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Molecular and structural characterization of barley vernalization genes

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Abstract

Vernalization, the requirement of a period of low temperature to induce transition from the vegetative to reproductive state, is an evolutionarily and economically important trait in the *Triticeae*. The genetic basis of vernalization in cultivated barley (*Hordeum vulgare* subsp. *vulgare*) can be defined using the two-locus *VRN-H1*/*VRN-H2* model. We analyzed the allelic characteristics of *HvBM5A*, the candidate gene for *VRN-H1*, from ten cultivated barley accessions and one wild progenitor accession (subsp. *spontaneum*), representing the three barley growth habits – winter, facultative, and spring. We present multiple lines of evidence, including sequence, linkage map location, and expression, that support *HvBM5A* being *VRN-H1*. While the predicted polypeptides from different growth habits are identical, spring accessions contain a deletion in the first intron of *HvBM5A* that may be important for regulation. While spring *HvBM5A* alleles are typified by the intron-localized deletion, in some cases, the promoter may also determine the allele type. The presence/absence of the tightly linked *ZCCT-H* gene family members on chromosome 4H perfectly correlates with growth habit and we conclude that one of the three *ZCCT-H* genes is *VRN-H2*. The *VRN-H2* locus is present in winter genotypes and deleted from the facultative and spring genotypes analyzed in this study, suggesting the facultative growth habit (cold tolerant, vernalization unresponsive) is a result of deletion of the *VRN-H2* locus and presence of a winter *HvBM5A* allele. All reported barley vernalization QTLs can be explained by the two-locus *VRN-H1*/*VRN-H2* model based on the presence/absence of *VRN-H2* and a winter vs. spring *HvBM5A* allele.

Abbreviations: gDNA, genomic DNA; InDel, insertion/deletion; LD, long day; MITE, miniature inverted-repeat transposable element; QTL, quantitative trait locus; RT-PCR, reverse transcription polymerase chain reaction; SD, short day; SNP, single nucleotide polymorphism; SSR, simple sequence repeat; UTR, untranslated region; VRN, vernalization

Introduction

Vernalization – the induction of flowering by exposure to an extended period of low temperature – is characteristic of many temperate zone plants, including 'winter growth habit' forms of the *Triticeae*. Vernalization requirement is of particular interest in the cereal crops due to its role in determining adaptation range and association with winter hardiness, i.e., the capacity of a genotype to survive the winter (Hayes *et al*., 1997). The principal components of cereal winter hardiness are low temperature tolerance, vernalization requirement, and photoperiod (day length) sensitivity. Relative to these trait combinations, the barley (*Hordeum vulgare* subsp. *vulgare*) germplasm can be divided into two broad winter hardiness growth habit classifications – winter and spring. In general, winter varieties are low temperature tolerant, photoperiod sensitive, and vernalization requiring; while we use the term 'requirement' for standardization with other cereal nomenclature, winter barley varieties are actually strongly vernalization responsive rather than requiring, as they will flower, eventually, without vernalization (Karsai *et al*., 2001). In contrast, spring varieties are essentially the opposite and have minimal low temperature tolerance capacity, do not require vernalization, and are typically insensitive to short day photoperiod. A third growth habit class – facultative – occurs in the barley germplasm which represents a subclass of the winter growth habit. The facultative growth habit lacks an unambiguous definition, but is typically utilized to refer to genotypes that are as low temperature tolerant as winter varieties, but lack a vernalization requirement.

The genetics of vernalization in Arabidopsis is well-characterized (see review by Henderson *et al*., 2003). While *Triticeae* vernalization genetics is not as complete, it appears that a different pathway leads to the same end phenotype relative to Arabidopsis (Takahashi and Yasuda, 1971; Karsai *et al*., 2001; Danyluk *et al*., 2003; Trevaskis *et al*., 2003; Yan *et al*., 2003, 2004a; Cooper *et al*., 2005). The *Triticeae* form a homogeneous genetic system with a high degree of synteny in which the genetic determinants of winter hardiness are conserved, and results of one species are frequently applicable to other members of the cereal tribe (Dubcovsky *et al*., 1998; Mahfoozi *et al*., 2000). The recent cloning of candidate genes in diploid wheat (*Triticum monococcum*) for *VRN-A^{m}1* and *VRN-A^{m}2* (Yan *et al*., 2003, 2004a) and hexaploid wheat (*T. aestivum*) for *VRN-1* (Danyluk *et al*., 2003; Trevaskis *et al*., 2003) has considerably advanced our understanding of vernalization in the economically important cereals.

Takahashi and Yasuda (1971) proposed a three-locus epistatic model for barley vernalization requirement where winter growth habit genotypes have the allelic architecture *Sh_sh2sh2sh3sh3*; all other allelic configurations lack vernalization requirement and yield the spring and facultative growth habits. Based on wheat:barley orthology, we use the standard *Triticeae* nomenclature, with an "H" to indicate the *Hordeum* genome: *Sh2 = VRN-H1* (chromosome 5H); *Sh = VRN-H2* (chromosome 4H); and *Sh3 = VRN-H3* (chromosome 1H), in the remainder of this report. Allelic variants at the *VRN-H3* locus are reported only in exotic barley genotypes (Takahashi and Yasuda, 1971), reducing the epistatic model to a two-locus (*VRN-H2* and *VRN-H1*) model in cultivated barley.

A molecular model explaining the *VRN-2*/*VRN-1* epistatic interaction in the *Triticeae* was proposed by Yan *et al*. (2004a) based on the positional cloning of *VRN-A^{m}1* and *VRN-A^{m}2* in *T. monococcum*, in which *VRN-A^{m}2* encodes a dominant repressor of flowering (*ZCCT1*) which inhibits the expression of the *VRN-A^{m}1* flowering gene *TmAP1* (*T. monococcum AP1*). Vernalization down-regulates *Vrn-A^{m}2* (*ZCCT1*) expression, allowing expression of *vrn-A^{m}1* in winter accessions, while no vernalization requirement is observed in accessions with a physical deletion of the *ZCCT* genes (*vrn-A^{m}2* locus), regardless of the allele at *VRN-A^{m}1*, as well as in genotypes that have *Vrn-A^{m}2* but lack a target binding site for the repressor in the *TmAP1* gene (i.e., dominant *Vrn-A^{m}1*). A deletion in the promoter region of *VRN-A^{m}1* that correlated with spring vs. winter growth habit was proposed as a possible *VRN-A^{m}2* target site (Yan *et al*., 2003). In subsequent work, we determined that in some spring wheat accessions, the promoter is invariant relative to winter accessions (Yan *et al*., 2004b). This suggests additional regulatory sites may be located intragenically, and that in wheat, *VRN-1* response to vernalization can be controlled either from the promoter and/or intragenically. In the remainder

of this work, we use the generic terms 'spring allele' to refer to alleles for the deleted *VRN-2* locus (recessive *vrn-2* allele) and the un-repressible *Vrn-1* allele and 'winter allele' to refer to alleles encoding the functional repressor of the *VRN-2* locus (dominant *Vrn-2* allele) and the repressible *vrn-1* allele. *HvBM5*, the barley ortholog of *TmAP1* (*VRN-A^{m}1*) (Yan *et al*., 2003), was cloned during a MADS-box screen by Schmitz *et al*. (2000) and its orthology with *TaVRT-1* (*VRN-B1*) and *WAP1* (*VRN-D1*) has been established; *TaVRT-1* (Danyluk *et al*. 2003) and *WAP1* (Murai *et al*., 1998; Trevaskis *et al*. 2003) represent *AP1* homoeologs from hexaploid wheat.

