Plant Breeding **132**, 563–570 (2013)

doi:10.1111/pbr.12086

Plant Breeding

Application of marker-assisted selection and genome-wide association scanning to the development of winter food barley germplasm resources

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With 6 figures

Received December 27, 2012/Accepted May 14, 2013
Communicated by E. Igartua

Abstract

Barley (*Hordeum vulgare*) is an important component of heart-healthy whole grain diets because it contains β-glucan. All current US barley varieties with high β-glucan are spring habit and have waxy starch. Winter varieties have agronomic advantages but require low-temperature tolerance (LTT). Vernalization sensitivity (VS) is associated with higher levels of LTT. To rapidly develop fall-sown varieties with LTT and higher grain β-glucan, we therefore used marker-assisted selection (MAS) at the *WX* and *VRN-H2* loci. The MAS-derived lines, together with unrelated non-waxy germplasm developed via phenotypic selection (PS), were used for a genome-wide association scan (GWAS). The panel was phenotyped for grain β-glucan, LTT and VS. It was genotyped with 3072 single-nucleotide polymorphisms (SNPs) and allele-specific primers. Marker-assisted selection fixed target alleles at both loci but only one of the target phenotypes (higher β-glucan percentage) was achieved. Variation for VS and LTT is attributable to (i) incomplete information about *VRN-H1* at the outset of the project and (ii) unexpected allelic variation at *VRN-H3* with a large effect on VS and LTT.

Key words: food barley — low temperature tolerance — marker assisted selection — betaglucan — vernalization — winter barley

There is renewed interest in barley as a food due to its nutritional properties, particularly grain β-glucan. The benefits of barley β-glucan consumption include lowering total and LDL cholesterol, blood sugar and the risk of cardiovascular disease and type II diabetes (Ames and Rhymer 2008). β-glucan is a soluble fibre and non-starch polysaccharide composed of mixed-linkage (1,3;1,4)-β-D-glucan. In barley, it is a major component of cell walls of the endosperm and aleurone. Breeding for higher grain β-glucan can be based on the positive pleiotropic effects of waxy starch on β-glucan (Xue et al. 1997). In the homozygous recessive condition, alleles at the *Waxy* (*WX*) locus encoded by granule-bound starch synthase I (*GBSS1*) alter the amylose/amylopectin ratio. Higher amylopectin content in *wxwx* genotypes is due to a deletion in the promoter of *GBSS1* (Domon et al. 2002, Patron et al. 2002). The promoter deletion in *GBSS1* provides a perfect marker for indirect selection for increased β-glucan.

Winter barley can have agronomic advantages over spring-sown barley, but winter hardiness is essential. Winter hardiness is a complex trait involving low-temperature tolerance (LTT), vernalization sensitivity (VS) and photoperiod sensitivity (PPDS) (Hayes et al. 1993). The roles of VS and PPDS in LTT relate to the timing of the vegetative to reproductive transition. Low-temperature tolerance requires acclimation, and maximum LTT is achieved in the vegetative stage. Once the plant transitions to reproductive growth, there is a rapid loss of LTT (Fowler et al. 1996). Vernalization sensitivity delays the transition until a sufficient input of low temperature is received, and PPDS delays the transition until daylength is sufficient (Mahfoozi et al. 2000).

The genetic bases of VS, PPDS and LTT in barley were recently reviewed by von Zitzewitz et al. (2011) and Fisk et al. (2013). Briefly, VS is determined by three loci located on three different chromosomes: *VRN-H1* (5H), *VRN-H2* (4H) and *VRN-H3* (7H). A perfect marker is available for *VRN-H2*; a deletion in the first intron of *VRN-H1* provides, theoretically, a perfect marker, but the large number of possible deletions challenges characterization; and only polymorphisms of possible functional significance are known for *VRN-H3*. Genes with roles in photoperiod response are *PPD-H1* (2H) and *PPD-H2* (1H). Perfect markers are available for both. Three major LTT QTLs are reported: *FR-H1, FR-H2 and FR-H3*. *FR-H1* and *FR-H2* are both on chromosome 5H (Francia et al. 2004, Skinner et al. 2005). In barley, *FR-H1* cosegregates with *VRN-H1* (Stockinger et al. 2006) and coincidence is likely due to a pleiotropic effect of *VRN-H1* (Dhillon et al. 2010). Therefore, direct selection for *VRN-H1* is expected to be effective as an indirect selector for *FR-H1* and therefore LTT. A cluster of C-repeat binding factor (*CBF)* genes coincides with *FR-H2* (Skinner et al. 2006, Francia et al. 2007), and recent evidence supports the candidacy of these genes as determinants of the LTT QTL effects (Fricano et al. 2009, von Zitzewitz et al. 2011) Therefore, tightly linked and/or associated markers for LTT are available at *FR-H2*, but not a perfect marker based on a functional polymorphism. As yet, no candidate genes are reported for *FR-H3*, on chromosome 1H (Fisk et al. 2013).

We designed a marker-assisted selection (MAS) project for winter food barley based on selection for VS and waxy starch, hypothesizing that VS, when configured with proper alleles at LTT QTLs, would maximize LTT and that waxy starch would increase grain β-glucan. Concurrently, in a different germplasm base, we had initiated phenotypic selection (PS) for normal

starch and the hull-less character using winter and facultative (*sensu* von Zitzewitz et al. 2011) parents. The MAS and PS lines we developed were used for genome-wide association scans (GWAS) under the auspices of the U.S. Barley Coordinated Agricultural Project (CAP) (available at http://www.barleycap.org/ [verified 19 Dec. 2012]). A number of recent reports attest to the utility of the GWAS approach using barley as a model (Waugh et al. 2009, Cuesta-Marcos et al. 2010, Hamblin et al. 2010, Roy et al. 2010, Wang et al. 2010, 2011, von Zitzewitz et al. 2011). In this report, we focus on three traits of primary importance to the development of winter food barley: LTT, VS and grain β-glucan percentage.

