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ORIGINAL PAPER

Structural and functional characterization of a winter malting barley

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Abstract The development of winter malting barley (*Hordeum vulgare* L.) varieties is emerging as a worldwide priority due to the numerous advantages of these varieties over spring types. However, the complexity of both malting quality and winter hardiness phenotypes makes simultaneous improvement a challenge. To obtain an understanding of the relationship between loci controlling winter hardiness and malt quality and to assess the potential for breeding winter malting barley varieties, we structurally and functionally characterized the six-row accession "88Ab536", a cold-tolerant line with superior malting quality characteristics that derives from the cross of NE76129/Morex//Morex. We used 4,596 SNPs to construct the haplotype structure of 88Ab536 on which malting quality and winter hardiness loci reported in the literature were aligned. The genomic regions determining malting quality and winter hardiness traits have been defined in this founder germplasm, which will assist breeders in targeting regions for marker-assisted selection. The Barley1 GeneChip array was used to functionally characterize 88Ab536 during malting. Its gene expression profile was similar to that of the archetypical malting variety Morex, which is consistent with their similar malting quality characteristics. The characterization of 88Ab536 has increased our understanding of the genetic relationships of malting quality and winter hardiness, and will provide a genetic foundation for further development of more cold-tolerant varieties that have malt quality characteristics that meet or exceed current benchmarks.

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Introduction

Barley (*Hordeum vulgare* L.) is cultivated throughout the world and its greatest economic impact is linked to the malting and brewing industry. Malting is the controlled germination and early seedling growth of barley grain and mainly involves processes of carbohydrate and protein hydrolysis (Bamforth and Barclay 1993). Currently, around one-third of the barley crop is used for malting (Baik and Ullrich 2008). There is no single definition for malting quality since malting and brewing practices, and consumer preferences, vary around the world. For example, in North America, the major brewers tend to prefer six-row malting varieties with higher protein and enzyme levels, while European brewers

prefer malt obtained from two-row barley varieties with higher starch content. A large number of parameters have been identified that contribute to malting quality, including: grain protein content, malt extract percentage, ratio of wort soluble protein to total malt protein, diastatic power, α-amylase activity, and malt β-glucan content. Many of these traits are interrelated (Burger and LaBerge 1985; Fox et al. 2003) and an initial understanding of the genetic control of malting quality is just beginning to emerge.

The genetics of malting quality is complex (Hayes and Jones 2000). Many quantitative trait locus (QTL) studies have been carried out to dissect the genetics of malting and 154 malting quality QTL associated with 18 traits have been recently summarized and placed on the Oregon Wolfe Barley (OWB) map (Szűcs et al. 2009). However, only a few of these genes have been fully characterized (Hayes et al. 2003; Szűcs et al. 2009). Large-scale gene expression technologies such as cDNA- and oligonucleotide-based arrays, and serial analysis of gene expression (SAGE) have proved to be useful to conduct transcript profiling during germination and malting (Lapitan et al. 2009; Potokina et al. 2002, 2004; Watson and Henry 2005; White et al. 2006), and to identify candidate genes for malting quality (Lapitan et al. 2009; Potokina et al. 2004, 2006).

As in the case of malting quality, the capacity to survive the winter (winter hardiness) also constitutes a complex phenotype that consists of three principal components: low-temperature (LT) tolerance, vernalization (VRN) response and photoperiod (PPD) sensitivity (Szűcs et al. 2006). Two major QTL on chromosome 5H are associated with LT tolerance in barley, *Frost resistance-H1* (*Fr-H1*) and *Fr-H2* (Francia et al. 2004). *Fr-H2* is coincident with a cluster of at least 11 C-repeat binding factor (*CBF)* genes, while *Fr-H1* co-segregates with *VRN-H1*, one of the three genes that control vernalization requirements in barley (Francia et al. 2004, 2007; Galiba et al. 2009; Stockinger et al. 2007). It is still unknown whether *Fr-H1* is a pleiotropic effect of *VRN-H1* or the effect of a physically linked gene (Galiba et al. 2009). *VRN-H2*, located on chromosome 4H, and *VRN-H3*, on chromosome 7H, are the other two genes that affect VRN response (Hemming et al. 2008 and references therein). *PPD-H1*, the major determinant of photoperiod-response under long-day conditions and located on chromosome 2H (Turner et al. 2005); and *PPD-H2*, which has been mapped to chromosome 1H and affects flowering time under short-day conditions (Faure et al. 2007), are the two genes that determine photoperiod sensitivity. Taken together, photoperiod, vernalization and LT tolerance pathways are highly interconnected (Galiba et al. 2009; Hemming et al. 2008; Karsai et al. 2008; Stockinger et al. 2007; Trevaskis et al. 2007).

Worldwide and especially in the USA, spring cultivars have been traditionally favored for malting. Due to the historical prevalence of spring malting barleys, the potential of winter malting varieties remains underexploited. Winter barley is an important crop alternative, providing advantages over the spring cultivars, such as increased yield and earlier maturation before the arrival of high temperature and water stress, which decreases the irrigation requirements (http://www.ambainc.org). For these and other reasons, the development of winter malting barleys is a high priority in the US. Recently, the American Malting Barley Association (AMBA) has recommended the first winter malting barley 'Charles', a two-row variety (Obert et al. 2006), as a malting variety (http://www.ambainc.org/ni/2009_Recommended.pdf).

Although there has been progress developing winter hardy malting quality varieties, combining winter hardiness with acceptable malting characteristics remains a challenge. To meet the needs of both producers and industry, barley breeders must simultaneously maintain or improve malting quality while improving low-temperature tolerance. Line 88Ab536 was cooperatively developed by the USDA-ARS and the Idaho Agricultural Experiment Station as a six-row winter barley with superior malting quality characteristics (Wesenberg et al. 1998). 88Ab536 (Morex/Ne76129// Morex) was derived from the backcross of the Nebraska line Ne76129 (winter habit feed barley) with the Minnesota line Morex (spring habit malting barley), where Morex was the recurrent parent. This line is unique in that it combines the best attributes of both parents: it is cold-tolerant and it has good malting quality characteristics. It was also the first and only US six-row winter barley to pass the AMBA plant scale malt testing. Although 88Ab536 was not ultimately approved by AMBA due to reasons other than malting quality (flavor concerns in brewery testing), it has been extensively used as a parent in the Oregon State University breeding program and it figures in the pedigree of many advanced winter malting lines. Therefore, characterizing the founder genotype 88Ab536 through defining regions of the genome determining malting quality and winter hardiness traits would assist breeders in targeting regions for MAS and accelerate variety release.