Prior to the cloning of candidate genes, quantitative trait locus (QTL) analysis tools were employed to dissect vernalization genetic traits in the *Triticeae*, which was simplified by the use of diploid barley and doubled haploid progeny. For example, in the Igri×Triumph (winter×spring) mapping population, Laurie *et al*. (1995) reported vernalization QTLs on chromosomes 4H and 5H that correspond to the predicted locations of the *VRN-H2* and *VRN-H1* loci, while the same location for *VRN-H1* was reported in the Nure × Tremois (winter × spring) mapping population (Francia *et al*., 2004). QTL for multiple low temperature and photoperiod winter hardiness-related traits were mapped in the Dicktoo × Morex (facultative × spring) population to chromosome 5H at the predicted position of *VRN-H1* (Hayes *et al*., 1997; Karsai *et al*., 1997). The availability of multiple barley winter hardiness QTL mapping populations segregating for vernalization requirement provides a unique opportunity to test the *VRN-1*/*VRN-2* model of Yan *et al*. (2004a) and to further explore the relationships of vernalization, photoperiod sensitivity, and low temperature tolerance in barley at the molecular level.

As part of our long-term objective of complete characterization of barley winter hardiness components (Cooper *et al*., 2005; Skinner *et al*., 2005), we focused on vernalization in this study and asked the question: ''Does the *VRN-1*/*VRN-2* epistatic model from *T. monococcum* apply to barley?'' Our specific goals were to determine whether (i) allelic variation at the promoter, introns, and/or coding region of *HvBM5* correlated with growth habit in a panel of eleven barley genotypes, (ii) the expression response of *HvBM5* to vernalization and photoperiod in representative growth habits follows the predicted pattern based on the *VRN-H1* and *VRN-H2* alleles combinations that are present, (iii) growth habit correlates with the presence/absence of *VRN-H2* in the same germplasm array, and (iv) allelic variation at the *VRN-H* loci explains QTL mapping results.

Materials and methods

Plant material and growth conditions

Ten *Hordeum vulgare* subsp. *vulgare* genotypes, representing both the three barley growth habit phenotype classes and parents from six primary barley winter hardiness QTL mapping populations, were utilized for allelic studies. These are (i) four winter habit genotypes: Kompolti korai, Nure, Strider, and Igri, (ii) two facultative habit genotypes: Dicktoo and 88Ab536, and (iii) four spring habit genotypes: Morex and Harrington (both North American spring habit germplasm) and Tremois and Triumph (both European spring habit germplasm). A *H. vulgare* subsp. *spontaneum* accession (OSU11; Caesarea 26–24) was also evaluated which represents an ancestral, vernalization-requiring form (Karsai *et al*., 2004) and is a parent of a set of important genetic stocks (Matus *et al*., 2003). An additional subsp. *spontaneum* winter growth habit accession (OSU6; Erez 8321) and three subsp. *vulgare* genotypes (two winter growth habit Spanish landraces (Albacete, and Pané) and the spring growth habit OWB-D (Oregon Wolfe Barley dominant)) were utilized for refinement of the *HvBM5A* intron 1 deletion region. Plants for genomic DNA (gDNA) extraction were grown under greenhouse conditions and supplemented with artificial light (16 h photoperiod; 18 °C/15 °C day/night temperature; mercury halide lighting, 225–250 μmol m^{-2} s^{-1} light intensity). For expression studies (April to June), Strider, Dicktoo, and Morex varieties were germinated and grown to the two-leaf stage (about 2 weeks) under the above greenhouse conditions and transferred to treatment conditions – unvernalized, short day (SD-) vernalized, or long day (LD-) vernalized – and bulk aerial tissue collected following 1, 2, 4, and 8 weeks of treatment exposure. Prior to treatment condition transfer, a pre-transfer (0 exposure) sample was collected from each variety. For unvernalized treatment,

plants were maintained under greenhouse conditions. For the SD- and LD-vernalized treatments, plants were transferred to an environmentally controlled cold room maintained at 2 °C on either an 8 h (SD) or 16 h photoperiod (LD) (Cool White Fluorescents, 50 μmol m^{-2} s^{-1} light intensity). For post-vernalization treatments, following 8 weeks vernalization, plants were returned to greenhouse conditions (LD) and samples collected after 2 weeks additional growth. Plants for *HvBM5A* cDNA isolations were grown and SD-vernalized for 4 weeks as above prior to tissue harvest.

Isolation of HvBM5A *alleles and genomic regions*

High density filters of the *H. vulgare* cv. Morex BAC library (Yu *et al.*, 2000) were screened with *TmAP1* and contig sets determined as previously described (Yan *et al.*, 2003). High quality BAC DNA for direct sequencing was prepared using a QIAGEN Large-Construct Kit (Qiagen, Valencia, CA). The sequence of the full-length spring growth habit Morex *HvBM5A* gene allele (AY750995) was determined from both strands of BAC clone 631P8. The sequence of the full-length winter growth habit Strider *HvBM5A* gene allele was determined from a set of cloned overlapping PCR amplicons using primer sets based on the *HvBM5A*-Mx sequence. To determine the respective *HvBM5A* promoter sequence, approximately 2.1 kb of sequence upstream of the starting ATG, based on the Morex allele (AY750995), was amplified and cloned from each remaining variety using primers HvBM5.027 (5′-aggcctattcgtttgcaatgc-3′) and HvBM5.006 (5′-atctcgtgcgccttcttgag-3′). The segment of *HvBM5A* intron 1 harboring the putative regulatory region was cloned using the primers HvBM5.042 (5′-gaaagctctacgagttctccac-3′) and HvBM5.043 (5′-ttctgcataagagtagcgctcat-3′) for genotypes Dicktoo, OWB-D, and Triumph, HvBM5.055 (5′-atgcatagaataattggctccagc-3′) and HvBM5.073 (5′-gtgaactgagtaaagacgtggaagg-3′) for genotypes 88Ab536, Igri, Kompolti korai, Nure, OSU 11, and Tremois, and HvBM5.055 and HvBM5.056 (5′-cagtaagcactacgatgatgataaac-3′) for genotypes Albacete, and Pané. For gDNA-based amplifications, total genomic DNA was extracted from a single plant of each variety using a DNeasy Plant Mini Kit (Qiagen) and high fidelity Takara *LA Taq* DNA polymerase (Takara Mirus Bio, Madison, WI) was used. *HvBM5A* cDNA alleles from each respective variety were PCR amplified from 4 week SD-vernalized first-strand cDNA, prepared using a RETROscript Kit (Ambion, Austin, TX), with the primers HvBM5.001 (5′-ccaaccacctgacagccatg-3′) and HvBM5.002 (5′-cgataggttaattcacagaaacaac-3′). For each gene/allele isolated via PCR, cloned amplicons of at least two independent PCR reactions were sequenced to confirm polymerase-based nucleotide substitutions had not occurred; only the non-primer portion was reported to GenBank (Table 1). Regulatory motif searches of the promoter and barley:wheat conserved intron 1 region were conducted using PlantCARE (http://intra.psb.ugent.be:8080/PlantCARE/), MOTIF (http://motif.genome.jp/), and PLACE (http://www.dna.affrc.go.jp/PLACE/) default sequence analysis functions.

