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Abstract approved:

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Fall-planted barley makes the best use of available precipitation in the Pacific Northwest of the United States. This growth habit is also suitable to many other areas of the world. A prerequisite for production in most of these areas is tolerance of low temperature during the vegetative growth stages. Fall-planted barley is often equated with winter habit barley. Winter habit type cereals require vernalization: a period of low temperature necessary to trigger the vegetative to reproductive transition. Facultative growth habit defines germplasm that is tolerant of low temperature but do not require vernalization. Cereals achieve their greatest cold tolerance during vegetative stages, and a vegetative condition can be maintained by vernalization sensitivity or sensitivity to short days. As global climate changes and

temperatures fluctuate without warning, vernalization sensitivity becomes an unreliable trait for maintaining a vegetative condition, and thus maximizing cold tolerance. Hence the interest in short day sensitive facultative types – provided that maximum cold tolerance can be achieved without vernalization sensitivity. Facultative cereals have the additional advantages that they can be fall or spring planted and they are amenable to rapid cycling breeding methods. In barley, winterhardiness loci have been identified using bi-parental QTL approaches. Candidates for the *FR-H1* and *FR-H2* QTL are *VRN-H1* and a cluster of *CBF* family members respectively. *VRN-H1*, which interacts epistatically with *VRN-H2* and *VRN-H3*, is also a major player in vernalization sensitivity. *FR-H1* and *FR-H2* are approximately 30 cM apart on the long arm of chromosome 5H. The candidate genes for the *PPD-H1* and *PPD-H2* photoperiod sensitivity QTL are *HvPRR7* on chromosome 2H and *HvFT3* on chromosome 1H respectively. Genome-wide association mapping provides a complementary or alternative approach to bi-parental mapping. In this research, we explored the genetics of winterhardiness in barley germplasm through genome-wide association mapping. We identified the same *FR-H1*, *FR-H2*, *VRN-H2*, *PPD-H1* and *PPD-H2* QTL identified via bi-parental QTL mapping. We found that *FR-H1*/*FR-H2*/*VRN-H2* haplotypes predict maximum cold tolerant facultative germplasm with high certainty and that facultative germplasm is as cold tolerant as vernalization sensitive germplasm.

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The Genetics of Winterhardiness in Barley: Perspectives from Genome-Wide Association Mapping

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

Jarislav von Zitzewitz, Author

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CONTRIBUTION OF AUTHORS

Dr. Patrick M. Hayes, initiated, advised, and supervised all aspects of this project. Dr. Alfonso Cuesta-Marcos and Dr. Federico Condon advised in association mapping and statistical strategies. Ann Corey, Kale Haggard, and Scott Fisk conducted much of the phenotyping and Tanya Filichkin conducted the in-house genotyping. Dr. Kevin P. Smith conducted the SPMN low temperature tolerance work. Dr. Ildikó Karsai and Dr. Otto Veisz conducted the controlled environment studies at MRI. Shiaoman Chao conducted the SNP genotyping. Dr. Ariel Castro Tabó and Dr. Lucia Gutierrez conducted essential edits.

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THE GENETICS OF WINTERHARDINESS IN BARLEY: PERSPECTIVES FROM GENOME-WIDE ASSOCIATION MAPPING

GENERAL INTRODUCTION

About 10,000 years before present, the wild ancestors of modern crops, including those of barley (*Hordeum* spp. *vulgare*), were selected and domesticated. Early agriculturalists saved the seed from plants with favored traits to be planted the next generation. Over time, these wild species were transformed into productive crops. The immediate ancestor of cultivated barley is *Hordeum* spp. *spontaneum* (C. Koch). Among the most important domestication traits is growth habit. *H. spontaneum*, in the wild, is a winter annual. That is, it has a winter growth habit. *H. spontaneum* that is not of winter growth habit has likely intercrossed with spring habit cultivated barley. Barley cultivation expanded to various latitudes in the world due to the selection and dispersal of spring growth habit types (Kole et al., 2006). Today, spring habit barley is far more prevalent than winter habit barley.

Growth habit is synonymous with vernalization response (or sensitivity). Vernalization sensitivity can be defined as the necessary induction of flowering by exposure of a plant to extended periods of low temperature. As reviewed by Michaels and Amasino (2000), the range of effective temperatures at which flowering is promoted by cold is usually between 1-7°C, and in some cases vernalizing temperatures can be as low as -6°C. Exposing the germinating seeds of winter

growth habit barley during the process of germination to temperatures between 3-5°C for a few days to several weeks (depending on the underlying genetics) will allow for the transition from the vegetative to the reproductive phase of development (Takahashi and Yasuda, 1971).

Candidates for the central genes regulating vernalization response in barley and other members of the *Triticeae* (*VRN1, VRN2, and VRN3*) have been cloned and characterized (Danyluk et al., 2003; Murai et al., 2003; Trevaskis et al., 2003; Yan et al., 2003; Yan et al., 2004b; von Zitzewitz et al., 2005; Yan et al., 2006). This has considerably advanced our understanding of this trait in economically important crop species. The genetics of vernalization are also well-characterized in *Arabidopsis thaliana* (reviewed by Amasino (2010)), where the central genes in the vernalization pathway are now known to be different than the central genes in the *Triticeae* (Yan et al., 2003; Yan et al., 2004b; Yan et al., 2006). It is well established that in the developmental processes leading to flowering in *Arabidopsis thaliana*, epigenetics plays a central role (Feng et al., 2010). In the *Triticeae*, winter wheat has been shown to be more highly methylated than spring wheat (Sherman and Talbert, 2002). These findings add to the complexity of vernalization genetics and suggest that epigenetic variation may be involved in “fast” population differentiation (Richards et al., 2010). Epigenetics may have been involved in the diversification of growth habit as barley was domesticated. However, there is a simpler explanation: gene deletion. The

complete deletion of the *VRN-H2* locus is common and sufficient to lead to a complete loss of vernalization sensitivity (Szűcs et al., 2009).

A key question is “does the loss of vernalization sensitivity lead to a loss of low temperature tolerance?” In general, barley genotypes that respond to vernalization (winter-types) are generally “winterhardy” whereas spring types are considered “non-winterhardy”. This presupposes that vernalization is involved in cold tolerance. In fact, a third growth habit type - facultative - negates this simple classification. Three traits need to be considered in the context of winter hardiness: low temperature tolerance, vernalization response, and photoperiod sensitivity (Hayes et al., 1993; Pan et al., 1994). In general, winter habit barleys are low temperature tolerant, highly responsive to vernalization, and vary in photoperiod sensitivity. Spring varieties have little to no low temperature tolerance, do not respond to vernalization, and sensitivity/insensitivity to short day photoperiod is not relevant if they are grown under long-day (spring planted) conditions. The term “facultative” is generally used to describe genotypes that are low temperature tolerant, are not sensitive to vernalization, and may be photoperiod sensitive. In an extensive characterization of a range of barley germplasm, Karsai et al. (2001) found that photoperiod sensitive facultative genotypes were the most cold tolerant. The variety “Dicktoo”, which is a model for winter hardiness research in the barley community (Pan et al., 1994; van Zee et al., 1995; Hayes et al., 1997; Choi et al., 2000; Mahfoozi et al., 2000; Fowler et al., 2001; Karsai et al., 2001) has facultative

growth habit: it is cold tolerant, sensitive to short photoperiod, and does not have a vernalization requirement.

Sensitivity to short photoperiod is an important attribute of low temperature tolerance. Maximum cold tolerance is achieved during vegetative, as opposed to reproductive, growth stages. Both vernalization sensitivity and photoperiod sensitivity can lead to maintenance of the vegetative condition during low temperature, short-day winter conditions. In a global climate change scenario - due to increasingly volatile fluctuations in temperature - vernalization sensitivity is an imperfect mechanism for maintaining a vegetative state and thus maximizing cold tolerance. Photoperiod sensitivity (to short day conditions) is a more effective strategy for postponing the vegetative-to-reproductive transition until the danger of low temperature stress is reduced. Facultative growth habit also has production and breeding advantages. For the former, facultative germplasm can be fall or spring planted and can be economically viable in either scenario. In terms of the latter, facultative germplasm can be subjected to accelerated cycles of generation advance (e.g. single seed descent or off-season nurseries). Nevertheless, there is not universal agreement that photoperiod sensitive facultative types can achieve the same level and duration of low temperature tolerance as winter habit types (Mahfoozi et al., 2001). Therefore, it is imperative to understand the genetics and physiology of vernalization, photoperiod sensitivity, and low temperature tolerance in order to

establish breeding objectives. Very different strategies will be required if trait associations are due to linkage or to pleiotropy.

The foundations for understanding the genetics of vernalization requirement in barley were established by Takahashi and Yasuda (1971), who proposed a three-locus epistatic model in which genotypes that respond to vernalization have the allelic architecture *Sh_sh2sh2sh3sh3*. All other allelic configurations lead to facultative and spring growth habits. Based on wheat: barley orthology, the *Sh* loci are now described using standard *Triticeae* nomenclature, with an "H" indicating the *Hordeum* genome, as follows: *Sh2* = *VRN-H1* (chromosome 5H); *Sh* = *VRN-H2* (chromosome 4H); and *Sh3= VRN-H3* (chromosome 7H).

Yan et al. (2003; 2004b) proposed a model explaining the *VRN1/VRN2* epistatic interaction in the *Triticeae*, based on the positional cloning and expression studies with the respective candidate genes in *T. monococcum*. Per this model, which ignores *VRN3*, *ZCCT1* (the candidate gene for *VRN2*) encodes a dominant repressor of flowering that binds to a *cis* element regulatory region of *AP1* (the candidate for *VRN1)* a MADS-box gene. Vernalization down-regulates the expression of *VRN2* allowing expression of *VRN1.* A lack of vernalization response occurs in genotypes with a deletion for *VRN2*, regardless of the allele at *VRN1* as well as in genotypes that do have *VRN2* but that lack a target binding site for the repressor in *VRN1.* A deletion was reported in the promoter region of *VRN-A1* that correlated with spring vs. winter growth habit (Yan et al., 2003; Yan et al., 2004a). This is not consistent with findings

in barley, where natural allelic variation for vernalization sensitivity is associated with deletions in the first intron of *VRN-H1* (Fu et al., 2005; von Zitzewitz et al., 2005). The candidate barley ortholog of *VRN-A1* was first described as barley MADS-box 5 (*HvBM5*) by Schmitz et al. (2000), but the work was not related to vernalization and *HvBM5* was not equated with *VRN-H1*. The detailed structural and functional characterization of *VRN-H1* with respect to vernalization was established by Fu et al., (2005) and von Zitzewitz et al., (2005). Additional evidence supporting the orthology of barley *HvBM5* with *T. monococcum AP1* (*VRN-A1*) is provided in expression studies reported by Danyluk et al. (2003), and Trevaskis et al. (2003). The model described by Yan et al. (2003; 2004b) fits conditions in which vernalization-sensitive germplasm is spring-sown and/ or grown under long-day conditions.

VRN-H3 was subsequently characterized by (Yan et al., 2006), who cloned the candidate gene *HvFT1*. However, phenotypic variation at this locus was considered rare and most barley germplasm (winter, spring, and facultative) was thought to be fixed for the recessive (winter) allele, as originally proposed by Takahashi and Yasuda (1971). An alternative model, with a more central role for *VRN-H3,* has been proposed by Trevaskis et al. (2007) and Hemming et al. (2008). According to this model, *VRN-H1* is a promoter of flowering that is activated by low temperatures. *FT1* (*VRN-H3*) mediates the long-day flowering response and is induced by long days to accelerate flowering. This long-day induction of *FT1* requires expression of *VRN-H1*, since in vernalization-responsive cereals, *FT1* is expressed only after plants have been

vernalized. *VRN-H2* is a repressor of flowering that is expressed in long days and delays flowering by suppressing long-day induction of *FT1*. *VRN-H1* also downregulates *VRN-H2* and provides a mechanism to allow long-day induction of *FT1* in vernalized plants.

Differences in the genetics of vernalization between the *Triticeae* and *Arabidopsis* - the "model plant for genetic analysis" - should be noted at this juncture because similar gene structures and names do not always equate to similar functions. The *Triticeae AP1* candidate for *VRN1* is similar to the Arabidopsis meristem identity gene *APETALA1* (*AP1*), but *Arabidopsis AP1* is not directly involved in vernalization (Murai et al., 2003; Trevaskis et al., 2003; Yan et al., 2003). In *Arabidopsis*, the vernalization-requirement phenotype is conferred by a combination of functional alleles at the *FRIGIDA* (*FRI)* and *FLOWERING LOCUS C* (*FLC*) loci (Burn et al., 1993; Koornneef et al., 1994). *ZCCT1*, the candidate for *VRN2*, is similar to *FLC* (as repressor), but belongs to a different family of transcription factors and contains a putative zinc finger in the first exon and a CCT domain in the second exon, in common with the *Arabidopsis CONSTANS* (*CO*) and *CO*-like proteins (Yan et al., 2004b). The CCT domain localizes *CO* in the nucleus (Robson et al., 2001), the central gene in the mechanism by which day length controls flowering (Valverde et al., 2004). *CO* and *FLC* proteins regulate *SOC1*, a flowering time gene, antagonistically via separate promoter motifs (Hepworth et al., 2002), suggesting that transcription factors involved in vernalization and photoperiod target upstream genes that are in

common to both pathways. In *Arabidopsis*, *VRN1* and *VRN2* are involved in the repression maintenance of *FLC*, clearly distinguishing their role from the *T. monococcum* genes with the same name.

Mapping of vernalization sensitivity genes (and other winter hardiness-related genes) in the *Triticeae* was first achieved using biparental mapping populations (Hayes et al., 1993; Pan et al., 1994; Laurie et al., 1995; Karsai et al., 1997a; Francia et al., 2004; Szűcs et al., 2006; Szűcs et al., 2007). For example, Laurie et al. (1995) reported vernalization QTL on chromosomes 4H and 5H in the Igri x Triumph (winter x spring) mapping population and the QTL positions correspond to the predicted locations of the *VRN-H2* and *VRN-H1* loci respectively. Francia et al. (2004), reported a vernalization QTL on 5H at the predicted position of *VRN-H1* in the Nure x Tremois (winter x spring) mapping population. QTL for multiple winter hardiness-related traits, including crown fructan content, photoperiod sensitivity and low temperature tolerance (Hayes et al., 1993) were mapped in the Dicktoo x Morex (facultative x spring) population to chromosome 5H that corresponds with the predicted position of *VRN-H1* (Karsai et al., 1997a).

The coincidence of low temperature tolerance and vernalization sensitivity QTL is not unexpected. Low temperature tolerance is an induced response: in order to reach maximum levels plants require a period of acclimation during which hundreds of Cold-Regulated (COR) genes are up- or down-regulated (Fowler and Thomashow, 2002). Cold acclimation, as it proceeds from fall to winter, occurs during

conditions of induction that determine vernalization response (low temperature) as well as photoperiod sensitivity (short days). Once the plant transitions to a reproductive state, the "acquired" cold tolerance level is gradually lost (Galiba et al., 2009).

The principal determinants of low temperature tolerance in the *Triticeae* are termed, as QTL, "*FR-1"* and "*FR-2"* (Francia et al., 2004; Skinner et al., 2005; Galiba et al., 2009). In barley, the two QTL are approximately 30 cM apart on the long arm of chromosome 5H. As with *VRN-H1*, the candidate for *FR-H1* is *HvBM5A* (Fu et al., 2005; von Zitzewitz et al., 2005). *FR-H2* candidate genes are one or more members of physically linked clusters of *C-repeat binding factor* (*CBF*) genes, also known as *DRE binding protein 1* (*DREB1*) (Francia et al., 2004; Skinner et al., 2005; Galiba et al., 2009). It is not established if the effects of *VRN-H1* on vernalization sensitivity and low temperature tolerance are due to pleiotropy or tight linkage. Dhillon et al., (2010) argue for pleiotropy. The timing of maximum cold tolerance coincides with the timing of vernalization saturation (Limin et al., 2007) and without vernalization the vegetative state is maintained. On the other hand, there is evidence that low temperature tolerance is independent of vernalization. As earlier noted, "Dicktoo", is not vernalization-sensitive. A low temperature tolerance QTL maps to *FR-H1* in the Dicktoo x Morex population, which does not segregate for vernalization sensitivity (Pan et al., 1994), and Dicktoo achieves a high degree of low temperature tolerance under short day conditions and without vernalization (Limin et al., 2007).

The vegetative to reproductive transition and flowering time is also controlled by photoperiod responses. As with vernalization and photoperiod sensitivity, genes determining photoperiod responses were first described as QTL and subsequently candidate genes have been identified. Allelic variation at *PPD-H1* on chromosome 2H is particularly important in spring barley as the recessive allele confers insensitivity to long-day conditions, allowing for a prolonged growing period and consequently higher yield. The candidate gene for *PPD-H1* is *HvPRR7* and differences in long-day sensitivity are attributed to amino acid changes in the CCT domain (Turner et al., 2005). A candidate gene has also been assigned to the short-day sensitivity QTL on chromosome 1H - *PPD-H*2. Allelic variation at *HvFT3* has been attributed to deletions of (or within) the gene in accessions that are sensitive (e.g. remain vegetative) under short-day conditions (Faure et al., 2007; Kikuchi et al., 2009). In previous QTL studies, *PPD-H2* was shown to have significant effects on flowering under short photoperiods and in autumn-sown field experiments (Pan et al., 1994).

The *Triticeae* are a very useful tool to study the genetics of winterhardiness because they form a homogeneous genetic system, in which results from one species are frequently applicable to other members of the cereal tribe (Dubcovsky et al., 1998). Within the *Triticeae*, barley is an economically important crop model for studying the genetics and physiology of the winterhardiness related traits. Barley is a simple genetic system due to its self-pollinating and diploid nature, and it displays genetic variation for the components of winterhardiness. In addition, an ever-

expanding set of tools exists for genetic and molecular analysis (Hayes et al., 2003; Szűcs et al., 2009), including multiple mapping populations, arrayed BAC clones (Yu et al., 2000), a large EST database, a microarray chip (Close et al., 2004), and an ever growing SNP database and genotyping platforms (Close et al., 2009; Szűcs et al., 2009). A consensus map built from all currently available SNP data has been developed by Close et al. (2009), and is available by downloading the 1.77 version of the barley HarvEST database (http://harvest.ucr.edu/). These tools can be very useful to rapidly develop marker assisted selection schemes at the single gene level or at genome-wide scales.