In the present work, we report the characterization of the founder genotype 88Ab536 at both structural and functional levels. Our objectives were to (1) determine the haplotype structure of 88Ab536 and define its malting quality and winter hardiness footprints; and (2) evaluate the potential of developing winter malting varieties.

Materials and methods

Plant material and micromalting conditions

The six-rowed facultative line 88Ab536 and the six-rowed spring cultivar Morex were used. 88Ab536 derives from a

Fig. 1 Barley seeds of Morex and 88Ab536 at time points "Out of steep" and "3d of germination" of the malting process

cross of NE76129/Morex//Morex. NE76129 was an experimental winter feed barley germplasm line from the University of Nebraska breeding program that was culled from the program and therefore not available for use in this study. Morex is a long established spring malting variety in the USA that appears in the pedigree of nearly all US six-row spring malting barley germplasm.

Grain samples of 88Ab536 and Morex were micromalted at the USDA-ARS Barley and Malt Laboratory of the Cereal Crops Research Units (CCRU) in Madison, Wisconsin, under standard CCRU micromalting conditions. Barley grains of 88Ab536 and Morex were steeped at 16°C for 24 and 29 h, respectively, to achieve an uniform steep-out moisture of 45%. Steeping involved alternating 4-h periods of immersion in water and air-rest. The hydrated grains were transferred to germinators maintained at 17°C and 100% relative humidity in the dark immediately after steeping. Samples were collected at the end of the steep period ('out of steep') and after 3 days in the germinator ('3d of germination') (Fig. 1). Three samples of each stage and genotype were harvested and placed in liquid nitrogen, and cleaned of emergent shoots and rootlets prior to microarray analysis. Finished malts derived from this experiment were subjected to malting quality analysis. A total of seven parameters commonly used to assess malt quality were measured following the standard ASBC (American Society of Brewing Chemists 2004) procedures: malt extract percentage (ME), grain protein content (GP), soluble protein (SP), ratio of soluble protein to total malt protein (S/T), diastatic power (DP), α-amylase activity (AA), and wort β-glucan concentration (BG). ME is one of the most important parameters for maltsters and brewers and measures the amount of soluble sugars and nitrogenous compounds obtained upon mashing malt into wort (Burger and LaBerge 1985). GP is a measure of the percentage of protein found in the grain and correlates with many of the other quality traits. SP is a measure of the amount of protein solubilized into wort after Congress mashing. S/T is a measure of the extent of protein mobilization reflecting proteinase activity, while both AA and DP measure the carbohydrate-degrading enzymatic activities. The latter includes the combined action of alpha-amylase, beta-amylase and the rest of the amylolytic enzymes (Burger and LaBerge 1985). Finally, BG reflects the amount of beta-glucan present in wort after Congress mashing. Beta-glucan is a byproduct of cell wall breakdown and provides a measure of the extent of malt modification.

Malting quality data corresponding to a common site and to the locations where the two genotypes were grown for the expression profiling experiments was compiled from historical data (http://www.ars.usda.gov/Research/docs.htm?docid=16513) and is shown in Supplemental Table 1.

Haplotype structure of 88Ab536 and QTL alignment

88Ab536 and Morex were genotyped with three 1536-plex pilot Oligonucleotide Pool Assays (pilot OPAs; POPA1, POPA2, POPA3) using the Illumina GoldenGate BeadArray SNP detection platform. The genotyping assays were conducted at the Southern California Genotyping Consortium at the University of California, Los Angeles. The SNP loci are designated by their POPA numbers (e.g., 1_1311), where 1 is the POPA number (POPA1 in this case) and the subsequent four digits correspond to the SNP order in the corresponding POPA. The locus designations can be directly referenced to assembly #35 unigene numbers, and linkage map position of each locus can be determined by referring to the barley SNP consensus map at HarvEST (http://harvest.ucr.edu and http://www.harvest-web.org). Many of those SNPs did not "survive" the quality checks.

Removal was based on low GenTrain score, missing data, and the lack of a consensus map position. Map orders and distances were transferred to an Excel spreadsheet, where each cell is 1 cM (Supplemental Table 2). For generating the final graphical haplotype of 88Ab536, all loci with allele calls in common between 88Ab536 and Morex were assumed to trace to Morex and are shown in blue. Allele calls that were different between 88Ab536 and Morex were assumed to trace to NE76129 and are shown in red. Common allele calls between 88Ab536 and Morex located in regions assumed to come from NE76129 are colored in pink. Only one locus was retained for each group of co-segregating loci. If all loci in the co-segregating group traced to the same parent, a single locus was selected at random to represent the map coordinate. To maximize our ability to detect small genome introgressions (or introgressions of any size in extensive regions of monomorphism), when there were one or more alleles in a cluster tracing to alternative parents, the locus with an allele call different from the two flanking loci was selected. Cells without mapped loci are shown in white.

Malting quality and winter hardiness-related genes and/or QTL were assigned to map positions based on their presence in POPA-3 or their position was inferred based on the OWB integrated map (Szűcs et al. 2009).

RNA isolation, microarray hybridization and data analysis

Total RNA from the 12 grain samples (two genotypes, two time points, and three replicates) was isolated using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) with phenol/chloroform/isoamyl alcohol (49:49:2) as a pretreatment, and passed through RNeasy columns (Qiagen, Valencia, CA, USA) for further clean up. RNA samples were quality assessed prior to labeling by means of an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). cDNA synthesis was conducted with 15 µg of total RNA and T7-Oligo(dT) primer (Proligo, Boulder, CO, USA) using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). The cDNA was purified with the Affymetrix GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA, USA). To produce biotinylated cRNA, the cDNA was transcribed in vitro using the Enzo BioArray HighYield RNA Transcript Labeling Kit (Enzo Life Sciences, Farmingdale, NY, USA) in the presence of biotinylated UTP and CTP. The biotin-labeled cRNA was purified with the Affymetrix GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA, USA). Labeled RNA (15 µg) was chemically fragmented using the Affymetrix GeneChip Sample Cleanup Module (Affymetrix), and used for hybridization. The chip hybridizations, washes, and data acquisition were conducted at the Biomedical Image Processing Facility at the University of Minnesota following standard Affymetrix procedures. Data files are available online from PlexDB's BarleyBase (experiment BB76, http://www.plexdb.org/modules/PD_browse/experiment_browser.php?expNo=BB76).