Genotyping of HvBM5A *intron 1 size and presence of* ZCCT-H *genes*

HvBM5A intron 1 size and presence of *ZCCT-H* genes were typed in the 11 primary barley genotypes. Both assays were conducted two or more independent times to verify reproducibility and a standard result is shown. To genotype the *HvBM5A* intron-based InDel polymorphisms, PCR amplifications were performed on gDNA using Takara *LA Taq* DNA polymerase (Takara Mirus Bio) and primers HvBM5.055 and HvBM5.056 (Figure 2, Set B) for the 5.2-kb intron 1 InDel, primers HvBM5.035 (5′-gaaaacttgaacaacaccagaacc-3′) and HvBM5.043 for the 42-bp intron 1 InDel, and primers HvBM5.80F (5′-gggatcagttcataccgacgc-3′) and HvBM5.81R (5′-ggaggcgcaactggtgacg-3′) for the 17-bp intron 2 InDel. Multiplex PCR using Qiagen *Taq* DNA polymerase was employed to simultaneously coamplify a conserved region of the two closely related candidate *VRN-H2* genes (*ZCCT-Ha* (AY485977) and *ZCCT-Hb* (AY485978)) with primers *HvZCCT*.001 (5′-cacatgatgtcgcccgttc-3′) and *HvZCCT*.002 (5′-ggactcg tagcggatttgc-3′) and the *VRN-H2* locus-proximal *HvSnf2* gene with primers *HvSnf2*.01F (5′-cctgaagcgagtatccatatgc-3′) and *HvSnf2*.03R (5′-gctgattgtttttgtggccagg-3′).

Table 1. Accessions of cloned HvBM5A allele regions.

HvBM5A allele	Growth habit	GenBank accession	Determined region
Strider	Winter	AY750993	Full gene
Morex	Spring	AY750995	Full gene
Morex	Spring	AY785825	EST HVSMEa0013C13
Kompolti korai	Winter	AY785835	cDNA
Strider	Winter	AY785830	cDNA
Igri	Winter	AY785833	cDNA
Nure	Winter	AY785834	cDNA
OSU11	Winter	AY785832	cDNA
Dicktoo	Facultative	AY785828	cDNA
88Ab536	Facultative	AY785827	cDNA
Morex	Spring	AY785826	cDNA
Harrington	Spring	AY785829	cDNA
Triumph	Spring	AY866486	cDNA
Tremois	Spring	AY785831	cDNA
Kompolti korai	Winter	AY785824	Promoter
Strider	Winter	AY785823	Promoter
Igri	Winter	AY785822	Promoter
Nure	Winter	AY785821	Promoter
OSU11	Winter	AY785820	Promoter
Dicktoo	Facultative	AY785817	Promoter
88Ab536	Facultative	AY785818	Promoter
Morex	Spring	AY785815	Promoter
Harrington	Spring	AY785816	Promoter
Triumph	Spring	AY866485	Promoter
Tremois	Spring	AY785819	Promoter
Kompolti korai	Winter	AY866487	Intron 1 critical region
Igri	Winter	AY866489	Intron 1 critical region
Nure	Winter	AY866488	Intron 1 critical region
OSU6	Winter	AY866493	Intron 1 critical region
OSU11	Winter	AY866492	Intron 1 critical region
Pané	Winter	AY866495	Intron 1 critical region
Albacete	Winter	AY866494	Intron 1 critical region
Dicktoo	Facultative	AY750994	Complete Intron 1
88Ab536	Facultative	AY866490	Intron 1 critical region
Tremois	Spring	AY866491	Intron 1 critical region
Triumph	Spring	AY871789	Complete Intron 1
OWB-D	Spring	AY750996	Complete Intron 1

RNA gel blot and RT-PCR expression analysis

Total RNA was isolated from all tissue sources using RNeasy Plant Kits (Qiagen). Gel electrophoresis and blotting of RNA (20 μg/lane) to Nytran nylon membranes (Schleicher and Schuell, Keene, NH) was performed as in Skinner and Timko (1998). RNA blots were probed and washed using Ultrahyb Solution (Ambion). For northern analysis, an *HvBM5A*-Mx probe fragment specific to the C-domain and 3′ UTR was used. An 18S rRNA fragment from Morex EST BF620814 was utilized as a constitutive loading control probe. Labeled probes were generated using a High Prime Labeling Kit (Roche, Indianapolis, IN). Diagnostic restriction-profile-based RT-PCR was employed to determine whether *HvBM5B* was expressed. The assay was conducted three independent times to verify expression and a standard result is shown. Dicktoo, Strider, and Morex pretreatment (0 exposure) and 4 week LD-vernalized RNA was used as a template for cDNA synthesis. First-strand cDNA for each sample was synthesized from 5 μg total RNA utilizing a RETROscript Kit (Ambion). Input

first-strand cDNA sample volumes for RT-PCR were first normalized for cDNA yield relative to a barley actin gene using the primers *HvActin*.001 (5′-tcgcaacttagaagcacttccg-3′) and *HvActin*.002 (5′-aagtacagtgtctggattggaggg-3′). For RT-PCR amplifications, the primers *HvBM5*.004 (5′-ggcgcagcaagatcaaactca-3′) and *HvBM5*.070 (5′-ggacctgaggctgcactgc-3′) (Figure 2, Set D) were used for *HvBM5A*/*B* analysis. This primer set coamplifies an analogous intron-spanning 3′ portion of the *HvBM5A* and *HvBM5B* coding regions, yielding differential-sized products following *Mwo*I digestion and simultaneously distinguishing products originating from cDNA vs. gDNA. To verify both *HvBM5A* and *HvBM5B* amplification would occur under the experimental conditions, and that *Mwo*I digestion of *HvBM5B* product had taken place, gDNA from Dicktoo, Strider, and Morex were also used as templates (not shown).