The application of these tools to identifying the genetic basis of complex traits can take several forms. As previously described, mapping of winterhardiness QTL in barley has been achieved using biparental mapping populations (Hayes et al., 1993; Pan et al., 1994; Laurie et al., 1995; Karsai et al., 1997a; Francia et al., 2004; Szűcs et al., 2006; Szűcs et al., 2007). This type of analysis involves lines derived from the cross of two inbred parents. Therefore, the lines have one of two parental alleles and all share the same ancestry. The two key elements of this approach are that the parents of the population have contrasting phenotypes for the trait to be analyzed and there are markers linked to the QTL. As described by Wang et al. (2005), statistical methods for QTL detection in biparental populations have been continuously improved. These approaches allow estimation of QTL positions (within a confidence interval), effects, and interactions between QTL without the necessity of a

high marker density (Piepho, 2000). There are, however, limitations to QTL detection in biparental populations which can compromise the success of marker assisted selection (MAS). These include: bias in the estimation of QTL effects due to reduced sample size (Vales et al., 2005), narrow genetic bases and consequent limited scope of inference (Crepieux et al., 2005), and broad confidence intervals for QTL positions and effects (Darvasi et al., 1993; Hyne et al., 1995).

Genome-wide association (GWA) mapping provides a complementary or alternative approach to biparental mapping. GWA can be done in different types of germplasm and the individuals in the analysis do not need to trace to a single cross. This provides some advantages over biparental QTL mapping: a wider genetic base, including actual breeding lines, can be sampled without having to spend generations and extra resources in developing populations (Flint-Garcia et al., 2003; Varshney et al., 2005). In addition, more than two alleles can be present at any given locus and, therefore, more alleles responsible for a specific trait can be identified in one analysis. QTL detection by means of GWA is based on the existence of linkage disequilibrium (LD), i.e. the non-random association of alleles at different loci, between QTL and markers. The extension of LD determines the marker density needed and the accuracy of estimates of QTL location. The higher the extension of LD, the lower marker density needed to detect QTL. However, in this case the estimation of QTL position is less accurate. Short-range LD requires high density of markers (Rafalski, 2002). High density genotyping can provide better estimates of the

actual polymorphisms in linkage disequilibrium with the QTL (Zhao et al., 2007) and the location of QTL can therefore be narrowed down to much smaller confidence intervals.

In biparental mapping populations, the non-random association of markers and QTL (or genes) is due only to linkage and LD extension depends only on the number of recombination events that occurred during the development of the population. In GWA many additional factors can be responsible for the extension of LD: mutation, admixture, different degrees of relatedness among individuals (kinship), genetic drift and selection (Flint-Garcia et al., 2003). All these processes may create an underlying structure in the populations that if not accounted for may lead to false positives and false negatives in the association analysis (Pritchard et al., 2000). There are examples in which high density markers and large population sizes have been used to accurately model for population structure and several methods have been implemented to increase computational speed in mixed-model GWA (Kang et al., 2008; Kang et al., 2010; Zhang et al., 2010).

Structured association was introduced by Pritchard et al. (2000) as a method for reducing confounding due to population structure with a java-based program called STRUCTURE. This program uses a model-based Bayesian clustering algorithm to execute all analyses conditional on the inferred assignments of individuals to subpopulations. The approach is extremely sensitive, but computationally intensive. This approach alone is not enough to control for spurious associations by integrating

the subpopulation assignments by STRUCTURE (the Q matrix) as fixed effects in the association. A mixed-model approach was introduced by Yu et al. (2006), where the Q matrix is introduced as a fixed effect in the model, and the individual random deviation from the phenotypic mean is controlled with the covariance between the individuals tested, assuming that the covariance is proportional to their relative kinship estimated with genome-wide marker data (K matrix of pair-wise kinship coefficients). The K matrix may capture features that the Q matrix does not, such as different levels of relatedness. Yu et al. (2006) and Zhao et al. (2007) argue that in most cases the K matrix alone is not sufficient to control for population structure.

Another approach that has been used to reveal and account for population structure is principal component analysis (PCA). However, this approach also has its drawbacks (November and Stephens 2008). Kang et al. (2008) developed an efficient mixed model association analysis method (EMMA) which corrects for population structure and relatedness based on the Yu et al. (2006) approach. An advantage of the EMMA algorithm is that it substantially increases computational speed by orders of magnitude and improves the reliability of the results. Furthermore, when inferring kinship with an identity-by-descent (IBD) similarity matrix using SPAGeDi or TASSEL, as proposed by Yu et al. (2006), the negative kinship coefficients are set to zero. According to Kang et al. (2008), converting this type of matrix to a positive semidefinite matrix is not mathematically correct, as this could generate “ill-defined” likelihoods in the estimation of variance components.

An R package implementation of EMMA (and web server) has been made available (Kang et al. 2008). EMMA includes the implementation of a simple genetic IBS similarity matrix to account for genetic relatedness in the mixed model analysis, which is at least as efficient as in previous methods and at the same time guaranteeing positive semidefiniteness. More recently, due to the large samples required to achieve statistical power to detect QTL with small effects, Kang et al. (2010) and Zhang et al. (2010) have created improved versions of the EMMA and TASSEL implementations respectively.

With the availability of dense genome-wide molecular marker coverage, linear mixed model association mapping has become a powerful tool for associating SNPs with traits of interest. As an extension of this approach, linear mixed models can be applied to predict genetic values with molecular markers in order to apply genomic selection in plant breeding programs (Meuwissen et al., 2001; Bernardo and Yu, 2007; Heffner et al., 2009; Piepho, 2009; Crossa et al., 2010; Heffner et al., 2010).

In this research, we used genome wide association mapping to address a long-term objective of the barley breeding program at Oregon State University: the development of low temperature tolerant varieties with superior malting and food quality. The genetics of quality traits are addressed elsewhere. In the research reported in this thesis, we focused on low temperature tolerance and vernalization sensitivity. Our hypothesis was that maximum levels of cold tolerance could be achieved with facultative growth habit via the deletion of *VRN-H2* and accumulation

of favorable alleles at other loci. Under the auspices of the Barley Coordinated Agricultural Project (CAP), 148 barley accessions were extensively phenotyped for low temperature tolerance, vernalization sensitivity, and flowering time. The same germplasm was genotyped with 3,072 single nucleotide polymorphism (SNP) markers. In addition, allele–specific markers were generated for 11 loci. The genotype and phenotype data were integrated via association analysis. In the next phase of this research, we will implement genomic selection for low temperature tolerance.

THE GENETICS OF WINTERHARDINESS IN BARLEY: PERSPECTIVES FROM GENOME-WIDE ASSOCIATION MAPPING

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ABSTRACT

Winterhardiness is a complex trait that involves low temperature (LT) tolerance, vernalization sensitivity and photoperiod sensitivity. Quantitative trait loci (QTL) for these traits were first identified using biparental mapping populations; candidate genes for all loci have since been identified and characterized. In this research we used a set of 148 accessions consisting of advanced breeding lines from the Oregon (USA) barley breeding program and selected cultivars that were extensively phenotyped and genotyped with single nucleotide polymorphisms. Using these data for genome-wide association (GWA) mapping we detected the same QTL, and genes, that have been systematically characterized using biparental populations over nearly two decades of intensive research. In this sample of germplasm, maximum LT tolerance can be achieved with facultative growth habit, which can be predicted using a three-locus haplotype involving *FR-H1*, *FR-H2*, and *VRN-H2*. The two LT tolerance QTL explained 25% of the phenotypic variation, offering the prospect that additional gains from selection can be achieved once favorable alleles are fixed at these loci. GWA mapping in larger sets of facultative germplasm using balanced data from field and controlled environment trials should be effective in identifying additional loci with small effects. In this germplasm, genomic selection should be effective in fixing, and validating, the effects of these alleles. This research was conducted using a small sample of related germplasm: abundant genetic resources in

germplasm collections and other breeding programs have yet to be mined for alternative alleles at known loci and at loci that have yet to be discovered.

INTRODUCTION

Winterhardiness in barley (and other members of the *Triticeae*) is a complex trait with a long history of quantitative trait loci (QTL) analysis in mapping populations derived from biparental crosses (Hayes et al., 1993; Pan et al., 1994; Karsai et al., 1997a; Francia et al., 2004; Szűcs et al., 2006; Szűcs et al., 2007). Low temperature (LT) tolerance, photoperiod (PPD) sensitivity and vernalization (VRN) sensitivity are the main components of winterhardiness (Hayes et al., 1993). Tolerance of low temperatures is an induced response: plants show maximum LT tolerance only after a period of acclimation during which hundreds of cold-regulated (COR) genes are differentially expressed (Fowler and Thomashow, 2002). Under field conditions, hardening typically occurs during conditions of induction that determine VRN response and PPD sensitivity (e.g. LT and short days). Once transitioned from a vegetative to a reproductive state, the level of LT tolerance diminishes (Galiba et al., 2009).

Frost Resistance-1 (*FR-1*) and *Frost Resistance-2* (*FR-2*) are the principal LT QTL on the group 5 chromosomes in the *Triticeae* (Francia et al., 2004; Skinner et al., 2005; Galiba et al., 2009). In barley, *FR-H1* and *FR-H2* are approximately 30 cM apart. The most likely candidate for *FR-H1* is *HvBM5A* (Fu et al., 2005; von Zitzewitz et al., 2005), a MADS-box protein similar to the *Apetala1* (*AP1*) gene in *Arabidopsis*. This gene was identified as the main source of allelic variation for the VRN response locus known as *VRN-1* in the *Triticeae* (Danyluk et al., 2003; Trevaskis et al., 2003; Yan et

al., 2003; von Zitzewitz et al., 2005). Recent evidence suggests that coincident *VRN-H1* and *FR-H1* QTL are the pleiotropic effects of *HvBM5A* (Dhillon et al., 2010). Further support comes from the observation that timing of maximum LT tolerance is usually coincident with the timing of VRN saturation (Limin et al., 2007) and an unsatisfied VRN requirement maintains the vegetative state. On the other hand, there is evidence that LT tolerance is not necessarily a function of VRN sensitivity. The barley variety “Dicktoo”, commonly used in LT research, is not VRN sensitive and LT tolerance QTL map to *FR-H1* in the Dicktoo x Morex population (Pan et al., 1994). “Dicktoo” achieves a high degree of LT tolerance under short day conditions and without VRN (Limin et al., 2007). The candidate genes for *FR-H2* are one or more members of two physically linked clusters of at least 11 family members of *C-repeat binding factor* (*CBF*) genes, also known as *DRE binding protein 1* (*DREB1*) (Francia et al., 2004; Skinner et al., 2005; Galiba et al., 2009).

Three loci (*VRN-H1*, *VRN-H2* and *VRN-H3*) interact in an epistatic fashion to determine VRN sensitivity. All allelic configurations except *Vrn-H2_/vrn-H1vrn-H1/vrn-H3vrn-H3* lead to a lack of significant VRN response (i.e. spring or facultative growth habits) (Takahashi and Yasuda, 1971). Deletions in intron I of *HvBM5A* are the functional polymorphisms of *VRN-H1* accounting for the VRN insensitive (dominant) alleles (Fu et al., 2005, von Zitzewitz et al., 2005, Szucs et al., 2007). A zinc finger−CCT (CONSTANS, CONSTANS-like, and TOC) domain transcription factor (*ZCCT*), encoding a flowering repressor down-regulated by VRN, is considered the

determinant of *VRN-H2* (Yan et al. 2004). Allelic variation at this complex locus (there are actually three tightly linked *ZCCT* genes in barley; *ZCCT-Ha*, *ZCCT-Hb* and *ZCCT-Hc*) is ascribed to loss-of-function mutations or complete deletion (Dubcovsky et al., 2005; Karsai et al., 2005). The candidate for *VRN-H3* is *HvFT1* (Yan et al., 2006; Faure et al., 2007). *HvFT1* expression is induced by long days and may mediate the long-day flowering response (Turner et al., 2005). Allelic variation at *HvFT1* is attributed to mutations in the first intron, with the relatively rare dominant alleles conferring very early flowering (Yan et al., 2006). According to Trevaskis et al. (2007) and Hemming et al., (2008), *HvBM5A* is a promoter of flowering that is activated by low temperatures. *HvFT1* mediates the long-day flowering response and is induced by long days. This long-day induction of *HvFT1* requires expression of *HvBM5A*, since in VRN responsive cereals, *HvFT1* is expressed only after plants have been vernalized. *ZCCT-H* is a repressor of flowering that is expressed in long days and delays flowering by suppressing long-day induction of *HvFT1*. *HvBM5A* also downregulates *ZCCT-H* and provides a mechanism to allow long-day induction of *HvFT1* in vernalized plants.

The role of VRN sensitivity in LT tolerance can be described in the context of the three growth habit types in barley: spring, winter, and facultative. Winter varieties are LT tolerant, highly responsive to VRN, and vary in PPD sensitivity. Spring varieties have little to no LT tolerance, do not respond to VRN, and sensitivity/insensitivity to short day PPD is not relevant if they are grown under long-day (spring planted) conditions. The term “facultative” is generally used to describe

genotypes that are LT tolerant, are not VRN sensitive, and may be PPD sensitive. Facultative varieties have "winter" allele haplotypes at the *VRN-H1* locus, and complete deletions of the *ZCCT-H* genes on 4H (Karsai et al., 2005; von Zitzewitz et al., 2005; Szűcs et al., 2007). "Winter allele" haplotypes have a full-length *HvBM5A* intron that includes a highly conserved 0.44 kb "VRN critical" region. This critical region is the putative binding site, under long day conditions, for the repressor encoded by *VRN-H2* (Fu et al., 2005; von Zitzewitz et al., 2005). Deletions of various lengths are associated with variation in flower time (Szűcs et al., 2007), with large deletions (~2.8 kb) characteristic of spring growth habit types.

In addition to the VRN genes, the vegetative to reproductive transition and flowering time are controlled by PPD sensitivity genes. Allelic variation at *HvFT3*, the candidate for the *PPD-H2* QTL on chromosome 1H, is due to deletions of (or within) the gene in accessions that are sensitive (e.g. remain vegetative) under short-day conditions (Faure et al., 2007; Kikuchi et al., 2009). In earlier QTL studies, *PPD-H2* was shown to have significant effects on flowering under short photoperiods and in autumn-sown field experiments (Pan et al., 1994; Cuesta-Marcos et al., 2008a). Allelic variation at *HvPRR7*, the candidate for the *PPD-H1* QTL on chromosome 2H, is particularly important in spring barley as the recessive allele confers insensitivity to long-day conditions, allowing for a prolonged growing period and consequently higher yield. Allelic variation at *HvPRR7* is attributed to amino acid changes in the CCT domain leading to the recessive allele (Turner et al., 2005).

Mapping of winterhardiness QTL in the *Triticeae* has been achieved using biparental mapping populations of inbred (or doubled haploid) lines. Statistical methods for QTL detection in biparental mapping populations have been continuously improved (reviewed by Wang et al., 2005). These methods allow the estimation of QTL positions (within a confidence interval), effects, and interactions between QTL without the necessity of a high marker density (Piepho, 2000). There are, however, limitations to QTL detection in biparental populations which can compromise the subsequent success of marker assisted selection. These include: bias in the estimation of QTL effects due to reduced sample size (Vales et al., 2005), narrow genetic base and consequent limited scope of inference (Crepieux et al., 2005), and broad confidence intervals for QTL positions and effects (Darvasi et al., 1993; Hyne et al., 1995).

Genome-wide association (GWA) mapping provides a complementary or alternative approach to biparental mapping. GWA mapping can be performed using different types of germplasm and the individuals in the analysis do not need to trace back to a single cross. Therefore, a wider genetic base, including actual breeding lines, can be sampled without having to develop populations (Flint-Garcia et al., 2003; Varshney et al., 2005). QTL detection by means of GWA is based on the existence of linkage disequilibrium (LD) between QTL and markers. In biparental mapping populations, associations between markers and QTL are only due to linkage, and the extension of LD depends on the number of recombination events that

occurred during the development of the population. In GWA many factors, other than recombination, can be responsible for LD: mutation, admixture, different degrees of relatedness among individuals (kinship), genetic drift and selection (Flint-Garcia et al., 2003). All these processes may create an underlying structure in the populations. Lack of consideration of population structure in the analysis may lead to false positives and false negatives (Pritchard et al., 2000). There are examples in which high density markers and large population sizes have been used to accurately model for population structure and several methods have been implemented to increase computational speed in GWA (Kang et al., 2008; Kang et al., 2010; Zhang et al., 2010).

The development of genotyping platforms with sufficient marker density has made barley GWA studies possible. GWA in barley is currently being implemented to identify and fine map traits directly in elite plant breeding material (Rostoks et al., 2006; Cockram et al., 2008; Gyawali et al., 2009; Beattie et al., 2010; Roy et al., 2010). We used the R software environment (R Development Core Team, 2009) with the efficient mixed model association (EMMA) package implementation (Kang et al., 2008) to empirically estimate the level of relatedness in our sample data and conduct GWA. Our objectives were to determine if mixed-model GWA using EMMA would be able to identify winterhardiness related QTL, and if so, would it provide new perspectives on the relationship between LT tolerance and VRN sensitivity.

MATERIALS AND METHODS

Germplasm

The germplasm consists of breeding accessions and cultivars, most of which originated from the Oregon State University (Corvallis, Oregon, USA) breeding program. There are two germplasm sets developed as part of the Coordinated Agricultural Project (http://barleycap.cfans.umn.edu; verified 22 November 2010): Oregon Barley CAP I and CAP II. Based on the phenotypic criteria described in Table 1, CAP I consists of 16 winter, 35 facultative, and 27 spring habit accessions. CAP II consists of 34 winter, 32 facultative, and 4 spring habit accessions. Of the total number of accessions (148), 39 accessions came from other breeding programs in the US. The remainder trace to crosses amongst six parents: Strider (winter, 6-row, feed type), Kold (winter, 6-row, feed type), 88Ab536 (facultative, 6-row, malt quality), Orca (spring, 2-row, malt quality), Legacy (spring, 6-row, malt quality), and Excel (spring, 6-row, malt quality). The two spring 6-rows were not included in the association analysis. The *Hordeum* Toolbox (THT) (http://hordeumtoolbox.org; verified 22 November 2010) contains genotype and phenotype data for all CAP accessions; data on the germplasm used for this study can be found in the THT database by searching for the Oregon State University breeding program and years 2006 (CAP I) and 2007 (CAP II). The identification number used for describing accessions in Figure 1 matches the “line synonym” number in the THT database.

Table 1. Criteria for defining spring, facultative, and winter germplasm groups in Oregon Barley CAP I+II based on phenotype. The trait and location are abbreviated as follows: LTT SPMN = Low temperature tolerance at St. Paul, Minnesota, USA; HD COR (S) = Heading date at Corvallis, Oregon, USA, spring planted.