Data analysis of GeneChip experiments was conducted using the software GeneSpring GX 9.0 (Silicon Genetics, Redwood City, CA, USA). Expression estimates were calculated using gcRMA algorithm implemented in GeneSpring. Quality control on samples was performed using the correlation coefficients among replicates which were over 0.98, and the hierarchical clustering algorithm. Only probe sets with a signal intensity value greater than the 20th percentile in all three replicates for at least one condition were considered reliable and included for subsequent analysis. Differentially expressed genes were identified by unpaired *t* test with Benjamini–Hochberg false discovery rate (FDR) correction (Benjamini and Hochberg 1995). Analysis was done using a FDR adjusted *P* value of 0.01 as the cutoff, followed by filtering for two-fold or greater changes. For annotation purposes, BLASTX (*E* score cutoff = e^{-10}) data was exported from HarvEST:Barley version 1.68 (http://harvest.ucr.edu).

Sequencing of the mapped differentially expressed genes

A total of six differentially expressed probe sets were sequenced in Morex and 88Ab536. Total gDNA was isolated from seeds of both genotypes using the DNeasy plant mini kit (Qiagen, Valencia, CA, USA). Gene-specific primers were designed by Primer3 software based on the blast consensus sequence of each unigene and are shown in Supplemental Table 3. Amplification reactions were carried out in a 50 µl reaction containing 50 ng of gDNA, 0.5 units of HotStarTaq DNA polymerase (Qiagen, Valencia, CA, USA), 1× PCR buffer, 0.2 mM of each dNTP and 0.3 µM of each primer. PCR reactions were optimized to 95°C for 15 min, 33 amplification cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension of 10 min at 72°C. Following PCR, products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and subsequently sequenced at the Biomedical Genomics Center (University of Minnesota). From 700 to 1,900 base pairs were sequenced for each fragment (see Supplemental Table 3).

Results

Malting quality characteristics

To examine the malting quality characteristics of 88Ab536 and Morex, seven primary parameters used to assess malting quality were measured (Table 1). Mean values

Table 1 Malting quality characteristics of Morex and 88Ab536

	ME (%)	GP (%)	SP (%)	S/T (%)	DP (°ASBC)	AA (20°DU)	BG (ppm)
A. Malting quality data summaries of Morex and 88Ab536 grown in Aberdeen, Idaho, during crop years 2003 to 2006							
Morex	78.7 ± 2.1	13.1 ± 1.8	5.16 ± 0.48	41.5 ± 4.8	157 ± 37	60.3 ± 8.2	216 ± 113
88Ab536	78.5 ± 1.6	12.9 ± 1.7	5.20 ± 0.76	41.9 ± 4.3	147 ± 23	65.0 ± 10.0	235 ± 61
Guideline	>79%	≤13.5%	5.2–5.7%	42–47%	>140	>50	<120
B. Historic malting quality data for Morex (Crookston, MN, USA) and 88Ab536 (Corvallis, OR, USA) for crop years 2001–2005 from locations at which the grain for the microarray experiments were produced							
Morex	77.9 ± 1.2	14.2 ± 0.7	5.84 ± 0.84	42.4 ± 5.6	162 ± 17	67.5 ± 4.5	214 ± 116
88Ab536	78.0 ± 1.9	11.6 ± 0.8	4.73 ± 0.65	43.4 ± 5.7	135 ± 22	65.1 ± 5.3	213 ± 95
C. Malting quality data for Morex and 88Ab536 (three biological replicates each) from the malting run used for microarray experiments							
Morex	77.6 ± 0.3	14.2 ± 0.0	5.58 ± 0.04	40.3 ± 0.0	173 ± 5	71.5 ± 2.0	199 ± 20
88Ab536	77.0 ± 0.3	11.7 ± 0.0	4.70 ± 0.08	42.1 ± 0.7	127 ± 6	63.7 ± 2.6	215 ± 19

Means and standard deviations for each of the seven malting quality traits are shown

Guideline AMBA Ideal Commercial Malt Breeding Guidelines for 6-row barley varieties (see http://www.ambainc.org/ni/Guidelines.pdf, accessed July 7, 2009)

ME malt extract, *GP* grain protein content, *SP* wort soluble protein, *S/T* ratio of wort soluble protein to total malt protein, *DP* diastatic power, *AA* alpha-amylase activity, *BG* malt beta-glucan content

corresponding to 4 years of submissions from a common site (Aberdeen, ID, USA) are presented to provide a general malt quality characterization of both genotypes in comparison to the AMBA ideal commercial malt criteria for six-row barley (Table 1A). As had been judged previously during AMBA evaluation, 88Ab536 generally meets or exceeds the established malt quality benchmarks, as does Morex. Both lines had slightly lower ME than the "ideal" and higher than desired beta-glucan content (BG) when malted according to the standard conditions at the CCRU. The similarity of the averaged quality characteristics is remarkable. Because the Morex and 88Ab536 barleys used for the expression profiling were not grown at the same location, historic data of each genotype produced at comparable locations are detailed in Supplemental Table 1 showing location-year variation in malting quality attributes. Values for the seven primary quality-assurance parameters in finished malt for the Morex and 88Ab536 series (three malting replicates each) used in microarray experiments (Table 1C) are compared to the averages of the historic values from the experimental locations in Table 1B. The data show that the malting quality performance of the Morex and 88Ab536 samples used in the microarray experiments is representative of the lines' normal malting quality performance measured over a number of years.

Structural characterization of 88Ab536

Haplotype structure

To construct the haplotype structure of 88Ab536, we genotyped this line and the recurrent parent Morex with the SNPs available from POPA 1, 2 and 3 (http://www.barleycap.org). Many of these 4,596 barley SNP markers were eliminated when both genotypes were compared in order to generate a more precise graphical haplotype (see "Materials and methods" for detailed information). Therefore, a total of 723 unique SNP-mapped loci, together with the 372 gaps with no mapped loci, resulted in a final map length of 1,095 cM (Fig. 2).

Genome regions where SNP loci were polymorphic between 88Ab536 and Morex were assumed, with confidence, to trace to NE76129. Monomorphic loci located in polymorphic regions were also considered to come from NE76129 since it is unlikely that two rounds of crossing would produce the recombination events to result in small regions of Morex alleles with a NE76129 block (Devaux et al. 1995; Sall et al. 1990). Monomorphic loci between the two genotypes were assumed to come from Morex. This assumption involves a certain degree of error since there could be common allele calls in regions where the two genotypes differ. However, 542 of the 723 loci in 88Ab536 had allele calls common to Morex, giving an estimated recurrent parent genome content of 74.97%. This value agrees with the expected value of 75% derived from a single backcross.