Apical meristem development

Shoot apical meristem development was determined in conjunction with the tissue harvest for the 0, 1, 2, 4, and 8 week expression analysis stages of the unvernalized, and SD- and LD-vernalized treatments from Dicktoo, Strider, and Morex; each genotype had visibly initiated heading at the 2-week post-vernalization treatment time point. For each time point, apical meristems of three individual plants from each genotype were dissected from the crown tissue and digitally captured; an average representative meristem from each relevant time point is presented. Meristem images were captured using a Nikon Coolpix 995 digital system and a Nikon SMZ-2T dissecting microscope. The phenological meristem transition to floral fate was monitored for via double-ridge formation as in Danyluk *et al.* (2003).

Linkage mapping and QTL analysis

Linkage map positions corresponding to *HvBM5A* (*VRN-H1*), *HvBM5B*, *ZCCT-H* (*VRN-H2*), *VRN-H2*-proximal *HvSnf2*, and *Bmy1* were assigned using either the Dicktoo×Morex (D×M) and/or the Nure×Tremois (N×T) doubled haploid mapping populations (Hayes *et al.*, 1997; Francia *et al.*, 2004). *HvBM5A*, *HvBM5B*, and *HvSnf2* were mapped in the D×M population using the respective primer sets: HvBM5.006/HvBM5.001 (Figure 2, Set A), HvBM5.032/HvBM5.035 (Figure 2, Set C), and Snf2.001/Snf2.002. *HvBM5A*, *HvSnf2*, *ZCCT-H*, and *BmyI* were mapped in the N×T population using the respective primer sets: HvBM5A.F/HvBM5A.R, Snf2.F/Snf2.R, HvZCCT.HcF/HvZCCT.HcR, and HdAMYB.F/HdAMYB.R. PCR reactions for mapping were performed using *Taq* DNA polymerase under standard conditions, and optimized for each locus and primer set according to the manufacturer's guidelines (Qiagen). Details on linkage mapping, mapping primer sequences, and QTL analysis are available in Online Supplemental Section S2.

Results

Identification and mapping of barley HvBM5 *as a candidate gene for* VRN-H1

High density filter screens of the *H. vulgare* cv. Morex BAC library (Yu *et al.*, 2000) with *TmAP1* identified six clones harboring the *HvBM5* genomic region, which were determined to form two contigs via fingerprinting and Southern hybridization strategies (data not shown). Single pass direct sequence analysis of the *HvBM5*-like 3′ region on a representative BAC clone of each contig (clones 631P8 and 315I20) revealed they contain closely related, yet distinct, *HvBM5*-like genes. The presence of two *HvBM5* genes in barley is in agreement with genomic blotting results (Schmitz *et al.*, 2000). We designated the *HvBM5* form present on BAC 631P8 as *HvBM5A* and the form on BAC 315I20 as *HvBM5B*. The Morex *HvBM5A* form (*HvBM5A*-Mx) is allelic to the cv. Atlas (*HvBM5-At)* cDNA (AJ249144) of Schmitz *et al.* (2000).

We mapped both *HvBM5* forms in the Dicktoo×Morex (D×M) population (GrainGenes: http://wheat.pw.usda.gov/ggpages/DxM/) to determine which form was the candidate for *VRN-H1*. *HvBM5A* maps to the long arm of chromosome 5H (Figure 1), and is localized in the same BIN as the vernalization requirement QTL reported for the Igri×Triumph (I×T) (Laurie *et al.*, 1995) and Nure×Tremois (N×T) (Francia *et al.*, 2004) barley mapping populations. This genome location corresponds to the positions of the *VRN-A^{m}1* and *VRN-D1* loci in *T. monococcum* and *T. aestivum*, respectively (Dubcovsky *et al.*, 1998; Yan *et al.*, 2003, 2004a).

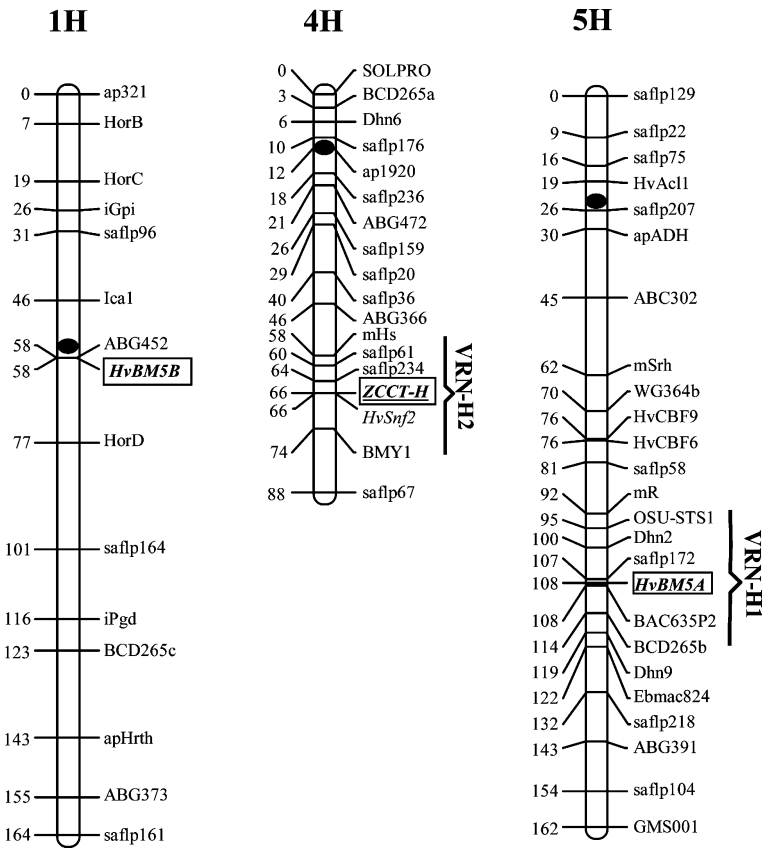

Figure 1. Linkage map positions of vernalization effect regions and candidate vernalization genes. Barley chromosomes 1H, 4H, and 5H are shown with marker distance in cM. Centromeres are denoted with a black oval and candidate *VRN-H* genes are boxed. All linkage marker positions are based on the D×M population except *ZCCT-H* (underlined). *ZCCT-H* position is assigned based on the *HvSnf2* position, which is inseparable from *ZCCT-H* in the N×T population. The QTL effect range of *VRN-H1* and *VRN-H2* are denoted with a peak centered at the most significant position (Laurie *et al*., 1995; Francia *et al*., 2004; Karsai *et al*. 2005).

