARLEY

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ABSTRACT

The requirements for brewing beer from barley (*Hordeum vulgare* L.) malt are specific and unique for each brewer. Anheuser-Busch InBev and Miller Coors Brewing Company (MillerCoors) are two major brewers in the United States that target different malt quality profiles for six-rowed barley malt. Two closely related cultivars developed by the University of Minnesota, Robust and Stander, differ greatly in agronomic and malt quality performance. Robust malt fits the requirements of MillerCoors and Stander malt has many of the parameters desired by Anheuser-Busch InBev. The close relationship between these two cultivars increases the chance of recognizing chromosome regions with the genes controlling malt quality traits. A total of 53 doubled-haploid (DH) lines (original population) and the parents from the Robust x Stander cross were grown at eleven locations in North Dakota and one location in Idaho the past six years. An additional 138 Robust x Stander DH lines were generated in 2009 and were evaluated alongside the original DH population in the summer of 2011 at two North Dakota locations. Agronomic data were collected at all locations and cleaned grain samples of the original population from six of the locations were micro-malted at NDSU. Three linkage maps were developed using the original and 191 DH line (entire) populations. The first linkage map was constructed using the original DH population, along with a total of 102 SNP, SSR, and DArT markers. The second and third linkage maps were developed using only 67 SNP markers, with the original and entire Robust x Stander DH population, respectively. The first map was used to identify QTL controlling malt quality and wort carbohydrate traits on chromosomes 4H, 5H, and 6H. The SNP map constructed using the original DH population was used to identify QTL controlling agronomic traits on chromosome 6H. The third map was used to

identify QTL controlling agronomic traits on chromosomes 4H and 6H. The ultimate goal for this research in years to come is to develop a genetic haplotype that helps distinguish six-rowed barley lines suitable for MillerCoors and Anheuser-Busch InBev.

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GENERAL INTRODUCTION

This dissertation contains four chapters. Chapter I includes a general introduction about the dissertation research, along with a literature review portion. Chapters II and III are written as two papers to be submitted for publication. Therefore, these chapters include an abstract, introduction, materials and methods, results and discussion, and references section. The references are specific for each chapter. Due to the similarity in genetic and statistical tools used, repetition does occur between chapters. Chapter IV highlights key findings and provides a general summary of this dissertation.

CHAPTER I

Introduction

Six-rowed barley (*Hordeum vulgare* L.) malt varies in enzyme level and functionality with different cultivars. The requirements of each brewer for brewing beer from barley malt are specific and unique. When looking at the two major brewers that utilize six-rowed barley malt in the United States (US), Anheuser-Busch InBev and MillerCoors Brewing Company (MillerCoors), the malt quality specifications of each company in the past were quite similar. However, since about 2000 the malt quality specifications have been quite different and this has resulted in breeding programs, such as the one at NDSU, having different breeding objectives for each brewer. MillerCoors desires cultivars with moderate protein modification levels during malting and moderate levels of enzymatic activity (Kay, 2005). On the other hand, Anheuser-Busch InBev desires cultivars with higher levels of protein modification during malting and high levels of enzymatic activity. These two categories of brewers' preferences can be represented by two cultivars developed by the University of Minnesota, Robust (Rasmusson and Wilcoxson, 1983) and Stander (Rasmusson et al., 1993). Robust fits the requirements of MillerCoors and Stander has many of the parameters desired by Anheuser-Busch InBev (R.D. Horsley and P.B. Schwarz, personnel communication, 2009). The pedigree of Robust is 'Morex'/'Manker' (Rasmusson and Wilcoxson, 1983) and the pedigree of Stander is Robust*2/3/'Cree'/'Bonanza'//Manker/4/Robust/'Bumper' (Rasmusson et al., 1993).

It is evident that Robust and Stander have a close pedigree relationship, which makes the development of a marker-assisted selection breeding strategy feasible with the

potential recognition of dissimilar occurrences by primers. The objectives of this project are to: 1) generate molecular marker linkage maps using the original 53 Robust x Stander DH lines and the entire DH population (191 lines); 2) phenotype the mapping population for agronomic, malt quality, and wort carbohydrate traits; and 3) identify quantitative trait loci (QTL) controlling these traits.

Literature Review

Barley

Barley, a founder crop of Old World agriculture, is one of the earliest cereals to be domesticated (Badr et al., 2000; Sang, 2009). Barley is considered the fourth most important cereal crop in the world in terms of production (FAOSTAT, 2009) and is adapted to survive in unfavorable conditions such as cold temperatures, drought environments, and alkali or saline soil types. The ability to handle such conditions, along with an early maturation date, allows this crop to be cultivated worldwide (Schulte et al., 2009). Barley has a higher adaptability to harsh climates than other cereal crops, shown by its ability to be produced farther within desert regions and at higher latitudes and altitudes (Baik and Ullrich, 2008). In a holistic view of annual crop production in the United States, barley ranks 17th among the other crops, with the United States ranking 9th out of all the barley producing countries (FAOSTAT, 2009).

The four major uses of barley grain include animal feed, malt, human consumption, and seed (Rasmusson, 1985). Not only is the grain important, the actual plant can be used for forage. The animal feed portion accounts for roughly two-thirds of global barley utilization, with the malting, brewing, and distillation companies constituting the remaining

third (Schulte et al., 2009). Approximately 2% of barley is used for human consumption world-wide (direct source of food) (Baik and Ullrich, 2008). Even though little barley is used for food, some cultures recognize the crop's nutritional benefits and use it as their primary food source.

Domesticated barley is a diploid with 2n=14 chromosomes (Reid and Wiebe, 1979). This annual cereal crop is an inbreeding grass species belonging to the family Gramineae, subfamily Festucoideae, and genus *Hordeum*. Triticeae is the name of the monophyletic tribe of which the genus *Hordeum* belongs (Reid and Wiebe, 1979). The *Hordeum* genus contains 31 recognized species (von Bothmer and Komatsuda, 2011). This tribe evolved within a subfamily of Poaceae around 12 million years ago, and not only includes barley, but also includes bread wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), and respective wild relatives (Gaut, 2002; Schulte et al., 2009). The genome size of barley is estimated to be close to 5200 Mbp, with highly repetitive DNA composing at least 80% (Close et al., 2009).

Domestication and US commercialization

Barley originated in the Fertile Crescent region (Rasmusson, 1985; von Bothmer et al., 2003; Morell and Clegg 2007). Based on archaeological evidence, remnants of barley found in this domestication region were dated roughly 8,000 B.C. (Badr et al., 2000). There is still a large debate as to whether the Fertile Crescent region represents the sole domestication event, or if other domestication occurrences exist for barley. Many scientists have collected data suggesting the existence of multiple origins, while others believe barley went through a single domestication event in the Fertile Crescent region and spread to other regions across the world (Badr et al. 2000; Sang, 2009). In 2007, a study conducted by

Morell and Clegg suggested the occurrence of at least two barley domestications. The results of this study were based on haplotype frequency differences between geographic regions at multiple loci. The authors were able to show the Fertile Crescent area being the largest contributor of genetic diversity in modern European and American cultivars by looking at frequencies of seven nuclear loci. A region approximately 1,500-3,000 km east of the Fertile Crescent was thought to be the second domestication site of barley. This area contributed most of the genetic diversity in cultivars of Central Asia and the Far East. Another group of scientists also believed in the multiple origin theory. With the use of chloroplast markers, it was proposed that multiple independent domestication events existed in the areas of Ethiopia and the western Mediterranean (Molina-Cano et al., 2005).

Domestication allowed for fundamental transitions between cultivated barley and its wild relative (*Hordeum vulgare* spp. *spontaneum*). The major changes include the development of a non-brittle rachis (reduction in grain shattering), higher seed weight, naked seeds (separation of hull from seed), decreased seed dormancy, and transition from two- to six-rowed spikes (Salamini et al., 2002; Pourkheirandish and Komatsuda 2007; Sang 2009). From a genetic standpoint, barley is considered one of the most diverse cereal grains due to the many different classification types. Examples of these morphological differences of the barley plant include spring or winter habit, the presence of fertile florets (two- or six-row), hulled or hulless, and whether the end-use of the grain is used for malting or feed (Baik and Ullrich, 2008). Barley can be further classified based on grain composition.

It is thought that Christopher Columbus carried barley with him on his second voyage, marking its introduction into the New World (Wiebe, 1979). In the United States,

it is believed that barley was first introduced both on the east coast and in the Southwest in the early 1600's. The English were known for growing barley on Martha's Vineyard and Virginia. It is speculated that the English brought two-rowed barley with them (Chevalier and Thorpe), while the Dutch brought the barley from continental Europe. The introduction into the Southwest regions took place during the Spanish colonization, in which the crop was first grown and introduced in Mexico in the 1600's. Throughout the settling period, it was found that six-rowed cultivars from Europe were better suited to the growing conditions than two-rowed cultivars from England (Wiebe, 1979). It wasn't until 1873 that the Wisconsin Agricultural Experiment Station introduced and distributed selections of the landrace Manshury to Wisconsin farmers (Weaver, 1943). Around 1894, the selected seed was distributed to Agriculture Experiment Stations in Minnesota and North Dakota. Manchuria barley was widely used across the Midwest and established near the end of the 19th century.

The susceptibility of barley to Fusarium head blight (FHB), incited by *Fusarium graminearum* Schwabe [teleomorph *Gibberella zea* (Schwein)] forced the growing region in the US to be pushed westward (Horsley and Harvey, 2011). In the time period of 1940s to 1990s, the Red River Valley region of North Dakota and Minnesota was considered the center of barley production. It wasn't until the 1990s when the Red River Valley region was impacted by the FHB disease. This was a favorable time period for the disease, because of the higher than average precipitation and increased production of maize (*Zea mays* L.). These factors along with changes to Federal farm programs caused a shift in growers' decisions, such that many quit growing barley. When looking at barley area in

the US today, north central North Dakota is the center of six-rowed barley production (Horsley and Harvey, 2011).

In the United States, barley was harvested on roughly 997,550 hectares in 2010 (USDA National Agricultural Statistics Service, 2011). When looking at the statistics for North Dakota, 271,139 hectares of barley were harvested in the same year, constituting roughly 27% of the total area harvested in the US. For 2010, the overall production value of the crop was around $690 million. In 2011, roughly 1.1 million hectares of barley were sown in the US, with North Dakota accounting for 17% of the total hectares seeded. Four regions of the United States make up the barley production area (Horsley and Harvey, 2011). These regions are the East, Upper Midwest, West, and Southwest. In most years, the largest barley producing state is North Dakota, which focuses production primarily on six-rowed malting barley cultivars.

When looking solely at the US, the primary breeding objectives are formed around traits that impact malting, feed and food, disease resistance, insect resistance, and abiotic stress resistance (Horsley and Harvey, 2011). There are many requirements and guidelines that need to be met when producing a cultivar specific for malting and brewing. This reason alone makes malting barley stand out among other crops, such that it needs to be stored on an identity preserved basis and under conditions that maintain its ability to germinate. Segregation among cultivars and even sometimes by production area or trait takes place in order to reach and maintain the high standards required by the malting and brewing industries. It is not unusual for a malting barley cultivar to be utilized in large proportions for malting and brewing for more than 15 or even 20 years in North America. Breeding methodology changes from trait to trait. A prime example is the comparison

between the two parameters malting and livestock feed. For malting barley, parents of high quality are usually chosen for the cross, which has led to a narrow germplasm base. As for livestock feed, parental selection is not as crucial.

Barley quality

The success of a malting barley cultivar is not controlled by one trait, but by many. Traits involved with determining barley quality, agronomic performance, disease resistance, and malt quality all contribute to the longevity of a cultivar's utilization. Some important agronomic traits analyzed in the field include heading date, plant height, and resistances to lodging, stem breakage, and disease. After the grain is cleaned, quality analyses are performed. The grain is analyzed for its 1000-kernel weight, plumpness, test weight, protein and moisture content, and kernel color. If the grain is deemed acceptable for malting, it will then be placed through further testing. Malt quality factors can be broken down into three categories: 1) modification, 2) congress wort, and 3) enzymes. Malt modification is measured through ß-glucan content, fine-coarse extract difference, Kolbach index, and viscosity. Congress wort is analyzed by determining soluble protein, extract, color, and free amino nitrogen (FAN). Finally, the enzyme portion includes testing of diastatic power (DP) and α-amylase (AMBA, Table 1).

Agronomic traits

Heading date, or commonly known as spike emergence time, is important primarily for two reasons: 1) the adaptation of cereal cultivars to certain environments, and 2) maximizing yield potential (Bezant et al., 1996). Plant height plays an important role in preventing yield loss from lodging and increasing the harvest index. Lodging is observed when the stems of barley are permanently displaced from their upright position after

exposure to environmental conditions such as wind, rain, or hail (Pinthus, 1973). The distribution and degree of lodging is usually not uniform, such that different areas of the field may experience a higher degree of lodging than others. Lodging severity is based on both the occurrence and degree. Lodging close to the maturity stage may not directly impact grain yield loss; however, since it interferes with harvest, a decrease in yield may be observed. Lodging also is responsible for increasing nitrogen and protein content in grain, due to decreased carbohydrate accumulation.

The major regions of the plant affected by barley diseases include the leaf and stem, spike and seed, and root and crown regions (Neate and McMullen, 2005). Diseases of the spike and seed region include smuts (incited by *Ustilago* spp.), ergot (incited by *Claviceps purpurea* (Fr.:Fr.) Tul.), and FHB. Examples of diseases found on the leaf and stem region are net blotch (incited by *Drechslera teres* (Sacc.) Shoemaker), spot blotch (incited by *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex Dastur), scald (incited by *Rhynchosporium secalis* (Oudem.) J. J. Davis), different rusts (incited by *Puccinia* spp.), and powdery mildew (incited by *Erysiphe graminis* DC. f. sp. *hordei* Em. Marchal). The major disease found in the root and crown region is root rot. In the northern Great Plains, the primary causal organism of root rot is *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex Dastur, the same organism that causes spot blotch. When considering malting as the end-product, the one disease of interest found on the spike and seed region is FHB. Many grasses serve as a host to this disease, including maize, wheat (*Triticum* spp.), and barley. This disease is found on the glumes or rachis of a barley plant, with the primary symptom being brownish lesions in minor infections. If severity increases, salmon-orange clusters of spores and head sterility may be observed. This disease can be controlled through use of

crop rotation, resistant cultivars, fungicidal treatment, and clean seed. The impact of *Fusarium* infection has been known to decrease overall malt yield, β-glucan content, and viscosity, while significantly increasing the content of soluble nitrogen, FAN, and wort color (Schwarz, 2003; Schwarz et al., 2002). Fusarium head blight reduces quality due to contamination by the tricothecene mycotoxin deoxynivalenol (DON). The pathogen F. *graminearum* primarily produces this mycotoxin (Schwarz et al., 2006; Salas et al., 1999). If FHB infected barley malt is utilized, key problems arise, such as changes in wort composition, existence of other mycotoxins and fungal metabolites, and beer gushing (Wolf-Hall, 2007). Gushing occurs suddenly after a container is opened, in which an over production of foam is released. Fungal organisms are also known to be involved with the production of off-flavors and lower germination rates (Schwarz, 2003).

1000-kernel weight

After the seed is cleaned, broken kernels and foreign material are removed and 1000 kernels are weighed to obtain the measurement (Schwarz and Li, 2011). In most cases, a seed counting device is utilized and the results are expressed on a moisture-free basis, with the final estimate being an average of dry kernel weight. Many scientists have determined that kernel weight and size are positively correlated with malt extract yield. Thus, a rough estimate of the overall malt quality of a sample can be predicted by knowing the size and weight of the kernel.

Plumpness

The measurement of kernel plumpness is obtained through the kernel assortment procedure (Schwarz and Li, 2011). This procedure tends to be performed and utilized more often than 1000-kernel weight in the US, due to the amount of time needed to count and

weigh each sample. For the assortment procedure, 100 g of barley seed is sorted mechanically on three stacked sieves. The stacked sieves have rectangular openings in sizes of 19.0 x 2.8, 2.4, and 2.0 mm. Kernels found on the 2.8 and 2.4 sieves are considered plump. Plumpness is based on a percent, so the kernels found on these top two sieves constitute the percent of plump kernels in the sample. Six-rowed malting barley samples should contain less than 3% thin and a minimum of 80% plump kernels (AMBA, 2008). It is important for the barley sample to have uniform kernel size; otherwise, the germination rate and water uptake of different sized kernels will vary and cause problems/fluctuations in the malting and brewing processes.

Test weight

Test weight is a kernel density measurement, which can be expressed in lbs bu^{-1} or kg hL^{-1} (Schwarz and Li, 2011). Test weight is determined by measuring the amount of seed needed to fill a Standard Winchester bushel (Bu) measure of 2150.42 in^3 (weighing a constant volume of seed). Test weight can be affected by a multitude of factors, including cultivar, environment where the barley was grown, or how well the sample was cleaned. The minimum test weight value for U.S. No. 1 Six-Rowed Malting barley is around 67 kg hL^{-1}.

Moisture content

This characteristic is expressed as a percent, representing the amount of barley dry matter and water found in the kernels (Burger and La Berge, 1985). By calculating moisture content, other quality factors can be estimated and expressed on a dry basis (db) and safe storage conditions can be ensured. This trait is an important selection criteria used for malting barley.

Protein

The total nitrogen content by weight represents protein. The ideal six-rowed malting barley protein content in the US is <13.5% (AMBA, 2008). Several methods can be used to estimate the protein content of a barley sample, including the traditional Kjeldahl method multiplied by the factor of 6.25 (DeClerck, 1958; Schwarz and Li, 2011), Near Infrared Reflectance (NIR) spectroscopy (Carroll et al., 1978), or combustion analysis (Foster, 1989). The most common procedure used today for estimating protein content is whole-grain NIR (Schwarz and Li, 2011). This analysis is non-destructive, requires little sample preparation, and is overall a quick procedure. Processing problems during malting and brewing have been linked with high protein content (Burger and La Berge, 1985). Examples of these problems include longer steep times, uneven germination rates, decreased malt extract yield, increased wort and beer color, and formation of haze in beer.

Kernel color

This parameter, also known as kernel brightness, is represented by the intensity of visible light reflected off the entire kernel surface. This trait is usually expressed as an L-value of the tristimulus color scale which is a measure of brightness (Shellhammer, 2009; Schwarz and Li, 2011). It is considered an objective measurement of hull discoloration (Burger and La Berge, 1985). These discoloration values help recognize rain weathering and microbial damage in the sample (Schwarz and Li, 2011). Culling of discolored samples is important for the malting and brewing process, because problems with uneven germination rates or water sensitivity can be avoided. The desired color for barley is a light yellow-straw color with a bright appearance.

Quality problems

Adverse environmental conditions, disease pressure, poor harvesting techniques, and improper storage and transport are just a few of the conditions that lead to poor seed quality (Schwarz and Li, 2011). Specifically, damaged kernels, sprouting, and fungal contamination or mycotoxin problems are a few examples of potential outcomes from the conditions listed above. Whether it be physical, heat, or frost damage, the kernels experience problems with the speed of germination. These problems ultimately result in lower malt extract, off-flavors, and reductions in processing performance. Rainfall or long periods of wet weather tend to initiate premature germination of grain in the field, commonly known as pre-harvest sprouting. Mycotoxin production is viewed as a major issue in the brewing industry, due to the food safety and consumer perception issues.

Malt quality

The major grain used in malting is barley. The three major steps in the malting process include steeping, germination, and kilning (Briggs, 1998). During the steeping process, the moisture content is increased in the barley seed to a predefined level, usually 42-47% (P.B. Schwarz, personnel communication). Throughout the germination phase, the aleurone cells and scutellum synthesize hydrolytic enzymes (Bamforth and Barclay, 1993; Macfadden et al., 1988; Ranki, 1990; Lapitan et al., 2009), which are then secreted into the starchy endosperm and convert proteins and carbohydrates into partially degraded biopolymers (Fincher, 1989). The grain at this stage of malting is referred to as “green malt”. The main goal of kilning is to remove the bulk of moisture from the germinated seed at 40-60°C. The temperature is gradually increased to 85-95°C (Briggs, 1998). In total, the kilning process of typical pale malt takes about 24 h (P.B. Schwarz, personnel

communication). During kilning, there is development of both malt flavor and color and the moisture is reduced to < 50 g kg^{-1} so the final malt can be safely stored (Burger and La Berge, 1985). After going through the three stage malting process, the final product (resulting malt) is a ready source of sugars, readily degradable starch, amino acids, and enzymes (Burger and La Berge, 1985).

Barley malt quality is influenced by both genetic and environmental factors. A few of the desirable grain characteristics for malt barley include uniform and plump kernels, moderate levels of grain protein, and high enzymatic activity. Uniform and plump kernels are necessary for a uniform rate of germination and higher malt extract production. Grain protein plays an essential role in the brewing process as a provider of nutrients for yeast during fermentation; however, excessive grain protein levels result in lower malt extract. Malt extract is a major economic factor for the brewing industry because it provides the potential production levels of beer from a given amount of malt (Burger and La Berge, 1985). Enzymes such as α- amylase, β-amylase, β-glucanase, and endo- and exo-proteinases play major roles in the determination of malt quality (Marquez-Cedillo et al., 2000). A major starch degrading enzyme is α-amylase, which attacks the starch granules and forms substrates that other enzymes can degrade. Another important amylase is β-amylase, with its main function involving the breaking of glucosidase bonds that result in the liberation of maltose (Marquez-Cedillo et al., 2000). The joint action of these two amylases, along with other enzymes known to degrade carbohydrates, is defined as DP (Pollock, 1962; Burger and La Berge, 1985).

Breeding for malt quality is difficult and complex because both malt quality and agronomic traits need to be selected for by the breeder simultaneously. It is important for

the breeder to try to avoid the antagonistic trends and negative linkages between genes governing important traits in the breeding program. For example, malt extract is an important trait in determining malt quality, but if it is negatively correlated to grain yield or positively correlated to lodging, then improving that trait is detrimental in a specific population. Therefore, malt traits such as malt extract, kernel plumpness, enzyme activity, along with grain yield, resistance to lodging, and seed shattering all need to be considered together (Rasmusson and Phillips, 1997). It is important to understand the impact of each trait and the interaction among all traits. However, determining malt quality generally involves expensive and time consuming procedures that cannot be performed until late stages in a breeding program when sufficient quantities of seed are available (Mather et al., 1997). Additionally, new malting barley cultivars almost always are derived from crosses between parents with acceptable quality. It is for these reasons that breeders concentrate on narrow crosses when trying to develop a new cultivar. Unadapted germplasm has been used more for development of disease resistant germplasm rather than lines for high malt quality characteristics (Horsley et al., 1995). Thus, in order for efficient development of improved populations with a broader germplasm base, the development of molecular markers is important; specifically, markers that are tightly linked to quantitative trait loci (QTL) controlling the desirable malt quality traits. The introduction of these markers would aid breeders in the process of adding new genes controlling all quality traits (agronomic or disease related), while keeping together the pool of favorable genes for specific traits already in their elite germplasm.

Understanding the genetic make-up of two closely related six-rowed barley cultivars (Robust and Stander) that differ substantially in malt quality would aid us greatly

in understanding the underlying genetics of malt quality. With this knowledge, the probability of developing high quality molecular markers for MAS and understanding the functional role of genes for malt quality traits would be greater. Cost reduction, less labor-intensive protocols, and less time are some of the main goals breeders strive for when creating the ideal malting barley cultivar. One potential solution for achieving this goal would be to incorporate MAS in the breeding scheme, which would produce an end product not only suitable for breeders and producers, but also for the malting and brewing industries. By utilizing markers and knowing the haplotype, or fingerprint, of the desirable six-rowed malting barley, new populations can be screened to identify progenies that have the desired genotype that corresponds to a particular brewer's needs.

Malt quality analyses

Degree of modification

The appropriate mashing schedule for obtaining the most extract is determined by the degree of modification. Only a single temperature rest for saccharification is required if the malt is well-modified.