Malting quality QTL

Malting quality QTL reported in the literature were assigned map positions on the 88Ab536 graphical haplotype based on the integrated OWB map (Szűcs et al. 2009). In this study, we focused on 40 of the 55 QTL for which Szűcs et al. (2009) assigned precise map coordinates based

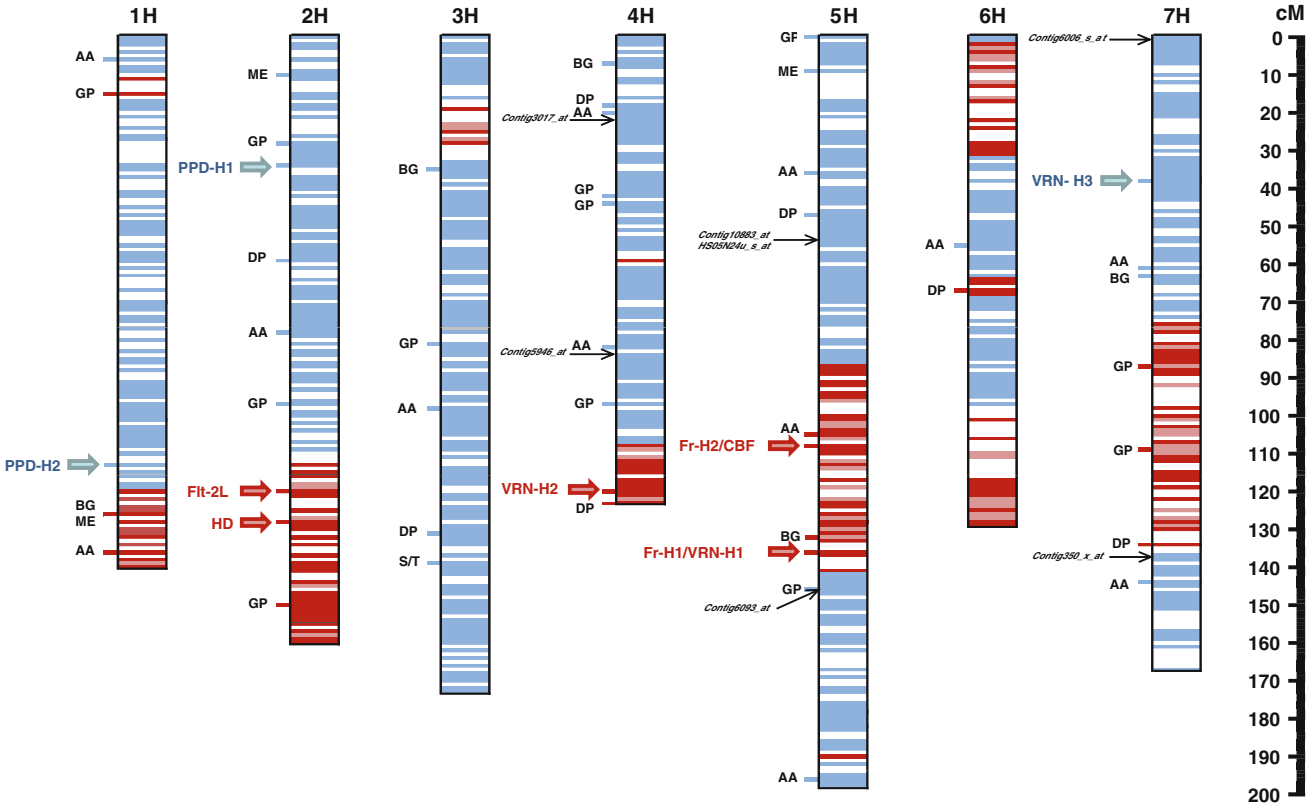

Fig. 2 Haplotype structure of 88Ab536. Regions that traces to Morex are colored in *blue*, while the *red color* represents regions that are derived from NE76129. Regions that are likely to come from the Nebraska parent are colored in *pink*. Map locations without a marker are colored in *white*. QTL of malting quality and winter hardiness traits are aligned. Differentially expressed genes with know map locations have been added to the 88Ab536 haplotype. *ME* malt extract, *GP* grain protein content, *S/T* ratio of wort soluble to total malt protein, *DP* diastatic power, *AA* alpha-amylase activity, *BG* malt beta-glucan content

on the presence of markers in common between the original QTL report and the integrated OWB map (Table 2). Only the QTL for the seven traits for which we have phenotypic measurements (Table 1) were placed on the map. As shown in Fig. 2, chromosomes 4H and 5H contained the highest number of QTL (8), while chromosome 6H had the least number of them (2).

Seventy percent of the malting quality QTL were located in regions where 88Ab536 alleles trace to Morex. Alpha-amylase (AA) activity and grain protein (GP) content were the parameters with the most QTL (12) placed on the haplotype structure. However, while most of the AA QTL were placed on regions derived from Morex, four of the GP QTL were located in genome regions tracing to NE76129. GP exhibits the highest number of QTL tracing to the feed barley parent. Diastatic power (DP) was also a well-represented trait, with seven QTL, of which three were located in genomic regions that traced to NE76129. There were five QTL for wort β-glucan content (BG), two of them traced to the feed barley parent. Three malt extract (ME) QTL were placed on the map, two of them on regions derived from Morex. Finally, the only QTL for the ratio of soluble protein to total malt protein (S/T) was located on chromosome 3H in a region tracing to the malting barley parent.

Winter hardiness QTL

Previously identified QTL associated with the capacity to overwinter were also aligned on the haplotype structure of 88Ab536 (Table 2). *Fr-H1* and *Fr-H2*, the main LT tolerance QTL (Francia et al. 2004), were located in an introgressed NE76129 region of chromosome 5H (Fig. 2). The candidate gene for *Fr-H2* is one or more members of two physically linked clusters of at least 11 *CBF* genes (Francia et al. 2007; Galiba et al. 2009; Skinner et al. 2005) whose expression is regulated by *Fr-H1* (Stockinger et al. 2007). *Fr-H1* is not only important for LT tolerance at the vegetative stage, but also for reproductive-stage frost tolerance (Reinheimer et al. 2004). *Flt-2L*, located in a region on chromosome 2H tracing to the cold-tolerant parent NE76129, is the other locus involved in this phenomenon of LT tolerance at the vegetative stage (Chen et al. 2009; Reinheimer et al. 2004).

