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Title: Breeding Food Barley: From Agronomic Assessment to Marker Assisted Selection

Abstract approved:

Patrick M. Hayes

This research was undertaken to provide barley growers, processors, and consumers with quality food barley. Because all currently available varieties of food barley are of spring growth habit, the first phase of the research involved agronomic assessment of these varieties under Oregon conditions. Because winter barley usually yields more than spring barley in these environments, the second phase of this research involved development of winter habit food barley germplasm. Grain β-glucan content is the most important attribute for food barley varieties. This trait is important because of the cholesterol-reducing properties of β-glucan. Thirty three cultivars and advanced lines reported to vary in β-glucan content were grown in 2006 and 2007 at two locations in northeastern Oregon under dryland cropping conditions. Seed yield, test weight, percentage of plump kernels, grain β-glucan and grain protein were measured on replicated samples from the four environments. Hulled, waxy starch varieties appear to have the greatest agronomic potential for dryland production as they combine high yield potential and grain β-glucan percentage. Marker assisted selection was used to rapidly develop barley germplasm with novel combinations of alleles at loci controlling starch synthesis and growth habit. The target loci were *WAX*, *VRN-H1*, and *VRN-H2*. The goal was to develop waxy starch, high grain β-glucan, and

winter habit germplasm. Pre-screening of candidate parental lines identified combinations with target vrn-H1 (winter) alleles. This allowed for immediate fixation of favorable alleles at this locus. *VRN-H1* is coincident with a major low temperature tolerance QTL (*Fr1*). Perfect markers were based on functional domains in *GBSSI* and *ZCCT-H*, the genes determining *wax* and *VRN-H2*. Because spring growth habit at *VRN-H2* is due to gene deletion, codominant alleles at a tightly linked locus (*HvSNF2*), were also used. Backcross-derived germplasm was genotyped in two stages. Homozygotes at the two loci were identified in one-step screening in the progeny of the double cross. BC1F3 and DCF3 lines were advanced to field testing in the Fall of 2007. In subsequent generations, these lines will allow for testing hypotheses regarding the pleiotropic effects of waxy starch on β-glucan and vernalization genes on low temperature tolerance.

Breeding Food Barley: From Agronomic Assessment to Marker Assisted Selection

by
Juan Ignacio Rey

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APPROVED:

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Juan Ignacio Rey

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CONTRIBUTION OF AUTHORS

Dr. Patrick M. Hayes initiated, advised, and supervised all aspects of the project. Ann Corey assisted with greenhouse and field trial data collection. Tanya Filichkin and Dr. Peter Szucs were involved in laboratory aspects of the research. Jennifer Kling helped in the statistical analysis. Caryn Ong and Jae Ohm helped in quality analyses. Dr. Steven Petrie, and Karl Rhinhart managed the spring barley field trials at Pendleton and Moro, Oregon. Drs. Steve Petrie, Andrew Ross, Peter Szucs, and Michael Flowers advised and helped with the manuscript preparation.

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In dedication to
Vanesa, my wife for her help, love and encouragement
Joaquin that this thesis serve as an example for his future
Juan Manuel and Gloria, my father and mother, for teaching me what is important in life

Breeding Food Barley: From Agronomic Assessment to Marker Assisted Selection

General Introduction

Marker-assisted-selection

The application of marker-assisted-selection (MAS) to plant breeding is expected to lead to greater efficiencies in terms of time, resource allocation, and response to selection. There is considerable interest and enthusiasm for MAS in the plant breeding community (Collard et al., 2005; Dubcovsky, 2004; Steele et al., 2006). For example, the prospect of increasing the adoption of MAS amongst US barley breeders was a fundamental justification of the USDA-CSREES Barley Coordinated Agricultural Project (CAP) (http://barleycap.cfans.umn.edu).

There are a number of papers describing MAS breeding schemes and comparing MAS vs. conventional breeding via simulations (Lande and Thompson, 1990; Dreher et al., 2003; Knapp, 1998; Kruchel et al., 2005; Van Berloo and Stam, 1999). There are fewer examples reporting results from MAS. Nonetheless, the available examples highlight some of the most promising applications. Using MAS to recover the recurrent parent genome (background selection), Ragot et al. (1995) showed that it was possible to gain up to two backcross cycles in the integration of the *Bt* transgene into different maize genetic backgrounds. Another application is resistance gene pyramiding (Huang et al., 1997; Hittalmani et al., 2000; Singh et al., 2001; Castro et al., 2003; Jiang et al., 2004). MAS can reduce linkage drag, which is of particular importance when introgressing genes from wild species. Lawson et al. (1997), for example, introgressed four chromosomal regions containing five QTLs for pest resistance from wild tomato into cultivated tomato. MAS is also useful in selecting for traits where phenotypic evaluation is very difficult and costly. For example, Han et al. (1997) were successful in configuring target alleles at two malting quality QTLs in barley and Toojinda et al. (1998) introgressing QTLs determining stripe rust resistance in barley.

It is useful to make a distinction between MAS using markers linked to a QTL and “perfect” markers designed from the specific differences in sequence among

the alleles of a gene. In the case of markers linked with QTLs, markers should be tightly linked and multiple markers flanking the QTL should be used in order to reduce the risk of losing target alleles due to recombination. The advantages of tight linkage, however, must be weighed against the ambiguity of estimates of QTL position. Perfect markers are based on allele sequence of the target gene, and ideally on the functional basis of allelic variation. In the case of selection for a null allele due to gene deletion, a combination of the perfect and linked markers will be necessary to rule out false positives (Knapp, 1998).

We chose MAS as a method for developing an entirely new class of barley: winter growth habit barley for human food. This serves as an excellent model for MAS because (i) there are perfect markers for target growth habit alleles and starch type, (ii) development of this novel germplasm would be a protracted task with conventional selection, and (iii) there is tremendous grower and industry interest in winter food barley due to its high yield potential and broad adaptation. Human food quality and winter growth habit will be addressed in order.

Human food barley

There is increasing interest in barley as a human food based on heightened awareness of the importance of both soluble and insoluble fiber in a healthy diet. Dietary fiber is a generic term for substances in plant material that resist human digestive enzymes. Potential health benefits of dietary fiber intake include reduction of bowel transit time (Feldheim and Wisker, 2000), prevention of constipation, reduction in risk of colorectal cancer (Faivre and Bonithon-Kopp, 1999), and lowering of blood cholesterol and regulation of blood glucose levels for diabetes management (Frost et al., 1999). β-glucans are an important component of dietary fiber (Newman and Newman, 1991b) and barley has the highest average β-glucan content of any cultivated grain (Aman and Graham, 1987). In recognition of the value of barley as a source of β-glucan, the FDA issued a health claim for barley in 2006 (FDA, 2006). To meet the claim, the barley food must contain 0.75 grams of soluble fiber per serving. β-glucan is a nonstarch

polysaccharide composed of ß-(1→4)–linked glucose units separated every 2–3 units by β-(1→3)–linked glucose. β-glucan and arabinoxylan are the two major constituents of barley endosperm cell walls (Izydorczyk and Biliaderis, 2000). Of course, consumption whole grain barley has benefits in addition to β-glucan: (Marlett, 1991; Newman and Newman, 1991; Berglund et al., 1994) demonstrate the benefit of barley as a whole grain and source in the human diet.

The FDA-approved health claim is expected to lead to expanding markets for human food barley with high levels of β-glucan. Selection of varieties for this trait is complicated by the expense of measuring the phenotype and a lack of knowledge regarding the genetic basis of the trait. Methods for determination of grain β-glucan include enzymatic assays (Hang et al., 2007), near-infrared reflectance spectroscopy and calcofluor flow injection (Andersson et al., 2004). The former is relatively expensive in terms of time and reagents: a skilled operator can reasonably process 300 samples per week with a reagent cost of $2.50 per sample, while the other two are not approved methods and the equipment required for the flow injection method is very expensive.

Powell et al., (1985) reported that β-glucan content in barley grain is controlled by a simple additive genetic system. Burton et al. (2006) recently cloned and characterized six members of the sub-family *CslF* of the *Csl* family (cellulose Synthase-like) genes, which are responsible for β-glucan biosynthesis, Although over expression of these genes in Arabidopsis led to β-glucan synthesis, it is not apparent if there is allelic variation at this complex locus in barley germplasm that would allow breeders to select for higher grain β-glucan.

An alternative approach to selecting for higher grain β-glucan is to capitalize on the pleiotropic effects of a recessive mutation in the *GBSSI* gene that leads to waxy starch phenotype (Patron et al., 2002). *GBSSI* encodes the granule bound starch synthase responsible for the synthesis of amylose in barley starch. A ~400-bp gene deletion in the promoter and 5'-UTR reduces levels of amylase, leads to higher levels of amylopectin in (Domon et al., 2002), and produces what is called a "waxy" starch. Positive correlations between β-glucan content and waxy starch are

documented (Xue et al., 1997). This positive association can be used to indirectly select for increased grain beta glucan via direct selection for the waxy (*wax*) allele of the *GBSSI* gene. Waxy starch has value in its own right as a preferred substrate for the development of modified starches for the food, papermaking, and adhesive industries (White, 1994). Therefore, the deletion in *GBSSI* provides the basis for design of a perfect marker for transfer of the *wax* allele to non-waxy cultivars, and this transfer is expected to lead to higher levels of grain β-glucan.

Winter hardiness

There is increasing interest in fall-sown barley in the Pacific Northwest of the United States due to its high yield potential and more efficient use of available moisture. For example, grain yield of fall-sown barley under the dryland conditions of the Columbia Basin is typically 20% higher than spring-sown barley at the same location. This is due to better synchronization of crop growth and development with winter rainfall patterns. Under irrigated conditions, yields of fall-sown and spring-sown barley are similar but the fall-sown crop can be produced with one or two fewer irrigations. The only drawback to fall-sown barley is the risk of crop losses due to low temperature stress.

Resistance to low temperature stress is also termed winter hardiness. It is simply a measure of the number of plants per unit area that survive the winter. The genetic and physiological factors that determine survival are, however, myriad and complex. In the Triticeae, this complexity can be partitioned into low temperature tolerance *per se* (Skinner et al., 2006), vernalization sensitivity, (von Zitzewitz et al., 2005) and photoperiod sensitivity (Szűcs et al., 2006).

These three characteristics are inter-related and the typical profile of a fall-sown barley "winter" barley is that it is cold tolerant, vernalization sensitive, and photoperiod sensitive. Maintenance of the plant in a vegetative phase until a sufficient period of cold exposure is attained (vernalization) or photoperiod reaches a certain critical length (photoperiod sensitivity) reduces the likelihood of

exposure of differentiated reproductive tissues to low temperatures. Furthermore, there is evidence, in the case of vernalization sensitivity, that maximum levels of low temperature tolerance are achieved coincident with sufficient exposure to low temperature to satisfy the vernalization requirement (Fowler et al., 1996). This suggests a causal role for vernalization in inducing low temperature tolerance (Fowler et al., 1996; Fowler et al., 1999; Limin et al., 2007). Photoperiod sensitivity has the same effect as vernalization in delaying the vegetative to reproductive transition. However, mapping of all known photoreceptor candidate genes did not reveal a coincident with low temperature tolerance QTL (Szucs et al., 2006).