HvBM5B mapped to chromosome 1H (Figure 1), confirming it is a distinct gene and not an *HvBM5A* allele. Interestingly, the *HvBM5B* map position is in the same genetic proximity of that predicted for *VRN-H3* (*Sh3*) (Takahashi and Yasuda, 1971); their relationship is currently under investigation. Based on single pass sequence walking approximately 2.0 kb 5′ to the last shared segment with *HvBM5A*-Mx, the Morex *HvBM5B* allele appears to be a 5′-truncated pseudogene and is not expressed in Morex, Dicktoo, or Strider (see below). Relative to the *HvBM5A*-Mx gene, *HvBM5B*-Mx only retains the region from exon 3 through the 3′ untranslated region (UTR), while the region 5′ to exon 3 is deleted except for remnants of introns 1 and 2 (Figure 2).

Vernalization requirement differences are not due to allelic variation of the HvBM5A *coding region*

We first investigated whether allelic variation of the HvBM5A polypeptide could be a source of barley vernalization requirement differences. We sequenced the spring growth habit *HvBM5A*-Mx (AY750995) allele on BAC clone 631P8, from approximately 2.1 kb upstream of the starting ATG through approximately 0.3 kb downstream of the termination codon. Numerous *HvBM5A* ESTs from multiple varieties, including Morex, are present in the GenBank EST database; no *HvBM5B* ESTs were noted however (data not shown), in support of it assignment as an unexpressed pseudogene (von Zitzewitz, 2004).

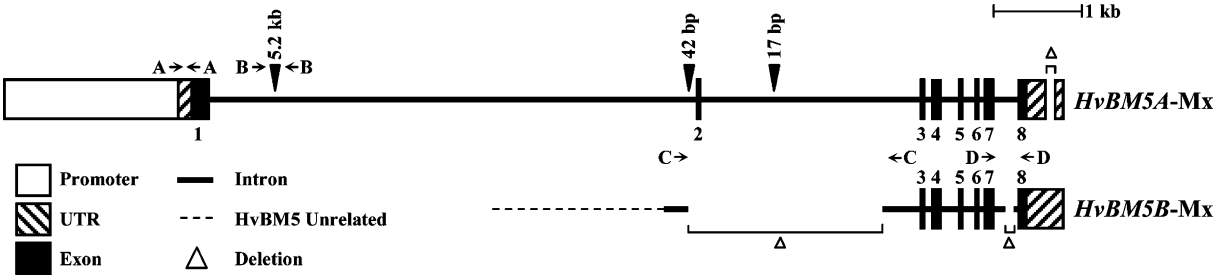

Figure 2. Comparative genomic structure of *HvBM5A*-Mx and *HvBM5B*-Mx. A schematic summary of the determined region of each gene is shown. Exons are numbered relative to *HvBM5A* structure. *HvBM5A*-Mx vs. *HvBM5B*-Mx InDels less than 20 bp are not indicated. The 5′ external region of *HvBM5B*-Mx with no homology to *HvBM5A* is denoted by a hatched line. The position and size of the three intronic deletion points in Morex relative to the *HvBM5A*-St winter habit allele are denoted by filled triangles. The position of primers (arrows) used for mapping of *HvBM5A* (Set A) and *HvBM5B* (Set C), determination of vernalization critical region presence (Set B), and RT-PCR assays (Set D), are indicated.

We completely sequenced one full length Morex *HvBM5A* EST clone (Accession BF624256) and confirmed the cloned cDNA (AY785826) is 100% identical to the exon and UTR segments of the BAC 631P8-derived genomic *HvBM5A*-Mx sequence. In conjunction with the promoter cloning (below), overlapping fragments to the remainder of the winter growth habit *HvBM5A* Strider allele were cloned and the full length *HvBM5A*-St gene assembled (Table 1). Both *HvBM5A* alleles have an intron/exon structure identical to *TmAP1* consisting of eight exons and seven introns; a prominent 5.2 kb deletion is present in *HvBM5A*-Mx intron 1 relative to *HvBM5A*-St (Figure 2).

Primers localized to the 5′ and 3′ UTRs of *HvBM5A*-Mx were used to amplify the corresponding *HvBM5A* cDNA from a panel of representative barley genotypes utilized in winter hardiness mapping populations (Table 1), which include: four winter (Kompolti korai, Nure, Strider, and Igri), two facultative (Dicktoo and 88Ab536), two North American (Morex and Harrington) and two European (Tremois and Triumph) spring growth habits, and a winter growth habit accession (OSU11; Caesarea 26–24) of the ancestor of cultivated barley (*H. vulgare* subsp. *spontaneum*) (Karsai *et al*., 2004). Sequencing of the *HvBM5A* cDNA alleles revealed only three UTR-localized polymorphisms, two single nucleotide polymorphisms (SNP) and one insertion/deletion (InDel)-based simple sequence repeat (SSR), are present (Figure S1). *HvBM5A* encodes a MADS-box transcription factor (Online Supplement Section S1; Figure S2) (Danyluk *et al*., 2003; Yan *et al*., 2003), and the coding region of each allele is 100% identical at the nucleotide level (Figure S1). Therefore, identical *HvBM5A* polypeptides are encoded for each genotype and polypeptide variation cannot account for differences in vernalization requirement among this panel of contrasting barley growth habit genotypes.

HvBM5A *expression correlates with growth habit*

The lack of polypeptide variation indicated that barley vernalization requirement differences are more likely due to differences in *HvBM5A* regulation. We therefore monitored *HvBM5A* expression in a representative spring (Morex), facultative (Dicktoo), and winter (Strider) barley genotype under unvernalized and short day (SD) and long day (LD)-vernalized conditions through eight weeks of treatment. At the initiation of the experiment (0 weeks), *HvBM5A* expression was readily detectable in the spring growth habit (Morex), while none was detected in either the facultative (Dicktoo) or winter (Strider) growth habits (Figure 3A). For unvernalized plants, the *HvBM5A* expression pattern varied with growth habit: expression was detectable in Morex at each sampling point, detectable in Dicktoo by week two and peaking by week four, and not detectable in Strider over the eight week time course. In the vernalization treatment, the *HvBM5A* expression pattern correlated with vernalization requirement, while the effect of SD vs. LD photoperiod varied with the genotype. Expression reached maximal levels in Morex by the second week, and photoperiod did not have a major effect on *HvBM5A*