VRN-H1 (synonymous with *HvBM5A*), one of the principal genes controlling VRN response in barley, is the

Table 2 Malting quality and winter hardiness QTL aligned on the 88Ab536 graphical haplotype (Fig. 2)

Chrom.	QTL	Trait	Pilot OPA	Position (cM)
1HS	AA (QAa.HaTR-1H.1)	Alpha-amylase activity	3_0933	6.0
	GP (QGpc.DiMo-1H)	Grain protein content	3_0955	15.3
1HL	PPD-H2	Photoperiod-H2	3_1526	112.9
	BG (QBgnm.StMo-1H.2)	Malt beta-glucan content	1_0207	126.5
	ME (QFge.HaTR-1H.2)	Malt extract	3_1387	127.4
	AA (QAa.StMo-1H)	Alpha-amylase activity	1_0041	135.6
2HS	ME (QMe.StMo-2H.2)	Malt extract	3_0775	10.1
	GP (QGpc.HaMo-2H.1)	Grain protein content	2_1261	28.4
	PPD-H1	Photoperiod-H1	2_1304	33.7
2HL	DP (QDp.StMo-2H)	Diastatic power	1_0883	59.2
	AA (QAa.StMo-2H.1)	Alpha-amylase activity	1_1388	78.0
	GP (QGpc.StMo-2H.2)	Grain protein content	1_1307	96.8
	Flt-2L	Flowering time-2L	2_0511	120.8
	HD	Heading Time	3_0041	127.6
	GP (QGpc.StMo-2H.3)	Grain protein content	2_0943	149.6
3HS	BG (QBgnm.HaTR-3H.1)	Malt beta-glucan content	3_0431	35.2
3HL	GP (QGpc.StMo-3H.1)	Grain protein content	2_1163	80.9
	AA (QAa.StMo-3H)	Alpha-amylase activity	3_0090	97.7
	DP (QDp.StMo-3H)	Diastatic power	2_0662	130.8
	S/T (QS/T.DiMo-3H)	Soluble/total protein	1_1328	138.8
4HS	BG (QBgnm.StMo-4H)	Malt beta-glucan content	2_0274	7.1
	DP (QDp.StMo-4H)	Diastatic power	3_0150	18.0
	AA (QAa.StMo-4H.1)	Alpha-amylase activity	2_0557	20.1
	GP (QGpc.DiMo-4H)	Grain protein content	3_0187	42.50
	GP (QGpc.HaTR-4H.1)	Grain protein content	3_0993	43.8
4HL	AA (QAa.StMo-4H.2)	Alpha-amylase activity	2_0197	81.7
	GP (QGpc.StMo-4H)	Grain protein content	3_0584	96.6
	VRN-H2 (OSU_VRN_H2_ZCCT_Ha_1430)	Vernalization-H2	1_0387	119.8
	DP (QDp.DiMo-4H)	Diastatic power	3_0824	125.4
5HS	GP (QGpc.HaTR-5H)	Grain protein content	3_0163	0
	ME (QFge.HaTR-5H.1)	Malt extract	2_1221	9.3
	AA (QAa.DiMo-5H)	Alpha-amylase activity	1_0580	35.7
	DP (QDp.StMo-5H)	Diastatic power	3_1492	47.4
5HL	AA (QAa.ChHa-5H)	Alpha-amylase activity	2_0403	105.2
	Fr-H2/CBF (OSU_HVCBF12_520)	Frost resistance-H2/C-repeat Binding Factors	3_0846	108.2
	BG (QBgnm.HaTR-5H.1)	Malt beta-glucan content	1_1472	132.5
	Fr-H1/VRN-H1 (OSU_VRN_H1_BM5A_intron1_vc_80)	Frost resistance-H1/ Vernalization-H1	1_0783	135.7
	GP (QGpc.DiMo-5H.2)	Grain protein content	1_0104	146
	AA (QAa.HaTR-5H.2)	Alpha-amylase activity	1_0322	196.1
6HL	AA (QAa.StMo-6H)	Alpha-amylase activity	2_1216	55.4
	DP (QDp.StMo-6H)	Diastatic power	2_0714	67
7HS	VRN-H3 (OSU_VRN_H3_HvFT1_585)	Vernalization-H3	3_0895	37.6
	AA (QAa.StMo-7H.3)	Alpha-amylase activity	3_0880	61.3
	BG (QBgnm.StMo-7H.2)	Malt beta-glucan content	1_0721	62.9

Table 2 continued

Chrom.	QTL	Trait	Pilot OPA	Position (cM)
7HL	GP (QGpc.HaMo-7H)	Grain protein content	2_1409	87.2
	GP (QGpc.DiMo-7H)	Grain protein content	2_0406	108.7
	DP (QDp.HaTR-7H)	Diastatic power	1_0861	133.8
	AA (QAa.HaTR-7H)	Alpha-amylase activity	3_1491	143.7

Locus designations from pilot OPAs and map positions are also shown. Names of QTL as they appear at the GrainGenes website (http://wheat.pw.usda.gov/ggpages/maps/OWB/) are added in parentheses

ME malt extract, *GP* grain protein content, *S/T* ratio of wort soluble to total malt protein, *DP* diastatic power, *AA* alpha-amylase activity, *BG* malt beta-glucan content

candidate gene for *Fr-H1* (Stockinger et al. 2007; von Zitzewitz et al. 2005). *VRN-H2*, encoding a zinc finger-CCT transcription factor that is a repressor of flowering (Yan et al. 2004), is the other key gene mediating VRN response. It is a complete *VRN-H2* deletion that makes 88Ab536 facultative (von Zitzewitz et al. 2005; Szűcs et al. 2007). Both *VRN-H1* and *VRN-H2* were located in regions derived from NE76129 (Fig. 2). Finally, *VRN-H3*, which encodes a promoter of the flowering orthologue to the Arabidopsis *FLOWERING LOCUS T* (*FT*) gene (Faure et al. 2007; Yan et al. 2006), was placed on a genomic region tracing to Morex (Fig. 2). There is limited allelic variation at the *VRN-H3* locus in most cultivated barley germplasm (Yasuda et al. 1993; Yan et al. 2006).

Regarding the two loci involved in photoperiod sensitivity, *PPD-H1* and *PPD-H2*, both of them were derived from the spring-variety Morex (Fig. 2). The fact that 88Ab536 carries the spring allele of *HvFT3*, the candidate gene for *PPD-H2*, indicates that this line is not sensitive to short-day photoperiods (Faure et al. 2007). As in the case of *PPD-H2*, the spring recessive allele at *PPD-H1* confers insensitivity to long-day conditions (Turner et al. 2005).

Functional characterization

To functionally characterize 88Ab536, we used the Barley1 GeneChip (Close et al. 2004) to identify the differentially expressed genes between this line and the recurrent parent Morex at two different time points of the malting process: "out of steep" and "3d of germination" (Fig. 1). Using a FDR adjusted *P* value of 0.01 and filtering by two-fold or greater changes, statistical analysis resulted in 40 differentially expressed transcripts at time point "out of steep" (Table 3) and 21 at "3d of germination" (Table 4). Five of these genes were found to be differentially expressed at both time points, therefore resulting in a total of 56 genes with significant differences in the expression level between 88Ab536 and Morex. Most of these genes (67.8%) had higher expression levels in 88Ab536 compared to Morex.