	LTT SPMN (% survival)			HD COR (S) (Days to Flower)		
	Spring[†]	**Facultative**[§]	**Winter**[‡]	**Spring**	**Facultative**	**Winter**
Maximum	40	85	80	195	199	250[¶]
Minimum	0	45	25	169	172	200
Mean	14.8	67.8	64.6	179.3	181.1	241.8
Standard deviation	13.3	9.2	11.9	6.4	7	16.5
Number of lines	31	67	50	31	67	50

[†]The spring check is Orca with LTT SPMN = 0% and HD COR (S) = 174 days
[‡]The winter check is Strider with LTT SPMN = 72.5% and HD COR (S) = 250 days
[§]The facultative checks are Dicktoo with LTT SPMN = 80% and HD COR (S) = 178 days; and Maja with LTT SPMN=75% and HD COR (S) = 180 days
[¶]A value of 250 days was assigned to those lines that never flowered in the experiment

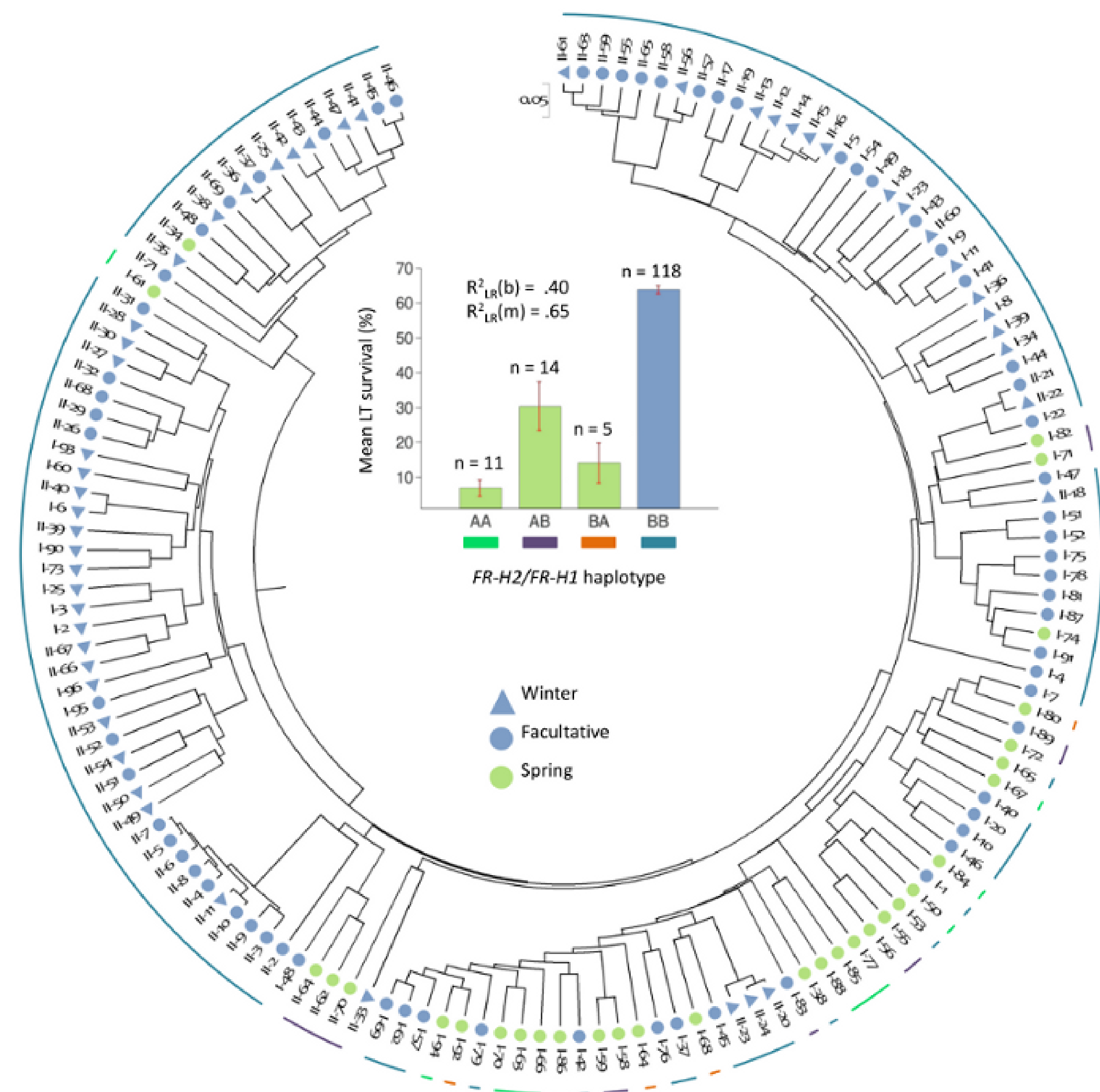

Figure 1. Genetic relationship among 148 barley CAP accessions (Continued).

Figure 1. Genetic relationship among 148 barley CAP accessions. The UPGMA tree is drawn to scale, with branch lengths representing the dissimilarities (1-shared alleles) from the same kinship matrix estimated for the association mapping analysis with R/EMMA. Roman numerals indicate the CAP germplasm set (I or II) and the numbers for each accession are the synonym identifications for the THT database (see materials and methods). Circles indicate vernalization insensitive accessions, while triangles indicate vernalization sensitive accessions. Accessions with a green label are low temperature susceptible, blue labeled accessions are low temperature tolerant. The inset panel displays the mean percent low temperature (LT) tolerance for the different *FR-H2/FR-H1* haplotypes with respective standard error bars. Within the panel is the likelihood-ratio-based R^2 for the model without (b=base) the markers and for the model with the markers (m = marker). The *FR-H2/FR-H1* haplotype for each accession is color coded in accordance to the inset panel with the outside tree brackets. *FR-H2* = marker 12_31236; *FR-H1* = marker *VRN-H1*a (Supplemental Table 1).

Phenotyping

Two winterhardiness traits were evaluated: LT tolerance and VRN sensitivity. LT tolerance was measured as the percentage of plants surviving LT stress in field and growth chamber tests. VRN sensitivity was measured under spring-sown field conditions and without VRN under greenhouse conditions. Flowering time *per se* was measured under fall-sown field conditions and under greenhouse conditions, with VRN. The various treatments, germplasm sets, years and environments during which the experiments were conducted are summarized in Table 2.

Table 2. Summary of phenotyping experiments conducted using Oregon Barley CAP I, CAP II, and CAP I+II germplasm.

	Environment and trait measured[†]									
	Field LT Tolerance[‡]			Controlled LT tolerance	Field VRN sensitivity (S)	Greenhouse VRN Sensitivity (V-)		Field flowering time (F)	Greenhouse flowering time (V+)	
Germplasm Set[§]	**%SURV**	**%SURV**	**%SURV**	**%SURV**	**HD**	**HD**	**FLN**	**HD**	**HD**	**FLN**
CAP I	POR 2006[¶]	FCCO 2006		MRI 2006		COR 2008	COR 2008	COR 2006	COR 2008	COR 2008
CAP II						COR 2009	COR 2009	COR 2007	COR 2009	COR 2009
CAP I+II			SPMN 2009		COR 2009					

[†] Environment and trait codes: Field = evaluated in the field; Controlled = evaluated in growth chamber with temperature and day length control; (V+) = vernalized; (V-) = not vernalized; (F) = Fall planted; (S) = Spring planted; LT = low temperature; VRN = vernalization

[‡] %SURV = percent survival; FLN = final leaf number; HD = heading date

[§] Germplasm code: lines submitted to the Barley Coordinated Agricultural Project in 2006 (CAP I) and 2007 (CAP II)

[¶] Location code and year of the trial: POR = Pendleton Oregon, USA; FCCO = Fort Collins, Colorado, USA; MRI = Martonvasar Research Institute, Hungary; SPMN = St. Paul, Minnesota; USA, COR = Corvallis, Oregon, USA

Controlled freeze tests were conducted at the Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, Hungary (MRI) as described by Skinner et al. (Skinner et al., 2006). Field assessments of winter survival were conducted at Fort Collins, Colorado, USA (FCCO), in Pendleton, Oregon, USA (POR), and in St. Paul, Minnesota, USA (SPMN). The trials were planted in the fall of 2006 (FCCO and POR) and 2009 (SPMN). At each location, plot sizes and experimental designs were in accordance with local practice. At SPMN, each accession was grown in a three meter, two-row plot, using a randomized complete block design with two replications. The percentage survival at all field sites was based on visual assessment when plots resumed growth after the winter.

For the greenhouse assessments, the VRN treatment consisted of maintaining seeds in moist soil in a growth chamber at a constant 4°C with no light for six weeks. Seedlings were then moved to a greenhouse maintained at 18°C/16°C day/night. A 16 h light/8 h dark light photoperiod was maintained using supplemental lights. Unvernalized plants were grown from seed planted one week prior to the removal of the vernalized treatments in the same greenhouse. The vernalized and unvernalized plants were grown in a two replicate randomized complete block design. On both vernalized and unvernalized plants, heading date (HD) and final leaf number (FLN) was recorded on the first stem to flower. The experiments were terminated 150 days after planting the unvernalized treatment. Plants that had not flowered by this time were assigned an HD value of 150. Fall- and spring-planted experiments were

conducted under field conditions at Corvallis, OR, USA (COR), using 1 m, one-row plots. Each entry was replicated twice using a randomized complete block design. The fall planted experiments were sown in 2006 (CAP I) and 2007 (CAP II). The spring planted experiments were sown in 2009. The experiment was terminated 250 days after January 1. Plants that did not flower were assigned an HD value of 250. Joint analyses of CAPI and CAPII for traits that were measured in different years (HD and FLN under controlled conditions and field flowering time under fall-sown conditions) were done using least squares adjusted means calculated with a set of common checks replicated in the different years.

Genotyping

Genomic DNA from each of the 148 CAP accessions was purified using Plant DNeasy (Qiagen, Valencia, CA, USA) kits starting with 100-300 mg of seedling leaves. Under the auspices of the Barley CAP project all accessions were genotyped for 3072 SNPs using two Illumina GoldenGate oligonucleotide pool assays (OPA). Details on the development of the OPAs are described elsewhere (Close et al., 2009; Szűcs et al., 2009). Briefly, SNPs detected in ESTs and sequenced amplicons were used to design three Illumina 1536-plex pilot Oligonucleotide Pool Assays (pilot OPAs; POPA1, POPA2, POPA3). SNPs were selected from three POPAs to generate two production barley OPAs (BOPA1 and BOPA2). The BOPA assays were conducted at the USDA-ARS Small Grains Genotyping Center in Fargo, ND. From the 3072 SNPs, 2126 were

informative in the combined CAP I and CAP II accessions. In addition, the *VRN-H1*, *VRN-H2*, *VRN-H3*, *PPD-H1*, and *PPD-H2* loci were genotyped using allele specific assays (Supplemental Table 1). The estimated positions of the SNPs are based on the consensus map developed by Close et al. (2009), and are available by downloading the 1.77 version of the barley HarvEST database (http://harvest.ucr.edu; verified 22 November 2010).

Statistical analysis

Linear mixed model

The linear mixed model approach used in the association mapping analysis, including the estimation of multiple levels of relatedness between accessions, was previously described by Yu et al. (2006). We additionally used the changes in algorithms and kinship estimation introduced by Kang et al. (2008). The vector of phenotypes, *y*, is modeled as:

$$y = X\beta + Qv + Zu + e$$

where X contains the marker data, β is a vector of marker allele effects to be estimated, Q contains the population assignments by STRUCTURE (Pritchard et al., 2000), v is a vector of subpopulation effects, Z is an identity matrix, u is the random

variance due to genome-wide relatedness, and e is the random variance due to error. The phenotypic covariance matrix is assumed to have the following form:

$$var(y) = 2K\sigma_g^2 + Z\sigma_e^2$$

where K is the matrix of kinship coefficients, σ_g^2 is the genetic variance from the genome-wide effects, and σ_e^2 is the residual variance.

Germplasm structure

The population assignment matrixes (Q matrices) for each of the CAP populations and for the combined set were generated with STRUCTURE following methods by Pritchard et al. (2000) and by using the linkage model described by Falush et al. (2003). A core set of 1527 SNP markers was selected after removing markers with minor allele frequencies and more than 10% missing data. The Kinship matrix was generated with R/EMMA (Kang et al., 2008). We determined whether the Q matrix would improve the fit to our vector of phenotypes significantly or if the Kinship estimation by EMMA would suffice as follows. Random SNPs are expected to be unlinked to the polymorphisms controlling the traits under study (H_0: no SNP effect). An approach that appropriately controls for type I errors is expected to show a uniform distribution of *p*-values (Yu et al., 2006). We chose the model that best explained our phenotypic data by plotting the cumulative distribution of the

observed *p*-values (generated with EMMA) for each model and population phenotypic data set against the expected, where the diagonal line in these cumulative plots represents the ideal distribution.

Association mapping

The phenotypic means per environment and for the combined environments and SNPs were subjected to an analysis with R/EMMA (Kang et al., 2008) using the publicly available (http://mouse.cs.ucla.edu/emma; verified 22 November 2010) package implementation. The association analysis was carried out by performing a linear mixed model association via *t*-test with restricted maximum likelihood estimates. After obtaining the *p*-values for each individual marker per phenotype, the threshold for the statistical significance was established by using the R /*q*-value, which measures the significance in terms of the false discovery rate (FDR) associated with each tested SNP (Storey, 2002; Storey et al., 2004). The q-value for a particular SNP is the expected proportion of false positives incurred when calling that fiture significant. In all our experiments we used a FDR α level equal to 0.05.

Phenotypic variation explained

The R^2 statistic is used in biparental QTL mapping to estimate the proportion of phenotypic variation explained by markers in the model. Linear mixed models have no well established R^2 calculation procedure. Sun et al. (2010) tested the

performance of several R^2-like statistics for linear mixed models and identified the previously described likelihood-ratio-based R^2 (R^2_{LR}) (Magee, 1990):

$$R^2_{LR} = 1 - \exp\left(-\frac{2}{n}(log\ L_M - Log\ L_0)\right)$$

where $log\ L_M$ is the maximum log-likelihood of the model of interest, $log\ L_0$ is the maximum log-likelihood of the intercept-only model, n is the number of observations. R^2_{LR} reduces to the regular R^2 and also provides a general measure for the QTL effects in linear mixed-model association mapping. The R^2_{LR} was used to calculate the variation explained by each individual significant marker.

As expected, all markers that are in high LD with each other will explain a similar proportion of the phenotypic variation. Therefore an LD heat map (r^2) was constructed with all markers with a *p*-value above the 0.05 FDR α level. The LD plot was created with R/snp.plotter (Luna and Nicodemus, 2007). One representative from each group of markers that were in complete LD ($r^2 = 1$) with each other was retained for further analysis. We further created a multi-marker model to test the number of QTL present within the remaining markers. For this, marker selection was carried out following a forward selection and backward elimination method, an approach regularly implemented in QTL detection (Basten et al., 1996; Cuesta-Marcos et al., 2008b). We used linear mixed models via *t*-test with restricted

maximum likelihood estimates implemented in R/nlme4 (Bates, 2005), using the markers as sources of variation. At each step, the marker with the lowest *p*-value of its *t*-statistic was added to the model. Markers with the lowest *p*-value of the *t*-statistic were then sequentially added to the model until no marker had a *p*-value below the 0.05 threshold. We then checked whether all markers included in the model were still significant. For the remaining markers we applied a backward elimination by sequentially removing markers with *p*-values above the 0.05 level until all markers left were significant and doing so we obtained the final model. With that final model, we conducted the likelihood ratio test (LRT) with ML estimates to find significant interactions between markers. Then we calculated the R_{LR}^2 of the model that included the significant markers and their significant interactions.

RESULTS AND DISCUSSION

Phenotyping

A comprehensive set of phenotyping experiments was conducted to measure LT tolerance and VRN sensitivity (Table 2). Flowering time *per se* was also measured; although this trait is not a major focus of this report, the data serve as a useful baseline for assessing the VRN sensitivity assays. The Oregon Barley CAP I and CAP II germplasm sets were measured together for percent winter survival at St. Paul, Minnesota, USA (SPMN) and for field VRN sensitivity at Corvallis, Oregon, USA (COR). Greenhouse assays were conducted separately for CAP I and CAP II and the data were combined by calculating the least square means based on the use of common checks. Throughout this report, we focus on these larger data sets, as population size is essential for greater detection power in association mapping studies (Yu et al., 2006; Zhao et al., 2007; Myles et al., 2009). However, as supporting evidence there are three independent measures of LT tolerance for CAP I – two field experiments: Fort Collins, Colorado, USA (FCCO) and Pendleton, Oregon, USA (POR) respectively, and one controlled environment at MRI, Hungary.

There is abundant phenotypic variation for LT tolerance, VRN sensitivity and flowering time in the Oregon Barley CAP I and II germplasm arrays (Figures 2 and 3, and Supplemental Figures 1 and 2). Starting with LT tolerance and the full population data set from SPMN, the range of values are representative of those reported in the literature for field survival in stress environments (Pan et al., 1994). The facultative

check Dicktoo was among the accessions with the highest survival (80%) whereas the survival for Orca, the spring growth habit check, was 0%. This differential winter injury was caused by minimum temperatures of -26°C (with 23 cm of snow cover) and -10°C (without snow cover). The percent survival for Maja, the facultative check used for the heading date studies, was 75%. Based on agronomic considerations, our definition of facultative growth habit includes high survival rates in target environments (Table 1). The minimum percent survival for facultative accessions was 45%. The results from the Oregon Barley CAP I germplasm tests at FCCO, POR, and MRI (Supplemental Fig. 1) corroborate the SPMN test. For these comparisons, the three individual CAP I tests are compared with the separate CAP I and CAP II data from SPMN. At FCCO the minimum temperature was -21°C (with snow coverage) -15°C (without snow coverage), and at POR the minimum temperature was -13°C (with snow cover) and -15°C (without snow cover). In each of these single germplasm array datasets, Dicktoo (facultative) is among the most cold tolerant and Strider, the winter check, has somewhat lower survival than Dicktoo. Within the individual environments Maja showed 90% survival at POR, 68% survival at FCCO, and a 78% survival at MRI. The MRI low temperature tolerance freeze tests support previous reports for Dicktoo and Morex (Spring check) (Hayes et al., 1993; Pan et al., 1994; Skinner et al., 2006). Using the same protocol, Skinner et al. (2006) report survival values for Dicktoo and Morex of 85% and 0% at −12.5°C, respectively. Overall, these data confirm that facultative accessions are as (or more) cold tolerant than winter

accessions. Karsai et al. (2001) reported similar findings in a survey of winter and facultative germplasm evaluated under controlled freeze tests at the MRI. Based on agronomic considerations, our definition of facultative growth habit includes high survival rates in target environments. As shown in Table 1, the maximum and minimum percent survival values for facultative and winter germplasm were 85% and 45%, and 80% and 25%, respectively.