Friability

This test is used to directly measure the modification of malt and is useful for controlling quality. The main goal of this test is to determine if the malt will fracture satisfactorily during the milling process (Briggs, 1998). The belief is that it should be easy to crush the endosperm of well-modified malt and hard to do so in a poorly modified malt. Thus, well-modified malt should produce small fragments and have a flour consistency (Schwarz and Li, 2011). A Friabilimeter is used for this method, in which a rubber roller enclosed inside a rotating screen crushes a 50 g sample. The calculation for friability is

based on a percentage and involves the subtraction of the amount of sample that did not pass through the screen from 100. The Friabilimeter can also be used to perform a second test that estimates the percent of unmodified malt (Schwarz and Li, 2011). An acceptable friability percentage for both two- and six-row malts is 78-82% (Briggs, 1998). Samples are considered well-modified with friability greater than 81%, and unacceptable with friability less than 75% (Kunze, 1999).

Growth count

During germination, there is full activation of the seedlings' metabolic systems (Burger and La Berge, 1985). The length of the acrospire is monitored during germination to ensure uniformity and estimate modification levels. In this analysis, 100 kernels are selected and acrospire lengths relative to the length of the entire kernel are recorded (Schwarz and Li, 2011). Five incremental categories are used for classifying lengths. These categories include 0-0.25, 0.25-0.5, 0.5-0.75, 0.75-1, and above 1/1, or commonly referred to as overgrown. The average acrospire length should be around 0.75 for brewer's malts; yet, well-modified malts used by U.S. brewers tend to have higher averages (Kunze, 1999; Schwarz and Li, 2011).

Fine-coarse extract difference

The degree of malt modification can be estimated by determining the difference between the fine-grind and course-grind extract values. It has been shown that finely ground malt gives a higher extract than coarsely ground malt in mashing procedures; thus, making it possible to estimate a difference value. In a finely ground malt, the majority of cell walls are disrupted, exposing starch granules and making them easily accessible to the diastatic enzymes in the mash (Briggs, 1998). In coarse-ground malt, under-modified

pieces of endosperm are present, making that portion of the sample resistant to amylases, and in turn, not allowing the entire sample to reach its potential extract yield. The AMBA currently accepts a maximum fine-coarse difference value of 1.2% (AMBA, 2008).

Extract

This trait is one the first parameters looked at by a brewer, and includes the measurement of soluble material collected after malt is mashed according to the standard ASBC procedure, where time and temperature incubations are controlled. Wort soluble materials are composed primarily of carbohydrates (roughly 90-92%), which can be further broken down into disaccharide maltose and branched dextrins (Burger and La Berge, 1985). The materials also contain amino acids, peptides, nucleic acid hydrolysis products, phenolic compounds, lipids, vitamins, and minerals. The end fermentation limit is determined by many factors, including fermentable sugars, malt enzymes, and free amino acids (Schwarz and Li, 2011). In order for six-rowed barley malt to be acceptable for the brewing industry, dry-basis extract should be greater than 79% (AMBA, 2008).

Diastatic power

Diastatic power represents a measurement of the malt's ability to convert starch to fermentable sugars. The process of starch being broken down into sugars and low molecular weight dextrins is largely completed by the complementary action of two enzymes, α-amylase and β-amylase. Random α (1→ 4) bonds are attacked along the starch polysaccharide chain by α-amylase, allowing for the release of dextrins, and various malto-oligosaccharides. The main function of β-amylase is to liberate the disaccharide maltose from the nonreducing ends of starch and dextrin chains. β-amylase completes this activity by splitting alternate α (1→4) interglucose bonds. The presence of these two enzymes is

imperative for obtaining a sufficient amount of fermentable sugars (Burger and La Berge, 1985; Schwarz and Li, 2011). Diastatic power is recorded in °ASBC, and the acceptable value by the AMBA is currently over 140 °ASBC (AMBA, 2008). It has been shown that DP values mainly represent the estimate of β-amylase activity in a malt sample (Burger and La Berge, 1985; Schwarz and Li, 2011).

Table 1. Malting barley criteria (Modified from AMBA, 2008).

Trait	Six-rowed barley
Barley quality	
Plump kernels†	>80%
Germination‡	>98%
Protein	≤13.5%
Malt Factors	
Total protein	≤13.3%
Beta-glucan (ppm)	<120
Kolbach index	42-47%
Viscosity (absolute cp)	<1.50
Malt enzymes	
Diastatic power	>140°ASBC§
Alpha amylase (DU)¶	>50
Congress wort	
Soluble protein	5.2-5.7%
Extract (fine-grind dry basis)	>79.0%
Color	1.8-2.5°ASBC
Free amino nitrogen	>200

†Sieve with slotted openings of 0.24 x 1.9-cm used to separate thin from plump kernels; based on percent of kernels that remain on the top of the sieve.
‡Based on a 72 hour germination period with 4 mL of water.
§°ASBC = Degrees American Society of Brewing Chemists.
¶Dextrinizing units at 20°C.

α-amylase

The importance of α-amylase (an endo-enzyme in brewing) is to reduce mash viscosity, and to provide additional substrate for β-amylase. This is achieved when α-

amylase reduces the size of starch and breaks down larger dextrins (Schwarz and Li, 2011). The activity of α-amylase is expressed in terms of 20° dextrinizing units (DU). This term can be easier thought of as the amount of α-amylase needed to dextrinize soluble starch in the presence of excess β-amylase at the rate of 1 g hr^{-1} at 20°C (Burger and La Berge, 1985). The AMBA requires over 50 DU for α-amylase content in acceptable six-rowed malts (AMBA, 2008). If cereal adjuncts are being used by the brewer, higher levels of α-amylase are necessary; however, many brewers generally don't consider this trait as important as other malt quality traits (Schwarz and Li, 2011).

Wort quality analysis

Wort viscosity

The filtration behavior of wort can be estimated with the results of wort viscosity. The concentration of higher molecular weight β-glucans influences the overall wort viscosity. Beer filtration is the main problem associated with insufficient β-glucan degradation (P.B. Schwarz, personnel communication). An undesirable glucan haze can also be detected in the finished beer product, if β-glucans are not degraded sufficiently (Burger and LaBerge, 1985). Due to potential slow lautering in the brewhouse, viscosities higher than 1.5 centipoises (cP) are deemed undesirable (Burger and LaBerge, 1985; AMBA, 2008).

Wort color

Wort color is analyzed with a spectrophotometer, using a single wavelength (430nm) (Schwarz and Li, 2011). The 430nm wavelength can be used to accurately analyze pale yellow or golden colored beers, but not darker and red hued products. In order for brewers to get more information for the darker colored products, tristimulus color

measurements can be used. The trait is recorded in °SRM, which closely matches the °Lovibond scale. Maillard reaction products are a large contributor to the overall color results; however, other reactions are also known to contribute to color such as pyrolysis, caramelization, and oxidation of polyphenols (Schwarz and Li, 2011). The acceptable color for congress wort according to AMBA is around 1.8-2.5°SRM (AMBA, 2008).

Free amino nitrogen

Free amino nitrogen analysis is utilized to help determine the amino acid composition of wort (Schwarz and Li, 2011). The reaction of ninhydrin with free α-amino nitrogen groups forms the basis of this method. The reaction results in a blue color, which is compared against a standard at 440 nm. This test is most sensitive to amino acids, due to the fact that the peptide and amino acids contain only a single free amino molecule/group (Schwarz and Li, 2011). The ideal FAN content for the AMBA is greater than 200 mg L^{-1} for six-rowed malt, to ensure adequate fermentation speed and to avoid the stimulation of high diacetyl levels (Fix, 1993; AMBA, 2008).

Kolbach index

This measurement, which is a ratio of soluble to total protein (S/T), directly estimates protein modification. The ratio includes wort soluble protein over total malt protein x 100 (Schwarz and Li, 2011). In some cases, barley protein levels are used instead of the malt protein in the ratio, because removal of rootlets is only thought to cause a small reduction in total nitrogen content. The Kolbach index tends to increase with extended periods of germination and high levels of malt modification (Schwarz and Li, 2011). Values around 42-47% are recommended for six-rowed cultivars in the US (AMBA, 2008).

ß-glucans

Carbohydrates in the endosperm cell walls are composed of approximately 70-75% β-glucan (Briggs et al., 2004). Besides the main starch derived (oligo)saccharides (maltodextrins, maltrotriose, maltose, and glucose), non-starch polysaccharides also play a major role in malt quality, and these include residual polymeric arabinoxylans and (1-3), (1-4)-β-D-glucans (β-glucans) (Vietor and Voragen, 1993). β-glucan is found in barley endosperm cell walls (Blake et al., 2011). During malting and mashing, β-glucanases degrade β-glucans to low molecular weight products (Vietor and Voragen, 1993). The enzyme endo-β-(1-3)(1-4)-glucanase develops during barley germination and is known to be the primary enzyme in hydrolyzing the β-glucan component (Kanauchi and Bamforth, 2001). β-glucans have been shown to affect viscosity and filtration in brewing. The reaction between β-glucans and calcofluor, a fluorescent brightener, is used to quantify the β-glucan content in a sample. This procedure is automated with the use of a flow injection analysis that detects variations in fluorescence (Schwarz and Li, 2011). In order to be deemed acceptable for six-rowed malt, the level of β-glucans should be below 120 mg L^{-1} (AMBA, 2008).

Wort carbohydrates

Starch is the source of wort carbohydrates. The two components that make up starch, amylose and amylopectin, are deposited in amyloplasts (Briggs et al., 2004). Roughly 1,600-1,900 residues long of linear α-(1,4)-linked chains of D-glucopyranose constitute the amylose component, whereas amylopectin is composed of highly branched molecules. In the complex structure of amylopectin, α-(1,4)-linked chains (26 glucose units long) are joined together by α-(1,6) branch points. On a dry basis, 58% of malt is

starch, and after it is broken down the breakdown products of starch make up a large portion of wort extract. Fermentable sugars and dextrins (breakdown products of starch) constitute the main source of extract. Starch is synthesized as granules in barley, which occur in two populations: A granules and B granules. The diameter for the A granules is 22-48 μm, whereas B granules are 1.7-2.5 μm. Roughly 10-20% of the granules are composed of A granules, which turns out to comprise roughly 85-90% of the weight (Briggs et al., 2004).

The saccharification time measurement is a rough estimate of the time needed to dextrinize starch, mainly dependent on the amount of α-amylase present in the malt sample (Briggs et al., 2004). When malt enzymes are mixed together to catalyze the hydrolytic breakdown of starch, 'diastase' is the term given to the mixture. Malt α-amylase can be observed in three classes in barley that has undergone germination. The three classes include α-amylase-I, α-amylase-II, and α-amylase-III. The first class, α-amylase-I, is found in small quantities in malt and can be inhibited by heavy metal ions. The second class, α-amylase-II, is the classical enzyme that is heat resistant, not completely stable in mashes, and can be broken down by calcium-binding chelating agents. The final class, α-amylase-III, is a complex between α-amylase-II and BASI (small protein limiting the enzyme's activity). Collectively, the α-amylase classes form the α-amylase mixture known to attack α-(1,4)-links located in the starch chains. Glucose, maltose, and a complex mixture of branched and unbranched oligosaccharides and dextrins are the products produced after extensive α-amylosis. The enzyme α-amylase releases dextrins, ultimately creating the substrate for saccharogenic β-amylase in the mashing process. β-amylase can exist in both insoluble and soluble forms in barley, and is known to be sensitive to heat and resistant to

acid conditions and chelating agents. This enzyme catalyzes the hydrolysis of the penultimate α-(1,4)-link found in amylose and amylopectin non-reducing chain ends, ultimately releasing maltose in the disaccharide form. Limit dextrinase's main function is to hydrolyze some of the α-(1,6)-linkages. Therefore, variable length dextrins are produced after α-amylase attacks the starch granule, and then β-amylase, limit dextrinase, and α-glucosidase are responsible for further degradation steps needed to obtain glucose (Briggs et al., 2004). The exo-acting enzyme α-glucosidase cleaves α-1, 4-linkages to produce glucose (Wang et al., 2006). Limit dextrinase cleaves α-(1-6) bonds of amylopectin, rendering the branched dextrins susceptible to further hydrolysis by β-amylase (MacGregor et al., 1994). Elevated levels of limit dextrinase may help increase total wort fermentability, by enhancing hydrolysis of un-fermentable branched dextrins into fermentable sugars. Residual dextrins are responsible for the mouthfeel of beer (Priest and Stewart, 2005).

The origination of fructose could be accredited to the hydrolysis of sucrose, or just the presence of free sugar initially found in the grist. During mashing, α-amylase, α-glucosidase, or β-glucosidase may be involved in the formation of glucose in malt. As for maltose, this sugar is mainly formed by β-amylase; however, a debranching enzyme and α-amylase may also play a role in its formation (Briggs et al., 2004). The brewer considers α-and β-amylases very important, and recognizes the effect of the mashing temperature on overall composition of wort (Schwarz and Li, 2011).

The carbohydrates (complex mixture) account for roughly 92% of the solids found in the wort solution. Glucose is the major constituent of most important sugars and dextrins produced during mashing, which is evidenced by the existence of D-

glucopyranose units joined by α-(1,4) links in maltose, maltotriose, maltotetraose, and maltopentaose. Monosaccharides such as glucose and fructose, disaccharides sucrose and maltose, and the trisaccharide maltotriose are considered fermentable by most yeast species (Briggs et al., 2004). Maltose, a disaccharide with two D-glucose molecules, is the most abundant fermentable sugar in brewer's wort, roughly comprising 50-60%. The second most abundant fermentable sugar is maltotriose, a trisaccharide with three D-glucose units, (15-20%), followed by glucose (10-15%) (Stambuk et al., 2006). Temperature, time, and pH levels all have an influence on the overall final composition of sugars in a wort sample. In order for yeast to continue with the process of alcoholic fermentation, fermentable carbohydrates must be present. High performance liquid chromatography (HPLC) is the instrument used to detect the quantity of fermentable sugars in wort. It is important to remember that fermentability is influenced by other factors besides fermentable sugars (Schwarz and Li, 2011).

Molecular research on malt quality

Malt quality has been a primary objective in many molecular studies throughout the years. However, due to the complexity of the traits and limited funding sources, there are still an abundance of grey areas that need to be answered before an effective MAS strategy is developed. Lapitan et al. (2009) utilized microarrays and expressed sequence tags (ESTs) to identify differentially expressed genes during the malting process in barley. In this study, between 11 to 102 genes showed correlation with six malting quality traits, but there was still a large number of genes with unknown function. This shows that malting quality traits are not only complex, but also not well understood on a molecular basis. Another study demonstrated the effectiveness of QTL pyramiding for the development of

elite germplasm with acceptable malting quality, disease resistance, and even desired grain plumpness (Emebiri et al., 2009).

A QTL analysis was conducted by Marquez-Cedillo et al. (2000), in which the results showed three phases of favorable alleles consistent at QTLs across three mapping populations (Harrington x Morex, Harrington x TR306, and Steptoe x Morex). In this study, malting quality QTL in the Harrington x Morex population coincided with the *Amy2* locus on chromosome 7H and the hordein loci on chromosome 1H. The authors also suggested that QTL information is pertinent in breeding programs in order to maintain specific configurations leading to a target quality profile, supplemented with markers. Many other studies throughout the years have investigated potential QTL being associated with malting quality traits. Specifically, malt extract was molecularly analyzed in three barley populations developed in geographical regions of Japan, Europe, and Canada, in hopes of recognizing associations between barley genomic regions and the trait of interest (Collins et al., 2003). The three mapping populations consisted of the Galleon/Haruna Nijo, Sloop/Alexis, and Chebec/Harrington. Out of these populations, Haruna Nijo, Alexis, and Harrington represent high malt quality varieties from Japan, Europe, and Canada, respectively. For the Galleon/Haruna population consisting of 112 F_1-derived DH lines, a linkage map consisting of 435 restriction fragment length polymorphism (RFLP) marker loci was constructed. For the next population consisting of 109 F_1-derived DH lines, the Snoop/Alexis, 187 amplified fragment length polymorphism (AFLP), 55 RFLP, and 62 microsatellite markers were used to construct the linkage map. With the incorporation of 120 F_1-derived DH lines for the Chebec/Harrington population, a linkage map was constructed from 259 RFLP, 47 AFLP, and 34 simple sequence repeat (SSR)

markers. A total of eight regions of the barley genome showed association with malt extract. Regions on chromosomes 1H, 2H, 3H, 4H, and 5H showed association with malt extract in the Sloop/Alexis population, whereas only regions on chromosomes 1H and 5H were detected in the Chebec/Harrington population. As for the Galleon/Haruna Nijo population, a region on chromosome 2H was found. From these eight regions, markers from six of them were looked at more closely in many breeding populations. A total of four regions proved to be significant at increasing malt extract for the allele from the high malt extract parent. These regions were found on chromosomes 2H (2 regions) and 5H (2 regions) (Collins et al., 2003).

In 2004, a study conducted by Emebiri et al. utilized 500 F_1-derived DH lines from the population VB9524 x ND11231*12. From these 500 lines, 180 were randomly selected for the construction of the linkage map. The QTL results for malt extract were found on chromosomes 2H and 7H, with a total of three loci at these chromosomes. A total of five QTL were detected for α-amylase, with the major QTL being on chromosome 6H, located relatively close to the previously mapped *Amy1* gene for α-amylase. There was only one QTL found on chromosome 1H, and that QTL was associated with β-glucanase. Overall, for all malt quality traits, one locus was detected on chromosomes 1H and 6H, while chromosome 2H proved to have a high concentration, totaling nine loci (Emebiri et al., 2004). Malt quality QTL resolution was further investigated in the Han et al. (2004) study that focused on a 28 cM distance on chromosome 7H, known to harbor QTL controlling malt extract, α-amylase, DP, and β-glucan. A total of 39 isolines from the Steptoe/Morex cross were developed through marker-assisted backcrossing. Through the use of composite interval mapping (CIM), QTLs were discovered. For the trait malt extract, one QTL was

identified. For other traits such as α-amylase, DP, and β-glucan, two QTL were identified. The interval for the resolved QTLs ranged from 2.0 to 6.4 cM for the CIM analysis, and 2.0 cM or less for the multiple interval mapping (Han et al., 2004).

The Steptoe/Morex population was further investigated by Gao et al. (2004) with the creation of DH lines, in which the North American Barley Genome Project was able to identify and map a malting-quality QTL complex known as QTL2 through interval mapping. This complex was found in the short-arm telomeric region of chromosome 4H. After this discovery, scientists felt it would be important to incorporate a fine mapping procedure in the same population to exploit QTL2. QTL controlling malt extract (3), DP (4), α-amylase (6), and β-glucan (2) were putatively mapped. An overall analysis, including all environmental factors, detected six QTL in a region from 0.7 cM to 27.9 cM. The telomeric region on chromosome 4HS located 15.8 cM away was highlighted as containing the majority of the identified QTLs and could potentially be used in marker-assisted breeding for malt quality (Gao et al., 2004). Validation of effective marker-based selection must be performed in a population other than that used for mapping. Ayoub et al. (2003) manipulated the α-amylase trait through a marker-based selection strategy in a barley breeding population. The Morex allele was selected at two PCR markers on the short arm of chromosome 5H near the centromeric region, effectively increasing α-amylase activity (Ayoub et al., 2003).

The β-glucan trait was further dissected in 170 DH lines derived from a cross between a moderately high β-glucan 2-rowed line (TR251) and a low β-glucan two-rowed semi-dwarf cultivar (CDC Bold) in the Li et al. (2008) study. Genotypic data were obtained for 88 of the lines using SSR, AFLP, and diversity array technology (DArT)

markers. These markers were integrated in the production of a 1,059 cM map. Simple interval mapping was used to detect seven genomic regions associated with low β-glucan content. Alleles from the 'CDC Bold' parent contributed to the low β-glucan content for the majority of the QTL regions except for two loci. The TR251 parent contributed alleles for these two loci on chromosome 5H. On chromosome 7H, a large effect QTL explaining 39% of the β-glucan content was identified in the centromere region (Li et al., 2008).

Schmalenbach and Pillen (2009) used wild barley introgression lines (39) in identifying QTL for malting quality traits. These lines were developed from the cross between 'Scarlett' (German spring barley) and a wild barley accession from Israel. To obtain the malt data, three different environments were used. A total of 40 QTLs were localized for eight of the malt quality traits, 35 proving to be stable across all environments. Six of the QTLs were associated with improved trait performance. When the authors compared the results with a previous study that involved the BC_2DH population S42, 18 of the 36 QTLs detected could be verified. Overall, eight new QTL effects were identified for the traits α-amylase, fine-grind extract, grain protein content, and Hartong 45°C. The Hartong 45°C trait is another way of expressing the fine grind over coarse grind value, by measuring the low-temperature extraction. Two QTLs on chromosome 4H (QAa.S42IL-4H.a and QAa.S42IL-4H.b) were sites of a favorable *Hsp* effect, which allowed for an enhanced α-amylase activity over the parent Scarlett. For grain protein concentration, four putative QTLs were identified on chromosomes 1H, 4H, 6H, and 7H, in which the region on chromosome 6H proved to cause an increase in the trait value when compared to the control (Schmalenbach and Pillen, 2009).

Current research using the Oregon Wolfe Barley (Szucs et al., 2009) population has integrated multiple types of markers into a single map, which consisted of 1,472 SNP, 722 DArT, and 189 prior markers. The scientists identified many QTLs involved with malt quality traits. Grain protein was controlled by the highest number of QTL (21), while α-amylase and malt extract were associated with 20 QTL. Chromosome 5H had the greatest number of QTL, whereas the least amount of QTL were found on chromosomes 3H and 6H (Szucs, 2009). In a more recent study, six-rowed malting barley lines from the University of Minnesota were genotyped with 1,524 SNPs (Munoz-Amatriain et al., 2010). These lines were also phenotypically and functionally characterized, with micro-malting procedures and the Barley1 GeneChip array. When looking at the most recent lines from the University of Minnesota program, a total of 49 differentially expressed genes were identified and showed association with at least one of the malt quality traits. It is important to concentrate on the differentially expressed genes in order to recognize future improvements of quality traits in the program. Serine-type endopeptidases were encoded by three of the candidate genes. The two traits with the greatest potential for improvement are α-amylase and malt extract. Correlated candidate genes were also found for grain protein, β-glucan, and Kolbach index. When crossing two elite lines, the smallest improvements would be for the DP, due to the small number of candidate genes (Munoz-Amatriain et al., 2010).

Significant QTL for malt quality, seed dormancy, and water sensitivity were detected by Castro et al. (2010) in the BCD47 (spring 2-rowed DH line developed at Oregon State University) x 'Baronesse' population on chromosome 5H. The malt quality traits associated with the significant QTL included malt extract, α-amylase, β-glucan

content, FAN, Kolbach index, wort turbidity, and protein content. Malt quality QTL were found on chromosomes 2H, 3H, 4H, 5H and 6H (Castro et al., 2010). Recently, Mikamo Golden (Japanese cultivar) and Harrington (North American cultivar) were crossed to produce 95 DH lines that were used in the construction of a high-density map with 550 markers in a study conducted by Zhou et al. (2011). Malt quality QTL were detected in the terminal region of chromosome 5HL. Traits controlled by QTL included malt extract, soluble nitrogen, and Kolbach index. An additional QTL was mapped on chromosome 2H for malt extract. The authors decided to develop cleaved amplified polymorphic sequence (CAPS) markers for malt extract, which proved to be effective for marker-assisted selection (Zhou et al., 2011).