Over 30% of these 56 genes had no match to any sequence in the UniProt, Rice and Arabidopsis databases, or encoded proteins with unknown function. Regarding the classified gene transcripts, most of them coded for proteins with different functions. Interestingly, we detected six histones, five of them up-regulated in 88Ab536 at time point "3d of germination" (Table 4). Three retrotransposon proteins up-regulated in 88Ab536 at "out of steep" (Table 3), and three jasmonate-induced proteins, two of them up-regulated at "3d of germination" (Table 4), were also found among the most numerous differentially expressed genes.

A total of seven of the differentially expressed genes were previously mapped to the barley SNP consensus map (http://harvest.ucr.edu) and their map locations are shown in Tables 3 and 4. Two of them (HS05N24u_s_at and Contig10883_at) mapped to the same position and had the same unigene sequence, indicating that they are the same gene. These six genes were placed on the haplotype structure of 88Ab536. As shown in Fig. 2, all of them were located in regions tracing to Morex, which suggests that other genes located in regions introgressed from NE76129 might control their expression (*trans*-factors) (reviewed by Kliebenstein 2009). To further explore this possibility, we sequenced these six genes in 88Ab536 and Morex. Sequencing showed that both genotypes had the same sequence for all the six mapped genes (data not shown).

Discussion

The development of winter malting barley varieties has emerged as a new priority for breeders. Winter varieties exhibit considerable advantages over spring barleys, including higher yield, lower irrigation requirements and possibility of production in areas where certain fungal diseases (e.g., Fusarium Head Blight) are not as serious a risk. However, the genetic control of malting quality and winter hardiness is extremely complex and is just beginning to be unraveled. Thus, developing winter malting varieties is

Table 3 Differentially expressed genes between 88Ab536 and Morex at time point "out of steep"

Probe set	Fold change (88Ab536 vs. Morex)	Regulation	*E* value	Putative function	Map location[a]
Contig8119_at	1,451.42	Up	–	No hit	
Contig11923_at	1,207.13	Up	2.00E-35	Retrotransposon protein	
Contig3707_at[b]	767.23	Up	1.00E-180	TMS membrane protein/ tumour differentially expressed protein (TDE)	
Contig7247_at[b]	503.13	Up	4.00E-40	Protein csAtPR5	
HS07O08u_x_at	228.20	Up	–	No hit	
Contig14284_at	200.13	Up	5.00E-34	Retrotransposon protein	
Contig3780_x_at	92.88	Down	3.00E-45	Lipid transfer protein-like protein	
Contig2060_s_at	72.40	Up	9.00E-45	Early nodulin protein	
Contig10109_at	59.88	Up	8.00E-61	Heat shock factor protein HSF8	
HVSMEl0003B06r2_at[b]	51.90	Down	–	No hit	
Contig8490_at	44.51	Up	1.00E-172	Protein monooxygenase	
Contig3017_at	38.52	Down	1.00E-118	Oxalate oxidase GF-2.8 precursor	4H (21.6 cM)
rbags22p06_s_at[b]	37.18	Up	–	No hit	
Contig350_x_at	35.04	Down	1.00E-59	Histone H2A	7H (136.6 cM)
Contig18336_at	29.65	Up	4.00E-87	Mitochondrial glycoprotein	
HU11O24u_at	22.52	Up	2.00E-20	Unknown	
Contig4028_x_at	19.80	Down	6.00E-29	Unknown	
Contig2243_at	19.65	Down	1.00E-48	Hypothetical protein wrsi5-1	
HI02J18u_at	17.38	Up	1.00E-11	Cytokinin-N-glucosyltransferase 1	
Contig17647_at[b]	16.00	Up	2.00E-29	Os08g0100400 protein	
Contig13847_s_at	15.29	Up	3.00E-85	Subtilisin-like serine protease	
Contig8511_at	13.48	Up	–	No hit	
Contig18232_at	12.07	Down	4.00E-39	Os01g0896300 protein	
Contig4009_at	8.57	Down	0	Enoyl-ACP reductase	
HVSMEf0016E08r2_at	8.02	Down	–	No hit	
EBpi01_SQ001_L06_at	7.89	Down	–	No hit	
Contig2899_s_at	6.49	Down	1.00E-163	Jasmonate-induced protein	
Contig6093_at	6.46	Up	1.00E-108	Seed maturation protein	5H (146.0 cM)
Contig26251_at	6.10	Up	3.00E-14	Protein retrotransposon protein	
HVSMEb0009H14r2_x_at	5.85	Down	1.00E-39	Wali3 protein	
Contig2967_at	5.63	Down	1.00E-157	Expansin EXPB9	
Contig5946_at	4.61	Up	9.00E-55	Embryonic protein DC-8	4H (82.4 cM)
Contig26482_at	4.54	Down	–	No hit	
Contig20856_at	4.29	Down	4.00E-16	Unknown	
HVSMEk0003I10r2_x_at	3.90	Up	2.00E-22	Low molecular weight oleosin	
Contig8368_at	3.70	Up	1.00E-135	Succinate dehydrogenase	
HV14C07u_at	3.68	Down	1.00E-21	Unknown	
Contig7672_at	3.16	Up	1.00E-137	Protein phosphatase 2C (PP2C)	
Contig18163_s_at	2.56	Up	9.00E-22	Unknown	
Contig7373_at	2.18	Up	8.00E-30	Protein SEP2	

[a] Map locations determined from http://harvest.ucr.edu

[b] Genes also found to be differentially expressed at "3d of germination"

difficult due to the lack of knowledge and the complexity of the traits. 88Ab536 is a winter barley with superior malting quality characteristics that has been extensively used as a founder genotype for developing winter malting varieties. To obtain an understanding of the genetic relationship between malting quality and winter hardiness, we

Table 4 Differentially expressed genes between 88Ab536 and Morex at time point "3d of germination"