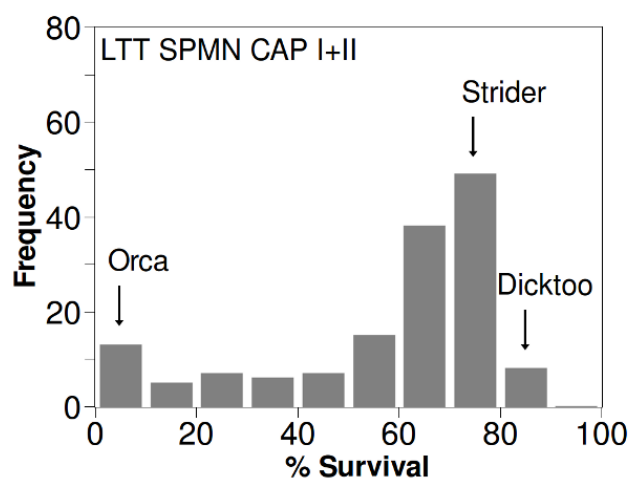

Figure 2. Phenotypic frequency distribution for low temperature tolerance evaluated in the Oregon Barley CAP germplasm. CAP I+II were evaluated for low temperature tolerance (LTT) at St. Paul, Minnesota (SPMN). Three checks used were: Orca = susceptible, spring growth habit; Strider = tolerant, winter growth habit; Dicktoo = tolerant, facultative growth habit.

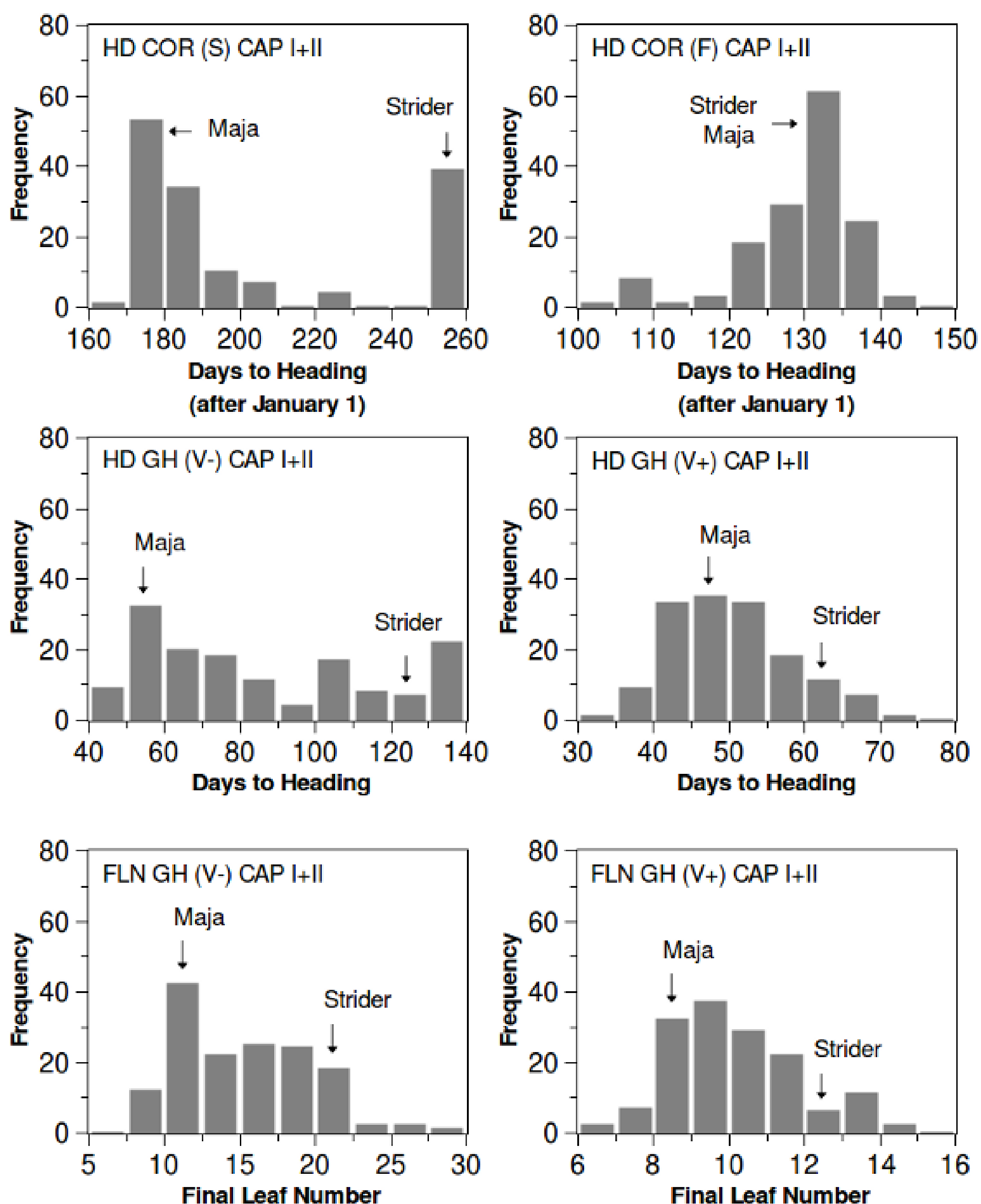

Figure 3. Phenotypic frequency distributions for heading date (HD) and final leaf number (FLN) evaluated in the Oregon Barley CAP germplasm. CAP I+II were evaluated for HD spring planted (S) and HD fall planted (F) under field conditions at Corvallis, OR, USA (COR). HD and FLN were measured with and without vernalization (V+, V-) under greenhouse (GH) conditions. Maja (facultative growth habit) and Strider (winter growth habit) were used as checks for the HD experiments.

Our definition of facultative growth habit is based on a lack of VRN sensitivity. We used three measures of vernalization sensitivity: (i) HD under spring planted field conditions at COR (ii) HD under greenhouse conditions, without VRN and (iii) FLN under greenhouse conditions, without VRN. HD and FLN under greenhouse conditions were measured on the same plants. Under field conditions, Strider and 38 other winter growth habit types did not flower (Figure 3). Maja (facultative) flowered 179 days after January 1. Average HDs for the spring checks Orca, Baronesse, Harrington, Robust, Tradition and Lacey were 174, 178, 176, 174, 174 and 175 days, respectively. There was a clear separation of winter vs. facultative/spring HDs under field conditions. There were a limited number of accessions with intermediate HDs. These accessions were either too late maturing for production agriculture (e.g. two weeks later than Maja) or they produced only a few tillers that flowered while the rest of the plant remained in a vegetative condition. Integrating the SPMN field survival data with the COR VRN sensitivity data we identified criteria for facultatives in this germplasm array as follows: there are 11 facultative accessions with winter survival values between 45% and 55% and 56 facultative accessions above 60%. Within the winter growth habit accessions there were 11 that flowered, under spring-sown conditions at COR between 200 and 229 days; the remaining 39 accessions never flowered.

Under greenhouse conditions, without VRN, a continuum of phenotypes is observed from insensitive to sensitive and there are lines out of the range of the

checks. Maja and Strider are constantly observed at opposite ends of the distributions for both HD and FLN. Maja and the spring habit checks had similar HD and FLN values, under greenhouse conditions and without VRN (Figure 3 and Supplemental Figure 2). For example, average HD values for Maja, Baronesse, Harrington, Robust, Tradition, and Lacy were 48, 48, 46, 46, 49, and 46 respectively. FLN values were 10, 10, 9, 9, 10 and 10 respectively. Over two-fold differences were observed between winters (e.g Strider) and facultatives/springs (e.g. Maja) for HD and FLN. The greenhouse data underscore the advantage facultative germplasm could have over winter germplasm: rapid cycling of generations as with springs. The highest correlation coefficients were observed between the three measures of VRN (Table 3 and Supplemental Table 2). HD under field conditions is most agronomically relevant and is necessary for determining if facultative germplasm will be appropriate for spring-sowing in any given environment. Under greenhouse conditions, HD is simpler to measure than FLN, but the latter is favored as an estimator of the transition from the vegetative to the reproductive phase of development (Limin et al., 2007; Cuesta-Marcos et al., 2008a; Cuesta-Marcos et al., 2008b). Similar distributions for greenhouse and field measures of vernalization sensitivity were observed for the separate Oregon Barley CAP I and CAP II germplasm arrays (Supplemental Figure 2).

Table 3. Pearson correlation coefficients among traits measured in the Oregon Barley CAP I+II germplasm. Trait and location codes are as follows: LTT = low temperature tolerance; HD = heading date; FLN = final leaf number; GH = greenhouse; SPMN = St. Paul, Minnesota, USA; COR = Corvallis, Oregon, USA; (S) = spring planted; (F) = fall planted; (V-) = without vernalization; (V+) = vernalized.

Trait and Environment†	HD COR (S)	HD GH (V-)	FLN GH (V-)	HD COR (F)	HD GH (V+)	FLN GH (V+)
LTT SPMN	0.31***	0.28***	0.31***	0.49***	0.15	0.09
HD COR (S)		0.86***	0.81***	0.42***	0.42***	0.40***
HD GH (V-)			0.93***	0.33***	0.48***	0.49***
FLN GH (V-)				0.31***	0.46***	0.53***
HD COR (F)					0.17*	0.16
HD GH (V+)						0.85***

* Significant at the 0.05 probability level
** Significant at the 0.01 probability level
***Significant at the 0.001 probability level

Although flowering *per se* is not a primary focus of this research, it is worth noting that HD under fall-sown field conditions shows a very low correlation with HD and FLN in the greenhouse conditions with VRN (Table 3 and Supplemental Table 2). This is likely due to the cumulative effects of changes in temperature and PPD duration under fall-sown conditions, whereas under greenhouse conditions a single long (16 h light/ 8 h dark) PPD was supplied, together with a consistent temperature profile of 20°C/10°C day/night. The distributions for fall-sown HDs for Oregon Barley CAP I compared with CAP II are very different, due to differing numbers of winter vs. facultative accessions. HD *per se* in barley is a complex topic in its own right (Karsai et al., 1997b; Hay and Ellis, 1998; Sameri and Komatsuda, 2004; Cuesta-Marcos et al., 2008b; Cuesta-Marcos et al., 2009).

Correlations between LT tolerance and VRN sensitivity measures (spring-sown and greenhouse without VRN) are low, but significant and positive. This can be explained by the fact that most (but not all) winter types have hight LT tolerance (Table 1). Likewise, fall-planted HD (*per se*) in the field is significantly and positively correlated with LTT. These patterns fit reports in the literature (Karsai et al., 2001). These patterns of positive correlations could be due to linkage or pleiotropy. In order to explore the genetic basis of these correlations we integrated the Oregon Barley CAP phenotype data with genotype data and conducted GWA mapping.

Structure and Association Mapping

The two Oregon Barley CAP germplasm sets are somewhat different in their composition. In CAP I, 66 lines trace to various crosses involving one or more of six parents (Strider, Kold, 88Ab536, Orca, Legacy, and Excel). Strider and Kold are winter growth habit types. 88Ab536 is a facultative. Orca, Legacy, and Excel are springs. All of these parental lines, except for Legacy and Excel, are also included in CAP I. The remaining eight accessions in CAP I do not figure in the recent pedigrees of other germplasm in the set. In CAP II, 31 lines trace to three parents (Strider, 88Ab536, and Kold). The remaining 39 accessions trace to other breeding programs and do not figure in the recent pedigrees of other Oregon CAP lines. Considering Oregon Barley CAP I+II, 97 lines share common parents and 51 (in terms of the short evolutionary history of a breeding program) are unrelated. The germplasm was selected according

to the Barley CAP objectives, which were directed towards characterizing barley germplasm relevant to breeding programs. The Oregon winter malting barley program is focused on the simultaneous improvement of winterhardiness and malting quality, as described by Muñoz-Amatriain et al. (2010), which leads to narrow pedigrees. Unrelated accessions from other breeding programs (e.g. USDA/ARS, Aberdeen, Idaho and Utah State University, Logan, Utah (both USA)) were included as possible sources of novel alleles for winterhardiness traits.

In order to reduce the number and likelihood of false positives in GWA mapping of winterhardiness traits we accounted for structure by determining the Q and the K matrices (see Materials and Methods). For all traits, we performed a linear mixed model association with restricted maximum likelihood estimates using the K matrix (estimated with EMMA), with the Q matrix (Q+K model) and without the Q matrix (K model). Per Supplemental Figure 3 we found that the expected and observed cumulative distributions of *p*-values obtained using the K and Q+K models were comparable. There were no consistent or notable differences in the number and identity of markers showing significant associations with the two models. Our results are in accordance with those of Zhao et al. (2007) who estimated the K matrix by defining kinship coefficients simply as the proportion of shared haplotypes for each pair of individuals. Zhao et al. (2007) showed that correcting for population structure with this simpler estimate of kinship was as effective in reducing the false-positive rate as using the Q + K model of Yu et al. (2006). We estimated the K matrix

using the Kang et al. (2008) method, which is similar to the method of Zhao et al. (2007), estimating a simple identical-by-state allele-sharing matrix . The K matrix estimated with EMMA also led to a biologically meaningful classification of the germplasm (Figure 1) as is discussed in greater depth later in this report. Therefore, we report the results of association mapping using only the K matrix to account for structure in the Oregon Barley CAP I + II.

We performed GWA mapping for all the data sets shown in Table 2. For LT tolerance we emphasize the SPMN results in this report due to the larger population size and simultaneous application of the LT stress to all the germplasm. The CAP II germplasm was assessed in field tests at POR and FCCO the year following the assessment of the CAP I, but due to milder winter conditions no differential winter injury was observed. Likewise, for the VRN sensitivity phenotype in this report we emphasize the Oregon Barley CAP I+II simultaneously spring planted at COR and the GH without VRN joint data sets. For low temperature tolerance at SPMN, all of the associations above the $\alpha = 0.05$ false discovery rate (FDR) were on chromosome 5H. A few markers on 3H, 4H, and 6H approached the threshold (Figure 4A). In the ~ 30 cM interval on 5H, 30 markers were significant (Figure 4B) and these markers occur in two linkage disequilibrium blocks corresponding to the reported positions of *FR-H1* and *FR-H2* (Francia et al., 2004).

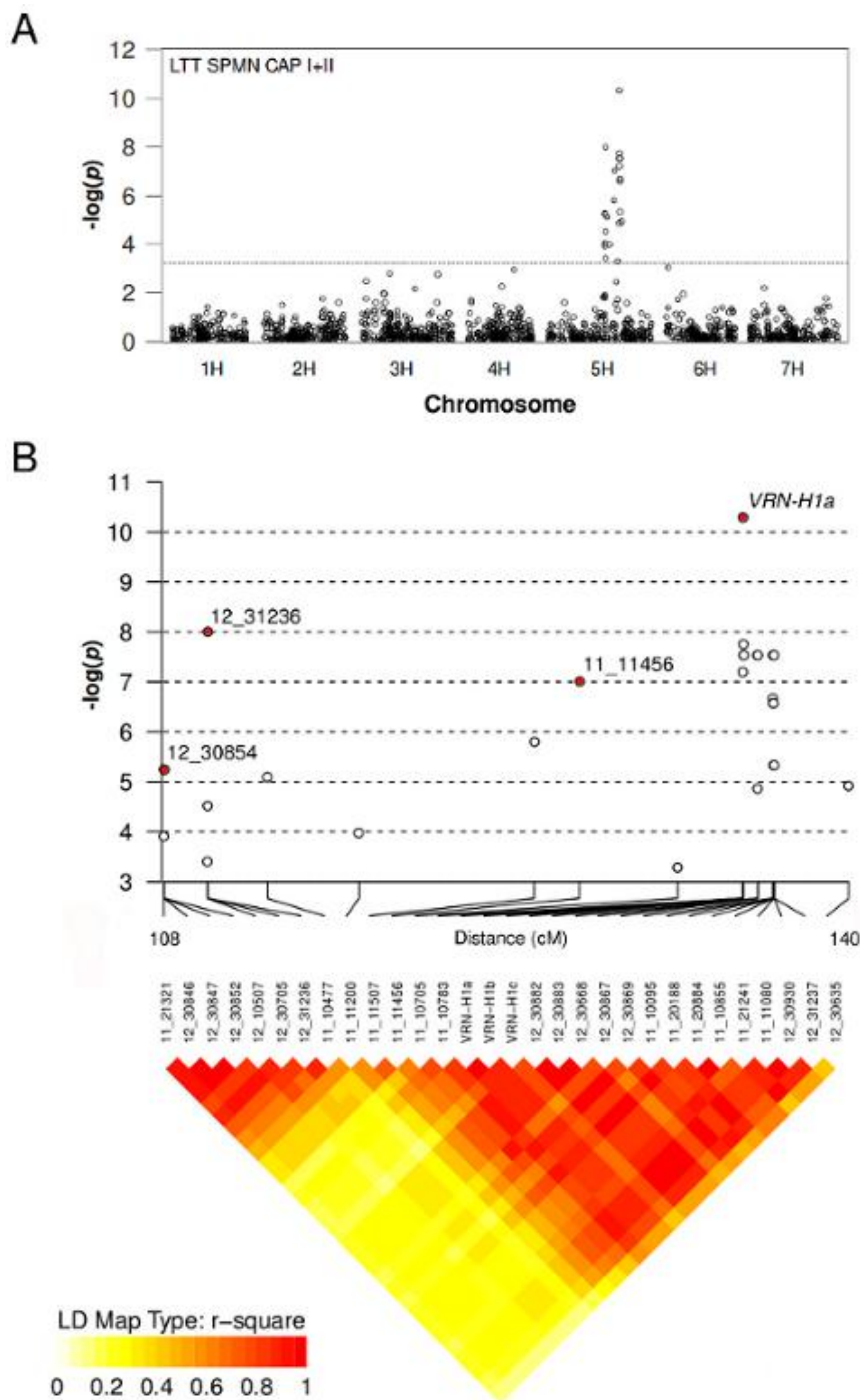

Figure 4. Low temperature tolerance (LTT) association scan for the Oregon Barley CAP I+II germplasm evaluated at St. Paul, Minnesota (SPMN) (Continued)

Figure 4. Low temperature tolerance (LTT) association scan for the Oregon Barley CAP I+II germplasm evaluated at St. Paul, Minnesota (SPMN). (A) Genome-wide association scan showing LTT QTL on 5H. The significance threshold was determined with a false discovery rate (FDR) α level of 0.05 (dashed line). (B) Close-up of the 5H chromosomal region (consensus map position from 108-140 cM) with r^2 LD plot of the significant markers. 12_30854 = SNP in HvCBF9; 12_31236 = SNP in barley heat shock transcription factor similar to Arabidopsis *HSFB2b*; 11_11456 = SNP in barley gene similar to Arabidopsis Glu-tRNA (Gln) amidotransferase subunit C; *VRN-H1*a = specific *HvBM5A* intron 1 amplicon (see Supplemental Table 1).