Many studies have looked at QTL associations between malt quality traits of α-amylase, β-glucan concentration, DP, and malt extract; however, few have focused on the associations with fermentability factors, such as wort carbohydrates. One study that focused on fermentability was published by Fox et al. in 2001. This study consisted of fifteen lines grown at four sites of the 1999 South Australian Research and Development Institute (SARDI) stage 4 trial and 70 lines from the 1998 'Galleon' x 'Haruna Nijo' mapping population. The lines were mashed to closely look at fermentable sugar profiles and recognize potential QTL regions. The QTL analysis for apparent attenuation limit was performed with QGENE on the Galleon*Haruno Nijo Mapping Population. Genomic regions associated with fermentability were identified on the long arm of chromosome 4H (*Bmy1* locus) and on chromosome 3H. Further mapping results showed an association between maltotetraose and the *Bmy1* locus. The fermentability QTL identified on chromosome 3H was not involved with starch synthesis or hydrolysis, but did match up to

the *denso* dwarfing gene location. The small branched dextrins were also shown to be associated with the *denso* locus on 3H (Fox et al., 2001). Previously, the denso locus and the *ari-eGP* dwarfing gene were found to influence fermentability on chromosome 5H. The association between fermentablility and the *ari-e*GP allele was positive (Swanston et al., 1999). The same allele was associated with higher hot water extracts, which led to faster modification rates (Swanston et al., 1990; Swanston et al., 1999).

Elia et al. (2010) looked at QTL associations of malt quality and fermentability traits in a cross between an European and North American cultivar. Triumph (European cultivar) and Morex (North American cultivar) were crossed in hopes of enhancing malt quality traits across a broad range of environments. Malt QTL were distributed across all seven chromosomes. Chromosomes 1H and 5H harbored the most influential QTL associated with hot water extract and alcohol yield across multiple environments. Three QTL were detected for malt extract in different locations. On chromosome 1H, a QTL for malt extract was detected in a region known to contain a cellulose-synthase-like gene *HvClsF9* (Elia et al., 2010; Burton et al., 2008). This gene has been recognized as a potential candidate gene for a β-glucan QTL in the Steptoe x Morex population (Han et al., 1995). Other authors also discovered this region on chromosome 1H in the Steptoe x Morex population (Hayes et al., 1993) and Sloop x Alexis population (Collins et al., 2003; Elia et al., 2010). For chromosome 5H, a region was detected for malt extract QTL in the Triumph x Morex population and the 'Dicktoo' x Morex population (Oziel et al., 1996; Elia et al., 2010). The malt extract QTL detected on chromosome 2H for this study also showed up in another study looking at the Harrington x Morex population, and is related

with the *vrs1* region (Marquez-Cedillo et al., 2000). QTL for fermentability were detected on chromosomes 1H, 2H, 3H and 7H. The fermentability QTL were located in the same genomic regions that had an effect on malt extract (Elia et al., 2010).

Previous NDSU research

In previous research on the NDSU barley breeding/genetics program, Pedraza-Garcia (2011) conducted molecular research utilizing a mapping population of 73 doubled-haploids from the Robust x Stander cross. Prior to 2009, both the population and parents were evaluated in four yield trial experiments in North Dakota and one experiment in Aberdeen, Idaho. After collecting phenotypic data and harvesting the test plots, the grain was micro-malted in the barley and malt quality laboratory of Dr. Paul Schwarz. Two of the North Dakota yield trial experiments were micro-malted in 2008 (Locations: Fargo 2006 and Fargo 2007). In 2009, an additional two locations from the 2007 growing season were micro-malted. These locations included one trial from both North Dakota and Idaho. DNA from the population was sent to Triticarte PTY Ltd. in Australia and genotyped using DArT marker analysis (Jaccoud et al., 2001). One hundred seventy markers detected polymorphisms between the parents and a linkage map with 11 linkage groups was constructed (Pedraza-Garcia, 2011). Three of the linkage groups could not be assigned to any of the seven known barley chromosomes. Pedraza-Garcia (2011) indicated that the large number of linkage groups was not unexpected, since Robust and Stander are very closely related and a large proportion of the genome is likely fixed, due to selection by breeders for the rigorous malt quality specifications a cultivar must meet in order for it to be recommended as a malting barley cultivar by the AMBA (Pedraza-Garcia, 2011; AMBA; Milwaukee, WI).

Pedraza-Garcia (2011) used over 200 SSR primers from the NDSU barley breeding program to identify polymorphisms between the parents. He found that 32 of the 200 primers identified polymorphisms, in which he added 14 of them to the previous DArT map. Fabio also used information from the USDA-CSREES Barley Coordinated Agricultural Project (CAP) to find 137 SNP markers from pilot OPA1 that identified polymorphisms between Robust and Stander. Many of the SNPs cosegregated to similar chromosome locations; thus, Fabio utilized a subset of 46 SNPs from different regions of the genome for development of PCR primers. To date, 32 new primers have been developed and they have been used to identify polymorphisms between the parents (Pedraza-Garcia, 2011).

Using the map consisting of DArT and SSR markers, QTL analyses were conducted using all agronomic data, malt quality data from two locations, and dormancy data from four greenhouse experiments (Pedraza-Garcia, 2011). Pedraza-Garcia (2011) discovered putative QTL for all malting quality traits, except fructose concentration. The results obtained from the Pedraza-Garcia (2011) research are important, but are thought of as preliminary due to the small population size utilized in the mapping study. Therefore, additional research with a larger population and more environments is necessary for collecting sufficient malt quality and agronomic data for the development of a MAS strategy specific for the Robust x Stander population.

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CHAPTER II. DEVELOPMENT OF A MOLECULAR MARKER MAP USING THE ROBUST X STANDER MAPPING POPULATION AND ITS APPLICATION IN MAPPING QTL CONTROLLING AGRONOMIC TRAITS

Abstract

Not only must malting barley (*Hordeum vulgare* L.) cultivars meet the quality specifications set by maltsters and brewers, but they must also possess the agronomic performance needed for growers to successfully grow the cultivars. Two closely related six-rowed barley cultivars that differ greatly in agronomic performance and malt quality are Robust and Stander. The close relatedness of these two cultivars should allow for determining the genetic basis of the differences. A total of 53 doubled-haploid (DH) lines (original population) and the parents from the Robust x Stander cross were grown in field trials at eleven locations in North Dakota and one location in Idaho the past six years. An additional 138 Robust x Stander DH lines were generated in 2009 and were evaluated alongside the original DH population in the summer of 2011 at two North Dakota locations. Agronomic data were collected at all locations and analyzed. Three linkage maps were developed using the original and 191 DH line populations. The first linkage map was constructed using the original Robust x Stander DH lines and a total of 102 SNP, SSR, and DArT markers. The second and third linkage maps were developed using only 67 SNP markers, with the original and entire Robust x Stander DH population, respectively. The second linkage map was used to identify QTL on chromosome 6H for plant height, lodging, and deciduous awns. The third map was used to identify QTL on chromosome 4H for heading date and chromosome 6H for plant height.

Introduction

Barley (*Hordeum vulgare* L.) is currently the fourth most important cereal crop in the world in terms of production (FAOSTAT, 2009). Barley is mainly used for animal feed, malt, human consumption, and seed (Rasmusson, 1985). Barley can be classified by spike morphology into two-rowed and six-rowed barley. Two-rowed barley is typically used for malting and brewing around the world, while six-rowed barley is most often used for livestock feed. However, due to adaptability and acceptable malt quality, six-rowed malting barley cultivars comprise over 90% of the barley produced in the Upper Midwest region. The six-rowed malting barley cultivars proved to be better adapted to dryland conditions (Upper Midwest region), whereas two-rowed cultivars show greater production in areas under irrigation (West region) (Horsley and Harvey, 2011). In the United States and Mexico, some large commercial brewers producing beers with adjunct use large proportions of malt made from six-rowed barley.

Currently in the US, there are 12 public institutions that have barley breeding programs, with seven of those concentrating solely on barley. Institutions working exclusively on barley are located in the northern part of the United States (Montana State University, North Dakota State University, Oregon State University, the University of California, Davis, the University of Minnesota, Washington State University, and the United States Department of Agriculture Agricultural Research Service at Aberdeen, Idaho) (Horsley and Harvey, 2011). Barley breeding programs, such as Busch Agricultural Resources, MillerCoors, and Westbred, comprise a large portion of the private sector in the US. Busch Agricultural Resources and MillerCoors develop malting barley cultivars, while Westbred focuses on feed barley (Horsley and Harvey, 2011). There are many

requirements and guidelines that must be met before a cultivar is used for malting and brewing. This reason alone makes malting barley stand out among other crops, such that it needs to be stored on an identity preserved basis and under conditions that maintain its ability to germinate. Segregation among cultivars, and even sometimes by production area or trait, occurs to ensure that only cultivars desired by the malting and brewing industries are purchased. It is not unusual for a malting barley cultivar to be utilized in large proportions for malting and brewing for more than 15 or even 20 years in North America.

Two cultivars developed by the University of Minnesota, Robust (Rasmusson and Wilcoxson, 1983) and Stander (Rasmusson et al., 1993), share a close pedigree relationship, but differ in many of the key agronomic and malt quality trait characteristics. The pedigree of Robust is 'Morex'/'Manker' (Rasmusson and Wilcoxson, 1983), and Stander is Robust*2/3/'Cree'/'Bonanza'//Manker/4/Robust/'Bumper' (Rasmusson et al., 1993). Even though the parents are closely related, they differ in three main agronomic traits. Stander is known to be more resistant to lodging, shorter in plant height (6 cm shorter), and higher yielding (6% higher) than Robust (Rasmusson et al., 1993).

The close relationship between Robust and Stander increases the chance of recognizing chromosome regions with the genes controlling malt quality traits. Before the use of such simple sequence repeat (SSR), diversity array technology (DArT), or single nucleotide polymorphism (SNP) markers, mapping using a population derived from closely related parents such as Stander and Robust would not have been possible because of the low polymorphism rate. In studies by Lin (2007) and Pedraza-Garcia (2011), the polymorphism rate between Stander and Robust using SSR and DArT markers was less than 10%. Current mapping studies of barley now have access to platforms with up to

9,000 SNPs (S. Chao, personnel communication). A limitation in the work of Pedraza-Garcia (2011) and Lin (2007) was that their mapping population consisted of less than 60 Stander x Robust DH lines. Recently, over 100 additional DH lines were generated and will be used for mapping. The original and new DH lines were genotyped using the Illumina 9,000 SNP platform. The objectives of this study were to: 1) generate molecular marker linkage maps using the original 53 Robust x Stander DH lines and the entire DH population (191 lines); and 2) identify QTL controlling agronomic performance, including heading date, plant height, lodging resistance, reaction to foliar and spike diseases, deciduous awns, yield, and test weight.

Materials and Methods

Plant material and field evaluation

A total of 53 lines from the Robust x Stander DH population and parents used by Pedraza-Garcia (2011) were grown in 2009 at three locations in North Dakota. The sites included dryland sites in Fargo and Langdon and an irrigated site near Ray (Nesson Valley). Grain from the Fargo and Langdon locations was micro-malted at NDSU. In 2010, the same population was grown at two dryland sites in North Dakota, Fargo and Osnabrock. Including the research done by Pedraza-Garcia (2011), a total of twelve environments of data for genetic analysis of agronomic traits were collected for the 53 Robust x Stander lines.

An additional 138 doubled-haploid (DH) lines from the Robust x Stander cross were generated by Dr. Paul Johnston of the New Zealand Institute for Plant and Food Research (Lincoln, New Zealand). With the addition of the new lines, 191 lines were

available for use in this project. Seed of the new and old DH lines was sown in the 2010 winter greenhouse for seed increase, and further increased in the 2010-2011winter at the New Zealand off-season nursery. In summer 2011, the entire DH population, parents, and check cultivars [Stellar-ND (Horsley et al., 2006), Morex (Rasmusson and Wilcoxson, 1979), Lacey (Rasmusson et al., 2001), Tradition, and Legacy] were grown and evaluated at Fargo, McVille, and Ray (Nesson Valley), ND. The experimental units included seven rows spaced 19.05 cm apart. The plot length was 2.44-m with a 1.52-m distance between adjacent plots. For all locations, a seeding rate of 63 kg ha^{-1} was used. Data were not collected at the 2011 Fargo location because the excessive rain and waterlogged soils killed the plants. A total of 12 environments of data for genetic analysis of agronomic traits were collected for the original 53 Robust x Stander lines, and two environments for the entire Robust x Stander DH population. The entire population obtained from the 2011 McVille, ND location is currently being micro-malted at NDSU.

Agronomic data collected from each field experimental unit included heading date, plant height, lodging resistance, deciduous awns, and any evidence of foliar disease. Heading date was recorded as the number of days after 31 May when 50% of the spikes emerged from 50% of the plants in an experimental unit. Plant height was measured in cm as the distance from the ground to the top of the plant (excluding awns). Lodging and deciduous awn severities were measured using specific scales. A 1-10 scale (1 = no lodging and 10 = severe lodging) was used to measure lodging, whereas a 1-5 scale (1 = awns intact and 5 = awns completely deciduous) was used to detect the presence of deciduous awns at harvest maturity. Once the barley reached maturity, the grain was harvested with a plot combine, dried down in a forced-air dryer to approximately 100 g

kg^{-1}, threshed, and cleaned. The cleaned grain was used to record measurements for yield (Mg ha^{-1}), and test weight (kg hL^{-1}).

Statistical analyses of phenotype data

Entries in the yield trial experiments were assigned to experimental units using an 8x9 rectangular lattice in 2009, a randomized complete block design in 2010, and a 14x14 simple square lattice design in 2011. Using SAS/STAT (SAS Institute, 2004), agronomic data from each environment were analyzed as a randomized complete block design and adjusted means were calculated using the LSMeans function of PROC GLM. The environments were treated as a random effect, whereas the DH lines (entries) were considered a fixed effect. *F*-tests were considered significant at $P \leq 0.05$. The denominator of the *F*-test for the environment x entry source of variation was the error mean square (MS) and the denominator of the *F*-test for the entries source of variation was the environment x entry MS. Mean separation was done using the PDIFF command in SAS/STAT (SAS Institute, 2004).

Genetic analyses

Genotyping

The entire population and Robust and Stander were sown in the fall 2011 greenhouse to provide leaf tissue for DNA extraction. The leaf tissue from one plant was sent to Dr. Shiaoman Chao's USDA-ARS laboratory in Fargo and genotyped using a high density SNP marker genotyping platform. The regenotyping of the population utilized by Pedraza-Garcia (2011) helped detect any admixtures, if present. The DNA was extracted using the procedure of Bodoslotta et al. (2008). The population was genotyped with a custom designed Illumina iSelect BeadChip platform containing 9,000 SNPs using the

Infinium assay (Illumina, 2008) developed by Illumina (San Diego, CA). The assay included three main steps: hybridization of total genomic DNA to beads, single-base extension, and allele specific detection methods. The BeadStation scanner was used to scan the bead chips and obtain hybridization intensity values, which were used as the basis for genotype calling. The software program GenomeStudio, developed by Illumina, was used to determine the genotype calls. Samples exhibiting the same genotype formed a cluster. The calling algorithm identified three clusters, each corresponding to one of the three genotypes (Figure 1). Due to the potential occurrence of cluster compression, each genotype call was manually checked and edited. After validating the genotype calls, the genotype data were exported from the software and used for further analysis.

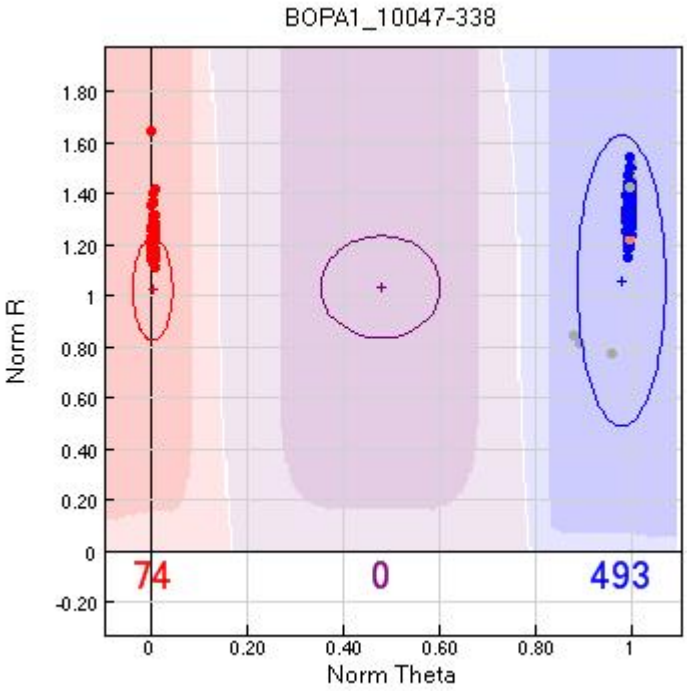

Figure 1. Genotype call output from GenomeStudio software. Three clusters representing AA, AB, and BB genotype classes (left to right).

Map construction

A map for the original population was obtained using 445 SNP markers that identified polymorphisms between Robust and Stander, and the 20 SSR and 153 DArT markers previously used by Pedraza-Garcia (2011). A second map was constructed using SNP genotype data from the original population. A third map was constructed using SNP data from the entire population. The software program MapDisto v.1.7.2.4 (Lorieux, 2006) was used to construct all linkage maps. Cosegregating markers were removed to obtain the best order of markers in all maps. The ordering of linked markers was determined by the seriation II method. The Kosambi mapping function was used to calculate the genetic distances in all maps. Markers significantly different at LOD≥ 3.0 were deemed to be unlinked. Additionally, the linkage groups were based on marker mapping distances no greater than 25 cM.

QTL mapping

The QTL analyses of the original population were based on agronomic phenotype data from 12 environments (Table 2). QTL analyses using the entire population were done using phenotype data from the 2011 McVille and Nesson Valley research sites. QGene 4.3.8 (Joehanes and Nelson, 2008) was used to statistically analyze and identify QTL for all traits. Single marker regression was performed first to identify significant marker-trait associations. To locate chromosomal regions associated with each trait, permutation tests (1000 iterations) were run to determine the minimum LOD value needed before a marker-trait association would be considered significant ($\alpha_{0.01}$ and $\alpha_{0.05}$ experiment-wide error). Composite interval mapping (CIM) was conducted, in which markers located outside the interval were treated as cofactors. The default parameters in QGene were used to select

and remove these cofactors. Each environment was analyzed separately in order to determine the percentage of environments with detected QTL. Those traits in which marker-trait associations were detected in >50% of the environments where a trait was measured were deemed QTL. Additional QTL analyses were performed using means averaged across environments for traits with QTL.

Table 2. Environment descriptions.

Environment	Location	Experiment type	Year	Phenotypic data utilized for mapping
1	Fargo	Yield trial	2006	Agronomic, Malting
2	Nesson Valley	Yield trial	2006	Agronomic
3	Fargo	Yield trial	2007	Agronomic, Malting
4	Idaho	Yield trial	2007	Agronomic, Malting
5	Osnabrock	Yield trial	2007	Agronomic, Malting
6	Fargo	Yield trial	2009	Agronomic, Malting
7	Langdon	Yield trial	2009	Agronomic, Malting
8	Nesson Valley	Yield trial	2009	Agronomic
9	Fargo	Yield trial	2010	Agronomic
10	Osnabrock	Yield trial	2010	Agronomic
11	McVille	Yield trial	2011	Agronomic
12	Nesson Valley	Yield trial	2011	Agronomic

Results and Discussion

Elimination of DH lines

Twenty DH lines were deemed to be heterozygotes based on the SNP data. These lines were not used in any of the analyses for descriptive statistics or mapping. The elimination of the lines and their accompanying data required the data to be analyzed as a RCBD and not a lattice.

Descriptive statistics

Mean values and descriptive statistics from the combined analyses of variance (ANOVA) across locations of the parents and the original DH lines for the agronomic traits of heading date, plant height, yield, and test weight are presented in Table 3. The parental lines (Robust and Stander) differed significantly ($P \leq 0.05$) only for plant height. Rasmusson et al. (1993) also observed height differences between Robust and Stander. The coefficient of variation for yield was 16.5% (Table 3). This value is greater than the desired value of the NDSU barley breeding program of no more than 15% and the preferred values of less than 12% (R. Horsley, personnel communication, 2012). The higher than desired CV value is indicative of field variation that was not controlled sufficiently by experimental design.

Mean values and descriptive statistics from the combined ANOVA of the parents and the entire DH population for heading date, plant height, and yield are presented in Table 4. Again, the two parents differed significantly ($P \leq 0.05$) only in plant height. Other agronomic and disease traits for the parents and the original DH lines are presented in Table 5. These results can be thought of as preliminary, as data were collected from no more than two environments. In these experiments, Robust and Stander differed significantly ($P \leq 0.05$) for lodging and deciduous awns.

For the original DH lines, the minimum, maximum, and mean agronomic values were not significantly different to those observed in both parents; thus, we cannot prove occurrence of transgressive segregation. For heading date, the range of the progeny was from 29.7 to 32.0, which is comparable to the values observed in the parents, 30.1 and 30.0 d after 31 May. The mean parental values for plant height were 82.5 cm for Robust and

77.6 cm for Stander, with the progeny ranging from 74.0 to 83.1 cm. Parental means were nearly identical for yield and test weight, around 4.7 T ha^{-1} and 67.5 kg hL^{-1}, respectively. The yield of the progeny ranged from 3.8 to 4.8 T. Test weight of the progeny ranged from 66.7 to 69.3 kg hL^{-1}. Results from the entire population from more than two environments are needed before a conclusion on transgressive segregation can be reached. For other agronomic and disease traits, such as lodging, deciduous awns, and foliar disease, the progeny means were similar to the parental means. Also, identification of which specific pathogens were causing the predominant foliar leaf spotting was not determined. It appeared that the predominant foliar disease seemed to be net form net blotch (incited by P*yrenophora teres* f. *teres* Drechsler).

Linkage map construction

Construction of the first linkage map using the original DH lines included 67 polymorphic SNP markers, along with 25 DArT and 9 SSR markers used previously by Pedraza-Garcia (2011). The polymorphism rate between Stander and Robust using SSR and DArT markers was less than 10% (Lin, 2007; Pedraza-Garcia, 2011), and 6.6% when using SNP markers. Fifteen linkage groups composed of 87 markers (67 SNP, 18 DArT, and 5 SSR markers) were obtained, covering chromosomes 1H to 6H. There was no linkage group for chromosome 7H. Linkage groups 4H-2, 6H-1, and 6H-2 represent relatively large portions of the respective chromosomes, while smaller segments make up the other chromosomes. Linkage group 4H-2 was 100.7 cM, 6H-1 was 99.63 cM, and 6H-2 was 109.7 cM. Based on estimated chromosome lengths from the Oregon Wolfe barley (OWB) consensus map (Szucs et al., 2009), linkage group 4H-2 comprised 80.3% of chromosome 4H, linkage group 6H-1 covered approximately 61.9% of chromosome 6H,

Table 3. Overall means and descriptive statistics of agronomic and quality traits for the parents and the original Robust x Stander doubled-haploid lines harvested at 12 locations across five years (2006-2007; 2009-2011).

	Heading date	Plant height	Yield†	Test weight‡
	d after 31 May	cm	T ha^{-1}	kg hL^{-1}
Parents				
Robust	30.1a§	82.5a	4.3a	67.4a
Stander	30.0a	77.6b	4.3a	67.6a
Population statistics				
Mean	30.7	79.0	4.3	67.9
Minimum	29.7	74.0	3.8	66.7
Maximum	32.0	83.1	4.8	69.3
Standard deviation	1.0	3.9	0.7	1.4
% Coefficient of variation	3.4	4.9	16.5	2.0

†Yield data were obtained from 11 locations.
‡Test weight data were collected from eight locations.
§Means for parents within a column followed by the same letter are not different at P=0.05.

Table 4. Overall means and descriptive statistics of agronomic traits for the parents and the entire Robust x Stander doubled-haploid population harvested at two North Dakota locations in 2011.

	Heading date	Plant height	Yield†
	d after 31 May	cm	T ha^{-1}
Parents			
Robust	37.8a	83.1a‡	2.9a
Stander	38.0a	75.3b	2.9a
Population statistics			
Mean	38.0	78.9	3.1
Minimum	35.8	72.1	2.2
Maximum	40.0	85.8	4.1
Standard deviation	1.3	3.7	0.4
% Coefficient of variation	3.5	4.7	12.9

†Yield data were obtained from 1 location.
‡Means for parents within a column followed by the same letter are not different at *P*=0.05.