Materials and Methods

The germplasm development process is shown in Fig. 1. The full germplasm array (n = 96) is identified as CAP IV. Entries within CAP IV are numbered consecutively based on the designation '09OR-01, 09OR-02', etc., where '09' indicated the year (2009) the germplasm was tested in the Barley CAP and OR refers to Oregon. Genotypic and phenotypic data on CAP IV are available at the *Triticeae* Toolbox (T3) (http://triticeaetoolbox.org [verified 19 Dec. 2012]). All germplasm was at the F_4 generation or higher. CAP IV consists of two types of germplasm, based on derivation and starch type. The MAS group (n = 64; 09OR-01 to 09OR-64) was developed by MAS in the BC_1F_1 and BC_1F_2 generations for the *wx* (recessive) allele at *GBSS1* and the winter allele (dominant) at *VRN-H2*. For validation, the MAS lines were genotyped at the BC_1F_4 generation using allele-specific primers. The MAS group consists of 54 two-row and 10 six-row barley types. There were four parents of the MAS lines: Luca (two-row, normal starch, hulled and winter growth habit), Merlin and Waxbar (two-row, waxy starch, hull-less and spring growth habit), and Strider (six-row, normal starch, hulled, winter growth habit). The PS group (n = 30; 09OR-65 to 09OR-94) was developed by PS for the hull-less trait and adaptation to Pacific Northwest conditions. All parents and progeny of the PS group are six-row types and have normal starch. Four parents were used to develop the PS lines: Strider (described above), Doyce (hull-less, winter growth habit), Maja (hulled, facultative growth habit) and Legacy (hulled, spring growth habit). The PS germplasm is not a formal control for comparison with the MAS population. Rather, it was unrelated food barley germplasm in the breeding programme that happened to be genotyped and phenotyped at the same time as the MAS population. To complete the panel of 96 lines, (64 MAS + 30 PS) Merlin and Waxbar were included as 09OR-95 and 09OR-96, respectively.

Field evaluations of the CAP IV were conducted over multiple years and locations. In this report, we focus on results from trials planted in the fall of 2009 at Hermiston (HER) and Corvallis (CVO), Oregon. HER was the only environment in which differential winter survival was observed, and CVO data from the same season was included for comparative purposes. The experimental design was a partially balanced lattice with three replications (CVO) and two replications (HER). Plot sizes were 7 m^2 and consisted of six rows. Seeding rates, fertility and weed management were in accordance with best local practice. At COR, there were 1091 mm of total precipitation between planting in 2009 and harvest in 2010. At HER, there were 233 mm of total precipitation between planting and harvest; therefore, irrigation was applied in accordance with