The candidate genes determining *FR-H2* are one or more members of a physically linked cluster of CBF family members (Skinner et al., 2005; Skinner et al., 2006). Marker 12_30854 is a SNP in *HvCBF9*. The *FR-H1* candidate is *HvBM5A* (von Zitzewitz et al., 2005; Dhillon et al., 2010). A specific amplicon in the first intron of *HvBM5A* (marker *VRN-H1a*; Supplemental Table 1) is the most significant marker for the LT tolerance association and additional significant markers are based on SNPs and INDELs in *HvBM5A* (Supplemental Table 1 and Supplemental Table 3). In the analysis of the SPMN data, and in the separate analyses of the CAP I data (see next section) *VRN-H1* markers based on functional polymorphisms in *HvBM5A* always showed more significant associations than SNPs elsewhere in the gene. In the *FR-H2* region, 12_31236 is more significant than 12_30854, the *HvCBF9* marker. Interestingly, the EST in which 12_31236 is located could be involved in LT tolerance, as it is annotated as a *heat shock transcription factor* (*HSF*). *HSF* genes are transcriptional activators of *heat shock proteins* (*HSP*s). *HSF*s and *HSP*s are numerous and are an interaction point between multiple stress response pathways, including heat, cold, salt, and

osmotic stress (Swindell et al., 2007). The highest homology with *Arabidopsis* of the protein encoded by the barley EST in which 12_31236 is located is *AtHSFB2b*. In Arabidopsis this gene is down-regulated more than two fold after 12 hours of exposure to 4°C (see supporting files from Swindell et al. 2007), and small HSPs have been shown to be induced by cold (Sabehat et al., 1998). Little is known about the low temperature responses of HSFs. The third most significant association in the 5H region was marker 11 _11456. The EST in which this marker is located encodes for a protein which shows the highest similarity to *Arabidopsis Glu-tRNA (Gln) amidotransferase subunit C*. This gene has no obvious relationship with low temperature tolerance. Other significant markers in the region could be of interest as candidate genes based on annotations in the HarvEST database with their respective unigene ID (Supplemental Table 3). However, in GWA mapping markers in high linkage disequilibrium with the functional/causal polymorphisms may show higher significance than the functional/causal polymorphism itself (Weigel and Nordborg, 2005). The resolution of our GWA mapping of LT tolerance cannot, therefore, establish causal relationships between the phenotype and candidate genes nor can we confirm or refute that *HvBM5A* is the determinant of *FR-H1*, or that one or more *HvCBF* members are candidates for *FR-H2*. What is of particular note is that in this GWA scan we identified the one chromosome region – and the two QTL therein – reported to be associated with LT tolerance in every biparental mapping study in the *Triticeae* reported over nearly two decades.

The GWA mapping results for the experiments involving Oregon Barley CAP I are consistent in identifying the same region on chromosome 5H (Supplemental Figure 4). In all cases, the most significant markers involve functional polymorphisms and SNPs in *HvBM5A* (Supplemental Table 3). The results from CAP I are at risk of false positive associations due to small population size. However, it is interesting that some significant associations are coincident with chromosome regions where genes/QTL are located that relate to the timing of the vegetative to reproductive transition. For example, the marker on the long arm of chromosome 4H showing the most significant association with LT tolerance at POR is 12_30824, a SNP in *HvBmy1,* which is ~4cM from *VRN-H2*. In CAP II SPMN, on chromosome 1H, 11_10686 approaches the FDR threshold and is ~20cM from *PPD-H2*. The EST in which marker 11_10686 is located has the highest similarity to Arabidopsis *ERF4* (*Ethylene Responsive Element Binding Factor 4*). Other significant associations are coincident or in the proximity of genes with possible roles in abiotic stress tolerance, e.g. *HvCBF8* on 2H. Annotations for other genes in which SNPs show significant, or nearly significant, associations in the CAP I and CAP I + II datasets do not have immediately apparent roles in abiotic stress resistance or growth and development. These associations may be due to false positives or to genes with effects too small to be detected as significant with the current germplasm, phenotype data, and marker density. Additional experiments involving GWA mapping in larger populations with balanced phenotype data and higher marker density, as well as genomic selection

(GS) will be necessary to validate these possible associations, and others, involved in LT tolerance.

The GWA scans for the three measures of VRN sensitivity (Figure 5) identify a specific amplicon of the *ZCCT-Hb* gene at the *VRN-H2* locus (Supplemental Table 1 and Supplemental Table 4) and SNPs within and/or near *ZCCT* gene family members. This validates the biparental QTL reports (Pan et al., 1994; Francia et al., 2004) and the functional models for the epistatic interaction of VRN genes in which, under long day conditions, the *VRN-H2* locus represses flowering (Yan et al., 2004; Trevaskis et al., 2007; Hemming et al., 2008). This finding provides a genetic explanation for the very high phenotypic correlation observed between the three measures of the VRN sensitivity phenotype (Table 3 and Supplemental Table 2). It further provides evidence that the complete deletion of *ZCCT-H genes* is sufficient to eliminate vernalization sensitivity. Neither *VRN-H1* nor *VRN-H3* individually were identified as significant determinants of vernalization sensitivity. There are a few markers approaching significance on 5H in the vicinity of *VRN-H1*, but these are not above the 0.05 FDR α level. The lack of a significant *VRN-H1* effect is probably due to a very small numbers of lines (n =13) that have spring alleles at *VRN-H1* and the epistatic inheritance of the trait. The *VRN-H3* situation is not as clear. It is likely that most, if not all, accessions in CAP I and II have recessive (winter) *VRN-H3* alleles. However, the assignment of dominant and recessive allele types at this locus is not possible at this point: the proposed functional polymorphism in *HvFT1* intron 1 (Yan et al., 2006)

is not valid in broader arrays of barley germplasm (Cuesta-Marcos et al. submitted). A significant effect is observed in the greenhouse data on chromosome 2H. This is due to a specific amplicon of the *HvPRR7* gene (*PPD-H1*) (Supplemental Table 1 and Supplemental Table 4), where some genotypes show later flowering under long days. The greenhouse was maintained at a 16 hr light/ 8 hr dark photoperiod for the duration of the experiment. *HvPRR7* nearly reaches the FDR threshold for FLN. The only individual data set in which *PPD-H1* shows a clear and significant effect is with the Oregon Barley CAP II GH HD data, without VRN (Supplemental Figure 5B). In this scan, marker 11_20074 (chromosome 7H) is also significant. Marker 11_20074 is a SNP within a *cinnamoyl CoA Reductase 1* gene similar to the wheat *Ta-CCR1*, which is associated with stem elongation (Ma, 2007), and hence could be affected by day length. Marker 11_20074 is approximately 17 cM from *HvFT1* (*VRN-H3*). The basis of the low phenotypic correlations between fall-sown HD and the vernalized GH treatments is apparent in the scans (Figure 5 and Supplemental Figure 5A, B). *PPD-H2* drives HD in the field due to short days over the winter, delaying the vegetative to reproductive transition in competent accessions. In contrast, *PPD-H1* drives flowering in the greenhouse due to the continuous long day photoperiods.

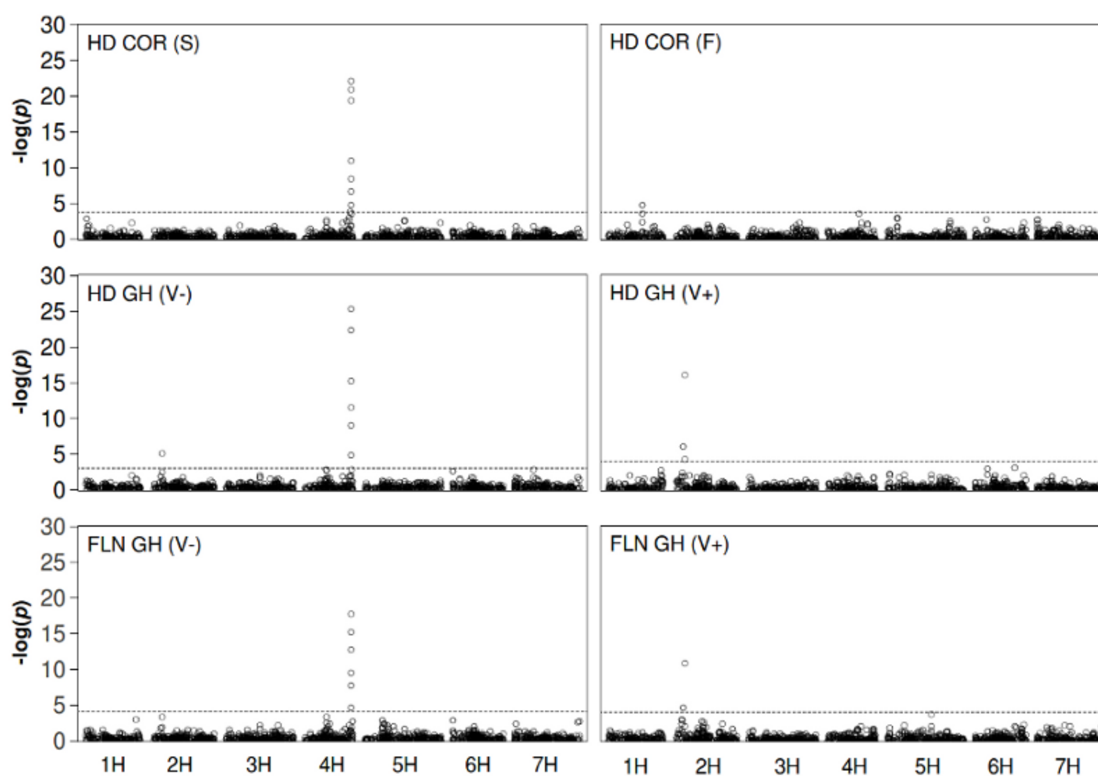

Figure 5. Heading date (HD) and final leaf number (FLN) association scans for Oregon Barley CAP I+II. Significance thresholds were determined with a FDR level of 0.05 (dashed line). Left panels show the vernalization sensitivity scans and right panels show the flowering time scans. Environments and treatments are coded as follows: COR = Corvallis, Oregon, USA; GH = Greenhouse; S = spring planted; (V-) = not vernalized; F = fall planted; (V+) = vernalized.

Based on the results of the association mapping of VRN sensitivity and LT tolerance, the strategy for developing facultative germplasm is apparent: fix “winter” alleles at *FR-H1* and *FR-H2* on chromosome 5H and the *ZCCCT-H* deletion on chromosome 4H. Photoperiod responses can be targeted to specific environments by selecting for appropriate alleles at *PPD-H1* and *PPD-H2*. Short day sensitivity is likely to be appropriate for all environments, as it will delay the vegetative to reproductive

transition. Long-day sensitivity could maximize yield by delaying maturity. However, the advantages may not be apparent in all environments. In some production zones, early winter barley avoids summer heat and water stress and in others earliness allows for relay cropping. All evidence, in this sample of germplasm, indicates that VRN sensitivity is not a prerequisite for maximum LT tolerance. Therefore facultative germplasm seems to be the growth habit of choice: it will allow for accelerated cycles in the greenhouse for specific breeding purposes and production of the same variety under fall or spring-sown conditions.

If breeding for facultative germplasm is the principal objective, the question that remains is whether to target *FR-H1* or *FR-H2* or both QTL. Interestingly, there are two distinct LD groups on chromosome 5H. In order to explore this issue in greater depth, we identified the phenotypic values of the four possible haplotypes at *FR-H2*/*FR-H1* and the proportion of phenotypic variance explained by the two-locus haplotype (Figure 1 and Table 4). This required an alternative approach to estimate the proportion of phenotypic variation explained by markers. The R^2 calculation is standard in biparental QTL mapping but it has not been well-established for linear mixed models. Sun et al. (2010) tested the performance of several R^2-like statistics for linear mixed models and reported that R^2_{LR} reduces to the expected R^2 and provides a general measure for QTL effects in linear mixed-model association mapping. We therefore used this approach and found an overall R^2 for the *FR-H2*/*FR-H1* haplotype on winter survival (using the SPMN data) of 0.25 (R^2_{LR} (marker model) –

R^2_{LR} (base model)) (Figure 1 inset and Table 4). The proportion of phenotypic variance accounted for by *FR-H2* alone is 9% and by only *FR-H1* is 15%. Francia et al. (2004), using a biparental mapping population, reported R^2 values of 0.22 and 0.37 for *FR-H2* and *FR-H1*, respectively, and an adjusted model accounting for both loci, of 0.63. The differences in our estimates are likely due to the germplasm and perhaps the QTL mapping methodology. Francia et al (2004) used a biparental population derived from a cross of two parents with extreme differences in LT tolerance.

Table 4 . Linear mixed model explaining the proportion of variation for low temperature tolerance in Oregon Barley CAP I+II. R^2_{LR} are calculated for each individual significant marker and interaction between markers. Marker 12_3123 is approximately 2 cM from the CBF gene cluster at *FR-H2* and is an SNP within a heat-shock transcription factor similar to Arabidopsis *HSFB2b.* Marker *VRN-H1a* is at *FR-H1*, and is a specific *HvBM5A* amplicon (see Supplemental Table 1). The effect of the interaction without the base model between *FR-H1* and *FR-H2* is R^2_{LR} = 0.25.

Model ID†	Model	Model form	-2(ML)	LRT	*p*-value	R^2_{LR}
1	Intercept	y=μ + e	1351.6			
2	Q	y=μ + Qυ + e	1309.0	42.6	<0.001	0.25
3	K	y=μ + Zu + e	1276.6	75.0	<0.001	0.40‡
4	Q + K	y=μ + Qυ + Zu + e	1273.2	3.5	0.327	0.41
5	12_31236 + K	y=μ + m_1 + Zu + e	1253.2	23.5	<0.001	0.49
6	*VRN-H1*a + K	y=μ + m_2 + Zu + e	1234.9	41.8	<0.001	0.55
7	12_31236 + *VRN-H1*a+K	y=μ + m_1 + m_2 + Zu + e	1205.9	29.0	<0.001	0.63
8	12_31236 × *VRN-H1*a + K	y=μ+m_1 + m_2 + $m_1 \times m_2$+Zu + e	1195.3	10.6	0.014	0.65§

† Model used for likelihood ratio test (LRT): alternative hypothesis 2 and 3 against null hypothesis 1; alternative hypothesis 4 against null hypothesis 3; alternative hypothesis 5 and 6 against null hypothesis 3; alternative hypothesis 7 against null hypothesis 6; alternative hypothesis 8 against null hypothesis 7

‡Proportion of the variation explained by the base model

§Proportion of the variation explained by the full model

We further used the R_{LR}^2 method proposed by Sun et al. (2010) with the Oregon Barley CAP I+II VRN sensitivity data (Table 5 and Supplemental Table 5). After applying the forward selection-backward elimination method and determining their R_{LR}^2 we found that the marker or markers that best explained the linear mixed model were: for COR spring planted *VRN-H2b* with an R_{LR}^2 = 0.29; for HD GH without vernalization *PPD-H1* and *VRN-H2b* with an R_{LR}^2 = 0.30; for FLN GH without vernalization *PPD-H1* and *VRN-H2b* and their interaction with an R_{LR}^2 = 0.25; for HD COR fall planted *PPD-H2* with an R_{LR}^2 = 0.05; for HD GH with vernalization *PPD-H1* with an R_{LR}^2 = 0.30; and for FLN GH with vernalization *PPD-H1* with an R_{LR}^2 = 0.19. Cuesta-Marcos et al. (2008b), using a biparental mapping approach, reported very similar R^2 values for markers within the same genes.

Table 5. Linear mixed model explaining the proportion of variation for flowering time for the Oregon Barley CAP I+II spring planted at Corvallis, Oregon, USA. R_{LR}^2 are calculated for each individual significant marker and interaction between markers. Marker *VRN-H2b* is a specific amplicon of *ZCCT-Hb* (see Supplemental Table 1). The effect of this marker is R_{LR}^2 = 0.29.

Model ID[†]	Model	Model form	-2(ML)	LRT	P-value	R^2_{LR}
1	Intercept	y=μ + e	1436.7			
2	Q	y=μ + Qυ + e	1392.2	44.5	<0.001	0.26
3	K	y=μ + Zu + e	1364.9	71.8	<0.001	0.38[‡]
4	Q + K	y=μ + Qυ + Zu + e	1364.4	0.5	0.327	0.39
5	*VRN-H2*b + K	y=μ + m_1 + Zu + e	1272.2	92.7	<0.001	0.67[§]

[†] Model used for the likelihood ratio test (LRT) = alternative hypothesis 2 and 3 against null hypothesis 1; alternative hypothesis 4 against null hypothesis 3; alternative hypothesis 5 against null hypothesis 3
[‡]Proportion of the variation explained by the base model
[§]Proportion of the variation explained by the full model

In our analysis, the winter allele haplotype (coded as “BB”) is preponderant in the Oregon Barley CAP I and II germplasm (118/148 accessions = ~ 80%). Accessions with this haplotype had an average winter survival at SPMN of 64%. The “BB” haplotype is found throughout the CAP I and II germplasm but only in winter (n = 52) and facultative (n = 66) accessions (Figure 1). In contrast, the few accessions with only one favorable allele (“AB” and “BA” haplotypes) had average survival values of 30% and 14% respectively. All of these accessions have spring germplasm in their pedigrees and persisted in the Oregon breeding program due to the fact that in the test environments routinely used by the program, no differential winter injury was observed during cycles of assessment and selection of this germplasm. Only one accession in the CAP I and II germplasm is a spring variety based on passport data (Orca). This variety and 10 other accessions had < 10% survival at SPMN. All of these had “AA” haplotypes at *FR-H2/FR-H1* (Table inset, Figure 1; Figure 6). Of the “AB” and “BA” haplotypes, all were classified as spring types, based on agronomic criteria except for two, which were classified as winter types, based on VRN sensitivity (Table 1). These classifications underscore the challenges of classifying germplasm by growth habit. In terms of phenotype, spring growth habit types can be classified based on their lack of VRN sensitivity and low probability of winter survival in target environments. Winter growth habit types can be classified as vernalization sensitive. Cold tolerance is implicit but not defined. Facultative types lie somewhere between. von Zitzewitz et al. (2005) proposed that the term “facultative” be used to describe

accessions with the *VRN-H2* deletion and a winter allele at *VRN-H1*. This two-locus haplotype corresponds to the "AB" + "BB" accessions identified in Figure 1 and the "AB(A)" + "BB(A)" in Figure 6. For most fall-sown environments in higher latitudes, we would amend this definition to specify the "BB" *FR-H2/FR-H1* haplotype, coupled with the "A" *VRN-H2* deletion haplotype ("BBA", Figure 6). By these criteria, 66 accessions in the Oregon CAP I and II germplasm would be described as facultative. All facultative accessions have *ZCCT-H* deletions. Based on a threshold cutoff of 45% survival at SPMN, 67 accessions would meet the definition of facultative based on phenotype. Thus, in this sample of germplasm, the three-locus haplotype is 99% effective in predicting the phenotype.