Table 5. Overall means and descriptive statistics of agronomic and disease traits for the parents and original Robust x Stander doubled-haploid population harvested at five North Dakota locations across the 2007-2011 growing seasons.

	Lodging†	Deciduous awns‡	Foliar disease
	1-10 scale	1-5 scale	1-10 scale
Parents			
Robust	4.0a§	3.5a	7.0a
Stander	2.8b	1.0b	6.8a
Population statistics			
Mean	3.4	2.3	6.6
Minimum	2.5	1.0	5.8
Maximum	4.8	5.0	7.3
Standard deviation	0.6	0.8	0.6
% Coefficient of variation	18.2	33.8	8.8

†Lodging and foliar disease data were collected from two environments.
‡Deciduous awns data were collected from one environment.
§Means for parents within a column followed by the same letter are not different at *P*=0.05.

and linkage group 6H-2 covered approximately 68.2% of chromosome 6H. The shortest chromosome segments were found on chromosome 5H. Linkage groups 5H-1, 5H-3, 5H-4, and 5H-6 were all shorter than 2% of the estimated length of the entire chromosome based on the OWB consensus map (Szucs et al., 2009). A concern that becomes apparent when estimating the percent of chromosome represented by each linkage group is that the sum of the lengths of linkage groups 6H-1 and 6H-2 is greater than 100%. This should not happen, especially since the linkage map constructed shows these two linkage groups to be unlinked. A couple of causes of this discrepancy include the small size of the original Robust x Stander DH population, 53 lines, and the fact that the overall chromosome lengths are based on a consensus map and not a single mapping population.

The detection of the partial chromosome segments (linkage groups) is not unexpected due to the close relatedness of the two parents. The malting and brewing industries have very strict malt quality requirements. Malting barley is purchased on an identity-preserved basis and is kept segregated by cultivar until after malting when cultivars may be blended. Any cultivar that is genetically deficient in even one of the quality parameters will not be purchased. This strict requirement for quality ultimately leads to fixed loci over large portions of the genome. Thus, by mapping and focusing on the polymorphic chromosome regions in Robust and Stander, the chance of determining the genetic basis for the differences between the two cultivars is greatly improved.

The second linkage map was constructed using the original Robust x Stander DH lines and only 67 polymorphic SNP markers. Thirteen linkage groups composed of 63 markers were obtained, covering chromosomes 1H to 6H. There was no linkage group for chromosome 7H. Again, linkage groups 6H-1 and 6H-2 represent relatively large portions

of the respective chromosomes, while the other chromosomes had much smaller linkage groups. However, the sum of estimated chromosome lengths of linkage groups 6H-1 and 6H-2 was 80.1%, not over 100% as was seen in the map with all markers. This indicates that inclusion of SSR and DArT markers was causing map expansion in the first map for chromosome 6H. Map expansion was also seen for segment 4H-2. In the SNP, SSR, and DArT map based on the original population, the length of linkage group 4H-2 was 80.3 cM. In the SNP only map using the 53 lines, linkage group 4H-2 was 15.1 cM in length.

Construction of the third linkage map included all of the Robust x Stander DH lines and 67 polymorphic SNP markers. Thirteen linkage groups composed of 60 markers were obtained, covering chromosomes 1H to 6H. Similar to what was found during the construction of the second linkage map, linkage groups 6H-1 (61.59 cM) and 6H-2 (61.40 cM) represented relatively large portions of the respective chromosomes, and no linkage group corresponded to chromosome 7H.

Linkage map comparison

Table 6 compares the linkage map constructed using the original Robust x Stander DH population and all three marker systems with the linkage map constructed using the entire DH population and only SNP markers. In the first linkage map based on the original DH lines and all three marker types, chromosome 5H was divided into six linkage groups, whereas only four linkage groups were detected in the linkage map based solely on SNPs. Two of the six 5H subgroups detected in the first linkage map were composed exclusively of DArT markers. This indicates that the SSR and DArT markers were mapping to locations not covered by the SNP markers. Thus, relying on only one marker type, such as SNPs, to construct a map using a population derived from two closely related parents may

not be a good strategy even if a large number of markers is available. Linkage group 5H-6, which is near the telomere of the long arm of chromosome 5H has been identified to be an important region containing many QTL controlling malt quality traits related to enzyme activity and protein modification (Pedraza-Garcia, 2011; Zhang et al., 2011) and dormancy and pre-harvest sprouting (Zhang et al., 2011; Lin et al., 2009; Lin, 2007). Based on the results from the map generated using the original DH lines and three different marker types, the full population of Robust x Stander DH lines should also be genotyped with the DArT and SSR markers used by Pedraza-Garcia (2011).

As mentioned in the previous section, map expansion was evident in the map of the original population using all markers. Linkage groups 1H, 4H-2, 6H-1, and 6H-2 were much longer than in the maps using only SNPs markers. Again, a prime example of this over estimation can be seen on the map for the original population for linkage groups 6H-1 and 6H-2, where chromosome 6H supposedly represented 130% of the consensus map. The downside in using all three marker systems in a small population is map expansion; however, on a positive note, more marker systems allow for new detection of linkage groups such as 5H-5 and 5H-6. In the linkage map utilizing the entire population and SNP markers, smaller linkage group regions were identified, ultimately corresponding more closely to the Oregon Wolfe Barley (OWB) OPA2008 map (Szucs et al., 2009). For the OWB population, the total length of all seven chromosomes was around 1279.47 cM. The two linkage maps (53 lines and all markers vs. 191 lines and SNP markers) presented in Table 6 comprise roughly 32% and 17% of that total length. For both maps, chromosome 7H was not correlated to any detected linkage group, which in itself leads to lower estimates of coverage. Map expansion and detection of new linkage groups composed of

DArT markers caused the slightly higher percentage value obtained for the first linkage map. It has been shown that genetic map expansion may be the result of excess heterozygosity (Knox and Ellis, 2002). In the present study utilizing DH lines, no heterozygosity was observed. Therefore, map expansion in our study could be due to suboptimal locus order (Wenzl et al., 2006), small population size, different marker systems, or poor estimation of recombinants. With the entire population and only SNP markers, map expansion compared to the map based on original lines and all markers was reduced by 8%. Based on the map of the entire population and SNP markers, the linkage groups represented roughly 12.2% of chromosome 1H, 3.1% of chromosome 2H, 5.8% of chromosome 3H, 35.7% of chromosome 4H, 5.8% of chromosome 5H, and 76.5% of chromosome 6H. The biggest difference was identified in chromosome 6H, with coverage values for the two maps being 130% and 76.5%, respectively.

Table 7 compares the linkage maps developed with only SNPs for both the original and entire Robust x Stander DH lines. Key findings include the absence of linkage groups 5H-5 and 5H-6 (previously detected in the linkage map using all three marker systems) (Figure 2), and chromosome 7H. For the OWB population, the total length of all seven chromosomes was around 1279.47 cM. The linkage map based on the entire population comprised roughly 17% (described above) of the total length, which is similar to that estimated from the linkage map developed using the original Robust x Stander DH lines (16%). The major differences identified for the entire DH population include some map expansion on chromosome 1H and detection of smaller regions on chromosome 6H. Slight differences in marker order and length also were observed. Differences detected in map length and marker order could be attributed to segregation distortion (Knox and Ellis,

Table 6. The first and third linkage map based on the original and entire Robust x Stander doubled-haploid lines in estimating length (cM), average marker interval length (cM), number of markers on each chromosome, and estimated percentage of consensus map.

	Original Robust x Stander DH population				Entire Robust x Stander DH population			
	All markers†				SNP markers			
Chromosome	Length‡	Average distance between markers	Number of markers	Estimated % of consensus map§	Length	Average distance between markers	Number of markers	Estimated % of consensus map
	---------cM--------				--------cM----------			
1H	17.9	6	4	11.50%	19.1	9.5	3	12.20%
2H-1	7.6	3.8	3	4.00%	2.6	2.6	2	1.40%
2H-2	5.9	2.9	3	3.10%	3.2	3.2	2	1.70%
3H-1	5.7	1.9	4	2.70%	7.3	2.5	4	3.50%
3H-2	5.8	1.9	4	2.80%	4.7	2.4	3	2.30%
4H-1	19.1	4.8	5	15.20%	22.5	5.6	5	17.90%
4H-2	100.7	9.2	12	80.30%	22.4	4.5	6	17.80%
5H-1	1.9	1.9	2	0.80%	1.6	1.6	2	0.70%
5H-2	7.6	3.8	3	3.10%	4.2	2.1	3	1.70%
5H-3	3.8	3.8	2	1.60%	4.2	4.2	2	1.70%
5H-4	3.8	1.9	3	1.60%	4.2	2.1	3	1.70%
5H-5	18.2	18.2	2	7.50%	-	-	-	-
5H-6	1.9	1.9	2	0.80%	-	-	-	-
6H-1	99.6	7.1	15	61.90%	61.6	4.7	14	38.30%
6H-2	109.7	5	23	68.20%	61.4	4.7	14	38.20%
7H¶	-	-	-	-	-	-	-	-

†Includes simple sequence repeat (SSR), diversity array technology (DArT), and single nucleotide polymorphism (SNP) markers.
‡LOD=3.0 and the Kosambi function were used to determine the order and length of each linkage group.
§Chromosome length (cM) estimation was based on the Oregon Wolfe Barley population (Szucs et al., 2009).
¶No polymorphic markers were detected for linkage group 7H.

2002). During the development of doubled-haploid lines, preferential gametic selection could have taken place, which could explain segregation distortion, if present (Costa et al., 2001). Genetic map expansion and shrinkage also is related to enhanced and suppressed recombination. The larger the population, the higher the map resolution will be.

QTL analyses

The two linkage maps developed with SNP markers were used to conduct QTL analyses in this chapter (Table 7). Those traits in which marker-trait associations were detected in >50% of the environments where a trait was measured were deemed QTL. The strength of analyzing each environment separately is that one can tell if a QTL is being detected in similar chromosomal regions in multiple environments. These types of associations are more likely due to "true" associations with genes and are more amenable to MAS. Additional analyses were performed for identified QTL using means averaged across environments. In all cases, the QTL regions detected in individual environments were similar to those detected using the overall means. Therefore, the detected QTL regions and coefficients of determination reported are those obtained from the analyses of means. Also, to facilitate comparisons of results from the original population and the entire population, the SNP only maps were used for the QTL analyses.

Original Robust x Stander DH lines

The QTL mapping software QGene 4.3.8 (Joehanes and Nelson, 2008) was used to analyze data from each environment and linkage group separately. Significant marker-trait associations were found for 17 agronomic traits; however, many of these associations were found in only one or two environments. QTL were detected in >50% of environments for plant height, lodging, and deciduous awns.

Table 7. The second and third linkage map based on the original and entire Robust x Stander doubled-haploid lines in estimating length (cM), average marker interval length (cM), number of markers on each chromosome, and estimated percent of consensus map.

	Original Robust x Stander DH population				Entire Robust x Stander DH population			
	SNP markers				SNP markers			
Chromosome	Length†	Average distance between markers	Number of markers	Estimated % of consensus map‡	Length	Average distance between markers	Number of markers	Estimated % of consensus map
	----------cM----------				----------cM----------			
1H	5.7	2.8	3	3.60%	19.1	9.5	3	12.20%
2H-1	3.8	3.8	2	2.00%	2.6	2.6	2	1.40%
2H-2	3.8	3.8	2	2.00%	3.2	3.2	2	1.70%
3H-1	3.8	1.9	4	1.80%	7.3	2.5	4	3.50%
3H-2	5.7	1.9	3	2.70%	4.7	2.4	3	2.30%
4H-1	19.1	4.8	5	15.20%	22.5	5.6	5	17.90%
4H-2	19	3.8	6	15.10%	22.4	4.5	6	17.80%
5H-1	1.9	1.9	2	0.80%	1.6	1.6	2	0.70%
5H-2	7.6	3.8	3	3.10%	4.2	2.1	3	1.70%
5H-3	3.8	3.8	2	1.60%	4.2	4.2	2	1.70%
5H-4	3.8	1.9	3	1.60%	4.2	2.1	3	1.70%
5H-5	-	-	-	-	-	-	-	-
5H-6	-	-	-	-	-	-	-	-
6H-1	64.7	5	14	40.20%	61.6	4.7	14	38.30%
6H-2	64.1	4.9	14	39.90%	61.4	4.7	14	38.20%
7H‡	-	-	-	-	-	-	-	-

†LOD=3.0 and the Kosambi function were used to determine the order and length of each linkage group.
‡Chromosome length (cM) estimation was based on the Oregon Wolfe Barley population (Szucs et al., 2009).
§No polymorphic markers were detected for linkage group 7H.

Table 8 provides a summary of the composite interval mapping (CIM) analyses using the original Robust x Stander DH lines. Single QTL for plant height, lodging, and deciduous awns were detected on chromosome 6H. The QTL for plant height and lodging were found on linkage group 6H-1 (Figure 3). The QTL for plant height (r^2=0.53) was detected in seven of the twelve environments in the individual CIM analyses. The plant height QTL had its peak LOD score near 58 cM on chromosome segment 6H-1 (Figure 4). The QTL for lodging (r^2=0.43) was detected in all environments where lodging data were recorded. Plants with the allele from Stander were shorter and had reduced lodging. The QTL for resistance to deciduous awns (Figure 3) was found on linkage group 6H-2 (r^2=0.43). This QTL was detected in all environments where deciduous awn data were collected.

Entire Robust x Stander DH lines

In the analyses using the entire Robust x Stander DH population, QTL were identified for heading date and plant height (Table 9 and Figure 5). The QTL controlling heading date was detected in linkage group 4H-1 (r^2=0.19) and the QTL controlling plant height was identified in linkage group 6H-2 (r^2=0.17). Similar QTL regions were detected for both traits in the individual environment CIM analyses. The QTL controlling heading date had its peak LOD score near 4 cM in linkage group 4H-1. The allele from Stander was associated with later heading. The peak LOD value for plant height was near 24 cM in linkage group 6H-2. The allele from Stander was associated with shorter plants.

Comparisons of the QTL analyses on populations of different size

As stated earlier, chromosome 6H harbored QTL for plant height, lodging, and deciduous awns in the original Robust x Stander DH lines. Pedraza-Garcia (2011) also

Table 8. Composite interval mapping analysis based on overall means from the original Robust x Stander doubled-haploid lines for plant height, lodging, and deciduous awns.

Trait	Linkage group	Bin‡	Linkage interval†						LOD score	r^2
			Left marker	Right marker	Length	Marker nearest peak LOD	Position	Additive		
					cM		cM			
Plant height	6H-1	60-63	2_0904	SCRI_RS_165945	7.5	1_0040	58	-1.26	8.72	0.53
Lodging	6H-1	63-79	1_0040	SCRI_RS_165945	3.7	1_0220	62	-0.32	6.37	0.43
Deciduous awns	6H-2	22-27	2_0745	1_0427	7.6	3_1485	36	0.51	6.44	0.43

†Significant QTL are described in terms of linkage interval, closest marker to the QTL, additive regression coefficient (Additive), LOD score, and the percent of variation explained by the QTL (r^2).
‡Bin location based on work Munoz-Amatriain et al. 2011.

Table 9. Composite interval mapping analysis based on overall means from the entire Robust x Stander doubled-haploid lines for heading date and plant height.

Trait	Linkage group	Bin‡	Linkage interval†						LOD score	r^2
			Left marker	Right marker	Length	Marker nearest peak LOD	Position	Additive		
					cM		cM			
Heading date	4H-1	43-86	2_0180	1_0010	7.3	1_0639	4	-0.43	8.62	0.19
Plant height	6H-2	27-32	1_0129	2_0745	9.4	1_0427	24	1.04	7.68	0.17

†Significant QTL are described in terms of linkage interval, closest marker to the QTL, additive regression coefficient (Additive), LOD score, and the percent of variation explained by the QTL (r^2).
‡Bin location based on work Munoz-Amatriain et al. 2011.

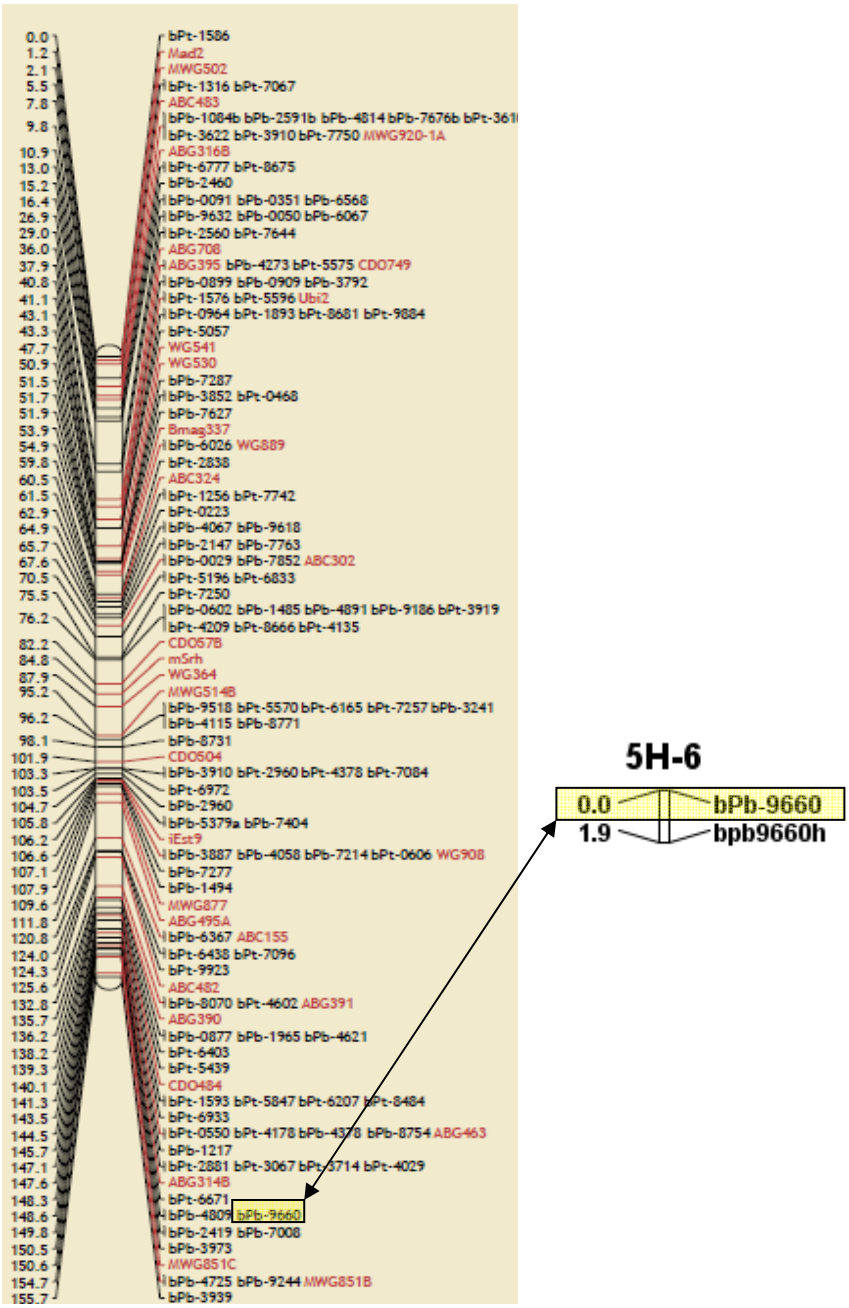

Figure 2. Linkage map of chromosome 5H of the Robust x Stander doubled-haploid population using DArT markers aligned with the corresponding chromosome linkage map of the Steptoe x Morex population obtained from Wenzl et al., 2006. The left map corresponds to the Steptoe x Morex linkage map and the right map represents the linkage group of the 53 Robust x Stander doubled-haploid lines. The arrow between the two maps indicates the common region. Common markers found between the two maps are highlighted with a rectangular box on the Robust x Stander map.

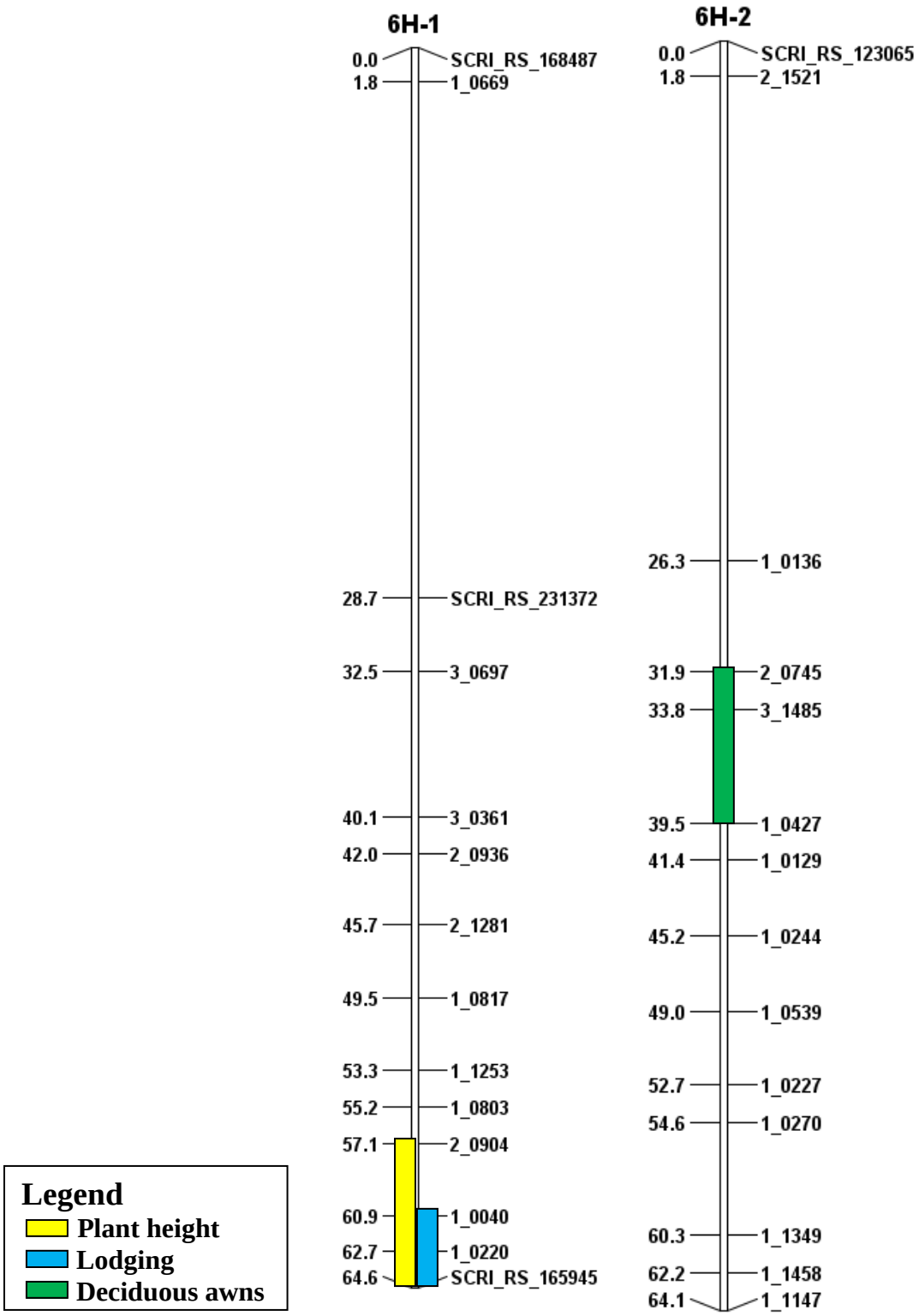

Figure 3. Linkage groups 6H-1 and 6H-2 obtained from the original Robust x Stander doubled-haploid lines. Cumulative distances in Centimorgans are located on the left hand side.