The coincidence of one of the two major low temperature tolerance QTL (*Fr1*) with *VRN-1* (Galiba et al., 1995) on the group 5 chromosomes of the Triticeae suggests that allelic variation at the latter locus could determine variation in low temperature tolerance. The second major low temperature tolerance QTL (*Fr2*) is 30 cM proximal to *Fr1*. One or more members of the cluster of *CBF* gene family members are candidates for the *Fr2* QTL (Skinner et al., 2006). This simple two-locus model for low temperature tolerance, however, belies the complexity of the relationship of vernalization and low temperature tolerance.

For example, "facultative" barley germplasm is not vernalization sensitive but it is cold tolerant. As demonstrated by von Zitzewitz et al. (2005), this combination of characters is due to the presence of "winter alleles" at the *Fr1* and *Fr2* candidate genes and a complete deletion of the *VRN-H2* locus on chromosomes 4H. *Vrn-H2* encodes a repressor of *vrn-H1* and deletion of *Vrn-H2* allows expression of *vrn-H1* – a meristem identify gene – from the juvenile stage onward (Szucs et al., 2007). "Spring" genotypes lack the binding site in *vrn-H1* for the *Vrn-H2*-encoded repressor; they may or may not carry the *Vrn-H2* deletion (Szucs et al., 2006). The low temperature tolerance QTL coincident with *Fr1* is significant in the Dicktoo (facultative) x Morex (spring) population. Low-temperature tolerance and photoperiod sensitivity QTLs map to the same region as the *Fr1* and *VRN-1* loci. Galiba et al. (1995) reported that the two loci were

divisible by recombination and Sutka et al. (1999) reported that there are two loci via physical mapping. This suggests that *vrn-H1* is not the determinant of *Fr1*. This implies that a gene, or genes, in relatively close physical proximity to *vrn-H1* may be the determinants of cold tolerance. However, these reports were based on a limited sample of germplasm that was not completely genotyped and exhaustively phenotyped, so it is not know with certainty if the coincidence of *Fr1* with *vrn-H1* is due to linkage or pleiotropy.

Because the functional basis of allelic variation at *VRN-H1* is known and this locus is coincident with *Fr1*, it provides a perfect marker for one of the vernalization genes that may have a pleiotropic effect on low temperature tolerance. Even if *vrn-H1* is not the determinant of *Fr1*, it is tightly linked to the causal gene (or genes). Likewise, since the functional basis of *Vrn-H2* is known and there is evidence that vernalization sensitivity may lead to maximum cold tolerance, this locus is also candidate for MAS. The situation at *Fr2* is not as clear: although the QTL is coincident with the *CBF* gene family cluster, there is little allelic variation at this complex locus between cold tolerant and cold sensitive genotypes (Skinner et al., 2005).

Prior to initiating the MAS program that is described in this report, we included a sample of waxy food barley germplasm - all described as "spring habit" - in our routine screening of *VRN* alleles. We discovered that two of the varieties - Waxbar and Merlin - are facultative, not spring. That is, they both have the winter allele at *vrn-H1* and the deletion of *Vrn-H2*. Because these two varieties are both high β-glucan, waxy (*waxwax*) types, we chose them as donors of the *wax* allele. Fortuitously, when in crosses involving Waxbar and Merlin with winter and facultative accessions, the *vrn-H1* winter allele will be fixed.

We chose a winter six-row -'Strider' - released by the Oregon State University Agricultural Experiment Station in 1997 and 'Luca' (an experimental winter two-row obtained from the Martonvasar Research Institute in Hungary) as recipient varieties for the *wax* alleles from 'Waxbar' and 'Merlin'. We hypothesized that introgression of the *waxwax* alleles from Waxbar and Merlin into Strider and Luca

would raise grain β-glucan levels and that configuring dominant (functional) alleles at *Vrn-H2* (from Strider or Luca) with the recessive (winter) allele at *VRN-H1* (from all parents) would lead to vernalization sensitivity. Vernalization sensitivity may confer higher low temperature tolerance and comparison of vernalization-sensitive progeny with the facultative parents will allow us to test the hypothesis that vernalization sensitivity increases cold tolerance. Creation of this novel germplasm will allow us to assess the utility of MAS for rapidly developing winter habit food barley germplasm that will meet the immediate needs and interests of barley growers, processors, and consumers.

Potential for Production of Dryland Barley for Human Food: Quality and Agronomic Performance of Spring Habit Germplasm in Oregon

Abstract

Grain β-glucan content is the most important attribute for barley (*Hordeum vulgare* L.) varieties destined for the human food market. This trait is important because of the cholesterol-reducing properties of β-glucan. High levels of grain protein, test weight and seed size may also add value. Seed yield potential, in part, determines the economic feasibility of producing human food varieties. In order to determine the potential of food barley production in the dryland production areas of the Pacific Northwest of the U.S.A, 33 cultivars and advanced lines reported to vary in β-glucan content were grown in 2006 and 2007 at two locations in northeastern Oregon under dryland cropping conditions. Seed yield, test weight, percentage of plump kernels, grain β-glucan and grain protein were measured on replicated samples from the four environments, allowing for assessment of average performance as well as genotype x environment interaction. Estimates of variance components showed that ~ 66% of the variability in β-glucan content was attributable to genotype. Cultivars and lines with waxy starch showed the highest average β-glucan values. We found significant two- and three-way interactions but these accounted for much less of the total variation in the measured phenotypes than the main effects of variety, year, and location. Hulless accessions were significantly lower yielding than hulled accessions, particularly in the second year of the study. Hulled, waxy starch varieties appear to have the greatest agronomic potential for dryland production as they combine high yield potential and grain β-glucan percentage.

Introduction

Consumption of whole grains helps to control obesity (Pauline and Rimm, 2003) and reduce the risks of Type II diabetes and coronary heart disease (Liu et al., 1999; Anderson et al., 2000; Liu et al., 2000). In 2006, the United States Food and Drug Administration approved a health claim for barley, based on the demonstrated reduction in risk of coronary heart disease resulting from consumption of whole grain barley and barley-containing products (FDA, 2006). To qualify for the health claim, the barley-containing foods must provide at least 0.75 grams of soluble fiber per serving. It is the β-glucan fraction of the soluble fiber that is reportedly responsible for lowering LDL and total cholesterol levels (Brown et al., 1999).

This new appreciation for the nutritional value of whole grain barley and barley β-glucan may increase the market for barley products (Bhatty 1995a, 1997) and provide new opportunities for β-glucan extraction and enrichment (Knuckles et al., 1992; Bhatty 1993b, 1995b). The traditional markets for barley are malt and animal feed. High β-glucan levels are a problem for brewing and as a consequence low levels of β-glucan in malt are specified by the malting and brewing industries (Bamforth and Barclay, 1993). Despite evidence that there is variation for feeding quality in barley (Hunt, 1996), a minimum test weight is usually the only quality parameter specified for ruminant feeds. Grain β-glucan is a problem for barley in poultry diets due to "sticky droppings" phenomenon. Solutions to the problem include application of exogenous β-glucanase to grain or over expression of β-glucanase in transgenics (Almirall et al., 1995; Lisbeth et al., 1996).

β-glucan is a nonstarch polysaccharide composed of ß-(1→4)–linked glucose units separated every 2–3 units by β-(1→3)–linked glucose and is found only in some grasses and cereals (Carpita, 1996). A health claim for oat β-glucan was issued in 1997 (FDA, 1997). Higher grain β-glucan levels can be achieved with barley than with oats (Aman and Graham, 1987). β-glucan and arabinoxylan are the two major constituents of barley endosperm cell walls and β-glucan levels are lower in the hull and outer layers of the grain (Henry, 1987a).

The genetic analysis of β-glucan has historically been approached with the practical objective of lowering malt β-glucan levels. Because malting involves germination, these analyses involve both β-glucan synthesis and β-glucan degradation by β-glucanases (Han et al., 1995). Three β-glucanases genes are known, but since barley products and β-glucan extraction will likely be based on un-malted grain, these genes will not likely be as important for the development of barley varieties for human nutrition as the genes involved in β-glucan synthesis. Powell et al. (1985) reported that β-glucan content in barley grain is controlled by a simple additive genetic system. Burton et al. (2006) recently cloned and characterized a family of genes responsible for β-glucan biosynthesis, members of the family of *CslF* (cellulose Synthase-like) genes. Although over expression of these genes in Arabidopsis led to β-glucan synthesis, it is not apparent if there is allelic variation at this complex locus in barley germplasm that would allow breeders to select for higher grain β-glucan.

Breeding for high grain β-glucan has been facilitated by the pleiotropic effects of the waxy starch allele at the *WAX* locus (Patron et al., 2002). Positive correlations between grain β-glucan and waxy starch are well-documented (Xue et al., 1997). As a consequence, most barley varieties developed for human nutrition are waxy. Many of the original waxy starch germplasm accessions were also hulless, leading to the "profile" that high β-glucan food barley varieties are waxy and hulless. GBSS and the Nud locus (which determines hull retention) are both on chromosome 7H, but they are far enough apart (80cM) to show independent assortment (Fedak et al., 1972). Hulless varieties may have higher β-glucan due to elimination of the "diluting" effects of the hull (Fox, 1981; Xue et al., 1997). Hulless types could be more appealing for human food uses since de-hulling (pearling) would not be required. There are, however, concerns with hulless varieties due to lower yield potential (Brown et al., 2005; Cavallero et al., 2004), lower vigor (D. Obert, USDA/ARS, Aberdeen, Idaho personal communication) and, in the U.S.A, the lack of a recognized Federal grade for hulless barley. This means that hulless barley is not eligible for marketing loans and that hulless barley can only be insured for the same value as feed barley.

There is keen interest in diversifying dryland crop production in the Pacific Northwest of the U.S.A. In 2007, there were 1.55 million hectares of dryland cereal production in Oregon, Washington and Idaho. There are demonstrated advantages to incorporating spring barley into the prevailing wheat-fallow cropping system, including suppression of certain diseases and nematodes (Young et al., 1994; Smiley et al., 1994). Data are available on spring malting and feed varieties adapted to the region, but not for food barley. In order to determine the potential for human food barley in the region, we assembled an array of spring cultivars and advanced breeding lines and evaluated them for grain β-glucan and other quality and agronomic characters at two representative locations in northeastern Oregon over a two year period.

Materials and Methods

Four cultivars and 31 advanced breeding lines were obtained from four breeding programs, as described in Table 1.1. 'Baronesse' and 'Camas' are hulled, non-waxy spring feed cultivars. They are the most widely-grown varieties in the growing areas sampled in this study. 'Salute' is a commercially available spring variety intended for the human food market. It, and two other spring experimental lines, is hulled and waxy. All the other spring experimental lines but one ('02WA-7037.9') are waxy and hulless. This germplasm collection was characterized for food quality traits (grain β-glucan and protein), grain physical quality traits (test weight and kernel plumpness) and grain yield in 2006 and 2007 at the Sherman and Pendleton stations of the Columbia Basin Agricultural Research Center located near Moro and Pendleton, Oregon, respectively. The Moro station is at 45° 48' latitude north and 120° 73' longitude west. The Pendleton Station is at 45° 75' latitude and 118° 63' longitude. The soil type at both sites is a Walla Walla silt loam (Typic Haploxeroll). Moro and Pendleton are non-irrigated test sites typical of Pacific Northwest dryland cereals production zones. The annual average precipitation at Moro and Pendleton is 280 and 420 mm, respectively; about 75% of the precipitation occurs between October 1 and May 1. Available moisture and temperature during the two years of testing are shown in Tables A1 and A2. The tests were conducted in the context of longer-term rotations at both locations. At Moro, the preceding component in the rotation was winter wheat in 2006 and fallow in 2007. At Pendleton, the preceding components were spring wheat in 2006 and 2007. More residues were left on the surface at both locations in 2007 in order to better reflect minimum tillage production conditions.