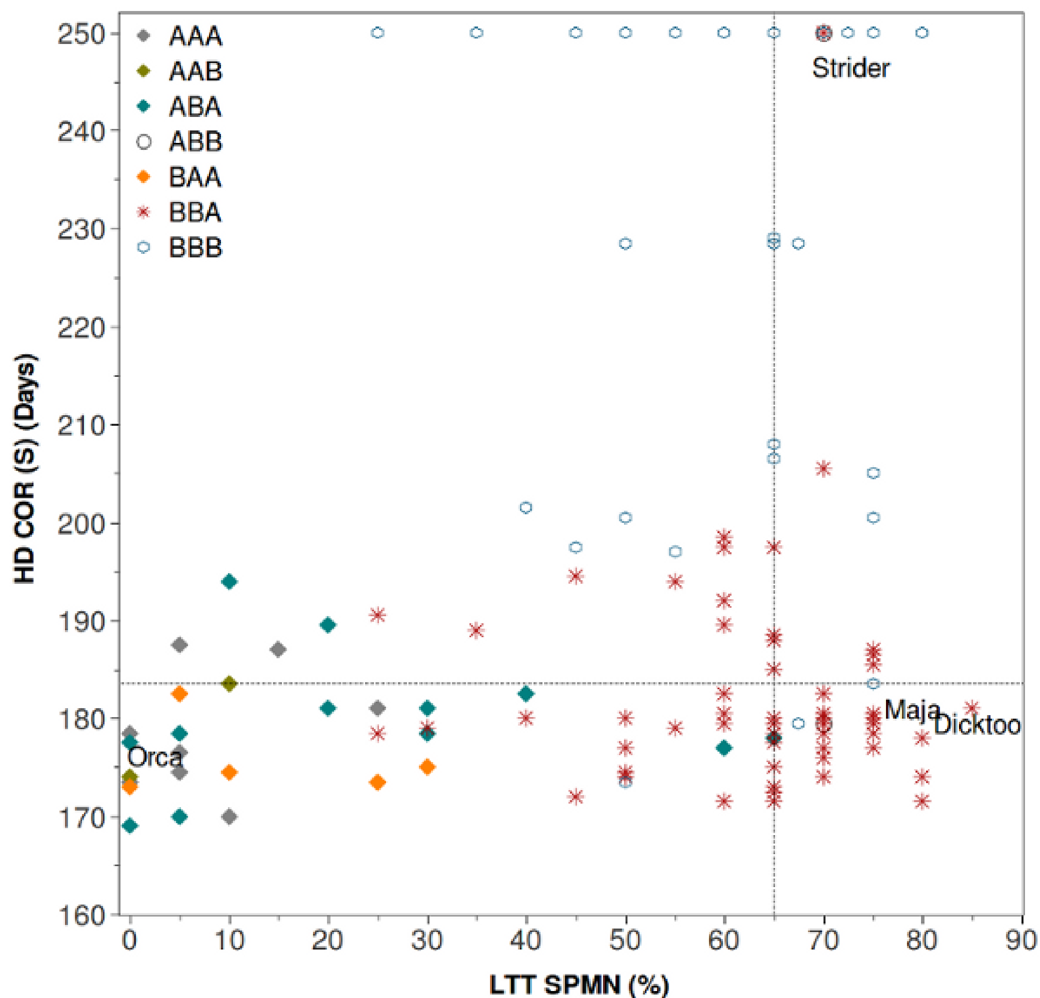

Figure 6. Biplot of vernalization sensitivity vs. low temperature tolerance for seven haplotypes at the *FR-H2*, *FR-H1*, and *VRN-H2* loci respectively in Oregon Barley CAP I+II germplasm. HD COR (S) = heading date, field spring planted, at Corvallis, OR, USA; LTT SPMN (%) = low temperature tolerance percent survival at St. Paul, Minnesota, USA. Dashed lines indicate the median for each trait. *FR-H1* = marker 12_31236; *FR-H1* = marker *VRN-H1*a; *VRN-H2* = marker *VRN-H2*b (Supplemental Table 1 and Supplemental Table 3).

This first use of GWA mapping for LT tolerance and VRN sensitivity in barley confirms that GWA is as effective as biparental QTL mapping for these traits. GWA mapping results were achieved using a sample of breeding lines, all of which, except for the spring variety Orca, had some agronomic potential (based on phenotype) in the Pacific Northwest of the USA. Subsequently, field survival at SPMN was effective in identifying a subset of accessions with the capacity to survive the low temperature stresses encountered at that location in the season during which the stresses were assesed. All biparental QTL populations used to date are based on winter/facultative x spring crosses in which at least 50% of the progeny are of no direct utility to the breeding program.

These results confirm that, in this sample of germplasm, maximum LT tolerance can be achieved with facultative growth habit. This presents opportunities for rapid cycling of germplasm and the option to use the same varieties under fall- and spring-planted conditions. Facultative growth habit can be predicted with very high accuracy based on a three-locus haplotype. Two of the loci define the *FR-H2/FR-H1* haplotype. Unless QTL for other traits are found in the interval between the two LD groups defining the *FR-H2* and *FR-H1*, selection could target the complete ~ 30 cM region on 5H. The third locus – *VRNH2* – is not necessary or sufficient for LT tolerance. These GWA mapping results were found using a population of modest size (Oregon Barley CAP I + CAP II = 148) and many of the same results were obtained using only CAP I and or CAP II. In terms of methodology, we found that the K matrix

alone was sufficient to account for structure in the sample of germplasm and that it generated a biologically meaningful classification of genetic relationships. The method of Sun et al. (2010) was effective in providing estimates of the proportion of phenotypic variance accounted for by significant associations. The modest estimate of R^2_{LR} (0.25) offers hope that additional gains from selection for LTT are possible in this germplasm. There are numerous regions in the genome where near-significant associations were observed. GWA mapping in larger sets of facultative germplasm in a balanced set of field and controlled environment trials should be effective in determining which of these possible associations are real and which are false positives. At the same time, genomic selection in this germplasm should be effective in fixing, and validating the effects of alleles with small effects. Finally, this research was conducted using a small sample of related germplasm: abundant genetic resources in germplasm collections and other breeding programs have yet to be mined for alternative alleles at the 5H loci and at as yet unknown loci.

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GENERAL CONCLUSIONS

Starting 10,000 years ago the ancestral winter growth habit of barley was selected against, leading to the present predominance of spring habit forms. Winter habit barley has persisted in isolated areas but breeding efforts are not at the scale of those applied to spring barley. As a consequence, winter barley varieties lack in cold tolerance, and their quality profiles may be deficient compared to spring varieties. Accelerated breeding of winter varieties is warranted by the advantages they offer in terms of risk avoidance, productivity and water use efficiency as compared to spring types. However, vernalization sensitivity complicates the accelerated development of such varieties. Our results confirm that, in this sample of germplasm, maximum low temperature tolerance can be achieved with facultative growth habit. This presents opportunities for rapid cycling of germplasm and the option to use the same varieties under fall- and spring-planted conditions.

These breeding cycles can be further accelerated, and gain from selection maximized, by using genetic information. Facultative growth habit can be predicted with very high accuracy based on a three-locus haplotype. Two of the loci define the *FRH1/FRH2* haplotype. Unless QTL for other traits are found in the interval between the two LD groups defining *FRH1* and *FRH2*, selection could target the complete ~ 30 cM region on 5H. The third locus – *VRNH2* – is not necessary or sufficient for LTT.

We have demonstrated that GWA mapping for LTT and vernalization sensitivity in barley is as effective as biparental QTL mapping for these traits. All biparental QTL populations used to date are based on winter/facultative x spring crosses in which at least 50% of the progeny are of no direct utility to the breeding program. Our results were obtained using breeding lines and current varieties. Nearly 20 years and multiple mapping populations were required to map *FRH1*, *FRH2*, *PPDH1*, and *PPDH2*. We identified all these loci using a population of modest size (Oregon Barley CAP I + CAP II = 148) and many of the same results were obtained using smaller subsets (e.g. only CAP I and or CAP II). Our results from GWA mapping are equally as effective in identifying candidate genes for QTLs as biparental populations and equally as ineffective at distinguishing between linkage and pleiotropy. The advent of cost-effective Next Generation sequencing technology should help to determine the actual genomic architecture in key regions, such as the ~ 30 cM region on 5H. This may reveal the presence (or absence) of genes in the vicinity of *VRNH1* that are actually the determinants of low temperature tolerance. Or *VRN-H1* may stand alone as the pleiotropic causal agent of low temperature tolerance and vernalization sensitivity. However, in the case of facultative types, it will be a *VRN-H1* unrepressed by *VRN-H2*.

In terms of fundamental understanding of abiotic stress physiology in the *Triticeae*, our results provide additional independent validation for the models accounting for the epistatic interactions of alleles at vernalization loci. Sensitivity to

short photoperiod is an important attribute of low temperature tolerance. Maximum cold tolerance is achieved with a delay in the transition from vegetative to reproductive stages. We found that short-day sensitivity was not a principal determinant of low temperature tolerance. Therefore, introgression and fixation of this attribute could be expected to have positive, or at least neutral, effects.

The results of this research should be of assistance in allowing the Oregon State University breeding program, and its cooperators, to address the long-term objective of developing low temperature tolerant varieties with superior malting and food quality. We tested our hypothesis that maximum levels of cold tolerance could be achieved with facultative growth habit via the deletion of *VRN-H2* and accumulation of favorable alleles at other loci and found it robust. This establishes the foundation for the next phases of this research in which GWA mapping in larger sets of facultative germplasm in a balanced set of field and controlled environment trials should be help us to determine which of the near-significant possible associations are real and which are false positives. At the same time, GS in this germplasm will allow us to fix, and validate, the effects of alleles with small effects. Finally, this research was conducted using a small sample of related germplasm: abundant genetic resources in germplasm collections and other breeding programs have yet to be mined for alternative alleles at the 5H loci and at as yet unknown loci.

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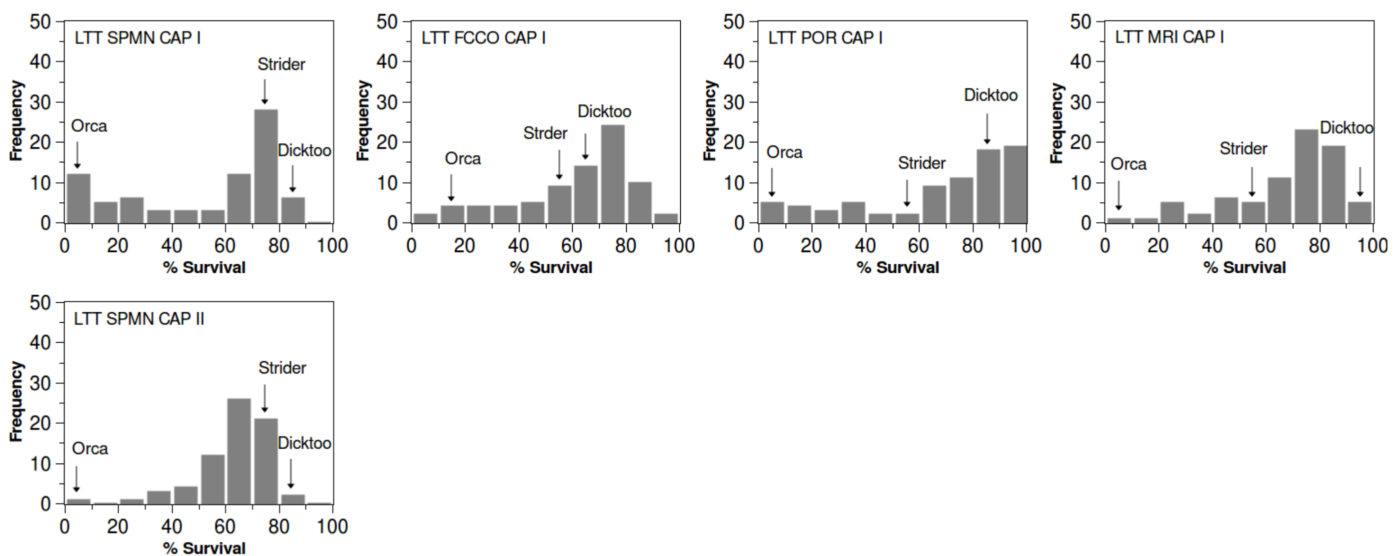
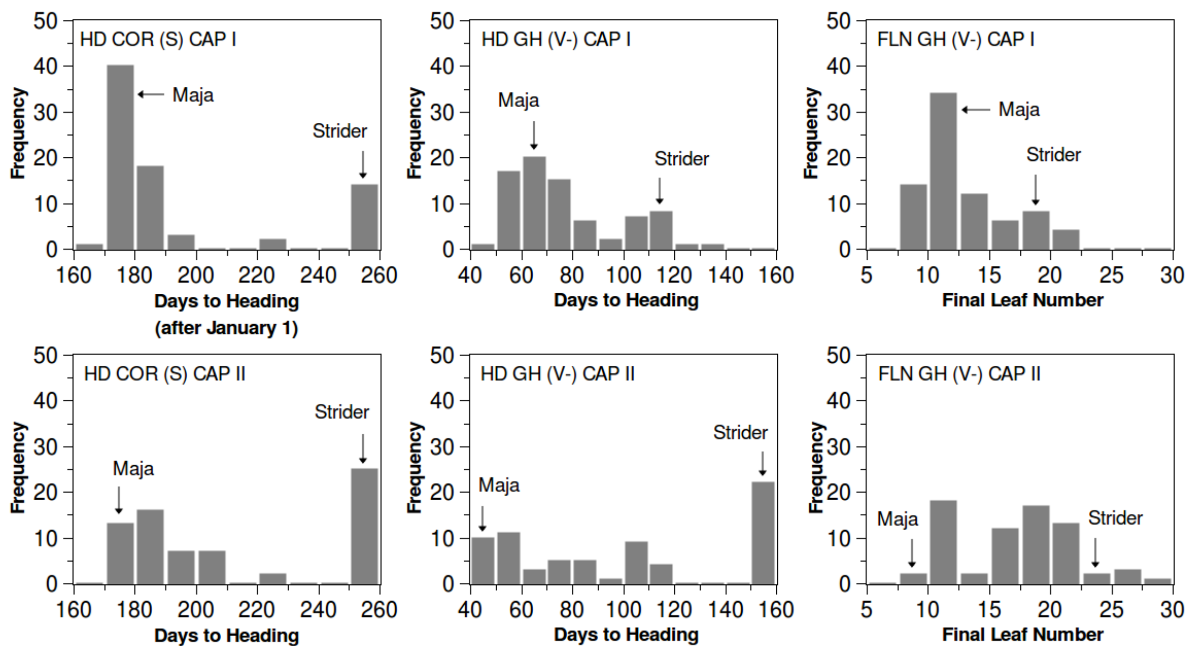
APPENDIX

Supplemental Figure 1. Phenotypic frequency distributions for low temperature tolerance (LTT) evaluated in Oregon Barley CAP I and CAP II germplasm. Three checks were used: Orca = susceptible, spring growth habit; Strider = tolerant, winter growth habit; Dicktoo = tolerant, spring growth habit. The locations are abbreviated as follows: SPMN = St. Paul, Minnesota, USA; FCCO = Fort Collins, Colorado, USA; POR = Pendleton, Oregon, USA; MRI = Martonvasar Research Institute, Hungary.

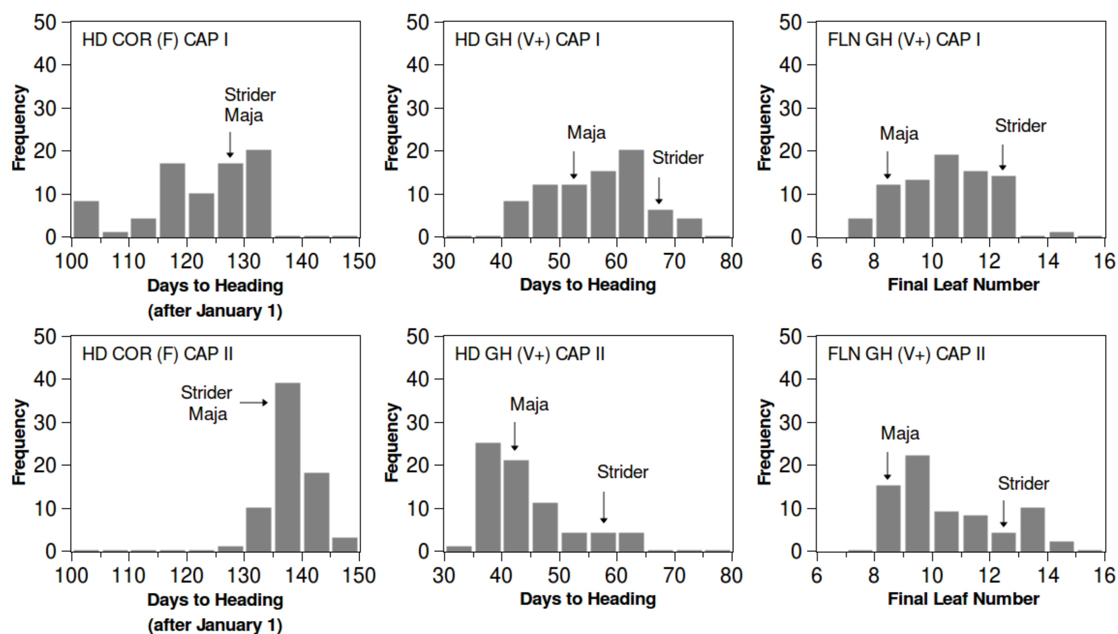


A



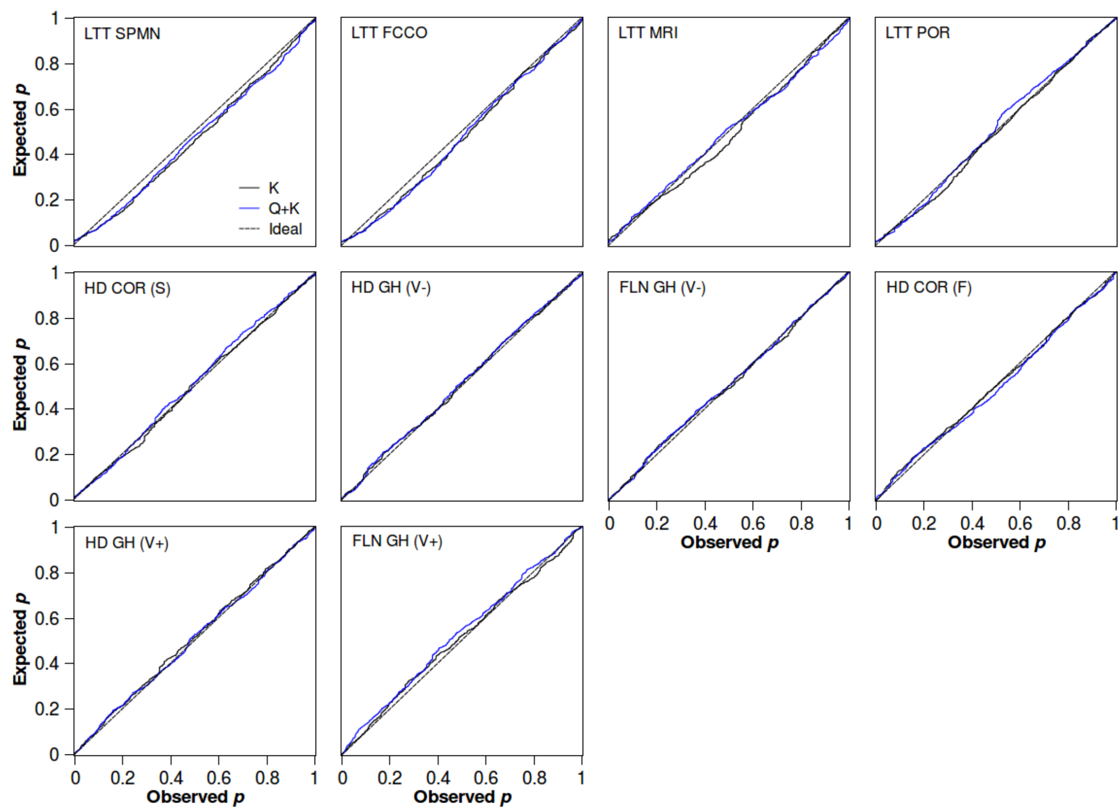
Supplemental Figure 2. Phenotypic frequency distributions for heading date (HD) and final leaf number (FLN) evaluated in the Oregon Barley CAP germplasm (Continued).