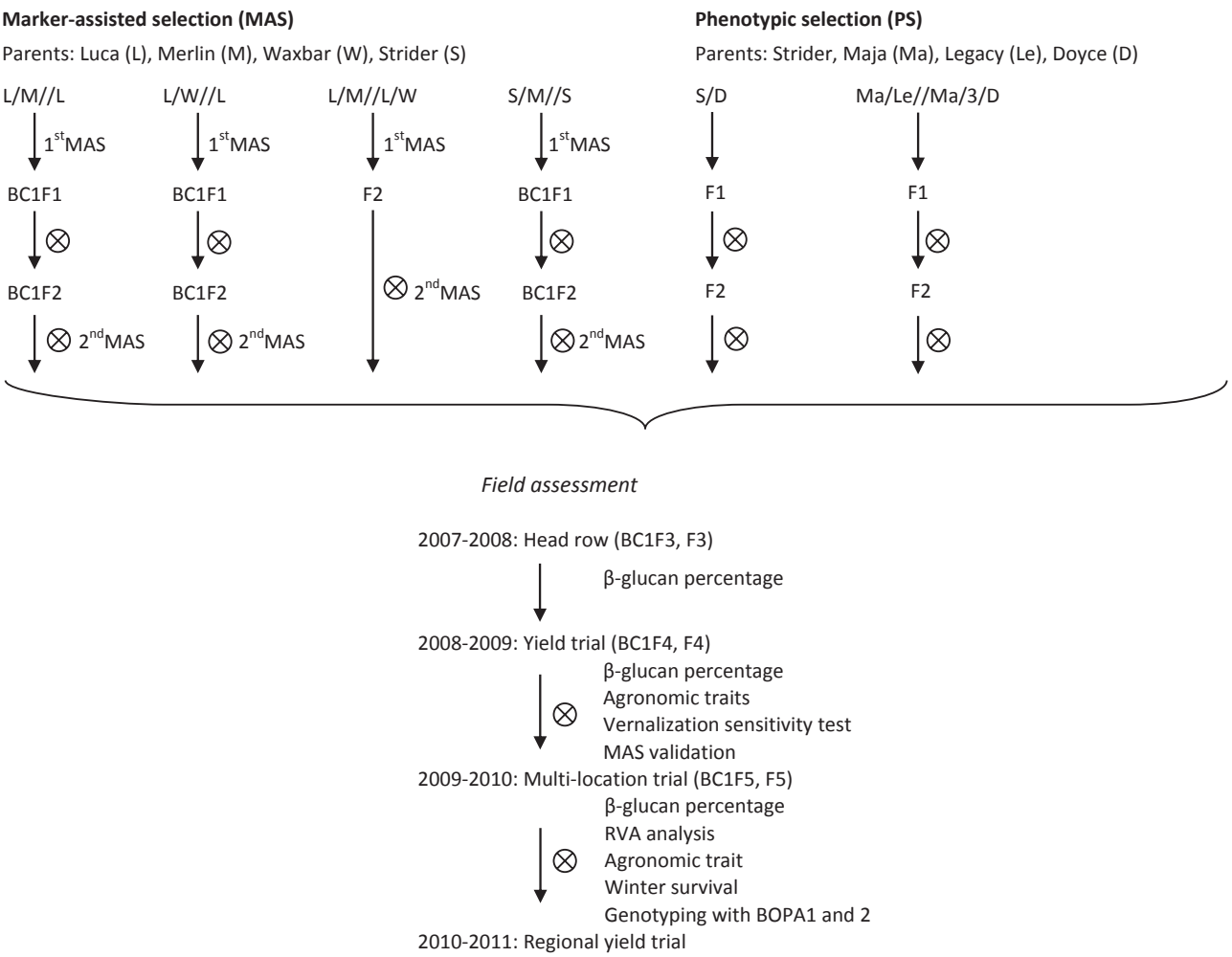

Fig. 1: Scheme for germplasm development of fall-sown barley with waxy and normal starch using marker-assisted selection and phenotypic selection

local practice via a centre-pivot system. Data were recorded for a range of agronomic and disease resistance traits, all of which are available at http://triticeaetoolbox.org [verified 4 Nov. 2012]. In this report, we focus on two traits from the field trials: grain β-glucan percentage and LTT. The latter was determined based on the visual assessment of the number of surviving plants when plots resumed growth after exposure to low temperature during winter. 'Luca', 'Merlin', 'Waxbar', 'Strider', 'Charles' [a winter-malting check selected by the American Malting Barley Association (AMBA)] and 'Eight-twelve' (the AMBA winter hardiness check) were included in the field trials.

Seed samples from each line from the first replication of each trial were ground in a Cleanmill. The resulting flour was used to determine the mixed-linkage β-glucan percentage following the method of Hu and Burton (2008) using Megazyme kits (Megazyme International Ireland Ltd.). β-glucan percentage was expressed on a dry-weight basis based on the McCleary method (McCleary and Codd 1991). Based on samples from CVO, the Rapid Visco Analyser (RVA) was used to categorize waxy and non-waxy starches as a function of paste viscosity and a peak time (Newport Scientific, Pty. Ltd., Warriewood, NSW, Australia). Tests were run according to a shortened profile of Crosbie et al. (2002).

Vernalization sensitivity was measured under greenhouse (GH) conditions using unvernalized plant material as described by von Zitzewitz et al. (2011). We also assessed flowering time with vernalization, as described in Data S1. There were two replications of each genotype. The number of days to flowering for each plant was recorded as the number of days from emergence until awns were first visible above the flag leaf sheath. Daily recording of flowering time was stopped 120 days after planting. Plants were kept an additional 30 days to ensure they would not flower in a normal fashion. Plants that did not flower were therefore assigned a days-to-flowering value of 150.

The CAP IV set was genotyped with 3072 SNP markers using Illumina GoldenGate Bead Array technology. The SNPs were designed from EST sequences and PCR amplicons and were organized into two Oligonucleotide Pool Assays (OPAs) known as Barley OPA1 and OPA2 (Close et al. 2009). In addition, the CAP IV set was genotyped for the *VRN-H1*, *VRN-H2*, *VRN-H3*, *PPD-H1* and *PPD-H2* loci using allele-specific assays, as described in von Zitzewitz et al. (2011), and for *GBSS1* using allele-specific primers described in Domon et al. (2002). Later, we developed an additional primer set of HvFT1.13F (5′CACCACGTCCCAAGAGTTTTC3′) and HvFT1.15R (5′GCGTACAACATCCACAGTCC3′) and product digestion with *AciI* to differentiate alleles at the promoter of *VRN-H3*.

Genome-wide association mapping was conducted with TASSEL V.3 (Bradbury et al. 2007) using three datasets: (i) the entire CAP IV set, (ii) the MAS population only and (iii) the PS population only. Luca, Strider and 'Eight-twelve' were included in the analysis for all three data sets. A linear mixed model was used to test for association between markers and traits of interest. Minor allele frequencies (MAF) lower than 0.05 were excluded. For the full CAP IV set, the population structure was determined using STRUCTURE 2.2 (Pritchard et al. 2000) with the linkage model described by Falush et al. (2003). Principal component analysis (PCA) was performed to determine population structure for the MAS data set using TASSEL V.3. For the full CAP IV set, better control of P-values was achieved using the Q matrix generated with STRUCTURE (QQ plots), whereas PCA gave us slightly better QQ plots for the MAS set.

The kinship (K) matrix was generated with TASSEL V.3 and was used to correct for relatedness of individuals. Only the K matrix was included in the model for the PS data set. For each GWAS, the cut-off P-value that controls the false discovery rate at $\alpha = 0.05$ was calculated using SAS v9.2 PROC MULTTEST (SAS Institute, Cary, NC, USA) and the method of Benjamini and Hochberg (1995). A linkage disequilibrium (LD) heat map for chromosome 5H was generated according to Shin et al. (2006). Two-way interactions between significant markers were tested with SAS v9.2 PROC MIXED using the restricted maximum-likelihood method. The Q matrix was included as a covariate, and the K matrix was the variance–covariance matrix of the random term (genotype). Using the same model, we also estimated the effects of individual markers.