Moro experiments were planted on March 15th in 2006 and 2007. The Pendleton experiments were planted on March 8th in 2006 and March 13th in 2007. The experiments were established using a Hege small plot drill in 2006 and a Fabro no-till small plot drill in 2007. The seeding rate was 236 seed per sq. meter in all experiments. The plot size was 1.64 X 6.6 meters in 2006 and 2.44 X 9.14 meters in 2007; the experimental design was a Randomized Complete Block (RCB) with four replications. The plots were harvested using a Wintersteiger plot combine.

In 2007, there were obvious differences in stand establishment between plots. "Vigor ratings" were made on May 7th at Moro and May 9th at Pendleton using a 1 to 10 scale; with 10 being a normal stand and 1 a poor stand. In order to determine if the vigor differences were related to differences in seed viability, the germination percentage of each cultivar/line was determined following the protocol of the Association of Official Seed Analysts (1993). Germination was measured on reserve seed of the same seed lot used for planting in 2007.

For the measurement of grain β-glucan and protein, samples were ground with a Udy Cyclone mill (Udy Corp., Fort Collins, CO) equipped with a 0.5 mm. screen. A subsample of this flour was used to determine mixed-linkage β-glucan percentage using the enzymatic assay procedure (EBC Method 3.11.1; Megazyme International Ireland Ltd.). Protein concentration was determined, using a subsample of the same flour, by nitrogen combustion (AACC-International, 2000). A subsample of the flour was oven-dried at 130 °C for 1 h. to calculate percent moisture. β-glucan and protein content were adjusted for moisture and expressed on a dry weight basis. The percentage of plump kernels was determined using a 100-g sample and a sieve with 0.24 - by 1.9-cm slotted openings per the protocol of the American Society of Brewing Chemists (1992). Grain test weight was calculated based on the weight of a sample of cleaned grain. Grain yield was determined based on the weight of grain harvested from each plot, adjusted for plot size.

Combined analyses of variance were performed across locations and years for all traits using the General Linear Models (GLM) procedure in SAS (SAS Institute, Cary, NC). To better understand environment and year interactions, data from of a sub-set of six representative cultivars/accessions were further analyzed by location and year using GLM. All effects were considered as fixed. The effect of hull type on quality traits was evaluated using orthogonal contrasts. F-protected LSD tests were used for mean separation. Simple correlation analysis was performed using the PROC CORR procedure in SAS. An across-location ANOVA was performed using the 2007 germination and plant vigor data. Contrasts were used to assess the effect of hull type on germination and vigor.

Results and Discussion

There were significant differences between cultivars and experimental lines for all traits (Table 1.2). Mean values for β-glucan varied from 3.2 to 7.0%, with '03AH2214' (a waxy hulless type) showing the highest percentage and Camas (a non-waxy, hulled type) showing the lowest. These values are similar to those reported by Hang et al. (2007) for waxy hulless and standard feed varieties grown in Idaho. The highest grain β-glucan value for barley reported in the literature is 10.7% (Bamforth and Martin, 1981). Minimum β-glucan levels of $\geq$ 5% are likely to be required by food barley processors (John Hamilton, Treasure Valley Renewable Resources, Ontario, Oregon personal communication). Thus it is apparent that acceptable β-glucan levels should be obtainable under dryland production conditions similar to those sampled at Moro and Pendleton in 2006 and 2007.

Mean values for grain protein ranged from 11.6 (Camas) to 14.2% ('01WA-1000.4'). These values are comparable to those reported by Hang et al. (2007) and are fairly typical of grain protein levels for spring barley under dryland conditions. There is genetic variation for grain protein content in barley; in an analysis of 1,400 accessions Polan (1968) found a range of 8.5-21.2%. The U.S. malting and brewing industry currently specifies a range of protein from 11.5 to 13.5% (AMBA, 2008). Although higher grain protein in animal feed and human should be a desirable attribute, there are currently no premiums paid for high protein, nor are specific protein fractions currently specified.

Mean values for grain yield ranged from 2864 (03AH3483) to 4656 kg ha^{-1} (Baronesse; Table 1.2). Compared to the feed barley check cultivars, the yields of the waxy, hulless cultivars were 15 to 38% lower. This yield reduction associated with the waxy, hulless trait has also been reported by others (Brown et al., 2005) and appears to be quite consistent across sites and years. Interestingly, no yield reduction was found when comparing the waxy, hulled cultivars to the feed barley checks. Therefore, cultivars such as Salute and 'BZ 502-563' may offer the best compromise for growers and processors looking for the waxy trait.

Test weights varied from 68 to 77 kg hL^{-1}. Among cultivars, the waxy hulless lines had significantly higher test weights than the hulled lines. Kernel plumpness ranged from 28 to 80%. In general, the hulled lines had a higher percentage of plump kernels compared to the waxy hulless lines.

The across years and locations ANOVA revealed that for all traits there were significant three-way interactions except test weight (Table 1.4). To explore these differences a subset of six representative cultivars were further examined. Table 1.3 reports the mean β-glucan, grain protein, grain yield, test weight, and kernel plumpness for each of these cultivars by location and year. In general, β-glucan content was quite stable across environments (Table 1.3). This overwhelming effect of genotype on β-glucan content is apparent in Table 1.5. Our results support the assertion that genetics is more important than environment in determining β-glucan content in grain (Gill et al., 1982). However, environment may also influence β-glucan content. Bendelow (1975) reported that dry conditions before harvest increase β-glucan. Savin et al. (1997) and Savin and Nicolas (1996) reported a decrease in β-glucan due to moisture stress during grain filling. In our study, the β-glucan content of Salute varied from 4.1 to 5.8% depending on environment (Table 1.3). If ≥5% β-glucan content is required by processors, then growing Salute under low rainfall dryland conditions such as those found in Moro may be a high risk practice.

Genotype had a lesser effect on grain protein: the location effect accounted for 57% of the total sums of squares. Average proteins were 14.5% and 11.8% for Pendleton and Moro, respectively. The environment is known to have significant effects on grain protein, with moisture and available nitrogen having major effects (Fathi et al., 1997). Torp et al. (1981), for example, reported that the protein content of barley of the same genotype varied from 8.1 and 14.7% at different locations with similar nitrogen fertilization levels.

Genotype x environment interaction for grain yield and yield components is the subject of extensive research in crops (reviewed by Manjit and Gauch., 1996) and in barley (e.g. Van Oosterom, 1993). In our experiments, the individual main effects of genotype, location and year were principal determinants for test weight, percentage

of plump kernels and grain yield (Tables 1.4 and 1.5). These results can be attributed to the preponderance of lower-yielding hulless lines vs. a limited number of higher-yielding hulled types.

There are two general reasons why hulless accessions may yield less than hulled types. The first is simply the weight of hulls, which estimated to be 11- 13% of the average grain yield (Bahtty, et al., 1975). Secondly, there is a much shorter history of breeding for agronomic performance in hulless type than for hulled types in the Pacific Northwest of US. Considerable improvement can be expected. In western Canada, for example, a serious effort at improving yield in hulless types was initiated in 1970 (Bhatty, 1999). In the specific case of our experiments, the yield of hulless lines was significantly lower the second year due to poor stand establishment.

At the time of seeding in the second year of the experiment, there was more surface residue on the surface of the soil at both locations in order to better represent the minimum/no tillage production systems of the Pacific Northwest. Visible differences in stand establishment were observed approximately one month after seeding at both locations. These differences were assessed using a subjective visual rating for vigor. There was a significant difference ($P<0.001$) for the contrast of hulless vs. hulled for vigor score. Hulless type may be more prone to physical damage to the embryo during harvest, leading to lower germination rates. However, in our experiments, differences in stand establishment were not due to seed viability. Germination percentages on reserve seed from the same lots used for seeding the 2007 trials were all greater than 96 %.

Additional research is warranted on the causes of poor stand establishment of hulless barley under reduced tillage. Potential causes of differences in stand establishment under reduced tillage include response to soil pathogens (Bockus and Shoyer, 1998) and response to lower temperatures during germination (Franzluebbers et al., 1995) It is important to understand the basis of poor stand establishment since the hulless types had, on average, significantly higher ($P<0.001$) grain β-glucan and grain protein. These significant differences, however, are due in part to the confounding effects of waxy vs. non-waxy starch and hulled vs. hulless in this sample

of germplasm. All but one of the hulless lines were waxy and would be expected to have higher β-glucan due to the pleiotropic effects of the waxy allele and hulless alleles on β-glucan content (Xue et al., 1991 and 1997). Three of the five hulled accessions have waxy starch (Salute, BZ-006, BZ 502-563). The average β-glucan level of these three accessions is significantly higher than those of the non-waxy hulled checks ($P<0.001$) and not significantly different from the average of the hulled waxy types.

β-glucan showed a modest positive correlation with grain yield (Table 1.6). Hang et al. (2007) found a modest negative correlation of β-glucan with grain yield and concluded that this could complicate simultaneous improvement for both traits. Their germplasm collection did not include high-yielding and high β-glucan varieties, such as Salute. We found a very high and positive correlation between grain protein and β-glucan. Fastnaught et al. (1996) and Hang et al. (2007) also reported positive relationships between the two traits.

Although the current market is most interested in β-glucan, there are potential markets for grain protein, making this positive correlation of potential economic value. Additional research, focusing on a set of high β-glucan cultivars and/or breeding lines, is needed to determine if this positive association would be useful for indirect selection: grain protein is simpler and cheaper to measure then grain β-glucan.

The approval of the barley health claim by the FDA, coupled with the increasing health consciousness of an American public faced with alarming rates of obesity and coronary heart disease, may increase interest in, and markets for food barley. To meet this demand, barley processors are likely to require production of waxy barley cultivars due to their high β-glucan contents. However, our research indicates that there are significant production problems and yield reductions associated with the hulless waxy trait. Therefore, waxy, hulled lines such as Salute and BZ 502-563 are likely the best alternative for growers and processors. Mechanical removal of the hull is easily accomplished by pearling. Because β-glucan levels are low in the hull and outer layers of the seed coat (Henry, 1987a), little value would be lost in this operation. Additionally, both Salute and BZ 502-563 show no production or yield

differences when compared to Baronesse and Camas, the hulled, non-waxy lines that are most widely grown in the dryland production zones of the Pacific Northwest. Thus, spring cultivars currently exist that are agronomically competitive with current feed barleys and have high β-glucan that could support a niche food barley market in the Pacific Northwest. Development of winter habit food barleys would provide growers and industry with even more choices.

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Table 1.1. Barley (Hordeum vulgare L.) genotypes tested for β-glucan, protein and other agronomic traits.