Probe set	Fold change (88Ab536 vs. Morex)	Regulation	*E* value	Putative function	Map location[a]
Contig3707_at[b]	927.71	Up	1.00E-180	TMS membrane protein/ tumour differentially expressed protein (TDE)	
Contig2905_s_at	658.05	Up	1.00E-152	Jasmonate-induced protein	
EBma03_SQ003_L08_s_at	525.54	Up	2.00E-73	Caffeic acid O-methyltransferase	
Contig30_at	481.82	Up	3.00E-53	Histone H4	
Contig119_at	358.20	Up	1.00E-59	Histone H2A	
Contig1177_at	331.72	Up	5.00E-61	Histone H2B.5	
Contig7247_at[b]	208.97	Up	4.00E-40	Protein csAtPR5	
HT07L18u_at	125.17	Down	─	No hit	
rbags22p06_s_at[b]	80.11	Up	─	No hit	
Contig124_at	66.71	Up	2.00E-70	Histone H3	
Contig6006_s_at	60.75	Up	0	Unknown	7H (0.6 cM)
HVSMEl0003B06r2_at[b]	56.10	Down	–	No hit	
Contig2905_at	55.70	Up	1.00E-113	Jasmonate-induced protein	
HS05N24u_s_at	53.77	Up	0	Vacuolar sorting receptor 1	5H (53.2 cM)
Contig17647_at[b]	21.07	Up	2.00E-29	Os08g0100400 protein	
Contig10883_at	17.33	Up	0	Vacuolar sorting receptor 1	5H (53.2 cM)
Contig14738_at	11.80	Up	1.00E-114	Integral membrane protein	
Contig1143_at	7.20	Up	8.00E-70	Histone H2B.2	
HVSMEl0007F21r2_s_at	6.31	Down	–	No hit	
Contig8732_at	3.28	Up	5.00E-60	15.9 kDa subunit of RNA polymerase II	
Contig20388_at	3.03	Up	1.00E-114	Unknown	

[a] Map locations determined from http://harvest.ucr.edu

[b] Genes also found to be differentially expressed at time point "out of steep"

characterized 88Ab536 using structural (4,608 pilot OPA SNP markers) and functional (22 k Affymetrix Barley1 GeneChip) genomic resources. This study provided the opportunity to (1) identify the genetic footprints of malting quality and winter hardiness; and (2) to assess the potential of developing winter malting barley varieties.

Malting quality footprints

88Ab536 exhibits malting quality characteristics comparable to its malting parent Morex (Table 1A), generally meeting the ideal commercial malt criteria established by AMBA (http://www.ambainc.org/ni/Guidelines.pdf). Since NE76129, the cold-tolerant parent of 88Ab536, is a feed barley with no reported favorable malting quality attributes, most favorable alleles for malting quality could be reasonably assumed to trace to the malting cultivar Morex. The high percentage of the malting quality QTL (70%) located in 88Ab536 regions tracing to Morex supports this assumption. There are, of course, examples in literature of favorable malting quality alleles tracing to parents with overall unfavorable phenotypes (Clark et al. 2003; Hayes et al. 1993).

Focusing on the specific traits, the allele at one out of the three ME QTL on the graphical haplotype was derived from NE76129 (Fig. 2). As the 88Ab536 values for ME were similar to the archetypical malting cultivar Morex (Table 1A), the NE76129 allele on chromosome 1H appears to have a neutral effect on the trait. In the case of GP, up to 4 of the 12 QTL were located in regions introgressed from NE76129 on chromosomes 1H, 2H, and 7H (Fig. 2). As in the case of ME, given the GP values for 88Ab536 and Morex (Table 1A), these four NE76129 alleles do not affect the trait. S/T values were also quite similar between 88Ab536 and Morex (Table 1A), which agrees with the observation that the only S/T QTL aligned on the haplotype structure was located in a region tracing to Morex (Fig. 2). For DP, 3 of the 7 QTL were located in genome regions of 4H, 6H, and 7H that traced to the feed barley parent. The QTL on chromosome 4H is coincident with the β-amylase gene *Bmy1* (Kreis et al. 1988). Re-sequencing of *Bmy1* in 88Ab536 confirmed the presence

of an *Sd4*-type allele whereas Morex is *Sd1* (Filichkin et al. submitted). No direct effects of *Bmy1* allele types on enzyme thermostability, enzyme activity, and diastatic power were found in an extensive characterization of North American six-row varieties and breeding lines, including 88Ab536 and Morex (Filichkin et al. submitted). Therefore, it is not possible to define the 4H DP allele tracing to NE76129 as positive or negative. However, the overall lower DP of 88Ab536 compared to Morex (Table 1A) suggests that substitution by the Morex alleles at one or more loci could be advantageous. Although only 2 of the 12 88Ab536 AA QTL traced to NE76129 (Fig. 2), their substitution by the Morex allele on chromosomes 1H and 5H could potentially improve AA (Table 1A). BG is the only malting quality trait in 88Ab536 that, due to its high value, did not fit specifications (Table 1A). High BG values are considered undesirable since they reflect a decreased level of modification during malting, leading to wort filtration difficulties (Bamforth and Barclay 1993). Two out of the three BG QTL were located in 88Ab536 genome regions tracing to NE76129 (Fig. 2). Although Morex had lower BG values (Table 1A), they are still too high for ideal performance at the brewery. As a consequence, future breeding effort should be directed towards the targeting and introgression of additional low BG alleles.

Samples of 88Ab536 and Morex from two key stages ('out of steep' and '3d of germination') in the malting process showed essentially identical morphologies (Fig. 1). Examination of their transcriptomes at those stages found that only a few genes were differentially expressed (Tables 3, 4). This similarity in the gene expression profiles of both genotypes is consistent with the similarity in their malting quality characteristics.

None of the 56 differentially expressed genes corresponded to proteins associated with mobilization of the major seed starch and storage protein reserves or cell wall carbohydrates during malting (Hayes et al. 2003; Potokina et al. 2004; White et al. 2006). One transcript (Contig13847_s_at) that was up-regulated in 88Ab536 compared to Morex at 'out of step' codes for a subtilisin-like serine endoprotease (Table 3). Serine endoproteinases shown to degrade malt beta-amylase have been hypothesized to indirectly affect DP (Schmitt and Budde 2007; Schmitt and Marinac 2008). However, no conclusive role in determining malting quality has been shown for the serine endoprotease corresponding to this transcript.

A few transcripts coding for histones, retrotransposon, and jasmonate-induced proteins were found among the differentially expressed genes, most of them up-regulated in 88Ab536 compared to Morex (Tables 3, 4). Differences in expression levels of histones have been found during seed germination in barley (Potokina et al. 2002) and their presence in this study could be due to differences in the cell division cycle activity between both genotypes. Many genes recently identified by Lapitan et al. (2009) as correlated to malting quality phenotypes coded for proteins involved in defense and stress response, among which jasmonate-induced proteins were the most abundant ones. Other previous studies on germinating seeds also identified these proteins (Watson and Henry 2005; White et al. 2006). Together with the three jasmonate-induced proteins found to be differentially expressed in 88Ab536, two genes encoding a heat shock factor protein HSF8 and an oxalate oxidase GF-2.8 precursor were up- and down-regulated in 88Ab536 at "out of steeping" (Table 3) were also found in the study of Lapitan et al. (2009).