Results

All of the 64 MAS-derived lines were homozygous dominant at *VRN-H2,* and 63 were homozygous recessive at *WX*. 09OR-16 is homozygous dominant for *WX*, indicating normal starch. All of the 30 PS progeny were dominant homozygotes at *WX* and had winter alleles at *VRN-H1*. At *VRN-H2*, 2 of 30 lines (09OR-65 and 09OR-68) were homozygous for the *VRN-H2* deletion (spring allele).

The RVA analysis identified two principal groups that correspond to the waxy (MAS) and normal starch (PS) germplasm (Fig. 2). The peak times of the waxy starch group were lower than those of the normal starch group. There were two lines (09OR-18 and 09OR-21) with intermediate peak times. Both are homozygous *wxwx*, suggesting that they have a waxy starch but have lower amylopectin/higher amylose content. 09OR-16 (homozygous *WxWx* based on genotyping) was in the normal starch group. The phenotypic distributions for grain β-glucan at CVO and HER were similar, bimodal and revealed phenotypic transgressive segregation (Fig. 3). Average grain β-glucan values were significantly ($P < 0.0001$) higher at HER than at CVO. At HER and CVO respectively, grain β-glucan percentage ranged from 5.0–7.0% to 4.1–6.3% in *wxwx* types. For *WxWx* types, the grain β-glucan percentages ranged from 3.5–5.0% to 3.0–4.5%.

All accessions in the panel, including Merlin and Waxbar, showed complete winter survival at CVO. The lowest minimum temperature was 2.3°C and occurred in December 2009 (Table S1). At HER, the temperature averaged ~0°C for 1 month and the minimum temperature (without snow cover) was −13°C. Considering the entire CAP IV set, the percent survival ranged from 0 to 100% (Fig. 3). Merlin and Waxbar were in the 0% group. Luca, Strider and 'Eight-twelve' had ≥95% survival. All PS lines were in the high survival group. Survival in the MAS lines ranged from 0 to 100%.

Without vernalization, there was a clear distinction between lines that did not flower and those that flowered (Fig. 3). Luca and Strider were in the non-flowering (VS) group. Merlin and Waxbar did not require vernalization to flower (FT = 46 and 64 days, respectively). Twelve of the MAS lines were VS, and the remainder (50 lines) showed a range of FT, from 31 to 91 days. All except two of the PS lines (09OR-65, 09OR-68) were VS.

The results of the structure analysis revealed four subpopulations ($K = 4$) for the full CAP IV data set (n = 96) plus checks (n = 3; 'Luca', 'Strider' and 'Eight-twelve'). For the MAS

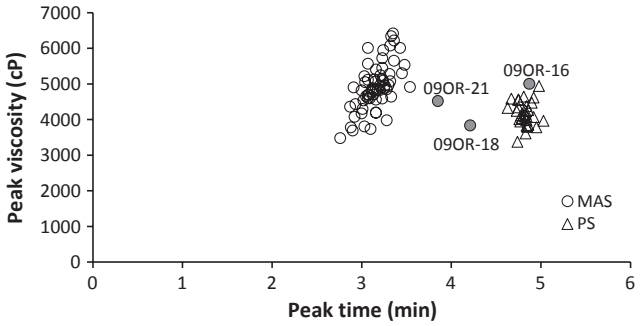

Fig. 2: Peak time vs. peak viscosity in barley CAP IV samples grown at Corvallis, Oregon. Triangle indicates the phenotypic selection group with non-waxy starch. Circle indicates marker-assisted selection (MAS) group with waxy starch. 09OR-18 and 09OR-21 are intermediate lines. 09OR-16 is a *WxWx* MAS line