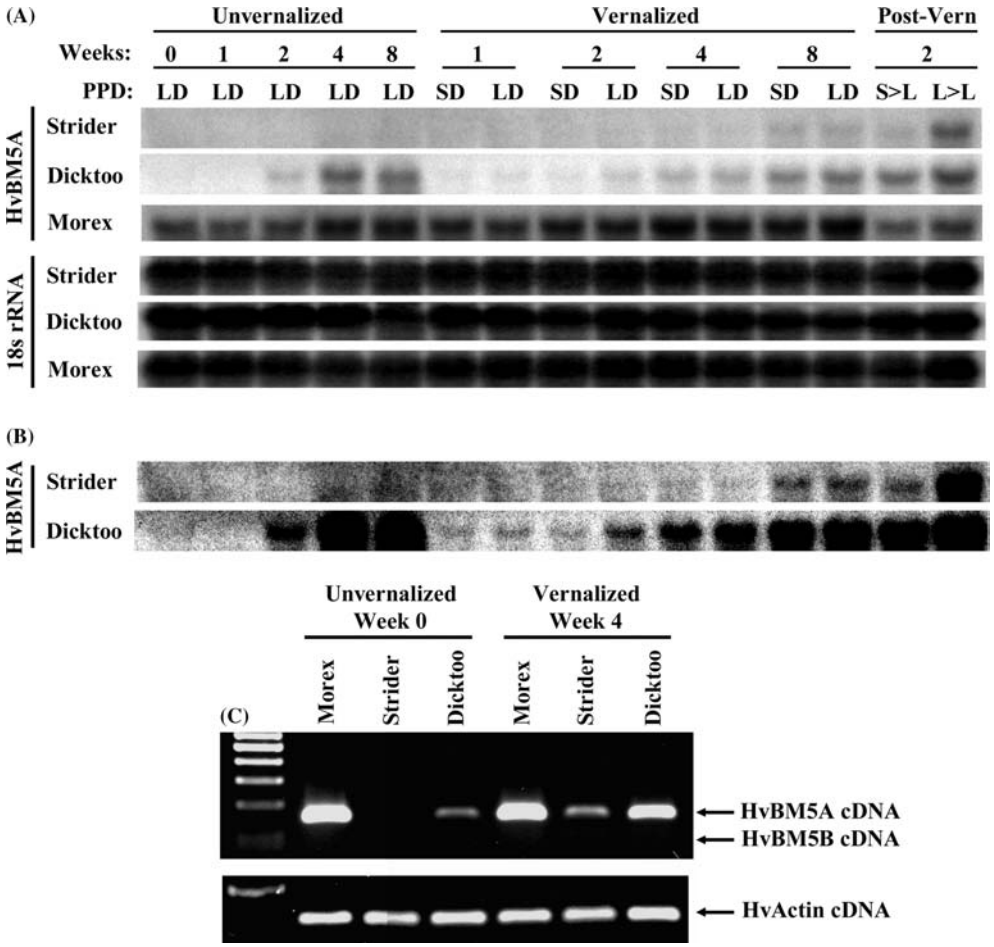

Figure 3. *HvBM5A* expression under unvernalized and vernalizing conditions. (A) *HvBM5A* expression in barley varieties Strider (winter), Dicktoo (facultative), and Morex (spring). See Methods for growth conditions and probe data. Weeks under growth condition and photoperiod length (SD: short day, LD: long day) are denoted. (B) Contrast enhanced images of the Strider and Dicktoo panels from (A) to show low abundance message levels. (C) RT-PCR analysis of *HvBM5A* vs. *HvBM5B* expression. A representative result is shown. cDNA was prepared from the same respective samples used for RNA gel blot analysis in panel A. The size of RT-PCR products following *MwoI* digestion corresponding to *HvBM5A* and *HvBM5B* are noted; no *HvBM5B* product is detected. *HvActin* cDNA was used as RT-PCR constitutive control.

transcript levels. A low level of *HvBM5A* expression is detectable in the photoperiod-sensitive Dicktoo genotype after one week of vernalization, which was slightly higher under LD, and is more evident at two weeks (Figure 3B). Dicktoo *HvBM5A* transcript was substantially less abundant relative to Morex after eight weeks vernalization under both photoperiod regimes. In contrast, no detectable *HvBM5A* expression was evident in the photoperiod-sensitive winter genotype Strider until eight weeks of vernalization, where *HvBM5A* transcript level was slightly higher under LD, but was still lower in abundance relative to Dicktoo and Morex (Figure 3B).

When eight week vernalized Morex, Dicktoo, and Strider plants were returned to LD, greenhouse (non-vernalizing) conditions, the resultant effect on *HvBM5A* expression patterns differed. *HvBM5A* transcript level decreased in Morex, and more so in SD-vernalized plants. In contrast, no change in *HvBM5A* transcript level was observed for SD-vernalized Dicktoo or Strider plants, while a noticeable increase occurred in LD-vernalized plants. These results highlight the complexity of photoperiod and vernalization interactions in the regulation of *HvBM5A* expression between winter, facultative, and spring growth habit barley genotypes.

We used a diagnostic reverse transcriptase PCR (RT-PCR) strategy to verify that the *HvBM5B* pseudogene is not expressed and the observed gel blot expression is that of *HvBM5A* alone. A conserved primer pair that amplifies an intron-spanning portion of the 3′ coding region of both *HvBM5A* and *HvBM5B* (Figure 2), and yields different size products following *Mwo*I digestion, was employed. This primer pair therefore distinguishes *HvBM5A* (149 bp) from *HvBM5B* (74 and 75 bp) cDNA products, as well as from larger intron-containing *HvBM5A* (399 bp) and *HvBM5B* (318 bp) products of gDNA origin if present. Analysis of RNA from Morex, Dicktoo, and Strider sampled at time 0 (pretreatment) and at four weeks LD-vernalization demonstrated that only the 149 bp *HvBM5A* cDNA product was present, and there was no evidence for the 74–75 bp *HvBM5B*-derived cDNA product (Figure 3C). The RT-PCR data paralleled our northern data for the three genotypes, except that the higher sensitivity of RT-PCR allowed detection of lower expression levels, e.g., low levels of *HvBM5A* expression at time 0 in unvernalized Dicktoo and after four weeks of vernalization in Strider.