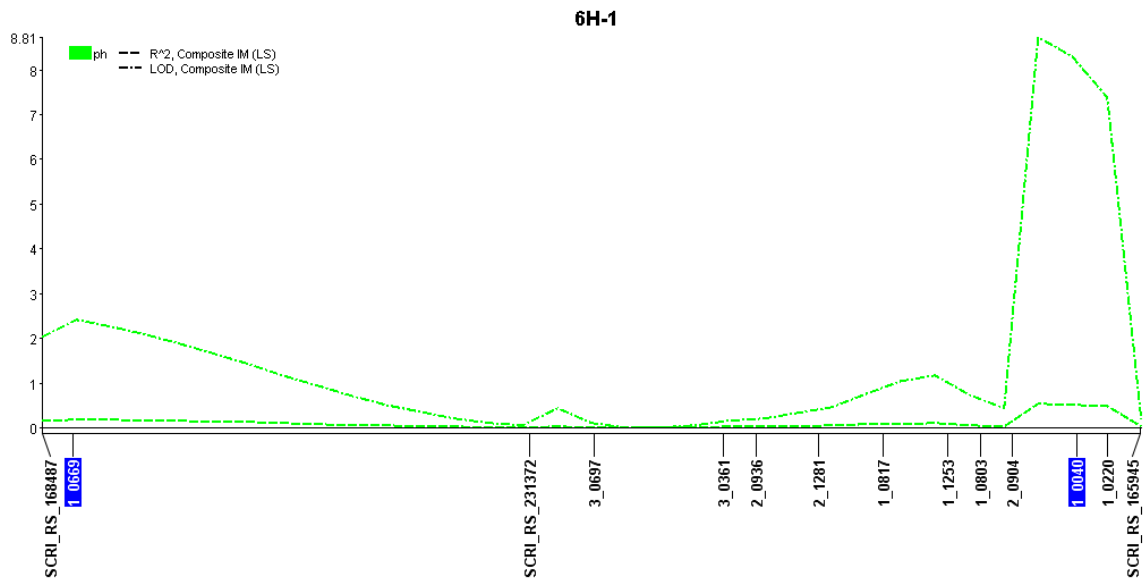

Figure 4. Distribution of the LOD value (Y-axis) in the linkage group 6H-1 (X-axis) for overall plant height using composite interval mapping in the 53 Robust x Stander doubled-haploid lines.

found chromosome 6H to harbor QTL controlling plant height in the Robust x Stander DH population. He found three QTL that mapped to separate bins. Another study focusing on DH lines in the Oregon Wolfe Barley population detected a plant height QTL near POPA2_0673 on chromosome 6H (Cistue et al. 2011). According to the Munoz et al. (2011) consensus map, POPA2_0673 is located in bin 67 at 78.52cM, which is relatively close to the same region in which the plant height QTL were detected in this study (74.65 cM).

A QTL associated with lodging was detected on chromosome 6H in the present study. This QTL mapped to a similar region as the QTL controlling plant height. This finding was not surprising because shorter plant height is often associated with reduced lodging. Additional research is needed to determine if the plant height and lodging effects are due to linkage or pleiotropy. Another study detected a QTL for lodging resistance in a

similar region (Figure 6) (Rostoks et al., 2005). Cakir et al. (2001) discovered a QTL associated with lodging on chromosome 6H in the Tallon/Kaputar DH population, which explained roughly 42% of the variation.

A QTL was detected for deciduous awns on chromosome 6H in bin 22 (Figure 7). To the best of my knowledge, mapping QTL for deciduous awns has not been done previously. Damage caused by deciduous awns falls under the category of skinned and broken kernels when the grain is evaluated at the point of sale. The Grain Inspection Handbook (http://www.gipsa.usda.gov/Publications/fgis/handbooks/grain-insp/grbook2/barley.pdf) defines skinned kernels as "Barley kernels that have one-third or more of the hull removed, or that the hull is loose or missing over the germ." Skinning of the kernel occurs when the awn breaks from the lemma, causing a tear in the lemma tissue. Grain with high levels of skinned kernels is unacceptable for malting; therefore, it is important to detect QTL controlling deciduous awns.

For the entire Robust x Stander DH population, a QTL controlling heading date was detected. In the individual CIM analyses for the original DH lines, 33% of the environments detected the same region on chromosome 4H being associated with heading date. Other studies have detected heading date QTL in the same region on the long arm of chromosome 4H (Figure 8).

Identification of favorable haplotypes

An ultimate goal for this research in the years to come is to determine the genetic basis for differences between Robust and Stander barley and to develop a genetic haplotype or "fingerprint" to differentiate six-rowed barley lines suitable for either MillerCoors or Anheuser-Busch InBev. The effect of substituting the favorable SNP allele 1_0040 on

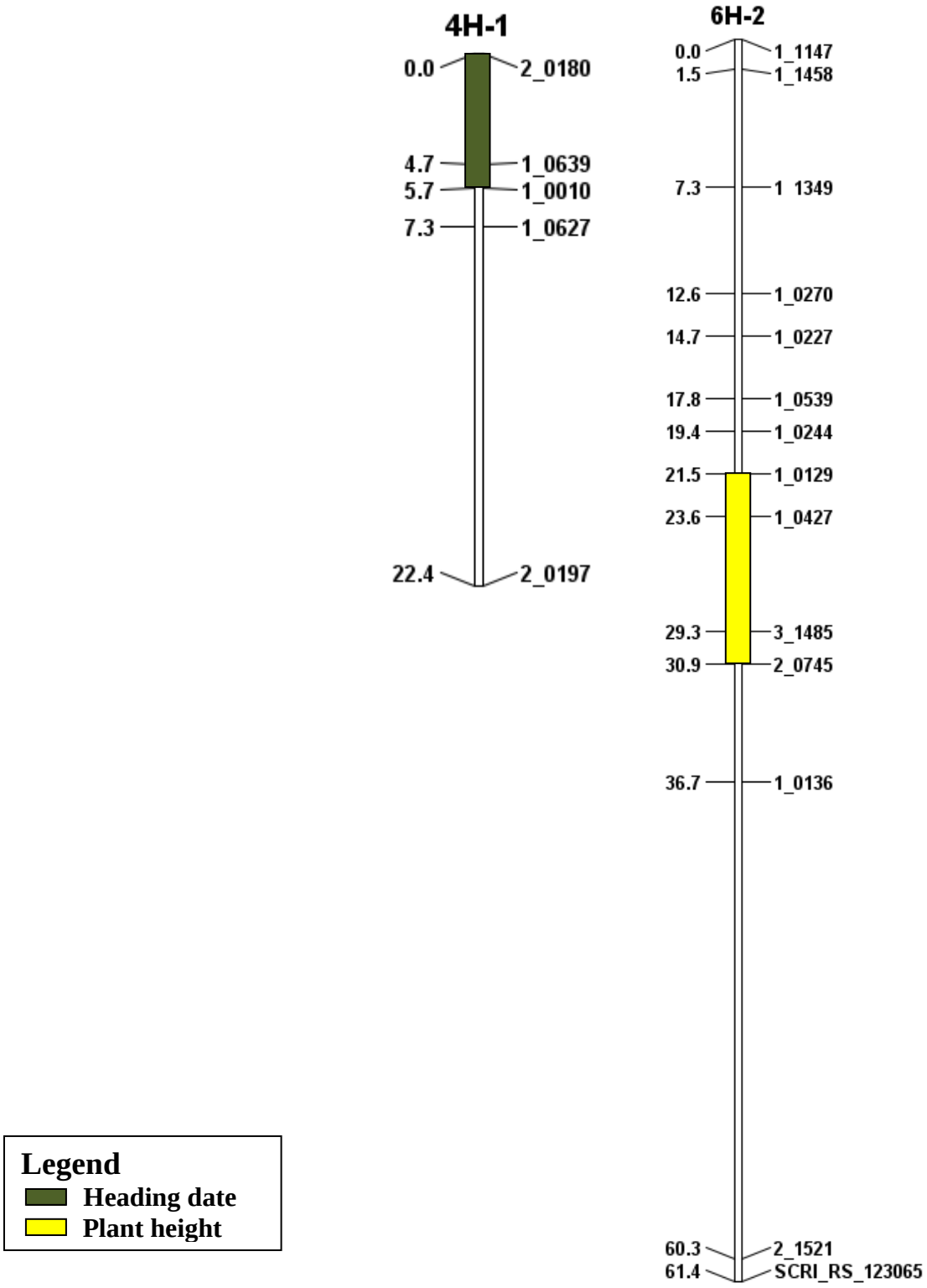

Figure 5. Linkage groups 4H-1 and 6H-2 obtained from the entire Robust x Stander doubled-haploid lines. Cumulative distances in Centimorgans are located on the left hand side.

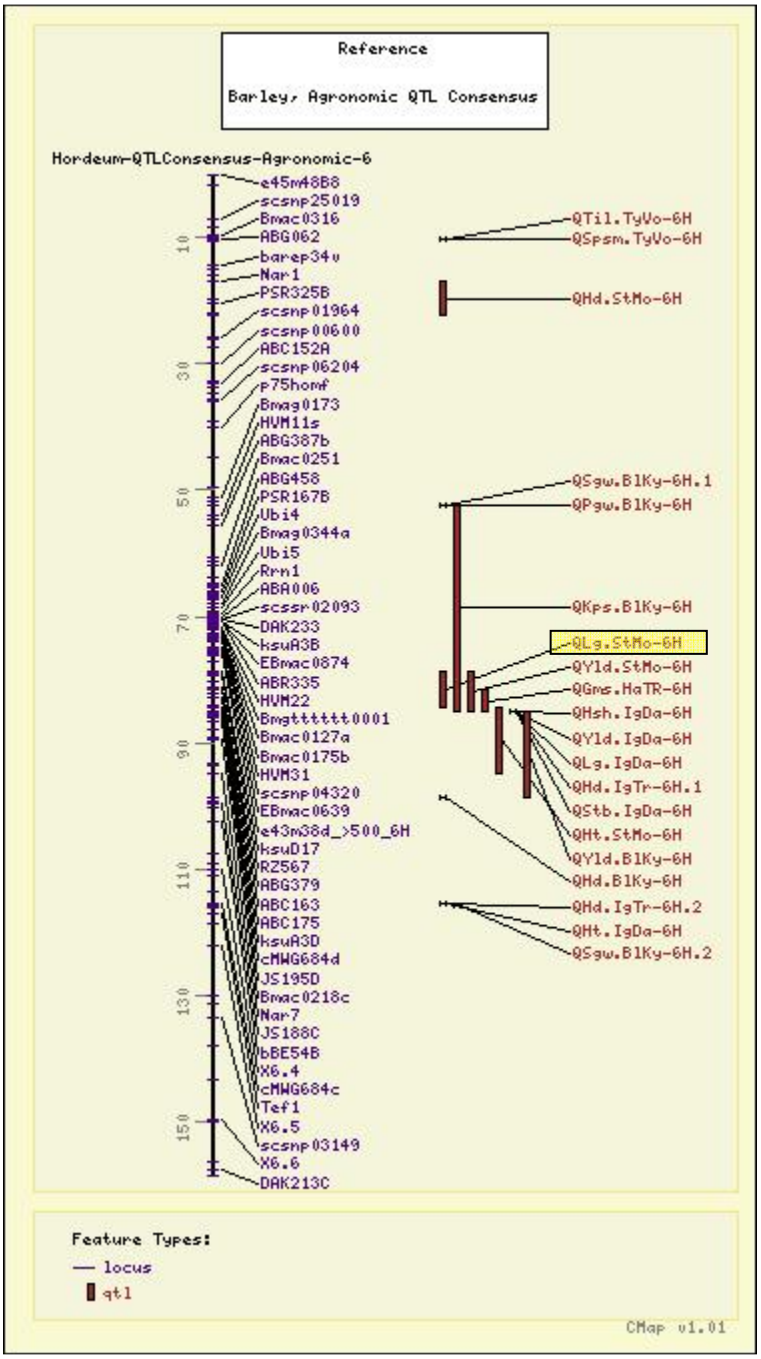

Figure 6. Consensus map obtained from GrainGenes 2.0, that highlights the detection of lodging on chromosome 6H in the Steptoe x Morex population (Rostoks et al., 2005).

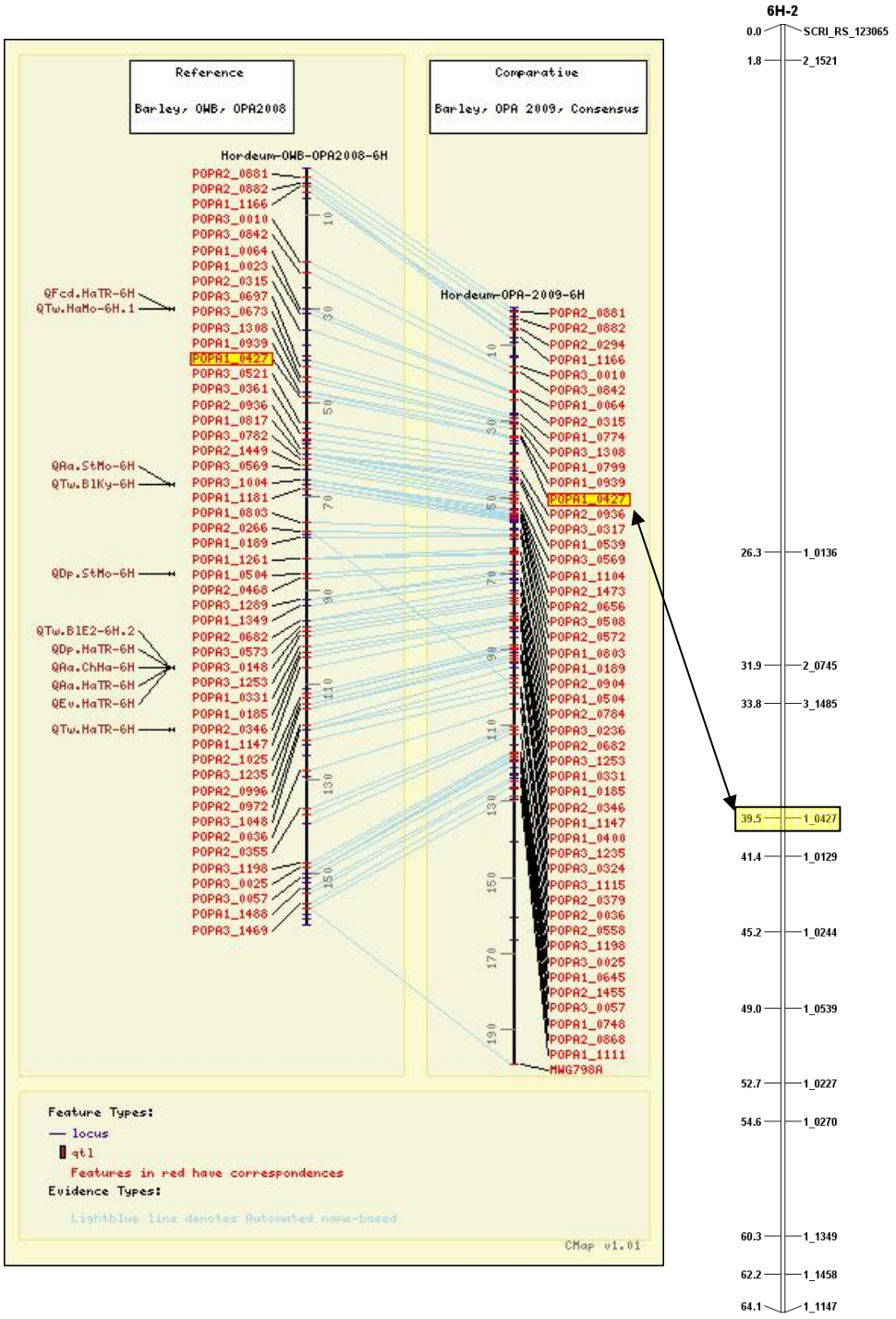

Figure 7. Linkage map of chromosome 6H of the Robust x Stander doubled-haploid population using SNP markers aligned with the corresponding chromosomes of two consensus maps obtained from GrainGenes 2.0. The two maps on the left correspond to consensus linkage maps and the right map represents the linkage group of the 53 Robust x Stander doubled-haploid lines. The marker nearest the peak LOD score POPA1_0427 for deciduous awns is highlighted in yellow on all three maps.

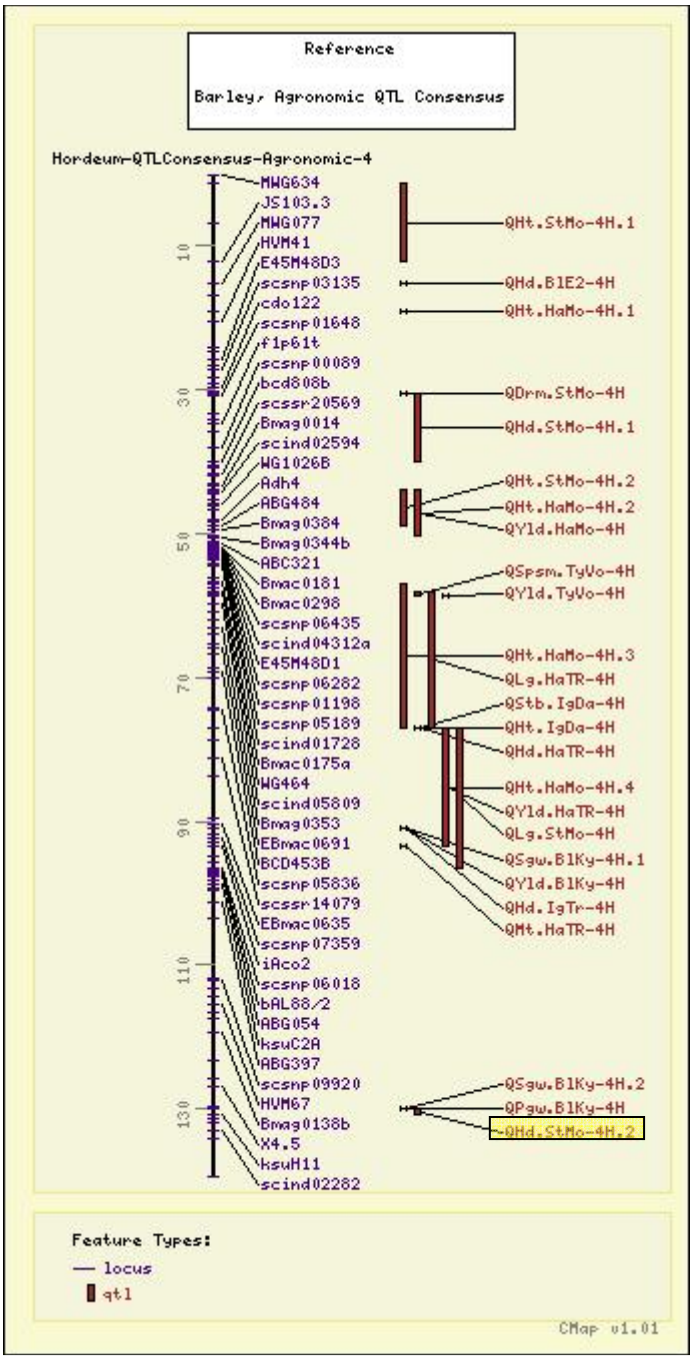

Figure 8. Consensus map obtained from GrainGenes 2.0, that highlights the detection of heading date on chromosome 4H in the Steptoe x Morex population (Rostoks et al., 2005).

plant height is presented in Table 10. The loci chosen were SNP markers found nearest the peak LOD value for each QTL. The AA alleles of 1_0040 were associated with reduced mean plant height of the progeny 2.7 cm over that of plants with the BB alleles. It is important to remember that a small population size was utilized in the development of these favorable allele tables. Table 11 shows the effect of substituting the favorable alleles for lodging at SNP locus 1_0220. The AA alleles of 1_0220 were associated with a reduced lodging score of the progeny by 0.6 over that of plants with the BB allele. The effect of substituting the favorable allele for deciduous awns at SNP locus 3_1485 is presented in Table 12. The BB alleles were associated with reduced deciduous awn score by 1.1 units over that of plants with the AA locus.

Before one can conclude that the identified haplotypes can be used for MAS, it needs to be determined if the same haplotypes are identified when mapping is done using the entire population. Once this is done, the effectiveness of the markers for MAS can be validated using the breeding lines evaluated in the USDA-CSREES Barley CAP project from 2006-2009. It also needs to be determined if the identified markers are diagnostic in all three Midwest six-rowed barley breeding programs (BAR, NDSU, and UM). Using a marker for seed dormancy developed using the original Robust x Stander population, Pedraza-Garcia (2011) found the marker was diagnostic in the UM breeding lines, but not in the BAR or NDSU breeding lines. It would not be surprising if similar results were found in this study because the Robust and Stander were both developed at the UM. However, it is possible that diagnostic markers specific to the NDSU or BAR programs may be identified in similar regions as the diagnostic markers found for Minnesota.

Table 10. Effect of allelic substitution at the SNP locus 1_0040 controlling mean plant height.

	1_0040	
	AA	BB
Robust	-	82.6±3.2
Stander	77.6±2.9	-
Progeny	***77.8±0.6†***	80.5±0.6

†Progeny means resulting from plants with favorable alleles are highlighted in bold italicized font.

Table 11. Effect of allelic substitution at the SNP locus 1_0220 controlling mean lodging score.

	1_0220	
	AA	BB
Robust	-	4.0±1.8
Stander	2.8±1.6	-
Progeny	***3.1±0.3†***	3.7±0.3

†Progeny means resulting from plants with favorable alleles are highlighted in bold italicized font.

Table 12. Effect of allelic substitution at the SNP locus 3_1485 controlling mean deciduous awns.

	3_1485	
	AA	BB
Robust	3.5±0.5	-
Stander	-	1.0±0.0
Progeny	2.9±0.2	***1.8±0.1***

†Progeny means resulting from plants with favorable alleles are highlighted in bold italicized font.

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CHAPTER III. IDENTIFICATION OF QTL CONTROLLING MALT QUALITY AND WORT CARBOHYDRATE TRAITS IN THE ROBUST X STANDER DOUBLED-HAPLOID MAPPING POPULATION

Abstract

The requirements for brewing beer from barley (*Hordeum vulgare* L.) malt are specific and unique for each brewer. Two major brewers that utilize six-rowed barley malt in the United States (US), Anheuser-Busch InBev and MillerCoors Brewing Company (MillerCoors), require different malt quality parameters for producing their products. Robust and Stander are two closely related cultivars that differ greatly in agronomic performance and malt quality. Robust fits the requirements of MillerCoors and Stander has many of the parameters desired by Anheuser-Busch InBev. A total of 53 doubled-haploid (DH) lines and the parents from the Robust x Stander cross were evaluated in field trials at eleven locations in North Dakota and one location in Idaho the past six years. Cleaned grain samples from six of the locations were micro-malted at NDSU. A linkage map of SNP, SSR, and DArT markers was constructed using 53 Robust x Stander DH lines. Using the map, QTL were identified on chromosome 4H for kernel plumpness, extract, and β-glucan; on chromosome 5H for Kolbach index, soluble protein, wort color, and glucose concentration; and on chromosome 6H for kernel color, kernel plumpness, α-amylase, and concentration of glucose and maltotriose.