Cultivar or line	***Pedigree***	***Hull type***	***Starch type***	***Source***
Camas	ND5976/ND7159	Hulled	Normal	UofI*
01WA-10001.4	Bear/SH97142	Hulless	Waxy	WSU**
01WA-12501.2	CDC Candle/Meresse	Hulless	Waxy	WSU
01WA-13860.10	SH97142/Merlin	Hulless	Waxy	WSU
01WA-13860-4	SH97142/Merlin	Hulless	Waxy	WSU
01WA-13860-5	SH97142/Merlin	Hulless	Waxy	WSU
02WA-7037.10	WA10314-97/SH97142	Hulless	Waxy	WSU
02WA-7037.25	WA10314-97/SH97142	Hulless	Waxy	WSU
02WA-7037.9	WA10314-97/SH97142	Hulless	Normal	WSU
WA 9892-99	Wanubet/Baronesse	Hulless	Waxy	WSU
Salute (BZ 598-095)	ND-187-636-2/ND-187-631-10//*2 Baronesse	Hulled	Waxy	WestBred, LLC
BG 006	Nebula/Stanuwax	Hulled	Waxy	WestBred, LLC
BZ 502-563	Nishino Hoshi/Meresse	Hulled	Waxy	WestBred, LLC
Meresse-2	Merlin/Baronesse	Hulless	Waxy	WestBred, LLC
YU 501-039 (HB 813)	Merlin/*2 Baronesse	Hulless	Waxy	WestBred, LLC
BZ 598-161 (HB 811)	Merlin/waxy Hector	Hulless	Waxy	WestBred, LLC
Baronesse	Mentor/Minerva//mutant of Vada/4/Carlsberg/Union//Opavsky/ Sale/3/Ricardo/5/Oriol/6153 P40	Hulled	Normal	WestBred, LLC

03AH1170	Baronesse/Azhul	Hulless	Waxy	USDA-ARS[§]
03AH2214	Azhul/Thuringia	Hulless	Waxy	USDA-ARS
03AH2215	Azhul/Thuringia	Hulless	Waxy	USDA-ARS
03AH2229	Azhul/Thuringia	Hulless	Waxy	USDA-ARS
03AH2616	Bear/Bowman//CDC Alamo	Hulless	Waxy	USDA-ARS
03AH2651	Azhul/CDC Alamo	Hulless	Waxy	USDA-ARS
03AH2689	Azhul/CDC Alamo	Hulless	Waxy	USDA-ARS
03AH2854	Bear/Bowman//CDC Alamo	Hulless	Waxy	USDA-ARS
03AH2873	Bear/Bowman//CDC Alamo	Hulless	Waxy	USDA-ARS
03AH3052	10/Azhul//CDC Alamo	Hulless	Waxy	USDA-ARS
03AH3054	10/Azhul//CDC Alamo	Hulless	Waxy	USDA-ARS
03AH3058	10/Azhul//CDC Alamo	Hulless	Waxy	USDA-ARS
03AH3483	C2-94-220-15-1/Azhul	Hulless	Waxy	USDA-ARS
03AH3491	C2-94-220-15-1/Azhul	Hulless	Waxy	USDA-ARS
03AH6481	CDC Alamo/Otis	Hulless	Waxy	USDA-ARS
03AH6482	CDC Alamo/Otis	Hulless	Waxy	USDA-ARS

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[**]Washington State University, Pullman, WA, U.S.A.
[§]USDA-ARS, National Small Grains Research Facility, Aberdeen, ID USA
Table 1.1. (Continued)

Table 1.2. Means of grain yield, test weight, seed plumpness, β-glucan and protein of 33 barley genotypes grown at two locations in 2006 and 2007.

Cultivar or line	*β-glucan content*	*Protein content*	*Grain yield*	*Test weight*	*Plump kernels*
	%		kg ha^{-1}	kg hL^{-1}	%
Baronesse	3.5	12.3	4656.5	68	63
Camas	3.2	11.6	4288.0	70	70
01WA-10001.4	4.9	14.2	3452.0	76	37
01WA-12501.2	5.6	13.0	3714.0	76	54
01WA-13860-10	5.3	13.6	3809.0	77	60
01WA-13860.4	5.6	13.8	3554.5	75	49
01WA-13860.5	4.9	13.7	3834.5	77	49
02WA-7037.10	4.1	13.0	3631.5	76	51
02WA-7037.25	4.0	12.8	3947.5	75	46
02WA-7037.9	3.9	12.9	3930.0	73	47
WA-9820-98	4.1	13.0	3732.5	75	28
Salute BZ-598-095	5.1	11.9	4429.0	69	80
BZ-502-563	5.4	12.4	4600.5	68	74
BZ 598-161(HB811)	5.2	13.0	3715.0	77	47
Yu 501-0039 (HB813)	5.5	12.7	3782.5	76	54
BG 006	6.5	12.5	3695.5	64	78
Meresse-2	5.9	13.8	3270.0	77	50
03AH1170	5.0	13.3	3499.0	75	57
03AH2214	7.0	13.5	3460.5	77	71
03AH2215	6.6	14.1	3460.0	76	59
03AH2229	6.2	13.8	3066.5	77	78
03AH2616	6.0	13.1	3588.0	77	50
03AH2651	6.0	13.5	3478.0	76	60
03AH2689	6.2	13.2	3442.5	77	63
03AH2854	4.2	13.1	3665.5	75	45
03AH2873	5.3	13.1	3647.0	77	58
03AH3052	6.4	13.4	3183.5	76	42
03AH3054	6.1	13.5	3172.0	76	43
03AH3058	6.5	13.9	3202.0	76	47
03AH3483	5.8	14.0	2864.5	74	57
03AH3491	6.1	13.3	3246.0	75	61
03AH6481	5.2	12.8	3956.5	77	71
03AH6482	5.2	12.9	3865.5	77	72
Mean	5.3	13.1	3661	74	57
CV%	10.8	6.4	10.9	2.4	13.7
LSD(0.05)	0.57	0.83	276	1.2	5.4

Table 1.3. Means of grain yield, test weight, seed plumpness, β-glucan and protein of six representative barley genotypes grown at Pendleton and Moro locations in 2006 and 2007.

Cultivar or line	β-glucan content	Protein content	Grain yield	Test weight	Plump kernels
	%		kg ha^{-1}	kg hL^{-1}	%
		2007 Pendleton			
Baronesse	3.3	14.8	3743.8	68.0	78.3
Camas	3.3	14.3	2790.3	70.8	83.3
Salute BZ-598-095	5.8	14.1	3157.3	68.5	87.1
BZ-502-563	5.4	14.7	3418.3	66.3	79.8
Meresse-2	5.9	14.7	2081.8	75.8	74.4
03AH6481	5.7	14.7	3392.8	74.5	81.5
Mean	4.9	14.5	3097.3	70.6	80.7
LSD (0.05)	0.5	2.3	785.9	2.3	7.1
CV%	3.6	6.2	16.8	2.1	5.8
		2006 Pendleton			
Baronesse	3.5	13.0	4364.3	68.5	66.2
Camas	3.4	13.1	4528.0	70.5	64.1
Salute BZ-598-095	5.4	12.3	4463.8	71.0	86.7
BZ-502-563	5.3	13.0	4656.8	67.8	78.4
Meresse-2	5.7	14.8	3979.3	78.5	49.0
03AH6481	5.6	14.5	3838.3	77.5	64.1
Mean	4.8	13.4	4305.0	72.3	68.1
LSD (0.05)	0.4	1.0	516.6	1.9	7.9
CV%	3.5	2.8	8.0	1.7	7.7
		2007 Moro			
Baronesse	3.5	11.7	4939.0	67.8	72.3
Camas	2.8	9.5	4136.0	68.8	74.3
Salute BZ-598-095	4.1	9.9	4413.5	68.5	86.6
BZ-502-563	5.8	9.7	4523.8	68.8	92.0
Meresse-2	6.4	14.1	2293.5	75.8	53.5
03AH6481	5.1	10.7	3342.3	74.5	80.0
Mean	4.6	10.9	3941.3	70.7	76.4
LSD (0.05)	0.8	2.7	528.2	2.2	12.6
CV%	6.5	9.6	8.9	2.0	10.9
		2006 Moro			
Baronesse	3.4	9.7	5577.8	65.8	38.5
Camas	3.2	9.6	5698.0	70.5	59.3
Salute BZ-598-095	5.0	11.5	5677.5	67.3	63.1
BZ-502-563	5.0	12.3	5803.0	69.3	46.7
Meresse-2	5.8	11.8	4724.5	79.8	23.7
03AH6481	4.5	11.3	5251.3	78.3	61.5
Mean	4.5	11.0	5455.3	71.8	48.8
LSD (0.05)	0.7	0.6	450.5	5.6	14.0
CV	5.7	2.0	5.5	5.1	19.1

Table 1.4. Estimates of variance components for traits measured in 33 barley genotypes for seed yield, test weight, seed plumpness, β-glucan and protein grown in two locations in 2006 and 2007.

Component of variance†	***β-glucan content***	***Protein content***	***Seed yield***	***Test weight***	***Plump kernels***
$\hat{\sigma}^2_G$	7.336**	3.024**	2729384**	178.500**	2586.088**
$\hat{\sigma}^2_L$	1.881*	471.469**	120075064**	24.613*	17057.956**
$\hat{\sigma}^2_Y$	9.435**	0.021ns	281959039**	184.363**	40500.285**
$\hat{\sigma}^2_{R(YL)}$	4.711**	8.652**	889439**	25.483**	1061.061**
$\hat{\sigma}^2_{GY}$	0.446ns	1.239*	487931**	20.047**	269.127**
$\hat{\sigma}^2_{GL}$	0.374ns	0.993ns	240716*	4.422ns	281.348**
$\hat{\sigma}^2_{YL}$	3.258*	2.927*	1548798**	9.818ns	6931.726**
$\hat{\sigma}^2_{GYL}$	0.616*	1.189*	386254**	4.767ns	155.974**

Significance ***** $P<0.05$, ****** $P<0.001$

†G, genotype; L, location; R, replicate and Y, year

Table 1.5. Contribution of factors to total variation obtained in the analysis of variance for traits measured in 33 barley genotypes for seed yield, test weight, seed plumpness, β-glucan and protein grown in two locations in 2006 and 2007.

	Contribution of factor %				
Source	***β-glucan content***	***Protein content***	***Seed yield***	***Test weigh t***	***Plump kernels***
Genotype	65.7	11.7	14.6	67.68	40.1
Rep.	5.3	4.18	1.78	1.63	2.2
Environment	0.5	57	20.04	0.32	8.3
Year	2.6	0.003	47	2	19.6
G*E	3.4	3.5	1.28	1.6	4.4
G*Y	4.0	0.4	2.6	0.2	4.2
G*E*Y	6.4	4.8	2.32	2.0	5.7

Contribution of each factor defined as (factor sum squares/total sum squares x100)

Table 1.6. Pearson's simple correlation coefficients and P values for traits measured in 33 barley genotypes grown in two locations in 2006 and 2007.