HvBM5A *transcription levels parallel the vegetative to floral meristem transition*

The formation of a double-ridge structure on the apical meristem of cereals is a phenotypic marker of meristem fate transition from the vegetative to reproductive state (Danyluk *et al*., 2003). In conjunction with the expression analysis tissue harvests, we accessed the apical meristem developmental state at each collection time point. As shown in Figure 4, the presence of the reproductive state double-ridge structure correlated with *HvBM5A* expression; only relevant time points are shown. For the Morex and Dicktoo genotypes, double-ridge formation was observed at the start of the experiment, indicating the pretrial exposure to LD greenhouse conditions was sufficient to initiate meristem transition. Based on the number of additional ridges formed post-double-ridge formation at time 0 (Figure 4), reproductive meristem transition had occurred earlier in Morex relative to Dicktoo and is in agreement with the higher *HvBM5A* expression level of Morex at this time point relative to Dicktoo (Figure 3A–C). Strider, which had no detectable *HvBM5A* expression at this time point, had not yet initiated double-ridge formation. Double-ridge formation was not observed in Strider until eight weeks of vernalization had transpired and is in agreement with the northern and RT-PCR analysis, where a low level of *HvBM5A* expression was only detectable at four weeks of vernalization via RT-PCR (Figure 3C).

HvBM5A *promoter differences most likely do not explain differential vernalization requirement*

We sequenced approximately 2.1 kb of upstream regulatory region from each of the eleven barley genotypes (Table 1) to investigate the basis of the *HvBM5A* expression differences and determine if the proposed CArG box difference of wheat (Yan *et al*., 2003) differed between winter and spring barleys. Alignment of the promoters revealed 37 polymorphic positions, consisting of SNPs, InDels, and SSRs, were present (Figure S3). With the exception of a length polymorphism in the number of repeats at a large SSR (AT repeat, 19–28 unit range), the promoter region of cultivated winter and facultative genotypes was otherwise identical. Thirty of the polymorphic positions were specific to a particular genotype or subset of genotypes and independent of growth habit, indicating they were not related to vernalization requirement. Among cultivated barleys, four polymorphic positions were consistent with the spring growth habit, but were also shared with the vernalization-requiring ancestral subsp. *spontaneum* OSU11 accession, indicating they also are likely to be unrelated to vernalization requirement. The remaining three polymorphisms, two tandem repeats and an SNP, were diagnostic of spring genotypes relative to the winter (and facultative) genotypes (Figure S3). While the differential presence of a known regulatory motif to explain the general growth habit-based vernalization effects on *HvBM5A* expression was not predicted at these three positions (data not shown), we cannot definitively exclude the possibility that an uncharacterized motif controlling this trait is present. Analysis of the conserved regions between the promoters of all the genotypes revealed a number of predicted regulatory motifs that may contribute to common *HvBM5A* expression properties (Table S1). These include, among others, multiple light-responsive, CArG, and low

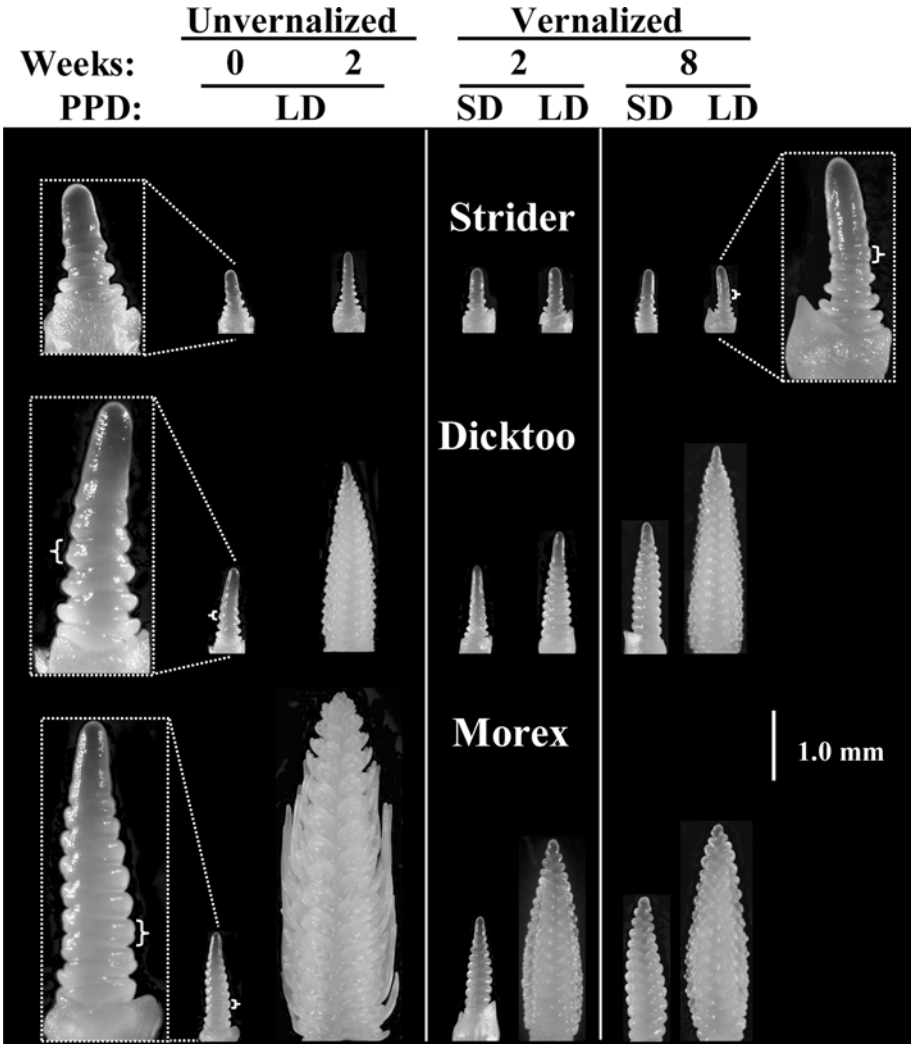

Figure 4. Apical meristem state analysis of Strider, Dicktoo, and Morex. Meristem fate was examined in conjunction with tissue collections for expression analysis (Figure 3). Only unvernalized week 0 and 2 and vernalized week 2 and 8 (under both photoperiods) are shown. Representative enlargements of respective meristems when the floral fate-diagnostic double ridge structure (bracketed) is first noted are indicated. PPD: photoperiod.

temperature-responsive CRT motifs. The putative CArG-related motif, proposed to be a basis of differential vernalization requirement of a subset of diploid spring wheat accessions (Yan *et al*., 2003, 2004b), is invariant among the 11 barley genotypes we studied and thus not a basis of differential vernalization requirement in barley (Figure S3).