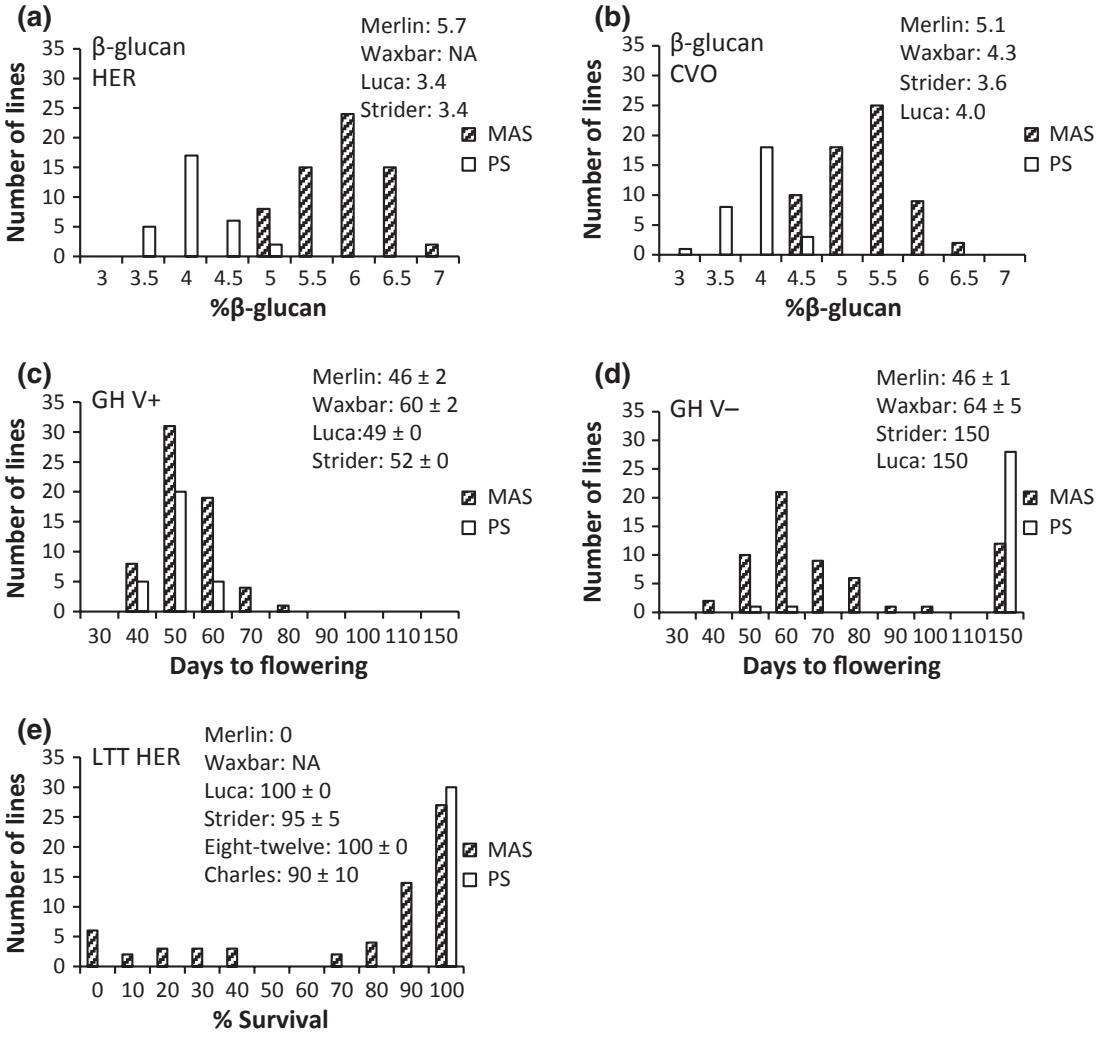

Fig. 3: Phenotypic frequency distribution for: (a) grain β-glucan percentage at Hermiston (HER), Oregon; (b) grain β-glucan percentage at Corvallis (CVO), Oregon; (c) Days to flowering under greenhouse (GH) conditions with vernalization (V+); (d) Days to flowering under greenhouse (GH) conditions without vernalization (V−); (e) Low-temperature tolerance evaluated at Hermiston (HER)

group, we accounted for structure using the first three principal components of the PCA analysis. The MAS group consists of n = 64 lines and 5 checks ('Luca', 'Strider', 'Eight-twelve', 'Waxbar' and 'Merlin'). For the PS group, there was no population structure detected, thus no correction was needed. The PS group consists of 30 lines and the same 5 checks as for MAS.

Considering the full CAP IV data set, GWAS revealed significant associations of markers on chromosome 7H with grain β-glucan at both locations (Figs 4 and S1). These significant markers include a perfect marker within the *GBSS1* gene and SNPs in LD with *GBSS1*. For the MAS lines, ~4 cM from *GBSS1*, there was also a strong association (P = 0.001) with marker 2_0227).

For LTT in the full data set, there were significant marker–trait associations on chromosomes 5H and 7H and a strong association on 6H (Fig. 5). The 5H and 7H associations are with *VRN-H1* and *VRN-H3*, respectively. As shown in Fig. 6, on 5H, the most significant markers (3_0590 and 1_1080) are 5 and 1 cM from *VRN-H1*, respectively, according to the consensus map (Close et al. 2009). The most significant marker on 7H was 1_0056, which is ~3 cM from *HvFT1* (*VRN-H3*). On 6H, 1_1455 approached the false discovery threshold. There are no genes associated with LTT reported in this region. In the MAS subset, there were associations on 5H and 7H that mirrored the full CAP IV set. There were no significant associations in the PS subset.

For VS in the full data set, there were significant marker–trait associations on chromosome 7H (Fig. S2). The significant markers were in the first intron of *HvFT1 (VRN-H3)*: 3_0894 and 3_0895, at positions 471 and 585, respectively. There was a strong association at 2_0119 (4H), which is 20 cM proximal to *VRN-H2*. The GWAS of the MAS group (Fig. S2) showed a significant association at *VRN-H3*, with the most significant marker defined by the primers HvFT1.03F/04R+*BclI* (Yan et al. 2006). Marker 3_0895, which showed a significant association in the full CAP IV set, did not approach the false discovery threshold in the MAS population. In the PS group, the GWAS shows a significant association between *VRN-H2* and VS. There was no significant association of *VRN-H3* markers with VS within the PS group. All PS lines are monomorphic for *VRN-H3* markers, except for 09OR-68, which showed a polymorphism at 3_0894. This line is not VS.

Haplotypes for the *FR-H1*, *FR-H2*, *FR-H3*, *HvFT1*, *VRN-H1*, *VRN-H2* and *VRN-H3*, *PPD-H2* and *Wx* regions for the 'best lines, in terms of LTT' from the MAS and PS populations, as well as the parents, are shown in Table S2.