B

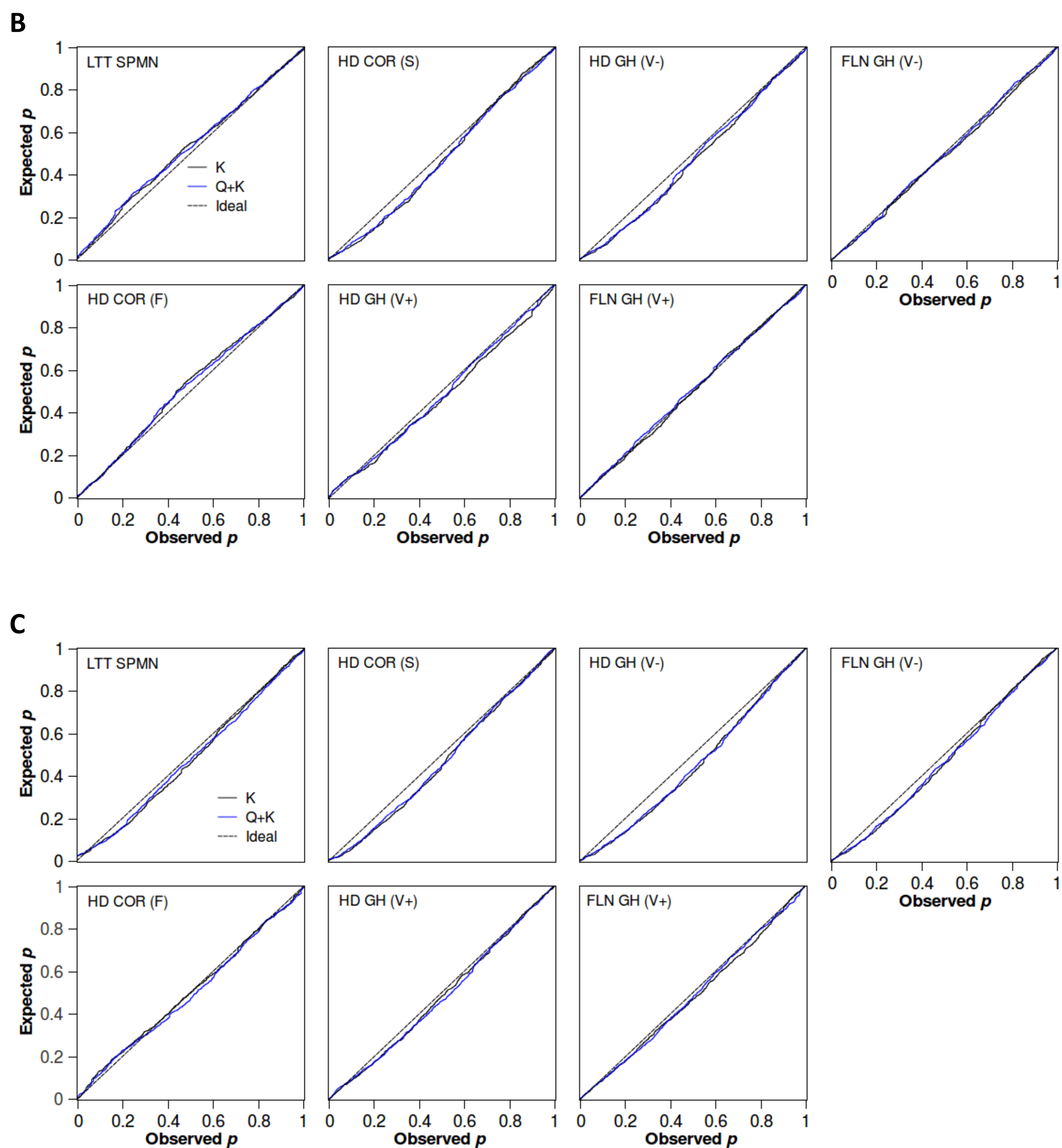


Supplemental Figure 2. Phenotypic frequency distributions for heading date (HD) and final leaf number (FLN) evaluated in the Oregon Barley CAP germplasm. (**A**) CAP I and CAP II were evaluated for HD spring planted (S) under field conditions at Corvallis, OR, USA (COR), and HD and FLN without vernalization (V-) under greenhouse (GH) conditions. (**B**) HD fall planted (F) under field conditions at COR, and HD and FLN were measured with vernalization (V+). Maja (facultative growth habit) and Strider (winter growth habit) were used as checks.

A

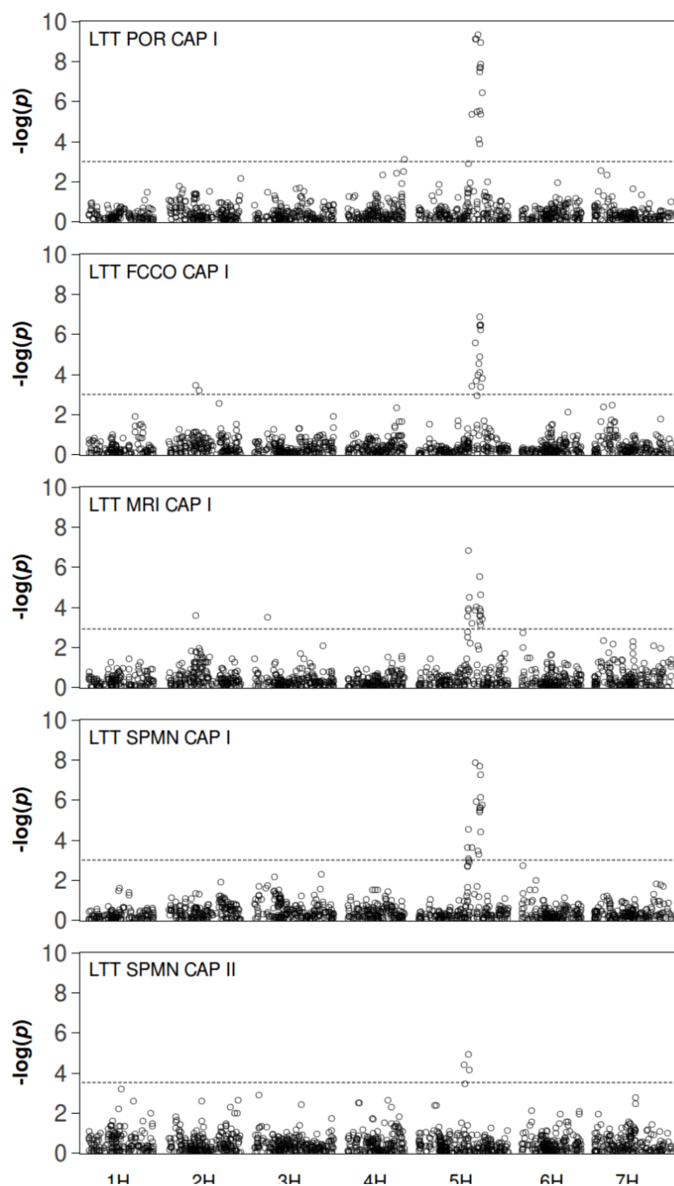


Supplemental Figure 3. Model fitting for each vector of phenotypes in the Oregon Barley CAP germplasm sets by including or excluding the Q matrix (Continued)



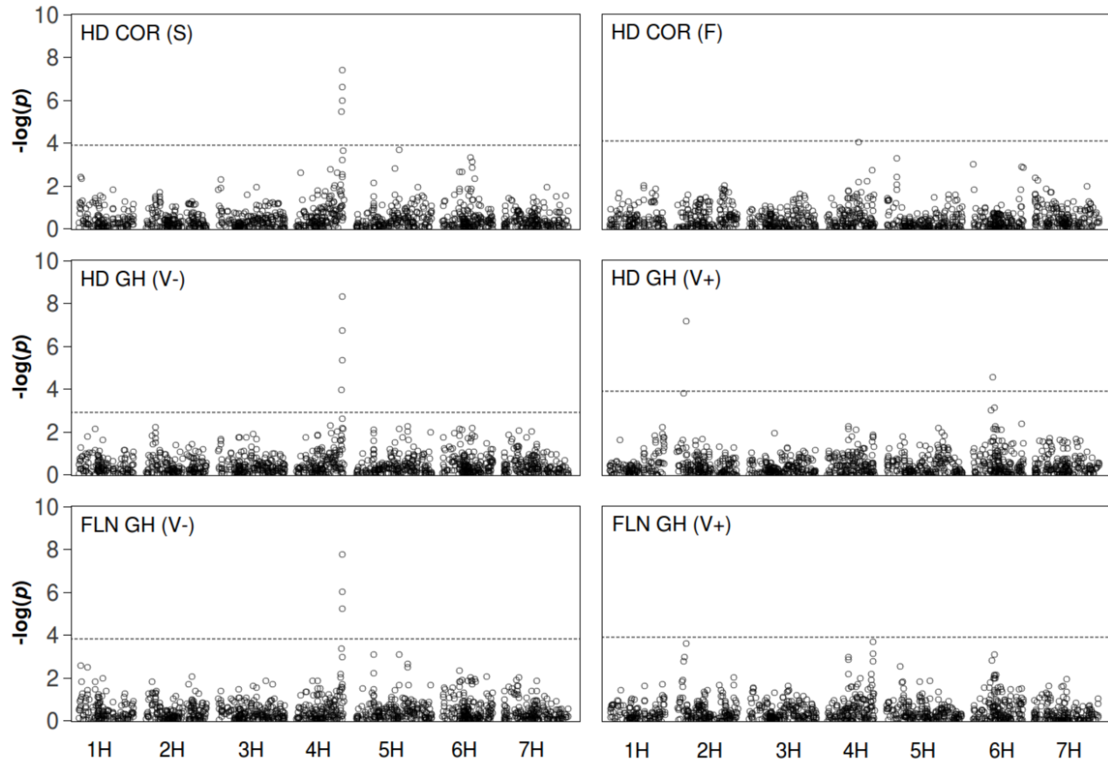
Supplemental Figure 3. Model fitting for each vector of phenotypes in the Oregon Barley CAP germplasm sets by including or excluding the Q matrix (Continued).

Supplemental Figure 3. Model fitting for each vector of phenotypes in the Oregon Barley CAP germplasm sets by including or excluding the Q matrix. Cumulative distributions of the observed *p*-values are plotted against the expected, where the diagonal line represents the ideal distribution of *p*-values. (A) CAP I, (B) CAP II and (C) CAP I+II. The traits are abbreviated as follows: LTT= low temperature tolerance, HD = heading date, FLN = final leaf number. Environments: POR = Pendleton, Oregon, US; FCCO = Fort Collins, Colorado, US; SPMN = St. Paul, Minnesota, US; COR = Corvallis, OR, US; MRI = Martonvasar Research Institute, Hungary. GH = green house; (V+) = vernalized; (V-) = not vernalized; (F) = fall planted; (S) = spring planted.



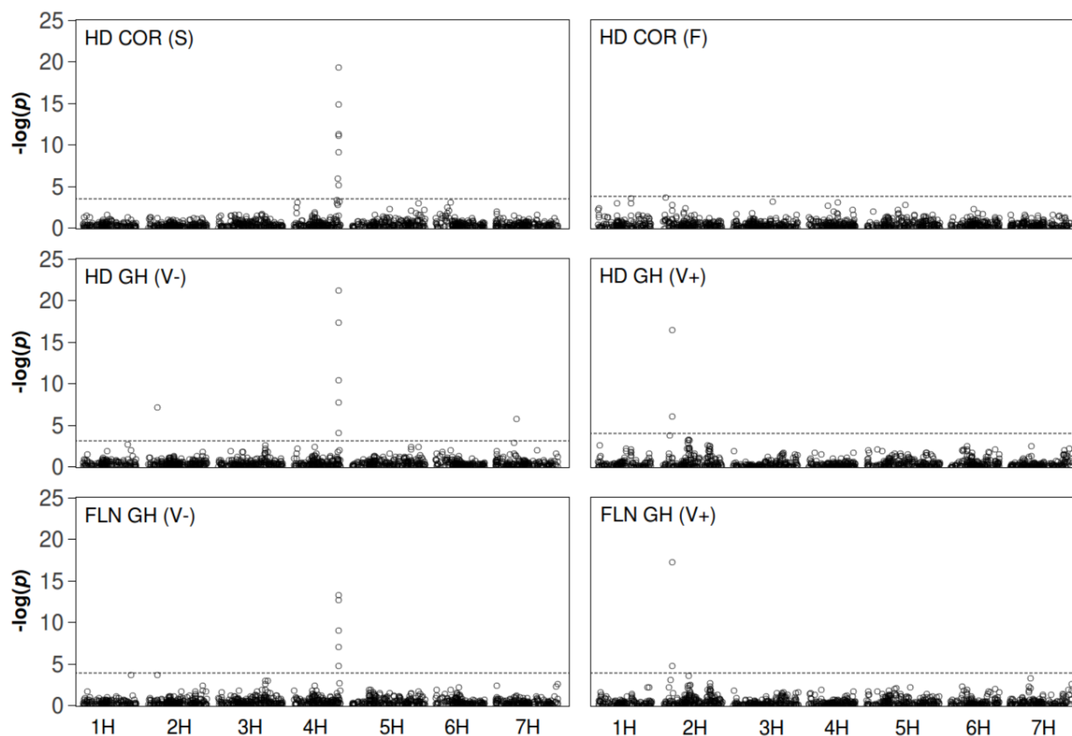
Supplemental Figure 4. Low temperature tolerance (LTT) association scans for Oregon Barley CAP I and CAP II germplasm evaluated at Pendleton, Oregon, USA (POR); Fort Collins, Colorado, USA (FCCO); Martonvasar Research Institute, Hungary (MRI); St. Paul, Minnesota, USA (SPMN). Significance thresholds were determined with a FDR level of 0.05 (dashed line).

A

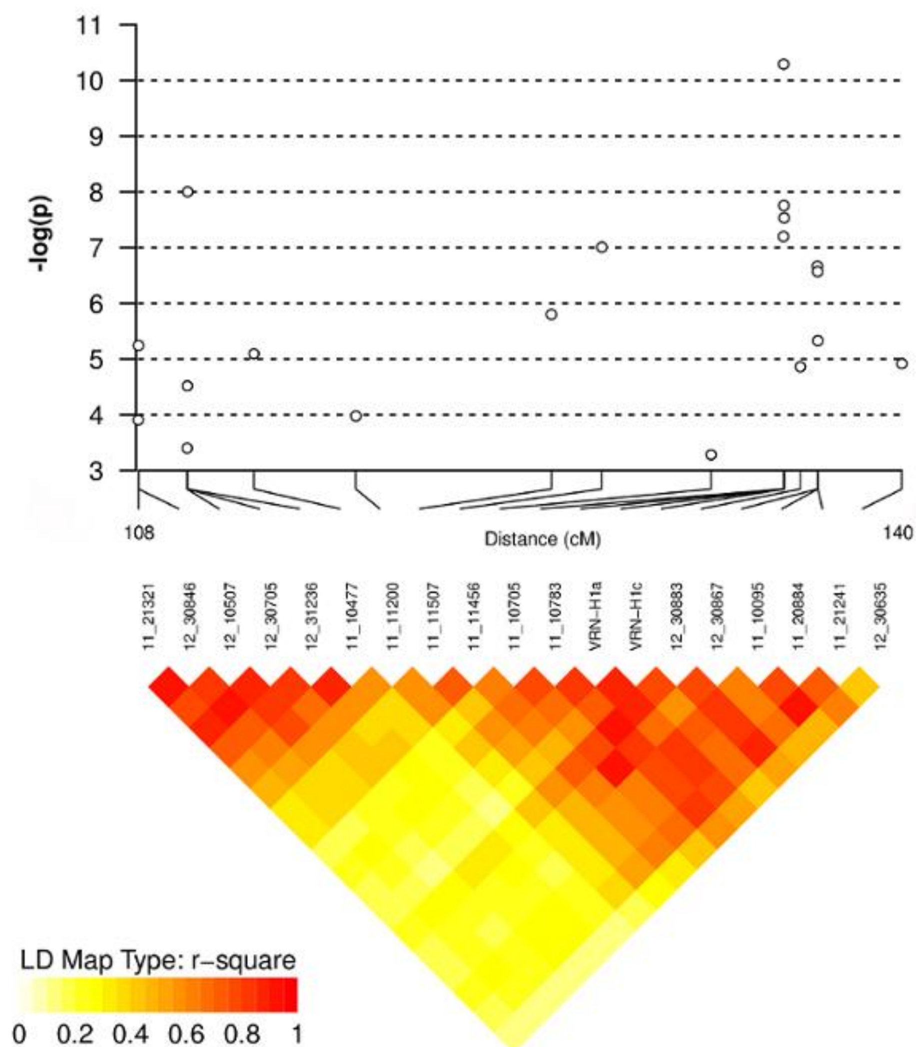


Supplemental Figure 5. Heading date (HD) and final leaf number (FLN) association scans for Oregon Barley CAP I (A) and CAP II (B) (Continued).

B



Supplemental Figure 5. Heading date (HD) and final leaf number (FLN) association scans for Oregon Barley CAP I (A) and CAP II (B). Significance thresholds were determined with a FDR level of 0.05 (dashed line). Left panels show the vernalization sensitivity scans and right panels show the flowering time scans. Environments and treatments are coded as follows: COR = Corvallis, Oregon, USA; GH = Greenhouse; S = spring planted; (V-) = not vernalized; F = fall planted; (V+) = vernalized.