Introduction

Six-rowed barley (*Hordeum vulgare* L.) malt varies in enzyme level and functionality with different cultivars. The requirements of each brewer for brewing beer

from barley malt are specific and unique. When looking at the two major brewers that utilize the six-rowed barley malt in the US, Anheuser-Busch InBev and MillerCoors, the malt quality specifications of each company were quite similar in the past. However, since about 2000 the malt quality specifications have been diverging and this has resulted in breeding programs, such as the one at NDSU, having different breeding objectives for each brewer. MillerCoors desires cultivars with moderate protein modification levels during malting and moderate levels of enzymatic activity (Kay, 2005). On the other hand, Anheuser-Busch InBev desires cultivars with higher levels of protein modification during malting and high levels of enzymatic activity. These two categories of brewers' preferences can be represented by two cultivars developed by the University of Minnesota, Robust (Rasmusson and Wilcoxson, 1983) and Stander (Rasmusson et al., 1993). Robust fits the requirements of MillerCoors and Stander has many of the parameters desired by Anheuser-Busch InBev (R.D. Horsley and P.B. Schwarz, personnel communication, 2009). The pedigree of Robust is 'Morex'/'Manker' (Rasmusson and Wilcoxson, 1983) and the pedigree of Stander is Robust*2/3/'Cree'/'Bonanza'//Manker/4/Robust/'Bumper' (Rasmusson et al., 1993). Even though the parents are closely related, they differ in malt quality traits, specifically α-amylase traits associated with protein modification. The levels of α-amylase have shown to be 36% higher in Stander when compared to Robust (Rasmusson et al., 1993).

Another important aspect of malting is the production of wort carbohydrates. The carbohydrates (complex mixture) account for roughly 92% of the solids found in the wort solution. Glucose is the major constituent of most important sugars and dextrins produced during mashing, which is evidenced by the existence of D-glucopyranose units joined by α-

(1,4) links in maltose, maltotriose, maltotetraose, and maltopentaose. Monosaccharides such as glucose and fructose, disaccharides sucrose and maltose, and the trisaccharide maltotriose are considered fermentable by most yeast species (Briggs et al., 2004). Maltose, a disaccharide with two D-glucose molecules, is the most abundant fermentable sugar in brewer's wort, roughly comprising 50-60%. The second most abundant fermentable sugar is maltotriose, a trisaccharide with three D-glucose units (15-20%), followed by glucose (10-15%) (Stambuk et al., 2006). Information on the genetics of wort carbohydrate production from barley malt is limited.

The close relationship between Robust and Stander increases the chance of recognizing chromosome regions with the genes controlling malt quality traits. Before the use of such simple sequence repeat (SSR), diversity array technology (DArT), or single nucleotide polymorphism (SNP) markers, mapping using a population derived from closely related parents such as Stander and Robust would not have been possible because of the low polymorphism rate. In studies by Lin (2007) and Pedraza-Garcia (2011), the polymorphism rate between Stander and Robust using SSR and DArT markers was less than 10%. Scientists mapping traits in barley now have marker platforms with up to 9,000 SNPs (S. Chao, personnel communication). This large number of SNPs should allow for mapping to be done in populations derived from closely related parents. In turn, the regions with markers that differentiate Robust and Stander are likely to be the regions where genes conferring phenotypic differences are located. The objectives of this project were to: 1) phenotype the Robust x Stander mapping population for the barley, malt quality, and wort carbohydrate traits of barley color, protein, plump kernels, malt extract, soluble protein, Kolbach index, free amino nitrogen (FAN), wort viscosity, wort ß-glucan,

α-amylase activity, diastatic power (DP); and concentration of glucose, fructose, maltose and maltotriose; and 2) identify quantitative trait loci (QTL) controlling these traits in the population.

Materials and Methods

Prior to 2009, 53 Robust x Stander doubled haploid (DH) lines and the parents used by Pedraza-Garcia (2011) were evaluated in four yield trial experiments in North Dakota and one experiment in Aberdeen, Idaho. Entries from the 2006 and 2007 Fargo experiments were micro-malted by Pedraza-Garcia (2011) in 2008. In 2009, entries from the 2007 Osnabrock and Idaho experiments and the 2009 Fargo and Langdon experiments were micro-malted. All micro-malting and related analyses were conducted in Dr. Paul Schwarz's barley and malt quality laboratory at NDSU.

Once the barley reached maturity in the field, the grain was harvested with a plot combine, dried down in a forced-air dryer to approximately 100 g kg^{-1}, threshed and cleaned. The cleaned grain was used to record measurements for percent of plump kernels (the kernels remaining on top of a 0.2 x 1.9-cm slotted opening sieve are defined as plump kernels; ASBC, 2009), and grain protein (g kg^{-1}). Near-infrared spectroscopy (NIR) with the Foss Tecator Infratec 1241 grain analyzer instrument was used to determine grain protein and kernel color (Perstorp Analytical Inc., Silver Spring, MD).

Micro-malting was conducted as described by Karababa et al. (1993). From each line, a 10 g (dry basis, db) sample was used in the pilot steeping procedure to determine the time needed for the sample to reach 437 g kg^{-1} moisture. After the times were determined, 80 g db of each sample was steeped. During the steeping process, a 1 h air-rest was

provided every 12 h and the water was aerated every 6 min hour^{-1} with compressed air. For the germination process, each sample in a 400 mL beaker was placed in a germination cabinet for 72 h, with constant conditions of 16°C and 95% relative humidity. The green malts were subjected to a 24 h kilning schedule. During this process, the temperature was sequentially ramped from 49 to 85°C as described by Karababa et al. (1993). Following kilning, the rootlets were removed.

Using the Malt-4 Method of the ASBC (ASBC, 2009), the malt samples were milled at a setting of 86 (coarse-grind) on a Bühler-Miag mill (Uzwil, Switzerland). After the samples were prepared, a chain-driven Weber-Ehrenfeld mashing apparatus (Chicago, IL) was used for mashing. The mashing procedure performed was a modification of the European Brewing Convention hot water extract (HWE) method (EBC, 1998), using 50 g of coarse-ground malt (Table 13). For 1 h, the samples were heated to 65°C.

The samples were analyzed for malt moisture (%), coarse-grind extract (% DB), soluble protein (%), Kolbach index (%), α-amylase activity (20^{o} dextrinizing units; DU), DP (^{o}ASBC), wort viscosity (cP), wort color (°L), wort FAN (mg L^{-1}), wort β-glucan content (mg L^{-1}), and wort carbohydrate concentration (g 100 mL^{-1}). Unless mentioned otherwise, all methods listed below as "Malt-" or "Wort-" followed by a number are described in the American Society of Brewing Chemists (ASBC) Methods of Analysis (ASBC, 2009). Malt moisture was analyzed as a percentage of total weight and was determined using a slight modification of ASBC standard Oven Drying method, Malt-3. The modification was that 4 g of malt sample was used to run the analysis instead of 5g. Coarse-grind extract also was measured as a percentage of malt db and was determined using ASBC Malt-4. Soluble protein was determined by a spectrophotometric procedure

(ASBC Malt-5), which consisted of determining the total nitrogen in the laboratory wort calculated back to % of malt (db). Alpha-amylase and DP were determined using the methods previously described by Karababa et al. (1993). Wort color was determined spectrophotometrically at 430 nm following the ASBC Wort-9. Wort ß-glucan was determined using flow injection analysis as described in Wort-18. Concentrations of fermentable sugars including fructose, glucose, maltose, and maltotriose were determined by high-performance liquid chromatography (HPLC) using a column (Aminex HPX-87, Bio-Rad Laboratories, Hercules, CA) consistent with the ASBC Method Wort-14B. Before the samples were processed, a slight modification was made to the Wort-14B procedure. Ten μL of a 10mg mL^{-1} solution of sodium azide (NaN_3) was added to the wort samples, serving as a preservative.

Table 13. Mashing schedule used for all malt samples.

Step	Temperature/Time
1	Hold at 45°C for 30 min
2	Ramping (1°C min^{-1} for 20 min)
3	Hold at 65°C for 50 min
4	Ramping (1°C min^{-1} for 5 min)
5	Hold at 70°C for 10 min

Statistical analyses of phenotype data

Entries in the yield trial experiments were assigned to experimental units using an 8x9 rectangular lattice in 2009 and a randomized complete block design in 2010. Using SAS/STAT (SAS Institute, 2004), barley quality, malt quality, and wort carbohydrate data

from each environment (Table 14) were analyzed as a randomized complete block design and adjusted means were calculated using the LSMeans function in PROC GLM. The environments were treated as a random effect, whereas the DH lines (entries) were considered a fixed effect. *F*-tests were considered significant at $P \leq 0.05$. The denominator of the *F*-test for the environment x entry source of variation was the error mean square (MS) and the denominator of the *F*-test for the entries source of variation was the environment x entry MS. Mean separation was done using the PDIFF command in SAS/STAT (SAS Institute, 2004). PROC CORR in SAS/STAT (SAS Institute, 2004) was used to calculate simple Pearson correlations for all pairs of barley quality, malt quality, and wort carbohydrate traits among the DH lines across environments. Correlation values were deemed significantly different from zero at $P \leq 0.05$.

QTL mapping

Detailed descriptions of methods used for DNA extraction, SNP genotyping, and map construction are described in Chapter II. QGene 4.3.8 (Joehanes and Nelson, 2008) was used to statistically analyze and identify QTL for all traits. Single marker regression was first performed to identify any significant marker-trait association. To locate chromosomal regions associated with each trait, permutation tests (1000 iterations) were run to determine the minimum LOD value that must be obtained before a marker-trait association would be considered significant ($\alpha_{0.01}$ and $\alpha_{0.05}$ experiment-wide error). Composite interval mapping (CIM) was conducted, in which markers located outside the interval were treated as cofactors. The default parameters in QGene were used to select and remove these cofactors. Each environment was analyzed separately in order to determine the percentage of environments with detected QTL. Those traits in which QTL

were detected in >50% of the environments where a trait was measured were chosen. QTL analyses were performed with the means averaged across environments for those selected traits.

Table 14. Environment descriptions.

Environment	Location	Experiment type	Year	Phenotypic data utilized for mapping
1	Fargo	Yield trial	2006	Agronomic, Malting
2	Nesson Valley	Yield trial	2006	Agronomic
3	Fargo	Yield trial	2007	Agronomic, Malting
4	Idaho	Yield trial	2007	Agronomic, Malting
5	Osnabrock	Yield trial	2007	Agronomic, Malting
6	Fargo	Yield trial	2009	Agronomic, Malting
7	Langdon	Yield trial	2009	Agronomic, Malting
8	Nesson Valley	Yield trial	2009	Agronomic
9	Fargo	Yield trial	2010	Agronomic
10	Osnabrock	Yield trial	2010	Agronomic
11	McVille	Yield trial	2011	Agronomic
12	Nesson Valley	Yield trial	2011	Agronomic

Results and Discussion

Elimination of DH lines

Twenty DH lines were deemed to be heterozygotes based on the SNP data. These lines were not used in any of the analyses for descriptive statistics or mapping. The elimination of the lines and their accompanying data required the data to be analyzed as a RCBD and not a lattice.

Impact of modified mash

The samples in our study were milled using a coarse-grind setting and exposed to 65°C for 1 hr, instead of 70°C as indicated in the protocol of the European Brewing

Convention HWE method (EBC, 1998). A study conducted by Schwarz et al. (2007) looked at the change in malt extract and wort analytical values when grind gravity and mash temperature profile were altered. Their study indicated that the magnitude of differences between samples may change with mash alterations, but not the overall rank of samples. Grind was shown to have the most impact out of all the operational parameters evaluated. Extract was affected the greatest by grind, followed by temperature profile. All analytical parameters were affected by the mash temperature profile; however, the greatest impact was observed for wort color, β-glucan content, and fermentable sugars.

Descriptive statistics

Mean values and descriptive statistics from the combined analyses of variance (ANOVA) across locations of the parents and the 53 DH lines for barley quality and carbohydrate modification traits of barley color, protein, kernel plumpness, coarse-grind extract, wort viscosity, and ß-glucan are reported in Table 15. The parental lines (Robust and Stander) differed significantly ($P \leq 0.05$) in barley color, barley protein, and extract. Stander had slightly darker kernels, lower barley protein, and higher extract than Robust. Stander was numerically higher in ß –glucan concentration than Robust; however, the difference was not significant. Levels of wort ß -glucan are known to impact wort viscosity and filtration in the brewhouse. As wort ß –glucan concentration increases, the wort viscosity is known to increase, ultimately slowing the filtration process (Schwarz and Li, 2011). Therefore, it was not surprising to see that Robust and Stander did not differ significantly for ß-glucan content, because brewers would not use barley cultivars with inherently high levels of ß-glucan concentration. The same rationale applies to the kernel plumpness trait. It is important for the barley sample to have plump kernels that are

uniform in size; otherwise, the germination rate and water uptake of different sized kernels will vary and cause problems/fluctuations in the malting and brewing processes.

Table 16 contains the mean values and descriptive statistics from the combined ANOVA across locations of the parents and the 53 DH lines for protein modification and enzymatic activity traits of soluble protein, Kolbach index, FAN, wort color, α-amylase, and DP. Robust and Stander differed significantly ($P \leq 0.05$) in soluble protein, Kolbach index, wort color, α-amylase, and DP. Stander had greater soluble protein, Kolbach index, and α-amylase; and darker wort color than Robust. The DP of Robust was greater than that for Stander and the two cultivars had identical values for FAN, 306 µg mg-1. The ideal FAN content for the AMBA is greater than 200 mg L^{-1} for six-rowed malt, to ensure adequate fermentation speed and to avoid the stimulation of high diacetyl levels (Fix, 1993; AMBA, 2008). The FAN values for Robust and Stander were found to be acceptable according to AMBA, but were not significantly different ($P \leq 0.05$). This result was somewhat surprising because the parents differed in grain protein and Kolbach index traits, which are known to be associated with FAN.

Mean values and descriptive statistics from the combined ANOVA across locations of the parents and the 53 DH lines for wort carbohydrate traits of maltotriose, maltose, glucose, fructose, and fermentable sugars are reported in Table 17. For the individual sugars, Robust and Stander differed significantly only in glucose levels. Stander had about a 22% higher concentration of glucose than Robust. For traits related to malting quality, AMBA provides guidelines on the desired levels of traits; however, for fermentable sugars the association has set no guidelines. In research, it has been reported that the most abundant fermentable sugars in wort are maltose, maltotriose, and glucose. A typical

breakdown of wort contains between 50-60% maltose, 15-20% maltotriose, and 10-15% glucose (Stambuk et al., 2006; Zastrow et al., 2000; Ernandes et al., 1993). Fructose concentrations are around 1-2%, with dextrin material composing 20-30% of wort (Ernandes et al., 1993). In this research, carbohydrate yields based on the total fermentable sugar composition for the parents (Robust and Stander, respectively) are as follows: maltotriose was 13.7% and 13.4%, maltose was 67.9% and 65.5%, glucose was 18.9% and 21.0%, and fructose was 2.0% and 2.4%, respectively. Thus, maltose and glucose levels in this study were greater than in a typical wort, while concentrations of maltotriose and fructose were close to the typical values.

The means of the parents were compared to the high and low values in the progeny for each trait to determine if there was transgressive segregation. The minimum and maximum values for kernel color and DP were close to those observed in both parents; thus, it appears that transgressive segregation did not occur for these traits. Transgressive segregation only in the negative direction was observed for one trait, glucose concentration. Segregation in the positive direction was observed for barley protein, Kolbach index, wort color, and α-amylase. Positive and negative transgressive segregants were found for coarse-grind extract.

Not only is it important to describe the progeny values in relation to the parents, but also in relation to the Ideal Commercial Malt Criteria suggested by the American Malting Barley Association (AMBA, 2008) (http://ambainc.org/media/AMBA_PDFs/Press_Releases/GUIDELINES.pdf; verified 15 Jan., 2012). For barley quality, the AMBA states that kernel plumpness and protein values should be greater than 80% and less than or equal to 13.5%. Both the progeny and the

parental means fit these desired parameters. For the malt factor traits of ß-glucan, Kolbach index, and viscosity, the desired values by the AMBA are ≤120 μg mg^{-1}, 42-47%, and <1.50 cP, respectively. Both the parents and progeny exhibited acceptable trait values for wort viscosity, but showed higher than acceptable values for both ß-glucan and Kolbach index. The desired values by the AMBA for the malt enzyme traits of α-amylase and DP are >50 DU and >140°ASBC, respectively. For α-amylase, the parents and progeny met the requirements by exhibiting values greater than 50 DU. Additionally, DP of Robust and the population had values in the desired range; however, Stander had values lower than desired. The final traits on the AMBA list are the congress wort traits of soluble protein, wort color, and FAN. The acceptable value for soluble protein should be between 5.2%-5.7%, the value for color should fall in between 1.8-2.5°ASBC, and FAN values should be above 200 μg mg^{-1}. The FAN levels from the population technically fit the acceptable guidelines set forth by AMBA, but were considerably higher than the value given, with mean values around 305 μg mg^{-1}. Both the parents and progeny had values outside the desired range for soluble protein, by exhibiting values higher than 5.7%. Wort color values were found to be acceptable for Robust and the DH population, but higher than desired for Stander.

QTL analyses

The QTL mapping software QGene 4.3.8 (Joehanes and Nelson, 2008) was used to analyze data from each environment and linkage group separately. Significant QTL were found for 14 malt quality traits (Appendix table A1). However, many of these QTL were found in only one or two environments. The strength of analyzing each environment separately is that one can tell if a QTL is being detected in similar chromosomal regions in

Table 15. Overall means and descriptive statistics of barley quality and carbohydrate modification traits of kernel color, protein, plumpness, extract, wort viscosity, and β-glucan for the parents and the Robust x Stander doubled-haploid lines harvested at 6 locations across 3 years (2006, 2007, 2009).

	Kernel color†	Kernel protein	Kernel plumpness	Extract	Wort viscosity	β-glucan‡
	L-value	%	%	%	cP	µg mg^{-1}
Parents						
Robust	52.9a§	13.0a	80.4a	77.3b	1.43a	202a
Stander	52.3b	12.6b	82.9a	78.1a	1.44a	194a
Population statistics						
Mean	52.6	13.0	81.3	77.8	1.43	188
Minimum	52.0	12.5	73.5	76.3	1.41	134
Maximum	53.2	13.6	88.8	79.0	1.48	249
Standard deviation	0.4	0.5	4.3	0.9	0.04	41
% Coefficient of variation	0.7	3.8	5.3	1.1	2.44	22

†Kernel color, plumpness, and protein data were collected from eight locations.
‡β-glucan data were collected from only four of the six locations.
§Means for parents within a column followed by the same letter are not different at *P*=0.05.

Table 16. Overall means and descriptive statistics of protein modification and enzymatic activity traits of soluble protein, Kolbach index, free amino nitrogen (FAN), wort color, α-amylase, and diastatic power (DP) for the parents and the Robust x Stander doubled-haploid lines harvested at 6 locations across 3 years (2006, 2007, 2009).

	Soluble protein	Kolbach index	FAN	Wort color	α-amylase	DP
	% of malt dry basis	%	μg mg^{-1}	°SRM	20^{o} DU†	^{o}ASBC
Parents						
Robust	5.8b	45.8b	306a	2.2b	61.3b	152a
Stander	6.1a‡	49.2a	306a	2.7a	74.0a	133b
Population statistics						
Mean	6.1	48.1	303	2.5	69.8	144
Minimum	5.7	44.5	269	2.0	60.2	128
Maximum	6.6	52.8	336	3.2	78.5	155
Standard deviation	0.4	2.7	42	0.4	4.8	19
% Coefficient of variation	6.4	5.7	14	15.8	6.9	13

†DU= Dextrinizing units at 20°C; °ASBC = Degrees American Society of Brewing Chemists
‡Means for parents within a column followed by the same letter are not different at *P*=0.05.

Table 17. Overall means and descriptive statistics of maltotriose, maltose, glucose, fructose, and total fermentable sugar concentration for the parents and 53 Robust x Stander doubled-haploid lines harvested at six locations across three years (2006, 2007, and 2009).

	Maltotriose†	Maltose	Glucose	Fructose	Total fermentable sugars
	g 100 mL^{-1}	g 100 mL^{-1}	g 100 mL^{-1}	g 100 mL^{-1}	g 100 mL^{-1}
Parents					
Robust	0.89a	4.43a	1.23b	0.13a	6.52a
Stander	0.90a	4.41a	1.41a‡	0.16a	6.73a
Population statistics					
Mean	0.91	4.38	1.32	0.16	6.62
Minimum	0.82	4.00	1.06	0.13	6.05
Maximum	1.00	4.59	1.46	0.21	6.89
Standard deviation	0.08	0.27	0.23	0.06	0.39
% Coefficient of variation	8.4	6.2	17.2	40.6	5.9

†Maltotriose data were collected from only five of the six locations.

‡Means for parents within a column followed by the same letter are not different at *P*=0.05.

multiple environments. These types of associations are more likely due to "true" associations with genes and are more amenable to MAS. Thus, only QTL that are detected in 50% or more of the environments are discussed. QTL that matched this criterion were found for kernel color, kernel plumpness, extract, soluble protein, Kolbach index, wort color, β-glucan content, α-amylase, glucose, and maltotriose concentrations (Table 18 and Figure 9). Additional analyses were performed for identified QTL using means averaged across environments. In all cases, the QTL regions detected in individual environments were similar to those detected using the overall means. Therefore, the detected QTL regions and coefficients of determination reported in this study are those obtained in the analyses of means. Descriptions of results will be presented in separate sections for barley quality, carbohydrate modification, protein modification, enzymatic activity, and wort carbohydrate QTL.

Barley quality QTL

A QTL region controlling kernel plumpness was detected on chromosome 4H in four of eight environments where the population was grown. On average, the QTL explained 54% of the variation in kernel plumpness in the progeny and the allele from Robust was associated with plumper kernels. Single QTL for kernel color (r^2=0.57) and kernel plumpness (r^2=0.52) were detected in different regions of linkage group 6H-1. QTL for kernel color was detected in five of the eight environments and the Stander allele was associated with darker kernel color. The kernel color QTL had its peak LOD score near 58 cM on chromosome segment 6H-1. The QTL for kernel plumpness was detected in four of the eight environments and it explained, on average, 52% of the variation in kernel plumpness. The kernel plumpness QTL had its peak LOD score near 66 cM on

chromosome segment 6H-1 and the allele from Robust was associated with plumper kernels. On linkage group 4H-1, a single QTL for kernel plumpness (r^2=0.54) was found in four the eight environments in the individual CIM analyses. Pedraza-Garcia (2011) detected QTL controlling kernel plumpness and kernel color in these same regions. On chromosome 6H, two major QTL for kernel discoloration were detected in a similar region identified for kernel color in this study, with one showing association to *Amy1* (de la Pena et al., 1999). Other studies (Backes et al., 1996; Marquez-Cedillo et al., 2001; Mather et al., 1997; Szucs et al., 2009) have found QTL controlling kernel plumpness on chromosomes 4H and 6H (Figure 10).

Carbohydrate modification QTL

Linkage group 4H-1 contained two closely linked QTL controlling extract (r^2=0.54) and β-glucan content (r^2=0.55) (Table 18). The QTL were detected in all environments for β-glucan and four of the six environments for extract. In the CIM analyses using means, the QTL controlling extract had its peak LOD score near 4 cM, while the peak LOD score for β-glucan was located near 6 cM. The allele from Stander was associated with increasing the mean percent coarse-grind extract and decreasing β-glucan content. Szucs et al. (2009) detected a QTL for β-glucan and extract on chromosome 4H (Figure 11) in a similar region as the QTL detected in this study. Other studies have found similar results. In a previous study focusing on the Steptoe/Morex population, two QTL were detected for β-glucan content on the short arm of chromosome 4H (Gao et al., 2004). Pedraza-Garcia (2011) detected a QTL controlling extract on chromosome 4H in a similar region as found in the present study; however, he did not detect the QTL for β-glucan content.

Protein modification QTL

Single QTL for soluble protein (r^2=0.60), Kolbach index (r^2=0.43), and wort color (r^2=0.80) were detected on chromosome 5H, in linkage group 5H-6. The QTL for soluble protein was detected in all six environments, and the QTL for Kolbach index and wort color were detected in four of the six environments. The allele from Robust was associated with decreasing the phenotypic value observed for all three traits. The peak LOD score was found near 0 cM for all three traits; however, the density of the map in this region does not allow for determining whether separate linked QTL or a single QTL with pleiotropic effects is present. The 5H-6 linkage group is only composed of two DArT markers and corresponds to the telomere region on the long arm of chromosome 5H. It is not surprising that a QTL for each trait was detected since each trait measures a component of protein modification. Pedraza-Garcia (2011) detected the same QTL responsible for controlling soluble protein, Kolbach index, and wort color that explained 23%, 32%, and 26% of the variation, respectively. Higher percentages of the phenotypic variation for soluble protein, Kolbach index, and wort color were explained in the present study than in Pedraza-Garcia's (2011), possibly because additional phenotypic data from four environments were included.