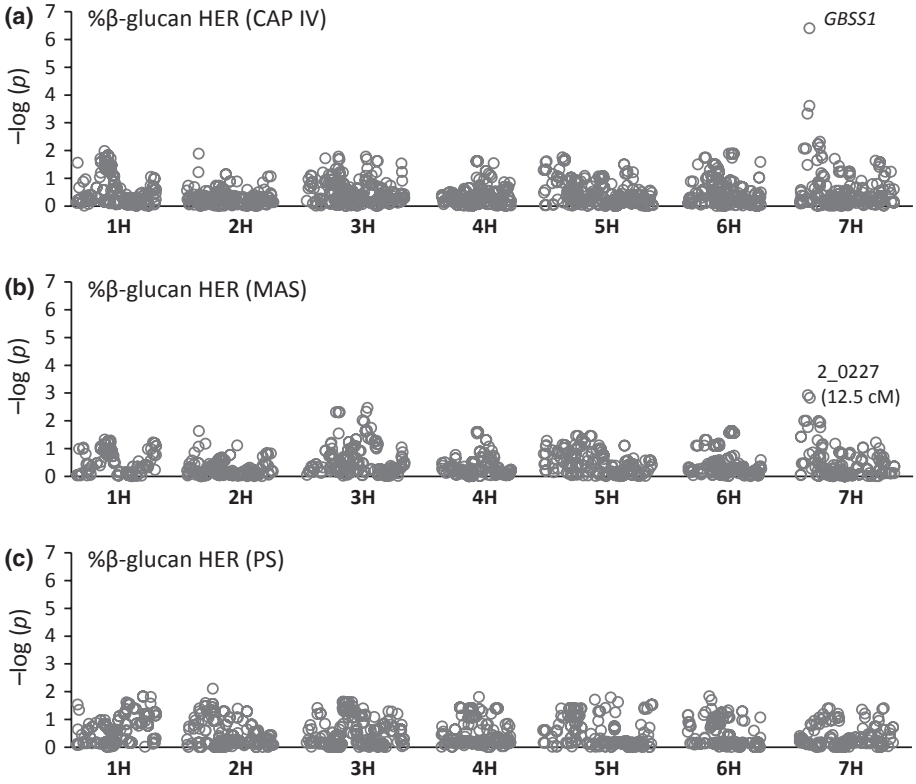

Fig. 4: Grain β-glucan percentage at Hermiston (HER), Oregon genome-wide association scans using three data sets. (a) full CAP IV data set (n = 99), (b) marker-assisted selection data set (n = 69) and (c) phenotypic selection data set (n = 65)

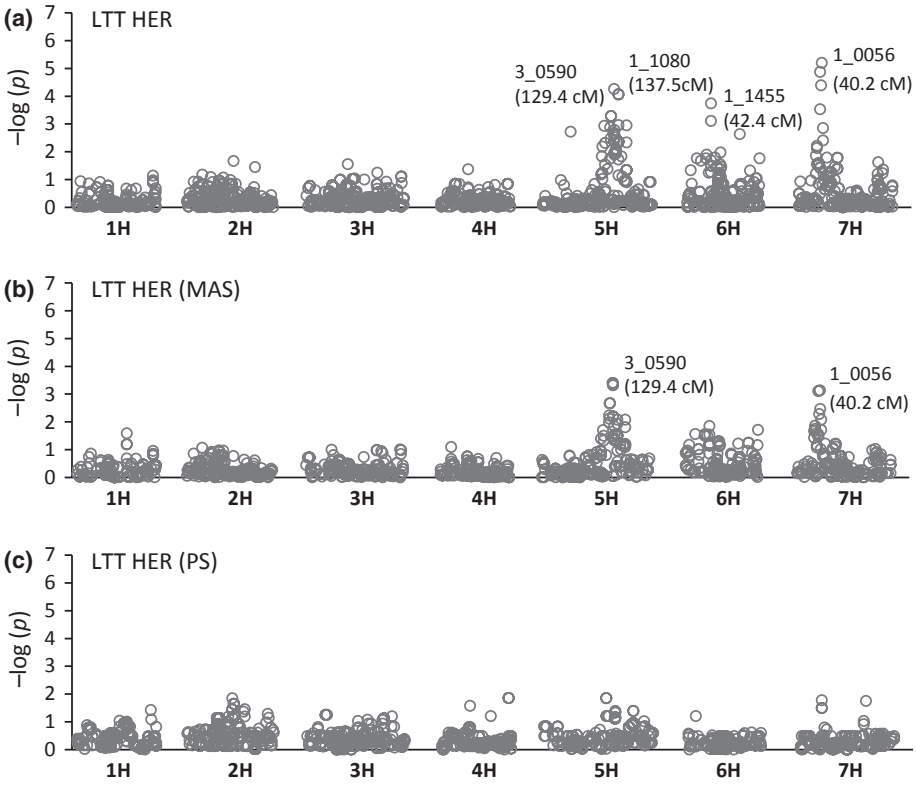

Fig. 5: Low-temperature tolerance at Hermiston (HER) genome-wide association scans using three data sets. (a) full CAP IV data set (n = 99), (b) marker-assisted selection data set (n = 69) and (c) phenotypic selection data set (n = 65)

Discussion

Marker-assisted selection was effective for fixing target alleles at *GBSS1* except for 09OR-16, which is homozygous dominant for *WX* and has normal starch, as predicted by genotype. This is due to an error in generation advance because records indicate that at the BC_1F_2, the antecedent of 09OR-16 was homozygous *wxwx*. On average, grain β-glucan percentage in *wxwx* types was significantly ($P < 0.0001$) higher than in normal starch types, confirming that selection for *wxwx* alleles was suc-