Supplemental Figure 6. Linkage Disequilibrium r^2 heat plot for 19 significant markers for low temperature tolerance at St. Paul Minnesota, USA, using Oregon Barley CAP I+II data. The 30 markers shown in Figure 2 at the *FR-H2*/*FR-H1* interval, above the 0.05 FDR α level were reduced to one representative per r^2=1 for a forward selection and backward elimination method (see Materials and Methods).

Supplemental Table 1. Allele specific primers for winterhardiness-related genes genotyped in the Oregon Barley CAP I and CAP II germplasm.

Gene	GWA ID[†]	Description	Primer ID	Forward Primer Sequence	Reverse Primer Sequence	References
VRN-H1	***VRN-H1a***	*HvBM5A* Intron 1 hypothesized regulatory region	BM5.88F/89R	5’ GAATGGCCGCTACTGCTTAG 3’	5’ GTCTGAGTCGGTTATATGCAGG 3’	von Zitzewitz et al. (2005)
VRN-H1	***VRN-H1b***	5' terminal region *HvBM5A* intron 1	BM5.42F/56R	5’ GAAAGCTCTACGAGTTCTCCAC 3’	5’ CAGTAAGCACTACGATGATGATAAAC 3’	von Zitzewitz et al. (2005)
VRN-H1	***VRN-H1c***	5' terminal region *HvBM5A* intron 1	BM5.42F/86R	5’ GAAAGCTCTACGAGTTCTCCAC 3’	5’ TCCCCCATTCTCGTCAAAAAGC 3’	Szűcs et al. (2007)
VRN-H2	***VRN-H2a***	*ZCCT Ha*	HvZCCT 14F/19R	5’ CAAGGAATATCAAGTACATATCTGC 3’	5’ CCGTATTTATTGAGTTGGTGGTG 3’	Szűcs et al. (2007)
VRN-H2	***VRN-H2b***	*ZCCT Hb*	HvZCCT 8F/11R	5’ GCATCAATGCACCCTACCTCTT 3’	5’ GGAAAACAATGGTGAGAGTAGTACAG 3’	Szűcs et al. (2007)
VRN-H2	***VRN-H2c***	*ZCCT Hc*	HvZCCT HcF/HcR	5’ CACCATCGCATGATGCAC 3’	5’ TCATATGGCGAAGCTGGAG 3’	Yan et al. (2006)
VRN-H3	***VRN-H3***	*HvFT1* intron 1 SNP	HvFT1. 03F/04R +BclI	5' CTTGCTCCCTCATACCCTAG 3'	5' GCTTAATTCGTGGCTGGCTTC 3'	Turner et al. (2005)
PPD-H1	***PPD-H1***	SNP in the CCT domain of *HvPRR7*	PRR7. 05F/08R +BstUI	5’ GATGGATTCAAAGGCAAGGAG 3’	5’ GCAGATACTCACACCGTCCA 3’	Faure et al. (2007)
PPD-H2	***PPD-H2a***	presence/absence of *HvFT3*	HvFT3. 01F/02R	5’ GTCTGTAGAGAGCTAGGTTGAC 3’	5’ GTGATGCAACATTACAGTACAGC 3’	Faure et al. (2007)
PPD-H2	***PPD-H2b***	Morex/ Triumph-like *HvFT3* polymorphism	HvFT3 F1/R1	5’ CTTAATACCACTAAGCATGCAGTTG 3’	5’CTGCACATTATTTGTGATGCAA 3’	Faure et al. (2007)
PPD-H2	***PPD-H2c***	Steptoe/Igri-like *HvFT3* polymorphism	HvFT3 F4/R1	5’ GGATGGATCGGATTATTATTGTATG 3’	5’ CTGCACATTATTTGTGATGCAA 3’	Faure et al. (2007)

[†]GWA ID = the marker designation used in the text

Supplemental Table 2. Pearson correlation coefficients among traits measured in the Oregon Barley CAP I and CAP II germplasm. Trait and location codes are as follows: LTT = low temperature tolerance; HD = heading date; FLN = final leaf number; GH = greenhouse; SPMN = St. Paul, Minnesota, USA; POR = Pendleton, Oregon, USA; FCCO = Fort Collins, Colorado, USA; MRI = Martonvasar Research Institute, Hungary; COR = Corvallis, Oregon, USA; (S) = spring planted; (F) = fall planted; (V-) = without vernalization; (V+) = vernalized.

Germplasm/Trait/Environment†	LTT POR	LTT FCCO	LTT MRI	HD COR (S)	HD GH (V-)	FLN GH (V-)	HD COR (F)	HD GH (V+)	FLN GH (V+)
CAP I-LTT SPMN	0.64***	0.67***	0.77***	0.31**	0.38***	0.43***	0.46***	0.22	0.12
CAP I-LTT POR		0.73***	0.67***	0.06	0.15	0.18	0.31**	0.13	0.00
CAP I-LTT FCCO			0.68***	0.18	0.25*	0.30**	0.37**	0.25*	0.12
CAP I-LTT MRI				0.30**	0.35**	0.42***	0.42***	0.25*	0.21
CAP I-HD COR (S)					0.87***	0.87***	0.45***	0.55***	0.57***
CAP I-HD GH (V-)						0.96***	0.42***	0.67***	0.68***
CAP I-FLN GH (V-)							0.37**	0.66***	0.72***
CAP I-HD COR (F)								0.18	0.09
CAP I-HD GH (V+)									0.81***
CAP II-LTT SPMN				0.15	0.03	0.07	0.34**	-0.07	-0.08
CAP II-HD COR (S)					0.85***	0.76***	0.21	0.30*	0.23
CAP II-HD GH (V-)						0.90***	0.11	0.37**	0.33**
CAP II-FLN GH (V-)							0.13	0.31**	0.35**
CAP II-HD COR (F)								0.27*	0.25*
CAP II-HD GH (V+)									0.92***

* Significant at the 0.05 probability level
** Significant at the 0.01 probability level
***Significant at the 0.001 probability level

Supplemental Table 3. Markers significantly associated with low temperature tolerance at FDR α = 0.05. The “-”symbol indicates no unigene identifier in HarvEST. See Materials and Methods for the location and germplasm codes.

HarvEST database descriptors[†]					-log (*p*) per location and germplasm set					
BOPA	**CH**	**cM**	**U32**	**U35**	**FCCO CAP I**	**MRI CAP I**	**POR CAP I**	**SPMN CAP I**	**SPMN CAP II**	**SPMN CAP I+II**
11_21321	5H	108.2	8344	3638						3.91
12_10844	5H	108.2	6444	18060						4.03
12_30854	5H	108.2	37585	46609						5.25
12_30846	5H	108.2	-	-		3.57		3.65		5.24
12_30847	5H	108.2	16913	23852		3.57		3.65		5.24
12_30852	5H	108.2	2208	15347		3.57		3.65		5.24
12_10507	5H	110.3	3641	15947		3.93				3.40
12_30705	5H	110.3	7467	3323		3.83		3.09		4.52
12_31236[‡]	5H	110.3	10975	18576		6.85		4.53	4.94	8.00
11_10477	5H	113.1	3478	14457		4.51			4.17	5.09
11_11200	5H	117.5	6135	2608	3.44	3.21	5.35	3.62		3.98
11_11507	5H	125.8	9761	4453	5.59	3.87	9.16	7.87		5.80
11_11456	5H	128.0	13510	21640	3.68	4.03	9.16	5.91		7.01
11_20003	5H	129.4	10047	19761			5.49			
11_11375	5H	130.8	14484	8573	3.99		9.36	3.44		
11_10705	5H	132.6	4795	16705	4.53		4.11	3.28		3.28
11_10783	5H	135.7	5571	17001	6.89	3.31	5.54	5.42		7.20
12_30882	5H	135.7	1501	687	6.44	3.60	7.70	5.63		7.53
VRN-H1a[‡§]	5H	135.7	-	-	4.89	5.55	7.50	7.70		10.29
VRN-H1b[§]	5H	135.7	-	-	4.89	5.55	7.50	7.70		10.29
VRN-H1c[§]	5H	135.7	-	-	4.13	3.96	3.91	5.51		7.75
12_30883	5H	135.7	1501	687	6.44	3.60	7.70	5.63		7.53
12_30668	5H	136.4	6933	16741	6.44	3.60	7.70	5.63		7.53
12_30867	5H	136.4	6929	3684	6.22	3.13	5.37	4.42		4.86
12_30869	5H	136.4	6929	3684	6.44	3.60	7.70	5.63		7.53
11_20884	5H	137.2	5156	17298	3.38	3.82	8.98	6.15		6.57
11_10855	5H	137.2	6553	16150	3.38	3.82	8.98	6.15		6.57
11_21241	5H	137.2	7523	17822	6.44	3.60	7.70	5.63		5.33
11_11080	5H	137.2	2723	15889	6.44	3.60	7.70	5.63		7.53
12_31237	5H	137.2	9335	18649	6.44	3.60	7.70	5.63		5.33
11_10095	5H	137.2	1394	397	6.49	4.62	7.87	7.28		6.67
12_30635	5H	140.8	6188	17489	3.81	3.43	6.43	5.77		4.92
11_20690	2H	62.8	4136	16459	3.46	3.58				
11_10265	2H	70.5	2284	15041	3.20				4.42	
11_20968	3H	28.4	5646	3028		3.52				
11_20013	4H	123.3	10273	20344			3.13			
12_30824	4H	123.3	954	14441			3.13			
12_30825	4H	123.3	954	14441			3.13			

[†] HarvEST Database descriptors: BOPA=Barley Oligonucleotide Pool Assay identification; CH= chromosome; cM = centi-morgan (consensus map can be downloaded with the database at http://harvest.ucr.edu/) ; U32/35 = unigene assembly identification searchable in the HarvEST database
[‡] Markers resulting from the forward selection backward elimination method
[§] Not in the HarvEST database. Specific gene amplicons (Supplemental Table 1)

Supplemental Table 4. Markers significantly associated with heading date traits at FDR α = 0.05. The "-"symbol indicates no unigene identifier in HarvEST.

HarvEST database descriptors[†]					-log (*p*) per treatment, environment and germplasm set														
					HD COR (S)			HD GH (V-)			FLN GH (V-)			HD COR (F)	HD GH (V+)			FLN GH (V+)	
BOPA	CH	cM	U32	U35	CAP I	CAP II	CAP I+II	CAP I	CAP II	CAP I+II	CAP I	CAP II	CAP I+II	CAP I+II	CAP I	CAP II	CAP I+II	CAP II	CAP I+II
*PPD-H2*a[‡]	1H	88.2[¶]	-	-										4.84					
12_10777	2H	21.6	5510	3218													6.14		4.69
12_30871	2H	26.6	13515	22237												6.14	4.35	4.87	
12_30872	2H	26.6	13515	22237												6.14	4.35	4.87	
PPD-H1[‡§]	2H	26.6	-	-					7.16	5.15					7.17	16.44	16.07	17.21	10.88
11_21130	4H	116.9	6689	18219		5.97	3.78												
11_21130	4H	116.9	6689	18219															
11_21210	4H	117.6	7221	14226	5.46		4.73	3.98											
11_20272	4H	119.1	1954	14780		5.14													
12_30884	4H	119.1	-	-		11.31	8.49		7.69	9.08		7.05	7.69						
12_30886	4H	119.1	-	-		11.31	8.49		7.69	9.08		7.05	7.69						
12_30889	4H	119.1	-	-	6.63	14.85	19.39	5.36	10.44	15.26	5.24	9.00	12.79						
12_30891	4H	119.1	-	-		9.09	6.65		4.08	4.88		4.72	4.67						
*VRN-H2*a[‡]	4H	119.1	-	-	5.99	19.32	20.93	6.76	21.19	22.33	6.03	12.72	15.29						
*VRN-H2*b[‡§]	4H	119.1	-	-	7.43	19.32	22.16	8.34	21.19	25.33	7.78	12.72	17.68						
*VRN-H2*c[‡]	4H	119.1	-	-		11.07	10.91		17.39	11.54		13.28	9.56						
12_30751	6H	49.4	8416	4733											4.58				
11_20074	7H	54.4	11387	19940					5.75										

[†]HarvEST Database descriptors: BOPA=Barley Oligonucleotide Pool Assay identification; Chromosome and Position= consensus map can be downloaded with the database at http://harvest.ucr.edu ; U32/35 = unigene assembly identification searchable in the HarvEST database for anotations and other usefull information

[‡]Not in HarvEST database. Specific gene amplicon (Supplemental Table 1)

[§]Markers resulting from the forward selection backward elimination method

[¶]*PPD-H2* is not included in the consensus map. The position is estimated from other maps

Supplemental Table 5. Linear mixed model explaining the proportion of variation for heading date traits in the Oregon Barley CAP I+II. R^2_{LR} are calculated for each individual significant marker and interaction between markers. (**A**) Trait = heading date (HD), greenhouse (GH), no vernalization ((V-)). Marker *VRN-H2b* and *PPD-H1* are specific *ZCCT-Hb* and *HvPPR7* amplicons respectively. The effect of *VRN-H2b* and *PPD-H1* is R^2_{LR} = 0.30. (**B**) Trait = final leaf number (FLN), GH, (V-). The effect of the interaction between *VRN-H2b* and *PPD-H1* is R^2_{LR} = 0.25. (**C**) Trait = DH, Corvallis, Oregon, USA (COR), fall planted ((F)). Marker *PPD-H2a* is a specific *HvFT3* amplicon. The effect of *PPD-H2a* is R^2_{LR} = 0.05. (**D**) Trait = HD, GH, vernalized ((V+)). The effect of *PPD-H1* is R^2_{LR} = 0.30. (**E**) Trait = FLN, GH, (V+). The effect of *PPD-H1* is R^2_{LR} = 0.19 (Continued).

A

Model ID[†]	Model	Model form	-2(ML)	LRT	P-value	R^2_{LR}
1	Intercept	y=μ+e	1416.1			
2	Q	y=μ+Qυ+e	1360.4	55.7	<0.001	0.31
3	K	y=μ+Zu+e	1312.5	103.6	<0.001	0.50[‡]
4	Q+K	y=μ+Qυ+Zu+e	1310.6	1.9	0.5894	0.51
5	PPD-H1+K	$y=\mu+m_1+Zu+e$	1292.8	19.7	<0.001	0.57
6	VRN-H2b+K	$y=\mu+m_2+Zu+e$	1198.0	114.5	<0.001	0.77
7	PPD-H1+VRN-H2b+K	$y=\mu+m_1+m_2+Zu+e$	1178.8	19.3	<0.001	0.80[§]
8	PPD-H1xVRN-H2b+K	$y=\mu+m_1+m_2+m_1xm_2+Zu+e$	1175.5	3.2	0.3580	0.80

[†] Model used for likelihood ratio test (LRT): alternative hypothesis 2 and 3 against null hypothesis 1; alternative hypothesis 4 against null hypothesis 3; alternative hypothesis 5 and 6 against null hypothesis 3; alternative hypothesis 7 against null hypothesis 6; alternative hypothesis 8 against null hypothesis 7
[‡]Proportion of the variation explained by the base model
[§]Proportion of the variation explained by the full model

B

Model ID[†]	Model	Model form	-2(ML)	LRT	P-value	R^2_{LR}
1	Intercept	y=μ+e	844.9			
2	Q	y=μ+Qυ+e	795.9	49.0	<0.001	0.28
3	K	y=μ+Zu+e	749.1	95.8	<0.001	0.48[‡]
4	Q+K	y=μ+Qυ+Zu+e	748.0	1.1	0.7791	0.48
5	PPD-H1+K	$y=\mu+m_1+Zu+e$	737.1	12.0	<0.001	0.52
6	VRN-H2b+K	$y=\mu+m_2+Zu+e$	670.1	79.0	<0.001	0.69
7	PPD-H1+VRN-H2b+K	$y=\mu+m_1+m_2+Zu+e$	661.6	8.5	0.0036	0.71
8	PPD-H1xVRN-H2b+K	$y=\mu+m_1+m_2+m_1xm_2+Zu+e$	652.5	9.1	0.0283	0.73[§]

[†] Model used for likelihood ratio test (LRT): alternative hypothesis 2 and 3 against null hypothesis 1; alternative hypothesis 4 against null hypothesis 3; alternative hypothesis 5 and 6 against null hypothesis 3; alternative hypothesis 7 against null hypothesis 6; alternative hypothesis 8 against null hypothesis 7
[‡]Proportion of the variation explained by the base model
[§]Proportion of the variation explained by the full model

Supplemental Table 5. Linear mixed model explaining the proportion of variation for heading date traits in the Oregon Barley CAP I+II.

C

Model ID†	Model	Model form	-2(ML)	LRT	P-value	R^2_{LR}
1	Intercept	y=μ+e	1031.7			
2	Q	y=μ+Qυ+e	989.5	42.3	<0.001	0.25
3	K	y=μ+Zu+e	919.2	112.5	<0.001	0.53‡
4	Q+K	y=μ+Qυ+Zu+e	919.2	0.01	0.9999	0.53
5	PPD-H2+K	$y=\mu+m_1+Zu+e$	904.7	14.5	<0.001	0.58§

† Model used for the likelihood ratio test (LRT) = alternative hypothesis 2 and 3 against null hypothesis 1; alternative hypothesis 4 against null hypothesis 3; alternative hypothesis 5 against null hypothesis 3
‡Proportion of the variation explained by the base model
§Proportion of the variation explained by the full model

D

Model ID†	Model	Model form	-2(ML)	LRT	P-value	R^2_{LR}
1	Intercept	y=μ+e	1030.3			
2	Q	y=μ+Qυ+e	1014.9	15.3	0.0015	0.10
3	K	y=μ+Zu+e	1003.1	27.2	<0.001	0.17‡
4	Q+K	y=μ+Qυ+Zu+e	1001.3	1.8	0.6210	0.18
5	PPD-H1+K	$y=\mu+m_1+Zu+e$	935.9	67.1	<0.001	0.47§

† Model used for the likelihood ratio test (LRT) = alternative hypothesis 2 and 3 against null hypothesis 1; alternative hypothesis 4 against null hypothesis 3; alternative hypothesis 5 against null hypothesis 3
‡Proportion of the variation explained by the base model
§Proportion of the variation explained by the full model

E

Model ID†	Model	Model form	-2(ML)	LRT	P-value	R^2_{LR}
1	Intercept	y=μ+e	568.0			
2	Q	y=μ+Qυ+e	545.4	22.6	<0.001	0.14
3	K	y=μ+Zu+e	514.6	53.4	<0.001	0.30‡
4	Q+K	y=μ+Qυ+Zu+e	512.1	2.5	0.4705	0.31
5	PPD-H1+K	$y=\mu+m_1+Zu+e$	469.7	44.9	<0.001	0.49§

† Model used for the likelihood ratio test (LRT) = alternative hypothesis 2 and 3 against null hypothesis 1; alternative hypothesis 4 against null hypothesis 3; alternative hypothesis 5 against null hypothesis 3
‡Proportion of the variation explained by the base model
§Proportion of the variation explained by the full model