Szucs et al. (2009) detected protein modification QTL across the entire length of chromosome 5H. Specifically, the telomeric region of chromosome 5HL is known for harboring important carbohydrate modification, protein modification, and enzymatic activity QTL (Mather et al., 1997; Marquez-Cedillo et al., 2000; Collins et al., 2003; Zhang et al., 2011). Further investigation of this region has shown the telomeric region of chromosome 5HL to be syntenic to the telomeric region of chromosome 3L in rice (Zhang et al., 2011). Zhang et al. (2011) conducted a candidate gene search with the use of rice

BAC (Bacterial Artificial Chromosome) sequences, in which barley expressed sequenced tags were identified and developed into primers that were used to amplify 33 genes in PCR. A total of seven co-dominant, gene-specific markers were mapped in three DH populations in the same order, with different genetic distances between markers. Only six of the seven markers were mapped in one the of the DH populations. For all three DH populations, the markers mapped within a 10cM region and showed significant correlations with malt quality traits (Zhang et al., 2011). The ultimate goal from the Zhang et al. (2011) research would be to implement a marker-assisted selection strategy in the current breeding scheme, that targets malt quality traits detected in the telomeric region of chromosome 5H. Major genes controlling those key malt quality traits in the 5HL region could also be identified and isolated, since gene-specific markers were utilized in their research (Zhang et al., 2011). Other studies have detected QTL controlling Kolbach index in the telomeric region of chromosome 5H (Figure 12) (Marquez-Cedillo et al., 2000; Szucs et al., 2009).

QTL associated with enzymatic activity

A single QTL for α-amylase (r^2=0.86) was detected on chromosome 6H, in linkage group 6H-1. The QTL was found in five of the six environments in the individual CIM analyses (Figure 13). The peak LOD score of the QTL was near 66 cM and the allele from Stander was associated with increased α-amylase. Pedraza-Garcia detected QTL controlling α-amylase in the same region of chromosome 6H that explained 73% of the observed variation. Additionally, other studies have detected QTL for α-amylase activity on chromosome 6H in this same region (Figure 14) (Szucs et al., 2009). The QTL region detected in the Szucs et al. (2009) study included the SNP marker POPA2_1025 and the peak marker associated with α-amylase in our study was POPA1_0220, which both can be

found on the consensus map constructed by Munoz-Amatriain et al. (2011). In the consensus map, POPA2_1025 is located at 102 cM and POPA1_0220 is located around 89 cM. The location of the peak marker detected in their study is near the region known to harbor the *Amy1* locus that controls α-amylase activity (Emebiri et al., 2004). It is possible that the QTL identified in this study is an allele of *Amy1*. Fine mapping and eventually sequencing the region of the Robust x Stander α-amylase QTL on chromosome 6H needs to be done to verify if the QTL actually is an allele of *Amy1*.

Wort carbohydrate QTL

A QTL for glucose (r^2= 0.17) was detected on chromosome 5H, in linkage group 5H-6. In the individual CIM analyses, the QTL was detected in three of the six environments, with the peak LOD score being located near 0 cM. Single QTL for glucose (r^2= 0.40) and maltotriose (r^2= 0.29) were identified on chromosome 6H, in linkage group 6H-1. In the combined analyses, the peak LOD values were near 62 cM for glucose and 66 cM for maltotriose. The QTL for glucose concentration was detected in three of the six environments in the individual CIM analyses, while the QTL for maltotriose was identified in three of the five environments. Few studies have focused on the genetic analysis of wort carbohydrates. To the best of my knowledge, this effort to define the genetic basis of sugar production in barley following a standard malting process and mashing procedure that focused on optimizing enzymatic activity is one of the first attempts. In 2001, a study was conducted that found genomic regions associated with fermentability located on the long arm of chromosome 4H (*Bmy1* locus) and on chromosome 3H. The fermentability QTL identified on chromosome 3H was not involved with starch synthesis or hydrolysis, but did map to the *denso* dwarfing gene locus (Fox et al., 2001). Another study found QTL for

fermentability on chromosomes 1H, 2H, 3H and 7H in the same genomic regions where QTL affecting malt extract were found (Elia et al., 2010).

Pedraza-Garcia (2011) found eight QTL controlling wort carbohydrate concentrations using the same Robust x Stander population. The locations of these QTL were on chromosomes 4H, 5H, and 6H. QTL controlling glucose concentration was found on all three chromosomes. Similarities between the Pedraza-Garcia (2011) study and the present study for wort carbohydrate levels includes the detection of QTL for glucose and maltotriose concentrations on chromosome 6H.

Relationship of QTL controlling different traits

Because many of the traits evaluated in this study are correlated, it is important to relate the QTL identified with phenotypic correlations (Appendix table A2) to obtain a better understanding behind the malt quality and wort carbohydrate trait relationships. β-glucan and coarse-grind extract were controlled by QTL in the same region on chromosome segment 4H-1. The correlation estimate between these two traits was significant and negative (-0.64). The associations between the traits above may be due to linkage or pleiotropy. However, it is not possible from the results obtained to determine the cause of the associations in this study. The same QTL region in linkage group 5H-6 was associated with soluble protein, Kolbach index, wort color, and glucose concentration. The correlation values between soluble protein, Kolbach index, and wort color were all >0.70; while the correlation values of these traits with glucose were all >0.40. Since soluble protein, Kolbach index, and wort color are related to protein modification, the strong associations (r>0.70) were not surprising. The relationship between these three traits and glucose concentration is not as clear. QTL detected in linkage group 6H-1

controlled kernel color, kernel plumpness, α-amylase, glucose, and maltotriose concentration. The association between α-amylase and glucose concentration was significant and positive (r=0.64). Negative associations were detected between α-amylase and maltotriose concentrations (r=-0.53). The strong relationships of α-amylase with wort carbohydrates is not surprising given α-amylase's role in cleaving α 1,4 bonds in starch into smaller molecules, such as dextrins and glucose. The associations of kernel color and kernel plumpness with α-amylase activity and concentrations of glucose and maltotriose is not as clear. Previous to the release of Stander, it was generally thought there was a negative association between α-amylase and kernel plumpness; however, the release of Stander was considered a breakthrough in overcoming this negative relationship (Horsley, personnel communication, 2011).

Identification of favorable haplotypes

An ultimate goal for this research in the years to come is to determine the genetic basis for differences between Robust and Stander barley and to develop a genetic haplotype or "fingerprint" to differentiate six-rowed barley lines suitable for either MillerCoors or Anheuser-Busch InBev. The effects of substituting the favorable allele at the SNP locus closest to the peak LOD value for each QTL are presented in Tables 19-21.

Before one can conclude that the identified haplotypes can be used for MAS, it needs to be determined if the same haplotypes are identified when mapping is done using the entire population. Once this is done, the effectiveness of the markers for MAS can be validated using the breeding lines evaluated in the USDA-CSREES Barley CAP project from 2006-2009. Also, it needs to be determined if the identified markers are diagnostic in all three Midwest six-rowed barley breeding programs (BAR, NDSU, and UM). Using a

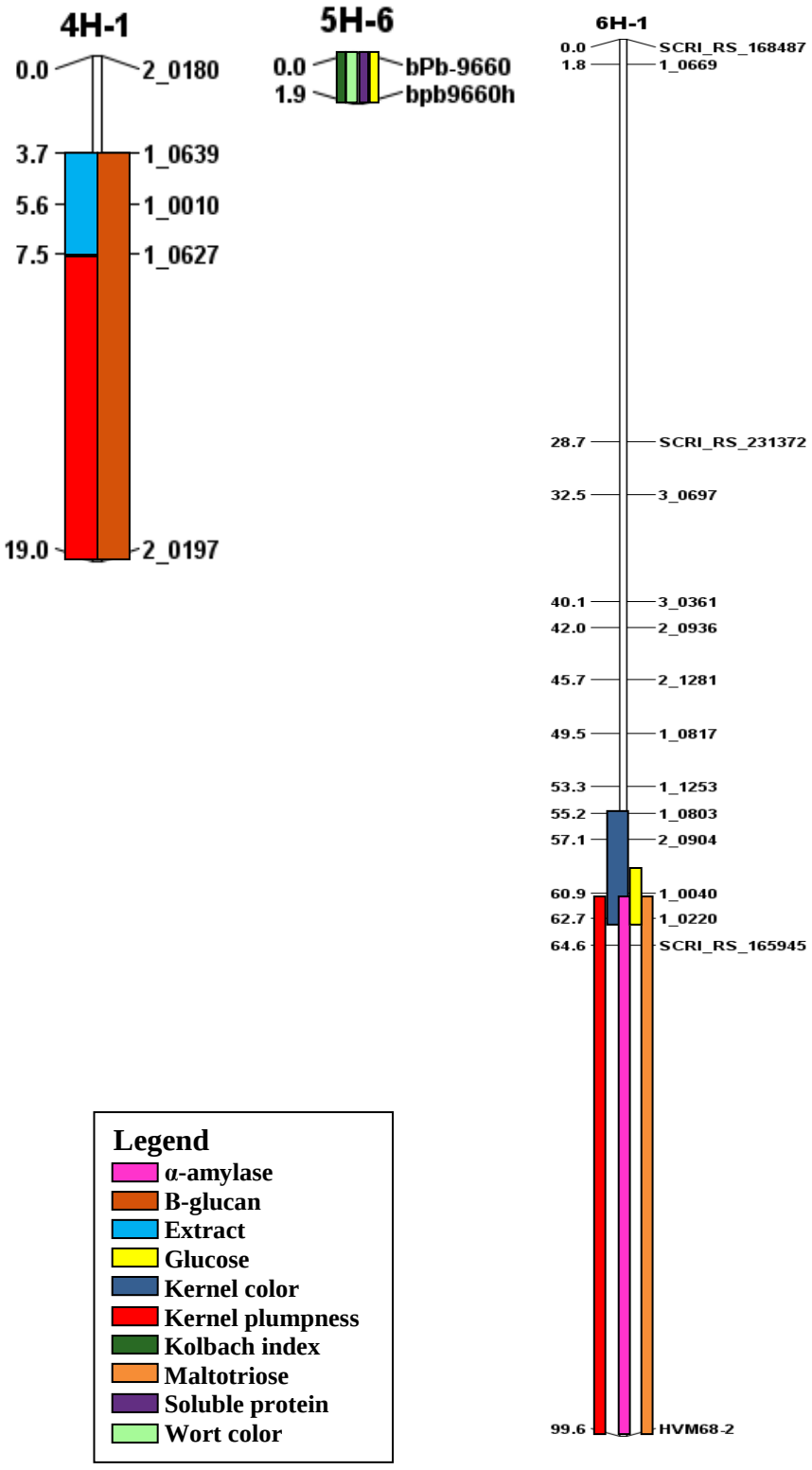

Figure 9. Linkage groups 4H-1, 5H-6, and 6H-1obtained from the Robust x Stander doubled-haploid lines. Cumulative distances in Centimorgans are located on the left hand side.

Table 18. Composite interval mapping analysis based on overall means from the original Robust x Stander doubled-haploid lines for kernel color, kernel plumpness, extract, β-glucan content, wort color, Kolbach index, soluble protein, α-amylase, glucose, and maltotriose concentrations.

			Linkage interval†							
Trait	Linkage group	Bin‡	Left marker	Right marker	Length	Marker nearest peak LOD	Position	Additive	LOD score	r^2
					cM		cM			
Extract	4H-1	83-90	1_0639	1_0627	3.8	1_0010	4	0.41	9.01	0.54
β-glucan	4H-1	83-103	1_0639	2_0197	15.3	1_0627	6	-17.76	9.14	0.55
Kernel plumpness	4H-1	90-103	1_0627	2_0197	11.5	2_0197	16	-1.97	8.91	0.54
Kolbach index	5H-6	-	bPb-9660	bPb-9660h	1.92	bPb-9660	0	-0.98	6.41	0.43
Soluble protein	5H-6	-	bPb-9660	bPb-9660h	1.92	bPb-9660	0	-0.15	10.41	0.60
Wort color	5H-6	-	bPb-9660	bPb-9660h	1.92	bPb-9660	0	-0.21	18.51	0.80
Glucose	5H-6	-	bPb-9660	bPb-9660h	1.92	bPb-9660	0	-0.03	2.10	0.17
Kernel color	6H-1	60-63	2_0904	SCRI_RS_165945	7.6	1_0040	58	-0.17	9.79	0.57
Glucose	6H-1	63-79	1_0040	SCRI_RS_165945	3.8	1_0220	62	0.05	5.91	0.40
Kernel plumpness	6H-1	79	1_0220	HVM68-2	36.8	SCRI_RS_165945	66	1.76	8.46	0.52
α-amylase	6H-1	79	1_0220	HVM68-2	36.8	SCRI_RS_165945	66	5.15	22.87	0.86
Maltotriose	6H-1	79	1_0220	HVM68-2	36.8	SCRI_RS_165945	66	-0.02	3.99	0.29

†Significant QTL are described in terms of linkage interval, closest marker to the QTL, additive regression coefficient (Additive), LOD score, and the percent of variation explained by the QTL (r^2).

‡Bin location based on work Munoz-Amatriain et al. 2011.

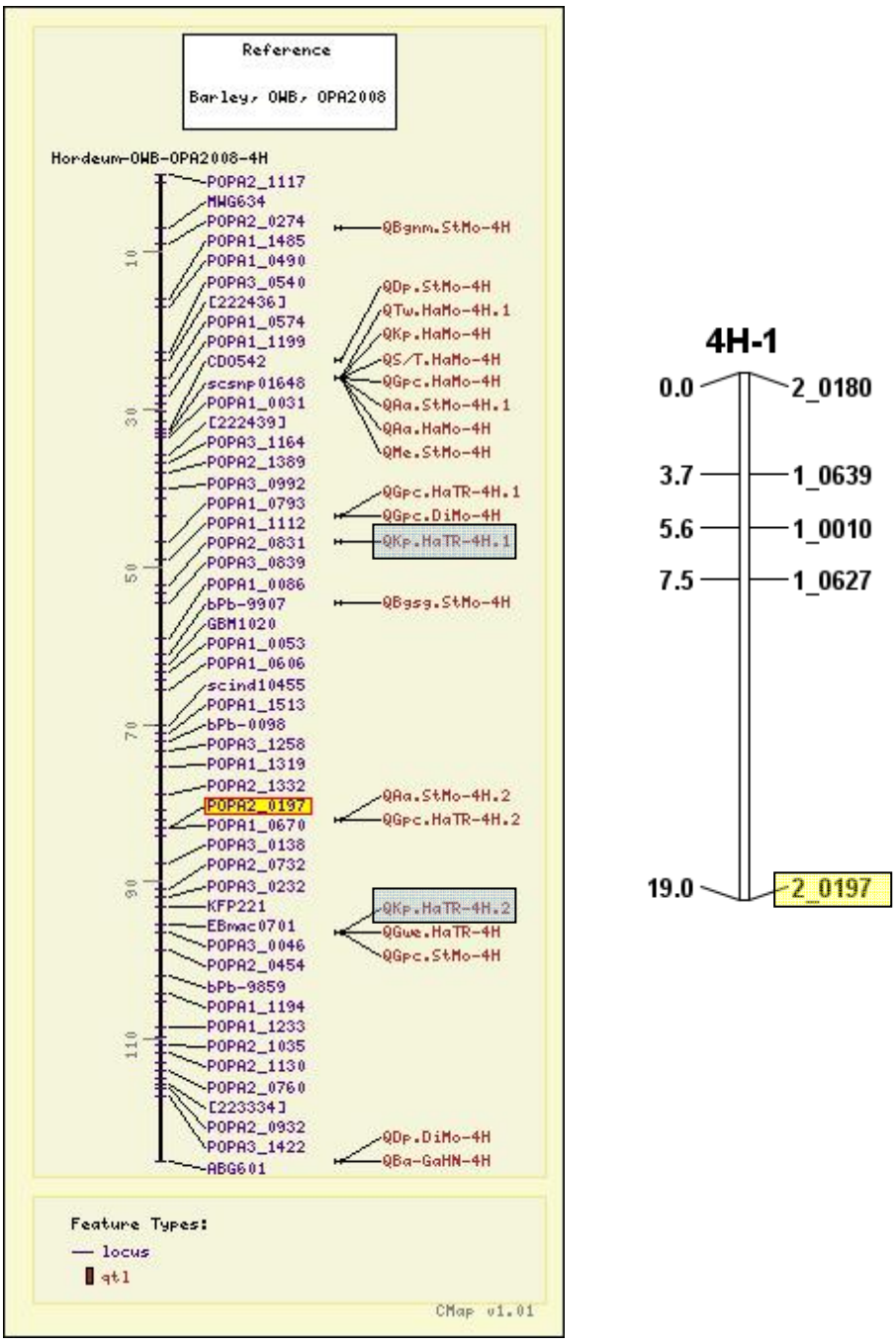

Figure 10. Linkage map of chromosome 4H of the Robust x Stander doubled-haploid population using SNP markers aligned with the corresponding chromosomes of the OWB OPA2008 map obtained from GrainGenes 2.0. The map on the left corresponds to the OWB population and the right map represents the linkage group of the 53 Robust x Stander doubled-haploid lines. The marker nearest the peak LOD score for kernel plumpness (2_0197) is highlighted in yellow on both maps. The identified QTL for kernel plumpness on the OWB map is highlighted in blue.

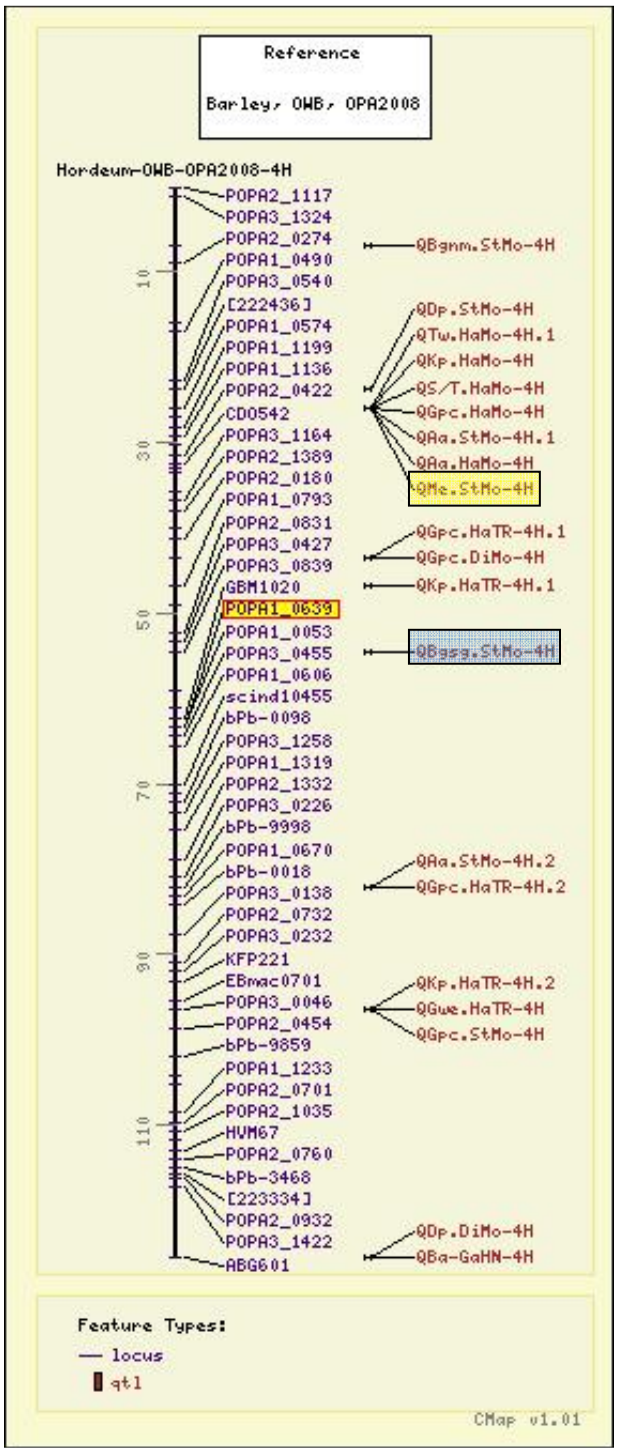

Figure 11. Picture obtained from GrainGenes 2.0 that highlights the detection of malt extract (yellow) and β-glucan (blue) on chromosome 4H in the Oregon Wolfe Barley population (Szucs et al., 2009). The SNP highlighted in yellow on the linkage map corresponds to the relative location of detected QTL controlling extract and β-glucan in the Robust x Stander doubled-haploid population in this study.

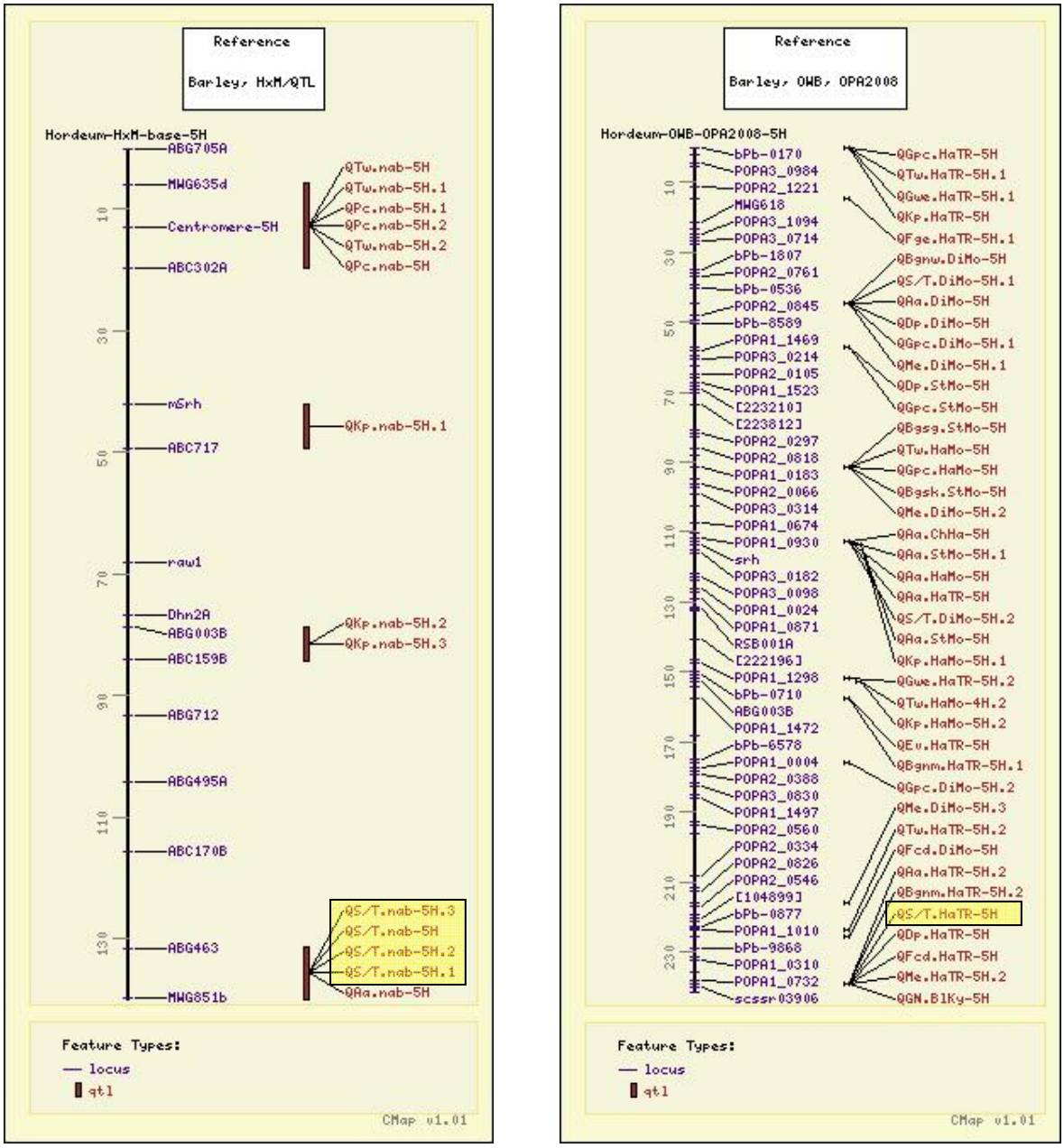

Figure 12. Two figures obtained from GrainGenes 2.0 that highlight the detection of Kolbach index (yellow) on the telomere region of chromosome 5H in the Harrington x Morex population (left-hand side) (Marquez-Cedillo et al., 2000) and the Oregon Wolfe Barley population (right-hand side) (Szucs et al., 2009).