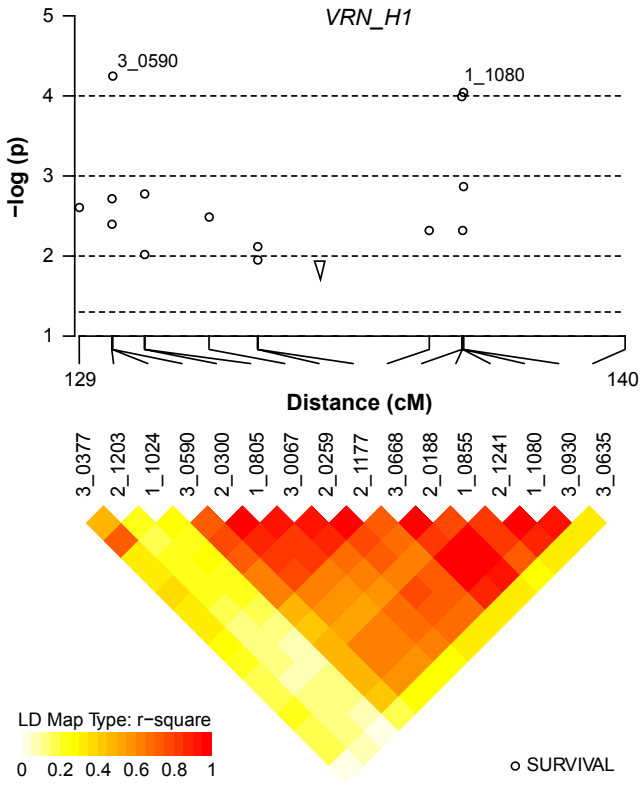

Fig. 6: Linkage disequilibrium heat plot for 11-cM region on chromosome 5H containing *HvBM5A* (*VRN-H1*). The consensus map position of *HvBM5A* is indicated. SNP 1_1080 and 3_0590 showed significant association with low-temperature tolerance

cessful in increasing grain β-glucan percentage, as was previously reported by Xue et al. (1997). The RVA analysis revealed two principle groups corresponding to waxy (MAS) and normal (PS) starch types. The peak times for the waxy starch group were lower than those of the normal starch group, in agreement with the results of Kim et al. (2003) who reported that the rapid swelling of waxy starch is due to the high amylopectin content. Two lines (09OR-18 and 09OR-21) had intermediate peak times; both are homozygous *wxwx*, meaning that they do have waxy starch, but may have lower amylopectin/higher amylose content. Examples of waxy high β-glucan lines with high amylose content have been reported by Islamovic et al. (2013).

Although phenotypic distributions for grain β-glucan at CVO and HER were similar, the average values were significantly higher at HER than CVO. The effect of environment on grain β-glucan has been reported (Zhang et al. 2002), with hot and dry conditions during grain filling favouring higher β-glucan (Ehrenbergerova et al. 2008). Neither moisture nor temperature during grain filling can account for the differences we observed as they were similar at both locations. The bimodal distributions indicate segregation of alleles at a single locus, and the transgressive segregation within each of the bimodal classes could be indicative of the effects of minor genes and/or experimental error. Minor genes for β-glucan content have been reported (Islamovic et al. 2013). This means that there can be different beta-glucan levels for the same GBSSI allele. However, in the GWAS for grain β-glucan percentage, we detected only *GBSS1*.

For LTT, GWAS revealed a significant effect for *FR-H1* and an effect for *VRN-H3. FR-H1* is most likely a pleiotropic effect of *VRN-H1,* and it is the most commonly detected determinant of LTT in barley (reviewed by von Zitzewitz et al. 2011). In the CAP IV, we did not expect to find a significant effect for *FR-H1* because (i) the winter parents (Luca, Strider and Doyce) all have similar levels of LTT and winter alleles at *VRN-H1*, and (ii) our initial genotyping of Waxbar and Merlin led us to believe that they had positive (winter) LTT alleles at *VRN-H1* based on genotyping with three sets of allele-specific primers: BM5.88F/89R, BM5.42F/56R and BM5.42F/86R (Szucs et al. 2006, 2007). However, subsequent to our initiating this project, at least ten different *VRN-H1* alleles were described in the literature (Hemming et al. 2009) that are specifically related to VS. The intron 1 allele-specific markers in *VRN-H1* do not show significant associations with LTT in the current study: the associations are with SNPs in LD with *VRN-H1*. Additional resequencing of *VRN-H1* alleles and adjacent genomic sequence will be required to resolve the gene (or genes) responsible for the significant associations in this region of the genome.

We found no effects of *FR-H2* on LTT in this germplasm, suggesting that favourable alleles are fixed, even in parental germplasm with poor LTT (e.g. Merlin and Waxbar). There is precedent in the facultative × spring (Dicktoo × Morex) mapping population where only *FR-H1* is a significant determinant of LTT (Skinner et al. 2006).

The significant effect of markers near *VRN-H3* implicates a role for this gene in cold tolerance. We genotyped two putative functional polymorphisms in *HvFT1* in the CAP IV panel and consider these findings in relation to the SNP data and the reported function of *VRN-H3*. Yan et al. (2006) reported that the functional domain in *HvFT1* is in intron I. This domain is represented as SNP 3_0895, and this marker was not significant in the GWAS. To further explore this lack of significance for *VRN-H3* itself vs. markers in LD with *VRN-H3*, we used allele-specific primers (HvFT1.03F and HvFT1.04R and product digestion with *BclI*) (Yan et al. 2006). By this method, two allele classes were identified: Merlin and Luca have the same allele and the 39 CAP IV lines with this allele had an average survival of 77%. Waxbar and Strider had the same allele and the 53 CAP IV lines had an average survival of 83%. Therefore, this polymorphism is not predictive, or causal, of the phenotypic variation in LTT observed in the CAP IV. An alternative functional polymorphism in *VRN-H3* is in the promoter at nucleotide position 927 (Cuesta-Marcos et al. 2010). Therefore, we resequenced the parental panel and developed the primer set HvFT1.13F (see Materials and Methods) to genotype the CAP IV. This primer set, due to the differences in restriction sites between parents, generated a total of four amplicons. Accordingly, these data were converted into four biallelic markers, and their effects were tested using SAS PROC MIXED with the REML method. The results revealed a unique allele in Luca. In the 11 individuals with this allele, the average survival was 52% compared with the average survival of the individuals with either of the three other alleles (83%, 87 individuals). All available data from three loci in and near *VRN-H3* point to Luca contributing the unfavourable allele at this locus. Considering the reported function of *HvFT1* in terms of VS and FT under long days (Yan et al. 2006, Trevaskis 2010), this association with LTT presents exciting challenges for further research.