	Trait			
Trait	***Test weight***	***Plump kernels***	***β-glucan content***	***Protein content***
Seed yield				
Pearson's correlation coeff.	-0.07778	-0.36244	0.2044	0.06664
P value	0.1227	<.0001	<.0001	0.1863
Sample size, no.	480	480	240	240
test weight				
Pearson's correlation coeff.		-0.18	0.01	0.01
P value		0.00	0.89	0.77
Sample size, no.		480	240	240
plump kernels				
Pearson's correlation coeff.			-0.1	-0.1
P value			0.0	0.3
Sample size, no.			240	240
β-glucan				
Pearson's correlation coeff.				0.9445
P value				<.0001
Sample size, no.				240

Development of Winter Food Barley: Marker-Assisted-Selection for Waxy Starch and Vernalization Sensitivity

Abstract

Marker assisted selection was used to rapidly develop barley germplasm with novel combinations of alleles at loci controlling starch synthesis and growth habit. The target loci were *WAX*, *VRN-H1*, and *VRN-H2*. The goal was to develop waxy starch, high grain β-glucan, and winter habit germplasm. Pre-screening of candidate parental lines identified combinations with target vrn-H1 (winter) alleles. This allowed for immediate fixation of favorable alleles at this locus. *VRN-H1* is coincident with a major low temperature tolerance QTL (*Fr1*). Perfect markers were based on functional domains in *GBSSI* and *ZCCT-H*, the genes determining *wax* and *VRN-H2*. Because spring growth habit at *VRN-H2* is due to gene deletion, codominant alleles at a tightly linked locus (*HvSNF2*), were also used. Backcross-derived germplasm was genotyped in two stages to sequentially select homozygotes at *VRN-H2* (BC1F1) and at *WAX* (BC1F2). Homozygotes at the two loci were identified in one-step screening in the progeny of the double cross. BC1F3 and DCF3 lines were advanced to field testing in the Fall of 2007. In subsequent generations, these lines will allow for testing hypotheses regarding the pleiotropic effects of waxy starch on β-glucan and vernalization genes on low temperature tolerance.

Introduction

Utilization of barley foods is increasing with awareness of the importance of fiber in a healthy diet. Health benefits of dietary fiber include reduction of bowel transit time (Feldheim and Wisker, 2000), lowering of blood cholesterol, and regulation of blood glucose levels for diabetes management (Frost et al., 1999). β-glucan is a complex carbohydrate that is a major cell wall constituent of barley endosperm (Izydorczyk and Biliaderis, 2000) and a component of dietary fiber. Barley has the highest average β-glucan content of any cultivated grain (Aman and Graham, 1987). In recognition of the value of barley as a source of β-glucan, the US Food and Drug Administration (FDA) approved a health claim for barley in 2006.

Selection for higher grain β-glucan is a first step toward developing varieties to meet the human food market. Burton et al. (2006) recently cloned and characterized barley *CslF* genes, candidates for β-glucan biosynthesis. Although over-expression of these genes in Arabidopsis led to β-glucan synthesis, it is not apparent if there is allelic variation at this complex locus in barley germplasm that will allow breeders to select for higher grain β-glucan. An alternative approach to selecting for higher grain β-glucan is to capitalize on a deletion (~ 400-bp) in the promoter and 5'-UTR of *GBSSI* that leads to a waxy starch with reduced levels of amylose and higher levels of amylopectin (Domon et al., 2002; Patron et al., 2002). A pleiotropic effect of this mutation is higher grain β-glucan (Xue et al., 1997) and this association can be used as a basis for to indirect selection. The deletion in *GBSSI* provides a perfect marker for transfer of the *wax* allele to non-waxy cultivars. Waxy starch has value in its own right as a preferred substrate for the development of modified starches for the food, papermaking, and adhesive industries (White, 1994).

All waxy starch barley varieties adapted to the Pacific Northwest of the US are spring habit. Winter habit varieties have higher yield potential in this region and make better use of scarce water resources. Winter-hardiness is a prerequisite for winter varieties. The genetic and physiological factors that determine winter-hardiness are myriad and complex. In the Triticeae, this complexity can be partitioned into low

temperature tolerance *per se* (Skinner et al., 2006), vernalization sensitivity, (von Zitzewitz et al., 2005) and photoperiod sensitivity (Szűcs et al., 2006).

These three characteristics are inter-related and the typical profile of fall-sown "winter" barley is that it is cold tolerant, vernalization sensitive, and photoperiod sensitive. Maintenance of the plant in a vegetative phase until a sufficient period of cold exposure is attained (vernalization sensitivity) or photoperiod reaches a certain critical length (photoperiod sensitivity) reduces the likelihood of exposure of differentiated reproductive tissues to low temperatures. Furthermore, there is evidence, in the case of vernalization sensitivity, that maximum levels of low temperature tolerance are achieved coincident with sufficient exposure to low temperature to satisfy the vernalization requirement (Limin et al., 2007). The coincidence of one of the two major low temperature tolerance QTL (*Fr1*) with *VRN-1* on the group 5 chromosomes of the Triticeae suggests that allelic variation at the latter locus could determine variation in low temperature tolerance (von Zitzewitz et al., 2005).

This model, however, is an oversimplification. "Facultative" barley germplasm is not vernalization sensitive, but it is cold tolerant (Karsai et al., 2001). As demonstrated by von Zitzewitz et al., (2005), this combination of characters is due to the presence of "winter alleles" at *VRN-H1* and a complete deletion of the *VRN-H2* locus on chromosomes 4H. *VRN-H2* encodes a repressor of *VRN-H1* and deletion of *VRN-H2* allows expression of *VRN-H1* – a meristem identity gene – from the juvenile stage onward (Szűcs et al., 2007). "Spring" genotypes lack the binding site in *VRN-H1* for the *VRN-H2*-encoded repressor; they may or may not carry the *VRN-H2* deletion (Szűcs et al., 2007). The low temperature tolerance QTL coincident with *Fr1* is significant in the Dicktoo (facultative) x Morex (spring) population (Skinner et al., 2006). This suggests that in facultative varieties *VRN-H1* may be regulated by a gene (or genes) other than *VRN-H2* and/or that a gene, or genes, in close physical proximity to *VRN-H1* are the determinants of cold tolerance. Because the functional basis of allelic variation at *VRN-H1* is known and this locus is coincident with *Fr1*, it provides a perfect marker for a gene that has a pleiotropic effect on low temperature tolerance and/or is tightly linked to the determinant gene (or genes). Likewise, since the

functional basis of *VRN-H2* is known and there is evidence that vernalization sensitivity may lead to maximum cold tolerance, alleles at this locus also provide perfect markers for marker assisted selection (MAS).

The application of MAS to plant breeding is expected to lead to greater efficiencies in terms of time, resource allocation, and response to selection (Collard, et al., 2005; Dubcovsky, 2004; Steele, et al., 2006). The availability of perfect markers for alleles at the *WAX* and *VRN* loci makes development of winter habit human food barley an ideal model for MAS. Prior to initiating the MAS program that is described in this report, we discovered that two waxy spring varieties – 'Waxbar' and 'Merlin' are actually facultative (unpublished data). That is, both have the winter allele at *vrn-H1* and the deletion of *Vrn-H2*. We hypothesized that introgression of the *waxwax* alleles from Waxbar and Merlin into the winter varieties 'Strider" and 'Luca' would raise grain β-glucan levels and that configuring dominant (functional) alleles at *Vrn-H2* (from Strider or Luca) with the recessive (winter) allele at *vrn-H1* (from any parent) would lead to vernalization sensitivity. Vernalization sensitivity, in turn, would lead to superior low temperature tolerance. Creation of this novel germplasm will allow us to test these hypotheses and to assess the utility of MAS for rapidly developing winter habit food barley germplasm that will meet the needs and interests of barley growers, processors, and consumers.

Materials and Methods

Germplasm and generation advance

Four barley accessions were used as parents. ‘Luca’ (P129/Dido) is a winter two-row experimental feed selection obtained from the Agricultural Research Institute (Martonvasar, Hungary). ‘Strider’ (OR1860164/Steptoe) is a winter six-row feed barley released by Oregon State University in 1997. ‘Waxbar’ (Male sterile 2-row population/Washonupana) and ‘Merlin’ (Male sterile 2-row population/BZ 585-85(Waxbar/TR-451)) are both two-row waxy starch varieties developed and marketed by Westbred, LLC. Waxbar and Merlin are described as “spring” habit varieties, but prior to the initiation of this study they were identified as carrying vrn-H1 alleles at VRN-H1 (unpublished data). The germplasm development process is outlined in Tables 2.1 and 2.2. Three single crosses were made between the parents (Luca/Waxbar; Luca/Merlin; and Strider/Merlin) in the winter of 2006. In the Summer of 2006, one double cross (DC) was made between the F1 of Luca/Waxbar and Luca/Merlin and each of the single crosses was backcrossed (BC) to the respective winter parent. The BC1F1 generation was grown in the Fall of 2006 and the BC1F2 generation in the Spring of 2007. MAS was applied at both the BC1F1 and BC1F2 generations. BC1F1 plants were screened with molecular markers (as described below) in order to select Vrn-H2Vrn-H2Waxwax genotypes. The BC1F2 generation (a target of eight plants per BC1F1 selection) was genotyped to identify waxwax homozygotes. As evident in Table 2.3, not all eight BC1F2 plants per selected BC1F1 plant germinated and were available for genotyping. For the double cross, MAS was applied at the F2 generation in two stages. The first set of 92 F2 plants was genotyped in the Fall of 2006 and the second set of 372 plants in the Spring of 2007. During each MAS cycle, F2 seedlings were screened with molecular markers in order to identify Vrn-H2Vrn-H2waxwax genotypes. Population sizes and the number of selected plants at each MAS cycle are shown in Tables 2.3 and 2.4. BCF1, BCF2, and double cross plants were grown in 55 cm3 pots. At the three leaf stage, seedlings were vernalized for seven weeks at 8° + 2°C with an 8h light/ 16 h dark photoperiod regime. After

vernalization, plants were grown under greenhouse conditions at 16° + 2°C day/night and with a 16 h light/ 8 h dark photoperiod regime.

Genotyping

DNA was extracted from seedling tissue using DNeasy plant maxi kits (QIAGEN Inc. California, USA). The allele-specific primers reported by Karsai et al., (2005) - ZCCT.HcF (5'-caccatcgcatgatgcac-3') and ZCCT.HcR (5'-tcatatggcgaagctggag-3') - were used for *Vrn-H2* genotyping. This is a dominant marker since genotypes with the winter (dominant) allele (Strider and Luca) have a 194-bp amplification product whereas the product is absent in spring genotypes with the *ZCCT-H* gene family deletion (recessive allele). A codominant marker based on *HvSNF2*, which is tightly linked to *VRN-H2* (Karsai et al., 2005), was used to corroborate the *ZCCT-HC* data and to distinguish heterozygotes from homozygotes at this locus (Szűcs et al. 2007). The primers were HvSNF2.02F (5'-cctggccacaaaaacaatcagc-3') and HvSNF2.04R (5'-gctgcattatagagaaacaacaacg-3'). The recessive (winter) *vrn-H1* allele was expected to be fixed in all cross combinations, but for corroboration, *VRN-H1* was assayed with BM5.88F (5'-gaatggccgctactgcttag-3') and BM5.89R (5'-gtctgagtcggttatatgcagg-3'). We designed these primers to amplify a ~800-bp fragment in the first intron of *BM5A* containing the "vernalization critical region" (Fu et al., 2005; Szűcs et al. 2007; von Zitzewitz et al., 2005). Primers waxP197.F (5'-caaacagacgacaagcggagaa-3') and waxP606.R (5'-tagaaaaagaaaacatcaagca-3'), amplify a 1000-bp fragment in the case of the dominant allele at *GBSSI* and a 600-bp fragment in the case of the recessive allele (Domon et al., 2002). In all cases, the PCR reaction mixture contained 40 ng templates DNA, 1 x Qiagen PCR buffer, 200 uM dNTPs, 0.6% Cresol Red, 1 x Q solution, 400 nM of each primer and 0.5 units of Taq DNA polymerase in a volume of 15 µl. Template DNA was initially denatured at 94°C for 2 min followed by 35 cycles of PCR amplification with the following parameters: 30 sec of denaturation at 94°C, 30 sec of primer annealing at 55°C and 1 min of primer extension at 72°C. A final 10 min of incubation at 72°C was applied at the end of the reaction. The amplified products

were separated on 1% agarose gels using 1 x TAE buffer, stained with 0.1 μg/ml of ethidium bromide, and visualized under ultraviolet light.