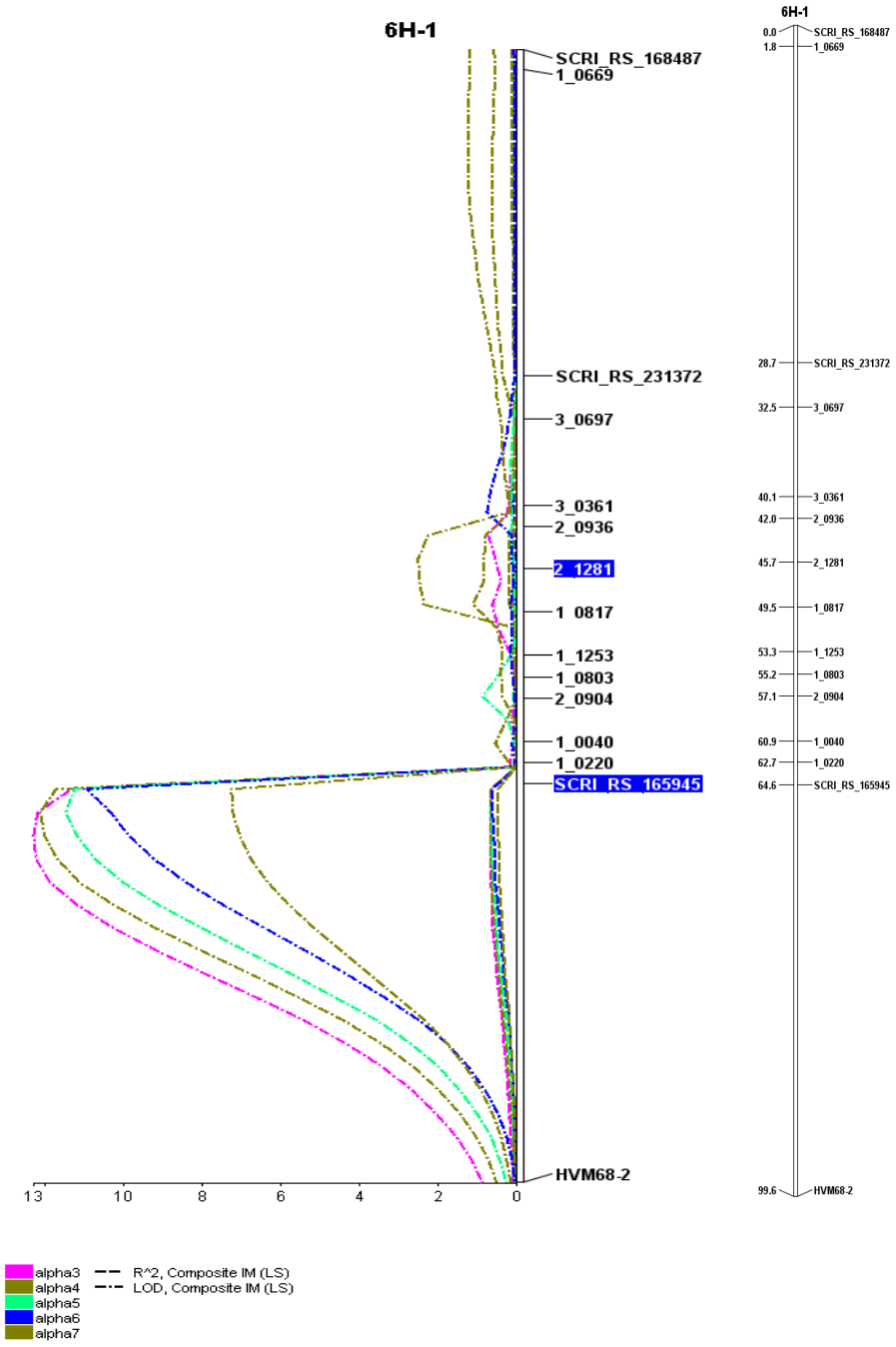

Figure 13. Distribution of the LOD value (X-axis) in the linkage group 6H-1 (Y-axis) for α-amylase detected in five of the six environments using composite interval mapping in the 53 Robust x Stander doubled-haploid lines (on the left). Linkage group 6H-1 from the Robust x Stander doubled-haploid lines is presented on the right-hand side. The figures are lined up to visualize the location of the detected QTL region controlling α-amylase in linkage group 6H-1.

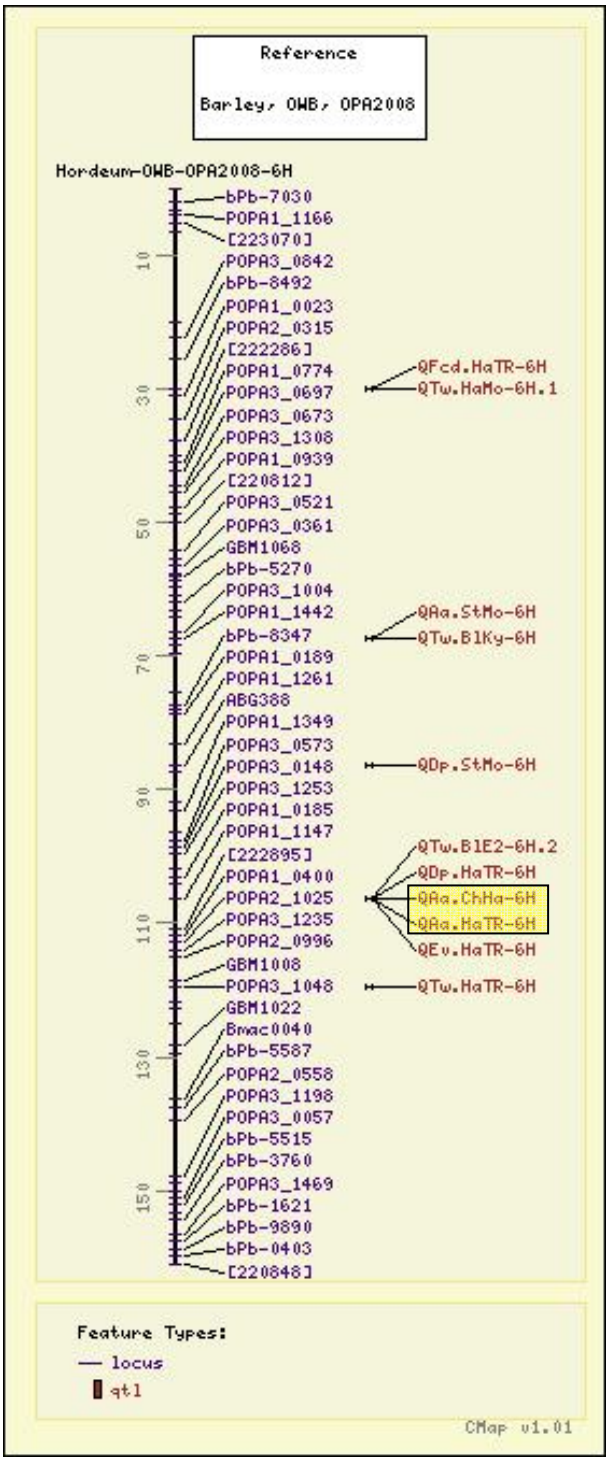

Figure 14. Picture obtained from GrainGenes 2.0, that highlights the detection of α-amylase on chromosome 6H of the OWB OPA2008 map (Szucs et al., 2009).

marker for seed dormancy developed using the original Robust x Stander population, Pedraza-Garcia (2011) found the marker was diagnostic in the UM breeding lines, but not in the BAR or NDSU breeding lines. It would not be surprising if similar results were found in this study because the Robust and Stander were both developed at the UM. However, it is possible that diagnostic markers specific to the NDSU or BAR programs may be identified in similar regions as the diagnostic markers found for Minnesota.

The AA allele of 1_0010 was associated with increased mean coarse-grind extract of the progeny by 0.8%. This value may not seem very big, but increasing malt extract has financial implications to the brewer. Higher malt extract allows the brewer to use less malt.

Research considerations for the future

Additional Robust x Stander DH lines have been developed and the total population size is now 191. Continued evaluation of the population at multiple environments and malting of the grain from these trials should allow for obtaining mapping results that are more reliable. However, it likely will not be possible to determine if the association between malt quality and wort carbohydrate traits is due to linkage or pleiotropy without fine mapping in the regions of question. Szucs et al. (2009) identified Chromosome 5H as having the greatest number of malt quality QTL, and chromosomes 3H and 6H with the fewest. Pedraza-Garcia (2011) detected the majority of QTL controlling malt quality in the telomeric region on the long arm of chromosome 5H and centromeric region of chromosome 6H. With the new set of markers (445 SNPs), chromosome 5H was split into seven relatively small linkage groups. The linkage group composed of two DArT markers located at the telomeric region of chromosome 5H harbored important protein modification and wort carbohydrate QTL.

Plans for the future using the Robust x Stander population are to conduct the mapping studies using a population of 191 DH lines. This population has been genotyped using SNPs, but it also needs to be genotyped with the same DArT and SSR markers used by Pedraza-Garcia (2011). Mapping done in the larger population will hopefully allow for consistent detection of QTL using CIM.

Based on the results of the present study, it is important to highlight chromosomes 4H, 5H, and 6H. These three regions harbored the majority of the detected malt quality QTL, such as coarse-grind extract, α-amylase, and plump kernels. These traits are the main ones that help differentiate malt barley cultivars acceptable for Anheuser-Busch InBev and MillerCoors.

For barley quality, malt quality, and wort carbohydrate traits, the ideal situation would be to implement a MAS breeding strategy. As can be seen in the materials and methods section, analyzing a sample for malt quality is labor intensive and time consuming. One way to reduce cost and time would be to select desired genotypes with molecular markers. Being able to discard those undesirable genotypes before submitting the samples to the malt quality laboratory allows the production of an end product not only suitable for breeders and growers, but also for maltsters and brewers.

Table 19. Effect of allelic substitution at the SNP locus 1_0040 controlling mean kernel color.

	1_0040	
	AA	BB
	---------- L-value ----------	
Robust	-	53.1±0.7
Stander	52.3±0.7	-
Progeny	52.5±0.1	***52.8±0.1†***

†Progeny means resulting from plants with favorable alleles are highlighted in bold italicized font.

Table 20. Effect of allelic substitution at individual loci controlling mean β-glucan and coarse-grind extract.

	1_0627		1_0010	
	β-glucan		Coarse-grind extract	
	AA	BB	AA	BB
	----------%----------		----------%---------	
Robust	-	182±31	-	77.4±0.5
Stander	194±31	-	78.1±0.3	-
Progeny	***168±6***†	206±6	***78.2±0.1***	77.4±0.1

†Progeny means resulting from plants with favorable alleles are highlighted in bold italicized font.

Table 21. Effect of allelic substitution at the SNP locus 1_0220 controlling mean kernel plumpness, α-amylase, glucose, and maltotriose concentration.

	1_0220							
	Kernel plumpness		α-amylase		Glucose		Maltotriose	
	AA	BB	AA	BB	AA	BB	AA	BB
	----------%---------		----------20^{o} DU----------		-----g 100 mL^{-1}-----		-------g 100 mL^{-1}-------	
Robust	-	79.6±5.5	-	61.6±1.4	-	1.2±0.1	-	0.88±0.06
Stander	82.9±4.4	-	74.0±3.0	-	1.4±0.1	-	0.91±0.06	-
Progeny	***83.0±0.9†***	79.3±1.1	***74.7±0.4***	63.1±0.3	***1.4±0.0***	1.3±0.0	0.90±0.01	***0.94±0.01***

†Progeny means resulting from plants with favorable alleles are highlighted in bold italicized font

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CHAPTER IV. RESEARCH HIGHLIGHTS

Developing a new malting barley cultivar is complex and difficult, since many quality parameters have to be met before maltsters and brewers consider a cultivar acceptable. Most quality testing is not performed until the later stages of the breeding program, when sufficient amount of seed is available. These malt quality analyses are not only labor-intensive and time consuming, they are expensive. With the use of markers or fingerprint information, screening of progeny and elimination of undesirable genotypes could take place prior to intensive malt quality testing. The inclusion of marker-assisted selection would especially be helpful in differentiating cultivars desired by major brewers.

The requirements of each brewer for malted barley are specific and unique. Two major brewers that utilize six-rowed barley malt in the US are Anheuser-Busch InBev and MillerCoors. Both companies target different malt quality parameters, with moderate levels of protein modification and enzymatic activity desired by MillerCoors and high protein modification and enzymatic activity levels preferred by Anheuser-Busch InBev. Two cultivars developed by the University of Minnesota, Robust (Rasmusson and Wilcoxson, 1983) and Stander (Rasmusson et al., 1993), share a close pedigree relationship but differ in many of the key agronomic and malt quality trait characteristics. Stander is known to be more resistant to lodging, shorter in plant height (6 cm shorter), higher yielding (6% higher), and contain higher levels of α-amylase (36% higher) than Robust (Rasmusson et al., 1993).

The close relationship between Robust and Stander increases the chance of recognizing chromosome regions with the genes controlling malt quality traits. The objectives of this research were to: 1) generate molecular marker linkage maps using the

original 53 Robust x Stander DH lines and the entire DH population (191 lines); 2) phenotype the mapping population for agronomic, malt quality, and wort carbohydrate traits; and 3) identify quantitative trait loci (QTL) controlling these traits.

Key findings discovered in this research included:

- To the best of our knowledge, the research reported in this dissertation is the first for mapping in barley using closely related parents and sufficient population size.
- Three linkage maps were constructed. The first linkage map was constructed using the original Robust x Stander DH lines, along with 102 SNP, SSR, and DArT markers, which covered 409.02 cM. The second and third linkage maps were developed using only 67 SNP markers, with the original and entire Robust x Stander DH population, respectively. The 67 SNP markers covered 206.58 cM in the second linkage map, and 218.85 cM in the third map.
- In the first linkage map, chromosome 5H was divided into six linkage groups, whereas only four linkage groups were detected in the linkage maps based solely on SNP markers. Two of the six subgroups identified in the first linkage map were composed of DArT markers, ultimately showing that DArT and SSR markers were mapping to locations not covered by the SNPs. Thus, it is not a good strategy to rely on only one marker type when developing a map using a population derived from two closely related parents; however, it is important to keep in mind that when more than one marker system is utilized in a small population, map expansion occurs.
- The first map was used to identify QTL controlling malt quality and wort carbohydrate traits on chromosomes 4H, 5H, and 6H. Chromosome 4H harbored

significant QTL controlling kernel plumpness, extract, and β-glucan concentration. QTL controlling Kolbach index, soluble protein, wort color, and glucose concentration were detected on chromosome 5H. QTL were identified for kernel color, kernel plumpness, α-amylase, glucose, and maltotriose concentration on chromosome 6H.

- To the best of my knowledge, our effort to define the genetic basis of sugar production in barley following a standard malting process and mashing procedure that focused on optimizing enzymatic activity is original and unique. The majority of QTL controlling production of fermentable sugars were located in the telomeric region of chromosome 5H and centromeric region of chromosome 6H. The telomeric region of chromosome 5H included QTL for glucose concentration, Kolbach index, soluble protein, and wort color. The centromeric region of chromosome 6H included QTL for glucose and maltotriose concentration, kernel plumpness, and α-amylase activity.
- The SNP map constructed using the original DH population was used to identify QTL controlling agronomic traits of plant height, lodging, and deciduous awns on chromosome 6H.
- The mapping of QTL for deciduous awns has not been reported previously.
- The SNP map constructed using all 191 lines was used to identify a QTL for heading date on chromosome 4H and plant height on chromosome 6H.
- QTL mapping analyses performed in biparental populations developed by crossing two closely related parents (Polymorphism rate = 6.6%) are informative.

- Continued evaluation of the entire Robust x Stander DH population at multiple environments and malting of the grain from these trials should allow for obtaining more robust mapping results.
- An ultimate goal for this research in the years to come is to determine the genetic basis for differences between Robust and Stander barley and to develop a genetic haplotype or "fingerprint" to differentiate six-rowed barley lines suitable for either MillerCoors or Anheuser-Busch InBev. To begin the development of a genetic haplotype, effects of allelic substitution at peak markers identified in the CIM analyses for agronomic, malt quality, and wort carbohydrate traits were shown.
- The utility of these SNP markers for marker-assisted selection needs to be validated using the 2006-2009 USDA-CSREES Barley Coordinated Project breeding lines from the University of Minnesota, North Dakota State University, and Busch Agricultural Resources six-rowed breeding programs.

Table A1. Composite interval mapping analysis based on overall means from the original Robust x Stander doubled haploid lines for kernel color, protein, plumpness, extract, β-glucan, wort color, free amino nitrogen, Kolbach index, soluble protein, α-amylase, diastatic power, glucose, maltose, and maltotriose concentrations.

				Linkage interval†							
Trait	% detection across environments	Linkage group	Bin‡	Left marker	Right marker	Length	Marker nearest peak LOD	Position	Additive	LOD score	r^2
						cM		cM			
Kernel protein	25%	4H-1	43-86	2_0180	1_0010	5.7	1_0639	2	-0.15	4.68	0.33
Extract	67%	4H-1	83-90	1_0639	1_0627	3.8	1_0010	4	0.41	9.01	0.54
β-glucan	100%	4H-1	83-103	1_0639	2_0197	15.3	1_0627	6	-17.76	9.14	0.55
Kernel plumpness	50%	4H-1	90-103	1_0627	2_0197	11.5	2_0197	16	-1.97	8.91	0.54
Kolbach index	67%	5H-6	-	bPb-9660	bPb-9660h	1.92	bPb-9660	0	-0.98	6.41	0.43
Soluble protein	100%	5H-6	-	bPb-9660	bPb-9660h	1.92	bPb-9660	0	-0.15	10.41	0.60
Wort color	67%	5H-6	-	bPb-9660	bPb-9660h	1.92	bPb-9660	0	-0.21	18.51	0.80
FAN	17%	5H-6	-	bPb-9660	bPb-9660h	1.92	bPb-9660	0	-8.37	3.46	0.26
β-glucan	25%	5H-6	-	bPb-9660	bPb-9660h	1.92	bPb-9660	0	10.67	4.15	0.30
Diastatic power	17%	5H-6	-	bPb-9660	bPb-9660h	1.92	bPb-9660	0	3.83	6.43	0.43
Glucose	50%	5H-6	-	bPb-9660	bPb-9660h	1.92	bPb-9660	0	-0.03	2.10	0.17
Maltose	17%	5H-6	-	bPb-9660	bPb-9660h	1.92	bPb-9660	0	0.06	3.68	0.27
α-amylase	33%	6H-1	34-52	2_1281	1_1253	7.6	1_0817	50	1.46	4.96	0.35
Kolbach index	17%	6H-1	34-53	2_1281	1_0803	9.5	1_0817	50	0.85	4.99	0.35
Kernel color	63%	6H-1	60-63	2_0904	SCRI_RS_165945	7.6	1_0040	58	-0.17	9.79	0.57
Glucose	50%	6H-1	63-79	1_0040	SCRI_RS_165945	3.8	1_0220	62	0.05	5.91	0.40
Kernel plumpness	50%	6H-1	79	1_0220	HVM68-2	36.8	SCRI_RS_165945	66	1.76	8.46	0.52

Table A1 (continued). Composite interval mapping analysis based on overall means from the original Robust x Stander doubled haploid lines for kernel color, protein, plumpness, extract, β-glucan, wort color, free amino nitrogen, Kolbach index, soluble protein, α-amylase, diastatic power, glucose, maltose, and maltotriose concentrations.

				Linkage interval†							
Trait	% detection across environments	Linkage group	Bin‡	Left marker	Right marker	Length	Marker nearest peak LOD	Position	Additive	LOD score	r^2
						cM		cM			
α-amylase	83%	6H-1	79	1_0220	HVM68-2	36.8	SCRI_RS_165945	66	5.15	22.87	0.86
Maltotriose	60%	6H-1	79	1_0220	HVM68-2	36.8	SCRI_RS_165945	66	-0.02	3.99	0.29

†Significant QTL are described in terms of linkage interval, closest marker to the QTL, additive regression coefficient (Additive), LOD score, and the percent of variation explained by the QTL (r^2).

‡Bin location based on work Munoz-Amatriain et al. 2011.

Table A2. Phenotypic correlation estimates among barley quality, malt quality, and wort carbohydrate traits of kernel color (CO), kernel protein (PR), kernel plumpness (P%), α-amylase (AA), diastatic power (DP), Kolbach index (KI), extract (EX), wort viscosity (WV), soluble protein (SP), wort color (WC), free amino nitrogen (FAN), β-glucan (BG), fructose (FR), glucose (GL), maltose (MA), and maltotriose (MT) concentrations for the original 53 Robust x Stander doubled haploid lines, evaluated across six environments.

	CO†	**PR**	**P%**	**AA**	**DP**	**KI**	**EX**	**WV**	**SP**	**WC**	**FAN**	**BG‡**	**FR**	**GL**	**MA**
PR	0.17														
P%	-0.26	0.29*													
AA	-0.61**§	-0.08	0.52**												
DP	0.20	-0.05	-0.21	-0.26											
KI	-0.34**	-0.23	0.05	0.48*	-0.43**										
EX	-0.10	-0.72**	-0.33**	0.18	-0.02	0.43**									
WV	0.01	-0.22	-0.13	-0.03	-0.01	0.00	0.25								
SP	-0.18	0.32*	0.24	0.40**	-0.37**	0.82**	0.05	-0.09							
WC	-0.34**	0.02	0.18	0.47**	-0.66**	0.72**	0.23	0.07	0.71**						
FAN	-0.13	0.06	0.24	0.33**	-0.33**	0.58**	0.16	-0.01	0.60**	0.63**					
BG	-0.02	0.42**	0.41**	-0.07	-0.01	-0.39**	-0.64**	-0.05	-0.20	-0.37**	-0.40**				
FR	-0.03	0.09	0.00	0.24	-0.02	-0.03	-0.12	-0.17	-0.01	-0.02	-0.05	0.05			
GL	-0.40**	-0.05	0.30*	0.64**	-0.31*	0.50**	0.20	-0.10	0.44**	0.50**	0.28*	-0.11	0.09		
MA	-0.01	-0.43**	-0.12	0.00	0.29*	-0.16	0.23	0.02	-0.41**	-0.49**	-0.20	-0.01	0.10	0.06	
MT¶	0.33**	-0.16	-0.38**	-0.53**	-0.02	-0.10	0.23	0.01	-0.20	0.00	0.01	-0.26	-0.07	-0.08	0.02

† Kernel color, protein, and plumpness data were collected from 8 locations.

‡ β-glucan data were collected from 4 locations.

§* and ** represent $P \leq 0.05$ and $P \leq 0.01$, respectively.

¶Maltotriose data were collected from only five of the six locations.