In terms of VS, GWAS revealed significant effects for markers near *VRN-H2* and *VRN-H3*. For VS there was a strong association at 2_0119 (4H), which is 20 cM proximal to *VRN-H2.* Given the distance between the two markers, it is not likely that this marker is in LD with *VRN-H2*. The annotation of this SNP does not directly implicate a role in flowering (HarvEST database, avail-

able at http://harvest.ucr.edu/HBarley178.exe [verified 7 Nov. 2012]). There are no reports of VS or FT genes in this region of the barley genome. It is most likely that this is a mathematical artefact attributable to the fact that *VRN-H2* is fixed in the MS lines and almost fixed in the PS lines. The alternative – that this effect is due to an unreported gene (or genes) determining vernalization sensitivity is unlikely. However, additional research will be needed to definitively choose between the two alternatives. In the PS group, the GWAS shows a significant association between *VRN-H2* and VS. This can be explained by the deletion of *VRN-H2* in 09OR-65, 09OR-68, Waxbar and Merlin. In the MAS group, there was a significant association with markers based on the functional polymorphism of *VRN-H3* reported by Yan et al. (2006). Cuesta-Marcos et al. (2010) reported that a polymorphism at position 927 (SNP927) in the promoter of *VRN-H3* differentiated phenotypes with dominant (T) and recessive (C) alleles in a limited set of genotypes with known *VRN-H3* alleles; however, when SNP927 was characterized on a broader array of germplasm, the T and C alleles did not show any association with flowering time. Likewise, Casas et al. (2011) assayed the C/T polymorphism in a set of Spanish barley cultivars and found no relationship with VS. Sequence alignment of the MAS parents (Fig. S3) revealed that Waxbar has the T allele at SNP927, whereas Luca, Strider and Merlin have the C allele. At position 928, Merlin contains T while all other genotypes have the C allele. Therefore, considering the haplotype defined by SNPs 927 and 928, Waxbar and Merlin can be differentiated from Luca and Strider. This classification corresponds to the observed growth habits of the MAS parents, but the association with VS in the MAS population cannot be determined until data are generated for allele type at SNP928.

The effect of the dominant *VRN-H3* allele is very early flowering (Takahashi and Yasuda 1971, Yan et al. 2006). Merlin and Waxbar were not among the earliest accessions to flower. Therefore, we hypothesize that Merlin and Waxbar have recessive alleles at *VRN-H3* and that the significant association of *VRN-H3* with VS is due to the effects of these uncharacterized recessive alleles. Allelic variation at *VRN-H3* would explain the observed segregation for VS in the MAS population: 12 of the 62 MAS lines are VS. This fits a 3 : 1 ratio ($P = 0.3$), which could be explained by the epistatic interaction of two loci: *VRN-H1* and *VRN-H3*.

In summary, we demonstrated successful MAS for target alleles at the *WX* and *VRN-H2* loci. The selection for *wxwx* was effective in raising grain β-glucan percentage, as expected. Fixation of dominant alleles at *VRN-H2* did not lead to VS and high LTT as expected. This was due to unexpected variation at *VRN-H1*/*FR-H1* and *VRN-H3*. Our characterization of the parents for the putative functional polymorphisms available at the time led to an erroneous conclusion regarding Merlin and Waxbar having favourable alleles at *FR-H1*. In the case of *VRN-H3*, we discovered unexpected allelic variation with effects on both LTT and VS. GWAS was useful for validating the effects of perfect markers (e.g. *WX*) and revealing the genome locations of determinants of target traits (e.g. *FR-H1* and *VRN-H3*).

Acknowledgements

This Project was supported by USDA-CSREES-NRI Grant No 2006-55606-16722 'Barley Coordinated Agricultural Project: Leveraging Genomics, Genetics, and Breeding for Gene Discovery and Barley' and by Agriculture and Food Research Initiative Competitive Grant no. 2009-85606-05701 from the USDA National Institute of Food and Agriculture. We appreciate the assistance of Dr. J. Von Zitzewitz (INIA, Uruguay) with generating the heat map shown in Fig. 6.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Grain β-glucan percentage at Corvallis (CVO), Oregon genome-wide association scans using three datasets.

Figure S2. Days-to-flowering genome-wide association scans under greenhouse (GH) conditions using three datasets and two treatments.

Figure S3. Sequence alignment among marker-assisted selection parents.

Table S1. Average monthly temperature and total monthly rainfall at two locations in Oregon during the growing period in 2010.

Data S1. Flowering time with vernalization.

Table S2. Haplotypes for the following regions: *FrH1, FrH2, FrH3, HvFt1, VRN-H1, VRN-H2, and VRN-H3, PPD-H2, and GBSSI* for the 'best lines' in terms of low-temperature tolerance, from the MAS and PS populations, as well as the parents.