Statistical analysis

Chi square (x^2) tests were used to compare observed and predicted allele segregation ratios at each population/locus/MAS cycle combination.

Results and Discussion

As shown in Tables 2.1 and 2.2, the development of this germplasm - from making the initial F1 to planting "F3" generation head rows in the field - required slightly less than two years (22 months). The two MAS cycles required a total of one year under accelerated greenhouse conditions. If the entire germplasm development process had been carried out under field conditions, where winter temperatures satisfy vernalization sensitivities, each generation would require one year. If the initial F1 had been made in the field in the Spring of 2006, the F1 generation would have been planted in the Fall of 2006, the BC1 made under field conditions in the Spring of 2007, and the BC1F1 planted in the Fall of 2007. The savings of two years that were achieved, as compared to strictly field advance of germplasm, were due to the off-season starting date of the experiment (crossing in December) and accelerated generation advance under greenhouse conditions.

A total of 1301 plants (Tables 2.3 and 2.4) were screened using a total of 3,752 PCR reactions. This number of PCR reactions is higher than was absolutely necessary since for validation purposes *BM5A* was assayed in all BC1F1 during the first cycle of MAS and in the DC population. All progeny were monomorphic for this locus, as expected, and accordingly genotyping for *Vrn-H1* was not continued. Both *ZCCT-Hc* and *HvSNF2* were used to genotype *VRN-H2* since *ZCCT-Hc* is a dominant marker and because it was necessary to distinguish between homozygotes and heterozygotes for MAS in the BC1F1. No recombinants were detected between *HvSNF2* and *ZCCT-Hc* in any of the populations. Therefore, genotyping with *HvSNF2* alone would have been accurate and more cost effective. At current prices, the approximate cost for DNA extraction is $2.00 per sample and each PCR reaction is $1.00. Therefore the estimated total cost of reagents for genotyping was approximately $6,440. If only the two minimum essential markers (*HvSNF2* and *Wax*) had been scored, the approximate cost would have been $4236. For example Dreher et al. (2003) selecting for the *o2* recessive allele at the *opaque2* locus reported a cost of $1.91 per sample for genotyping. Dreher et al. (2003) and Moreau et al. (2000) concluded that the cost of using MAS per sample will vary depending on economies of scale.

The amplicon (allele) sizes for the four loci in the four varieties are shown in Table 2.5. Using the published primers for *GBSSI*, we found that our waxy accessions (Merlin and Waxbar) and non-waxy accessions (Strider and Merlin) had the same amplicon allele sizes as reported for the two types of germplasm by Patron et al. (2002) and Domon et al. (2002). This suggests a conservation of function in the Waxy allele and a common basis of the mutant (waxy) allele. In the case of the waxy allele, this is not surprising, since the cultivar Washonupawa, which were included in the survey by Domon et al. (2002) are in the pedigrees of Waxbar and Merlin. In the case of *BM5A*, the sequence of the vernalization critical region from Strider (AY750993), Merlin (EU331773), Waxbar (EU331774) and Luca (EU331772) were identical although Luca has a 118 bp deletion in 3` orientation to the vernalization critical region. The pre-screening of the waxy germplasm for allele types at *BM5A* increased the efficiency of our MAS project by allowing us to select facultative waxy accessions and thus fix target alleles at the *VRN-H1* locus, which is coincident with (or tightly linked to) *Fr1*, one of the major cold tolerance QTL. In the case of the *ZCCT-Hc*, the presence/absence of the gene corresponds with growth habit.

The observed segregation at *GBSSI* and *HvSNF2* in the BC1F1 generation fit expected 1:1 ratios in all three populations (Tables 2.6 and 2.7). In the BC1F2, the ratios for *GBSSI* fit the expected 1:2:1 ratio (Table 2.8). In the double cross F2 population, the observed ratios for *HvSNF2* and *GBSSI* both fit the expected 1:2:1 ratios (Tables 2.9 and 2.10) and the observe ratios for *ZCCT-Hc* fit the expected 3:1 ratio (Table 2.11). The perfect fit to expected ratios underscores the robustness of these perfect markers. The complete lack of missing data also justifies the use of more expensive DNA extractions and quality control procedures used for PCR. Higher throughput extractions and assays can increase efficiency, but these gains need to be weighed against the possibility of higher numbers of missing data points or unreliable allele calls (Forte et al., 2005; Mahuku, 2004).

This MAS experiment generated 120 BC1F3 lines, and 28 double cross-derived F3 lines, that are homozygous for target alleles at two key loci: *GBSSI* and *Vrn-H2*. These 148 lines are also homozygous for target alleles at *vrn-H1*. It is highly

unlikely that these same lines would have been identified without MAS. Furthermore without the genotype information on *VRN-H1* in Waxbar and Merlin, other waxy genotypes might have been selected that have spring alleles at this locus. In that case, selection for target alleles at *VRN-H1* would also have been required. This would have necessitated even larger populations and genotyping at an additional locus. If no selection for vernalization sensitivity or waxy starch was applied during generation advance, much larger populations would have been necessary at each generation to ensure that comparable numbers of F3 generation lines, homozygous for these target loci, were advanced to field tests.

Phenotypic selection for vernalization sensitivity and waxy starch is possible, but it would be expensive and would delay the process. Under fall-sown field conditions, it is not always possible to select for vernalization sensitivity: under these conditions, facultative and vernalization-sensitive genotypes flower at similar times. Confirmation of vernalization sensitivity requires a spring-sown progeny test, which would further delay generation advance. Selection for vernalization sensitivity under greenhouse conditions would require progeny tests or propagation of segregating generations for ~ 100 days in order to identify vernalization-sensitive lines (Szűcs et al., 2007). Both options would delay cycle time and require expensive controlled environment space. Phenotypic selection for waxy starch is possible during early generations, either by tactile assessment or iodine stain (Pedersen et al., 2005). However, neither method will identify heterozygotes nor both would be time-consuming. Likewise, direction measurement of grain β-glucan by enzymatic assays (Hang et al., 2007) is possible, but it would be expensive and hampered by the limited amount of seed available from individual plants, particularly plants grown in small containers under greenhouse conditions. A comprehensive comparative economic analysis of MAS vs. conventional selection is beyond the scope of this project. Likewise, the limited number of crosses sampled does not allow for generalizations on the merits of using backcross or double cross populations. However, the estimated total genotyping cost of $6,440 in the two types of populations seems justifiable, given the complications and uncertainties that would arise from not applying MAS.

It is even more difficult to quantify the indirect benefits of MAS: germplasm of know allelic architecture is a valuable resource for advancing knowledge of the genetics, physiology, and biology of target traits. The germplasm we have created will allow us to proceed, in subsequent generations, to test several important hypotheses. In the case of *GBSSI*, we will be able to empirically determine what, if any, increase in grain β-glucan is attributable to "converting" Luca and Strider to waxy types. This can be initiated in the Summer of 2008 with direct measurement of grain β-glucan in the F4 generation seed of selected lines. The grain β-glucan of these lines (and the variation between lines) can be compared to that in the four parents. In the case of vernalization genes, will be able to address, in the F4 generation, the question of whether coupling *Vrn-H2* with *vrn-H1* leads to superior cold tolerance. This will be done in the Winter of 2008/2009 in controlled freeze tests of selected lines. The percent survival of selected lines (and the variation among lines) can be compared to the case of the winter allele only at *vrn-H1* (e.g. in Waxbar and Merlin) vs. both alleles (e.g. Strider and Luca). Using the same *BM5A* primers, we can also determine if the *vrn-H1* winter allele from Luca, when coupled with *Vrn-H2* leads to different levels of cold tolerance than when coupled with the *vrn-H1* alleles from Merlin and Waxbar. These same primers will not differentiate between Strider and Merlin; additional allele sequencing in *BM5A* will be necessary to determine if the four varieties differing in other regions of this gene.

In summary, this research generated winter habit, waxy germplasm resources that should be useful for the development of winter hardy food barley varieties that will benefit growers by providing an alternative cropping and marketing options, industry with an abundant supply of quality food barley, and consumers with a nutritional tool for better health. These resources should also benefit science and breeding by providing genetic stocks for addressing fundamental questions regarding β-glucan content and winter hardiness.

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Table 2.1. Timeline for development of BC1F3 winter growth habit waxy starch barley germplasm using marker assisted selection (MAS).

Time	***Generation***	***Activity***
Winter 2006	Parental	Cross spring waxy x winter non-waxy
Spring 2006	F1	Backcross F1 x winter non-waxy
Fall 2006	BC1F1	First cycle MAS. Targets *Vrn-H2Vrn-H2 Wxwx*
Spring 2007	BC1F2	Second cycle MAS. Target *wxwx*
Fall 2007	BC1F3	Field assessment - head rows

Table 2.2. Timeline for development of double cross F3 winter growth habit waxy starch barley germplasm using marker assisted selection (MAS).

Time	***Generation***	***Activity***
Winter 2006	Parental	Cross waxy spring x winter non-waxy
Summer 2006	F1	Cross F1 x F1
Fall 2006	F2*	MAS. Targets: *Vrn-H2Vrn-H2 wxwx*
Spring 2007	F2*	MAS. Targets: *Vrn-H2Vrn-H2 wxwx*
Fall 2006	F3	Field assessment - head rows

*The F2 generation seed was divided into two lots and each lot was screened separately.

Table 2.3. Population sizes (numbers of plants) used for marker assisted development of 120 BC1F3 winter growth habit waxy starch barley lines.

Generation/MAS cycle	***Population***			***Activity***
	L/M//L*	L/W//L**	S/M//S***	
BC_1F_1	113	127	114	Screened
1st MAS Cycle	23	25	27	Selected
BC_1F_2	130	194	160	Screened
2nd MAS Cycle	32	49	39	Selected

*Luca/Merlin//Luca; **Luca/Waxbar//Luca; ***Strider/Merlin//Strider

Table 2.4. Population sizes (numbers of plants) used for marker assisted selection development of 28 double cross-derived winter growth habit waxy starch barley lines.

Generation/MAS cycle	***Population***	***Activity***
	Luca/Merlin//Luca/Waxbar	
F2 Fall 2006	91	Screened
MAS	8	Selected
F2 Spring 2006	372	Screened
MAS	28	Selected

Table 2.5. PCR-product sizes (bp) using allele-specific markers for WAX, BM5A, ZCCT-Hc and HvSNF2 in the barley varieties Luca, Strider, Merlin and Waxbar.