Six of the differentially expressed genes had been previously mapped to the SNP consensus map and were placed on the haplotype structure (Fig. 2). Interestingly, all of them were located on regions tracing to Morex, which suggested that these genes are regulated in *trans* (Kliebenstein 2009). The sequencing of these six genes in both 88Ab536 and Morex supported this assumption since both sequences were identical for all the mapped genes.

Winter hardiness footprints

Since Morex is a very susceptible variety to winter injury, most favorable alleles of 88Ab536 for LT tolerance could be reasonably assumed to trace to NE76129. The graphical haplotype supported this assumption since the *Fr-H1/VNR-H1* and *Fr-H2/CBF* regions on 5H in 88Ab536 traced to NE76129 (Fig. 2). This also provides additional evidence for the importance of this genomic region in cold tolerance. The chromosome 4HL introgression accounted for the facultative growth habit of 88Ab536, it includes a complete deletion of *VRN-H2* (von Zitzewitz et al. 2005; Szűcs et al. 2007). The fact that Ne76129 was facultative due to a deletion of this locus was not entirely unexpected since we have also found *VRN-H2* deletion lines in germplasm arrays where most individuals are *VRN-H2* dominant (i.e. the gene is present) (Szűcs et al. 2008). The genomic region including *VRN-H3* allele traced to Morex (Fig. 2). The functionally recessive (winter allele) allele is present in most cultivated barley accessions; the dominant "spring" alleles confer very early flowering (Yasuda et al. 1993; Yan et al. 2006). Therefore, a "Morex" allele at this locus would not be unexpected in a "winter" genotype. The *Flt-2L* QTL involved in LT tolerance at the reproductive stage (Chen et al. 2009; Reinheimer et al. 2004) was placed on the introgressed NE76129 region of chromosome 2H. Although in principle this is a distinct phenomenon and this region has not been reported in vegetative LT tolerance, interestingly it has been retained in 88Ab536, suggesting an effect of the LT tolerance at the vegetative stage. Both *PPD-H1* and *PPD-H2* were located in genomic regions where 88Ab536

traced to Morex (Fig. 2). The late-flowering *PPD-H1* spring allele is recessive due to a mutation in the CCT domain and it is particularly important when a variety is sown in the spring since the long-day insensitivity conferred by this allele allows a prolonged growing period and, consequently, a higher yield (Turner et al. 2005). The fact that 88Ab536 also has the spring allele at *PPD-H2* (Szűcs et al. 2008), which confers insensitivity to short days (Faure et al. 2007), is not a desirable feature for a winter barley. Short-day photoperiod sensitivity would be better for a fall-sown variety because it will keep plants at the vegetative state longer (Pan et al. 1994), perhaps through the maintenance of the expression of genes conferring low-temperature tolerance (Fowler et al. 2001). Therefore, the substitution of this spring allele by the winter one would be desirable for greater cold tolerance that allows expanding the potential winter malting barley acreage.

Future prospects for developing winter malting varieties

The simultaneous alignment of the coincident malting quality and winter hardiness QTL reported in the literature on the foundation line 88Ab536 allows the identification of the alleles that are critical for the development of new winter malting barley varieties. Although 88Ab536 possesses most of the alleles that are needed for a cold-tolerant variety that fits commercial malting quality criteria, there is still room for improvement. Regarding winter hardiness, 88Ab536 carries the favorable winter alleles for the two main genomic regions involved in cold tolerance (*Fr-H1* and *Fr-H2*). Maintaining the 88Ab536 haplotype for this region of 5H will ensure a minimum level of cold tolerance. However, the level of cold tolerance displayed by 88Ab536 is still not sufficient to allow commercial production in all target environments. Ongoing efforts will reveal additional genomic regions involved in cold tolerance and/or alternative alleles at Fr-H1 and/or Fr-H2. The facultative growth habit of 88Ab536 is advantageous. Facultative varieties may be sown either in fall or spring, simplifying variety distribution and giving the option of re-planting with the same variety in the spring. Under controlled environmental conditions, facultative germplasm is amenable to accelerated cycles of breeding as it does not require a vernalization treatment (typically 6 weeks) between each generation. However, 88Ab536 has the spring allele at *PPD-H2*, the major photoperiod-response gene responsible for the short-day sensitivity (Fig. 2). Its substitution by the winter allele would be feasible and desirable to ensure that varieties do not initiate a vegetative-to-reproductive transition too early in the growing season. The favorable allelic state at *PPD-H1* will vary depending on target environments. One of the advantages of winter varieties in many environments is that they ripen prior to the onset of maximum summer temperatures. Under such situations, long-day sensitivity may be desirable. In environments where moisture is not limiting and full yield potential can be realized, long-day insensitivity would be desirable. Concerning malt quality characteristics, 88Ab536 approaches commercial expectations for malting quality parameters. The line was not commercialized due to beer flavor issues. No data are available for genetic dissection of beer flavor. Industry preferences and specifications for GP and DP may change with time. However, it is likely that ME cannot be too high and BG too low. The favorable allele for ME tracing to NE76129 may allow for future improvements in ME. Marker-assisted selection should be particularly useful for breaking the repulsion linkage between BG and winter hardiness due to the proximity of the BG QTL with *Fr-H1* and *Fr-H2* (Fig. 2). Since QTL mapping studies generally underestimate the number of QTL associated with quantitative traits, there are likely other undetected loci important for quality that will be segregating in breeding crosses made with 88Ab536. Thus, MAS at QTL for the favorable alleles identified in this study that are not linked in repulsion followed by phenotypic selection, should take advantage of both mapped and unmapped loci and result in improved malting quality and winter hardiness.

88Ab536 is testimony to the power of persistent plant breeding and phenotypic selection. These efforts have made possible this first and only six-row selection that combines winter hardiness and an AMBA-approved level of malting quality. Its structural and functional characterization has allowed us to understand how this breakthrough was achieved, letting us to create a template (footprint) for future variety development efforts by ensuring that the desired alleles are maintained at key regions of the genome. This will also facilitate the introgression of alleles determining other essential traits (e.g. agronomic performance, disease resistance) while maximizing the likelihood of meeting threshold values for winter hardiness and malting quality. The breeding approaches for future improvement will depend upon available resources, breeding strategies within individual programs, and accessibility to cheap, robust and high-throughput markers.

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