Gene	***Variety***			
	Luca	Strider	Merlin	Waxbar
GBSSI	1000	1000	600	600
BM5A	700	800	800	800
ZCCT-Hc	200	200	absent	absent
HvSNF2	500	500	700	700

Table 2.6. Observed and expected numbers of plants, and p values, from chi square tests for segregation at the WAX locus in BC1F1 populations derived from Luca (L), Merlin (M), Strider (S), and Luca (L). The PCR amplicon allele sizes are defined in Table 2.5.

Population	***Allele size (bp)***				***p-value***
	1000/1000		1000/600		
	Numbers of plants				
	Expected	Observed	Expected	Observed	
L/M//L	56.5	58	56.5	55	0.777
L/W//L	63.5	64	63.5	63	0.929
S/M//S	57	54	57	60	0.574

Table 2.7. Observed and expected numbers of plants, and p values, from chi square tests for segregation at the *HvSNF2* locus in BC1F1 populations derived from Luca (L), Merlin (M), Strider (S), and Luca (L). The PCR amplicon allele sizes are defined in Table 2.5.

Population	***Allele size (bp)***				***p-value***
	500/500		500/700		
	Numbers of plants				
	Expected	Observed	Expected	Observed	
L/M//L	56.5	58	56.5	60	0.510
L/W//L	63.5	63	63.5	64	0.929
S/M//S	57	60	57	54	0.574

Table 2.8. Observed and expected numbers of plants, and p values, from chi square tests for segregation at the WAX locus in BC1F2 populations derived from Luca (L), Merlin (M), Strider (S), and Luca (L). The PCR amplicon allele sizes are defined in Table 2.5.

Population	***Allele size (bp)***						***p-value***
	600/600		1000/600		1000/1000		
	Numbers of Plants						
	Expected	Observed	Expected	Observed	Expected	Observed	
L/M//L	32.5	32	65	58	32.5	40	0.287
L/W//L	48.5	49	97	95	48.5	50	0.955
S/M//S	40	39	80	76	40	45	0.653

Table 2.9. Observed and expected numbers of plants, and p values, from chi square tests for segregation at the *WAX* locus in F2 populations derived from Luca (L), Merlin (M), Strider (S), and Luca (L). The PCR amplicon allele sizes are defined in Table 2.5.

Population	***Allele size (bp)***						***p-value***
	600/600		1000/600		1000/1000		
	Numbers of Plants						
	Expected	Observed	Expected	Observed	Expected	Observed	
1^{st}L/M//L/W	22.75	25	45.5	43	22.75	23	0.834
2^{nd} L/M//L/W	93	90	186	188	93	94	0.937

Table 2.10. Observed and expected numbers of plants, and p values, from chi square tests for segregation at the HvSNF2 locus in F2 populations derived from Luca (L), Merlin (M), Strider (S), and Luca (L). The PCR amplicon allele sizes are defined in Table 2.5.

Population	***Allele size (bp)***						***p-value***
	500/500		500/700		700/700		
	Numbers of Plants						
	Expected	Observed	Expected	Observed	Expected	Observed	
1st L/M//L/W	22.75	22	45.5	47	22.75	22	0.951
2nd L/M//L/W	93	96	186	185	93	91	0.929

Table 2.11. Observed and expected numbers of plants, and p values, from chi square tests for segregation at the ZCCT-Hc locus in F2 populations derived from Luca (L), Merlin (M), Strider (S), and Luca (L). The PCR amplicon allele sizes are defined in Table 2.5.

Population	***Allele size (bp)***				***p-value***
	200/200 and 200/0		0/0		
	Numbers of Plants				
	Expected	Observed	Expected	Observed	
1st L/M//L/W	68	63	23	28	0.223
2nd L/M//L/W	279	271	93	101	0.333

General Conclusions

This research has demonstrated that there are commercially available varieties of spring barley (e.g. Salute and BZ 502-563) that, under dryland conditions in northeastern Oregon, can produce high levels of grain β-glucan and acceptable yields. Therefore, farmers and processors could immediately proceed with commercialization of these varieties to meet a niche food barley market in the Pacific Northwest. Across America, the obesity epidemic rages, diabetes is rampant, and heart disease is a leading cause of mortality. The FDA has stated that barley can be an important part of comprehensive dietary programs that lead to better health. Therefore, American consumers need to eat more barley.

Development of winter habit food barleys would provide growers and industry with even more choices. An accelerated program of marker-assisted development of winter food barley germplasm was initiated in order to provide these choices. The pre-screening of the waxy germplasm for allele types at *BM5A* increased the efficiency of our MAS project by allowing us to select facultative waxy accessions and thus fix target alleles at the *VRN-H1* locus, which is coincident with (or tightly linked to) *Fr1*, one of the major cold tolerance QTL. Marker assisted selection therefore focused on alleles at the *WAX* and *VRN-H2* loci. A total of 1301 plants tracing to three different backcrosses and one double cross were screened using a total of 3,752 PCR reactions at an estimated reagent cost of $6,440. This generated 120 BC1F3 lines, and 28 double cross-derived F3 lines, that are homozygous for target alleles at the two key loci. It is highly unlikely that these same lines would have been identified without MAS. These lines entered field trials in 2007 – laying the foundation for development of waxy germplasm resources that that will benefit growers by providing an alternative cropping and marketing options, industry with an abundant supply of quality food barley, and consumers with a nutritional tool for better health.

The germplasm we have created will allow us to proceed, in subsequent generations, to test several important hypotheses. In the case of *GBSSI*, we will be able to empirically determine what, if any, increase in grain β-glucan is attributable to "converting" Luca and Strider to waxy types. This can be initiated in the Summer of

2008. In the case of vernalization genes, will be able to address, in the F4 generation, the question of whether coupling *Vrn-H2* with *vrn-H1* leads to superior cold tolerance. This will be done in the Winter of 2008/2009 in controlled freeze tests of selected lines.

Longer term, this germplasm will also allow for assessment of the role of *VRN-H3* in low temperature tolerance, since we recently discovered that Waxbar is homozygous for spring (*Vrn-H3)* alleles at this locus, whereas Strider, Luca and Merlin are winters (*vrn-H3vrn-H3*) (unpublished data). Because this locus was not targeted in the development of this germplasm, all winter/waxy germplasm that is advanced should be genotyped for this locus and phenotyped for vernalization response concomitant with the low temperature tolerance phenotyping.

In preliminary controlled environment freeze test experiments conducted after the development of this germplasm, we also found that Waxbar and Merlin are much less cold tolerant than Dicktoo. After freeze testing at -13.5^{0}C, the percent survival values for Merlin, Waxbar, and Dicktoo were 5, 18, and 88%, respectively (unpublished data). As reported in this thesis the three varieties have the same critical sequences in *BM5A* that are assumed to define a winter allele at this locus. This suggests that a winter type vernalization critical region in *BM5A* may be necessary, but not sufficient, for low temperature tolerance. Therefore, this germplasm should be more fully characterized for its haplotype on chromosome 5HL for an extended region starting proximal to *Fr2* and ending distal to *Fr1*. Starting points for such an ambitious undertaking could include comparative re-sequencing of the *HvCBF* gene cluster (*Fr2*) as well as the complete *BM5A* gene. The results of sequencing of 5HL BAC clones spanning *Fr1* and *Fr2* (in progress) may also reveal additional candidate genes for characterization.

In summary, this project has been of practical, educational, and scientific utility. Barley is a unique model system that allows for simultaneous benefits to agriculture and science.

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Appendix

Table A1. Mean monthly rainfall during the growing period in the two locations in 2006 and 2007.

	Monthly Precipitation (mm)					
	Pendleton-2006	***Pendleton-2007***	***Average Pendleton†***	***Moro-2006***	***Moro-2007***	***Average Moro****
September	1.5	18.5	18.3	1.3	0.5	14.2
October	34.8	21.3	34.8	46.0	19.6	23.4
November	41.7	89.7	52.6	47.8	80.5	42.9
December	54.4	58.7	52.1	92.7	63.8	42.4
January	87.6	16.3	50.0	67.8	21.3	40.9
February	25.4	44.7	38.6	26.7	19.8	29.2
March	63.5	41.7	43.9	16.0	16.8	24.4
April	72.1	27.9	39.4	45.7	23.6	20.3
May	39.9	24.1	38.4	46.5	8.6	21.6
June	55.4	29.2	31.2	37.8	14.0	17.3
July	2.8	8.1	8.4	1.5	10.2	5.6
August	0.0	9.1	11.7	0.5	0.0	7.1
Total	479.0	389.4	419.4	430.3	278.6	289.3

*Average 77 years

Table A2. Mean monthly temperature during the growing period in the two locations in 2006 and 2007.

	Monthly Temperature (°C)					
	Pend-2006	***Pend-2007***	***Average Pendleton†***	***Moro-2006***	***Moro-2007***	***Average Moro****
September	14.7	16.4	15.8	15.0	16.1	16.4
October	11.1	9.2	10.0	10.3	10.0	10.6
November	4.2	5.8	4.4	2.8	3.9	4.4
December	-0.3	0.6	1.4	-1.9	-0.8	1.1
January	6.4	-0.6	0.0	3.6	-0.8	-0.3
February	2.2	3.6	2.8	1.4	3.1	2.5
March	6.4	7.8	6.1	4.7	7.0	5.8
April	9.5	8.6	9.5	8.6	7.8	9.2
May	13.3	13.6	13.6	13.1	13.1	13.1
June	17.8	17.8	17.2	17.0	16.4	16.7
July	22.2	23.1	21.1	22.2	22.2	20.9
August	19.5	19.5	20.6	19.5	18.3	20.3

*Average 77 years

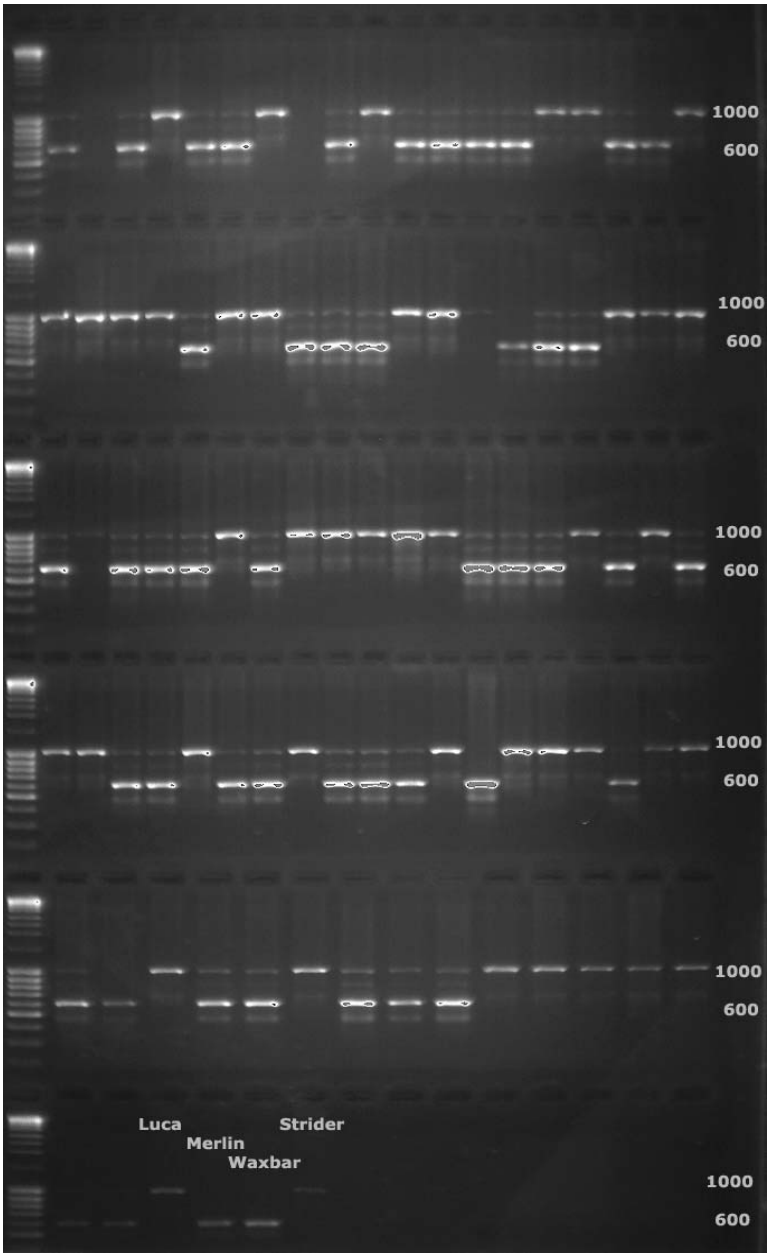


Figure A1. PCR analysis using primer waxP197.F and waxP606.R for *wax* gene in Strider x Merlin population. Plants showed a band of 600-bp identical to that of its waxy parent Merlin and these plants were, therefore assumed to carry *wax* gene in homozygous state. Similarly, plants were also selected from others BC_1F_2 populations.

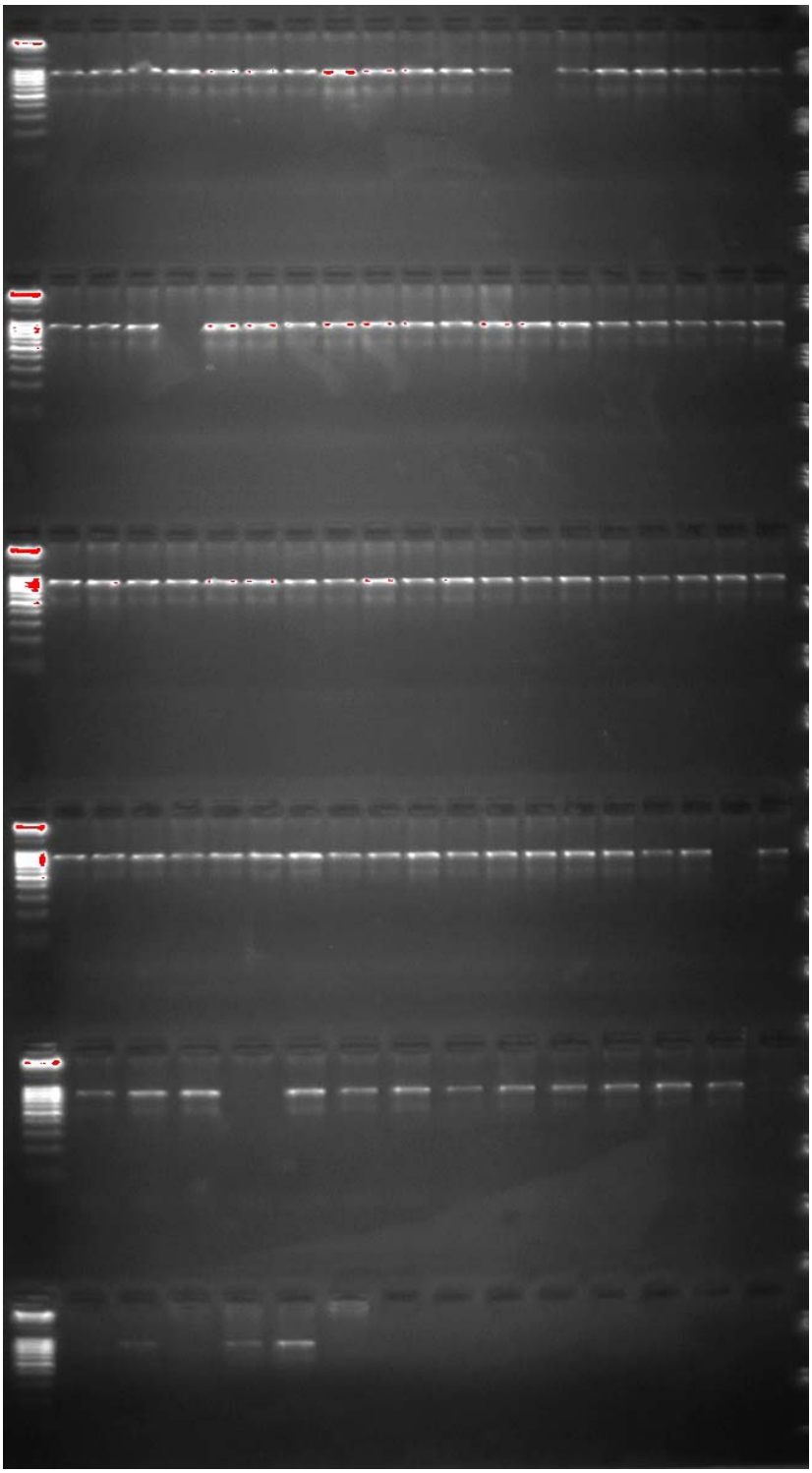


Figure A2. PCR analysis using BM5.88F and BM5.89R primers for vrn-H1 gene, the presence of the gene in a plant is recognized for a 1000-bp band, the absent of a band in the gel are gaps that then were confirmed as positive. The markers for vrn-H1 were run just in the first cycle of selection to confirm the presence of the vrn-H1 gene.

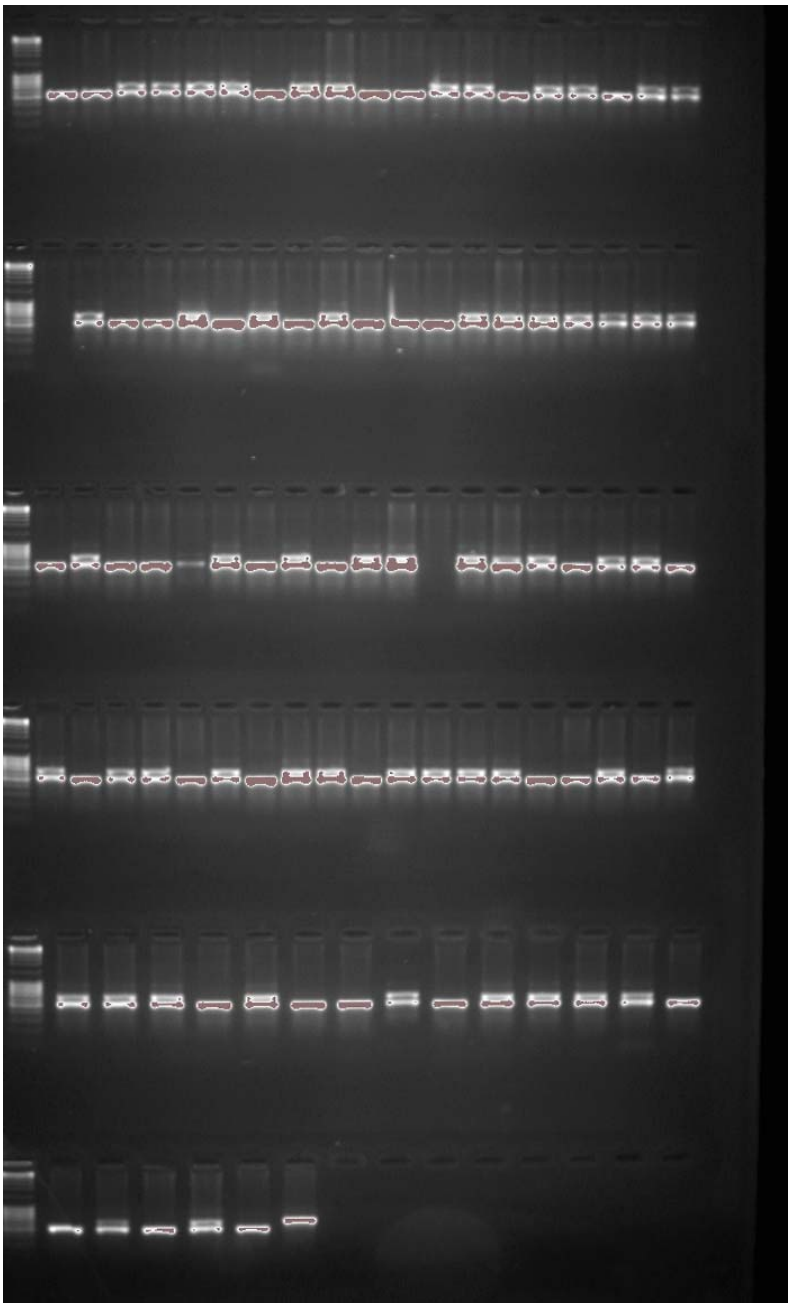
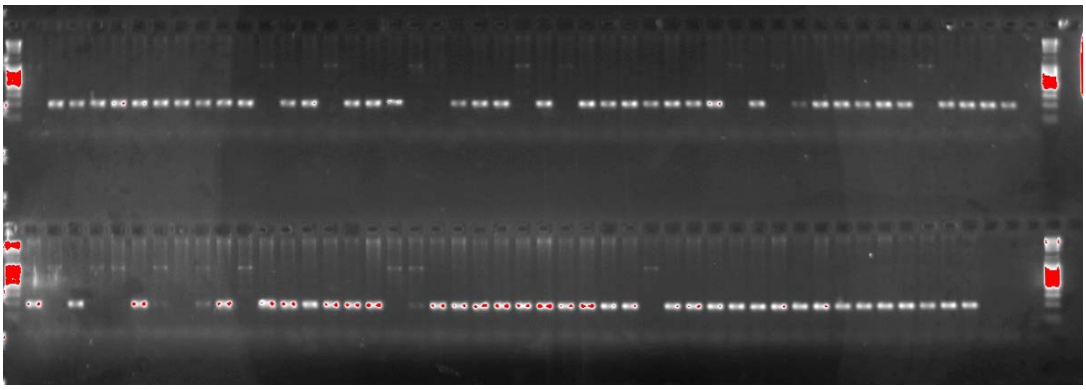


Figure A3. PCR analysis using HvSNF2.02F and HvSNF03R primers for the gene HvSNF2. We use these markers due to its tight linkage with the Vrn-H2 gene. Because the ZCCT-H marker is dominant HvSNF2 primers allow us to check if the individuals positive for the presence of the ZCCT-Hc gene are heterozygous or homozygous. The homozygous and the heterozygous were scored and goodness of fit was tested for wax and HvSNF2 loci for each MAS cycle (Tables 2.1, 2.2 and 2.3). Numbers of plants screened and selected for each MAS cycle are shown in table 2.4.



Figures A4. PCR analysis using ZCCT.HcF and ZCCT.HcR primers for Vrn-H2 gene, the presence of the gene in a plant is recognized for a 194-bp fragment.