A length polymorphism in HvBM5A *intron 1 is correlated with growth habit*

In Arabidopsis, intron-based regulation of MADS-box genes related to flowering and vernalization has been reported for *AG* (Sieburth and Meyerowitz, 1997) and *FLC* (He *et al*. 2003; Bastow *et al*. 2004). Accordingly, we analyzed the intragenic regions of *HvBM5A* for polymorphic segments that could be a source of vernalization requirement control. Comparison of the full-length spring *HvBM5A*-Mx and winter *HvBM5A*-St alleles revealed three prominent InDel events are present in the intragenic regions, a 5.2 kb and a 42 bp InDel in the first intron, and a 17 bp InDel in the second intron (Figure 2); in each case the spring allele has the deletion. Using primers that flank these InDels, we determined

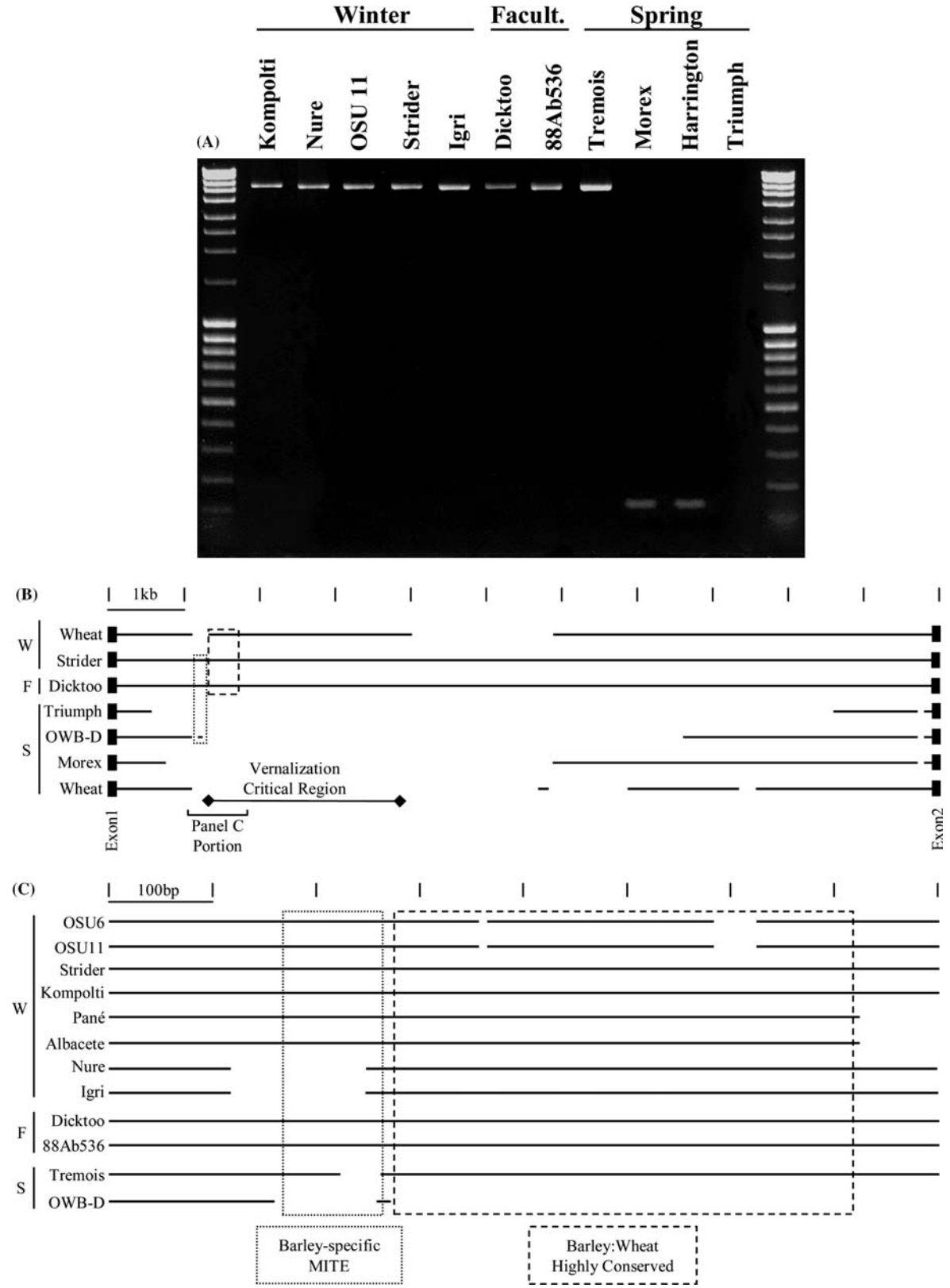
Winter
Facult.
Spring
(A)
Kompolti
Nure
OSU 11
Strider
Igri
Dicktoo
88Ab536
Tremois
Morex
Harrington
Triumph
(B)
1kb
W
Wheat
Strider
F
Dicktoo
S
Triumph
OWB-D
Morex
Wheat
Vernalization
Critical Region
Exon1
Panel C
Portion
Exon2
(C)
100bp
W
OSU6
OSU11
Strider
Kompolti
Pané
Albacete
Nure
Igri
F
Dicktoo
88Ab536
S
Tremois
OWB-D
Barley-specific
MITE
Barley:Wheat
Highly Conserved

Figure 5. *HvBM5A* Intron 1 analysis of regions critical for vernalization response in representative barley genotypes. Genotypes and growth habit are denoted. (A) PCR amplification of the region harboring the 5.2 kb intron 1 InDel relative to Morex InDel position. InDel size was examined in the primary barley parental genotype panel and a representative gel run is shown. Triumph contains a larger deletion (see Panel B) resulting in no amplification due to primer annealing site loss. (B) Schematic representation of the total *HvBM5A* intron 1 region present in five barley genotypes determined by sequencing. Edges of exons 1 and 2 are indicated by flanking black boxes. Physical gaps represent deletions relative to the full-length Strider allele. A representational summary of the minimal winter and maximal spring wheat *TmAP1*/*TaVRT1* intron 1 regions found in a screen of wheat accessions (Fu *et al*., 2005) is also shown. The minimal barley:wheat vernalization critical region and the portion analyzed in Panel (C) are denoted. (C) The 0.44 kb minimal vernalization critical and flanking region. The indicated regions were cloned and sequenced from the respective barley genotypes. A barley MITE (dotted) absent from wheat, and the 0.44 kb minimal critical vernalization region (dashed), are boxed in Panels (B) and (C). Abbreviations: W: Winter, F: Facultative, S: Spring.

that the winter, including OSU11, and facultative genotypes, always contain the larger size polymorphism, while the spring genotypes Morex, Harrington, OWB-D, and Triumph have the truncated form (Figure 5A and B, data not shown). The spring growth habit Tremois, interestingly, contains the larger size polymorphism at each position indicative of winter and facultative genotypes however. In separate work, we determined the large intron 1 InDel is also observed between spring and winter wheat *TmAP1*/*TaVRT1* alleles, with the deleted form present in the vernalization unresponsive wheat alleles (Fu *et al*., 2005). Based on intron 1 deletion point comparison of barley and wheat accessions, a minimal 2.8 kb common region was deduced that is consistently present in vernalization requiring winter barley and wheat growth habits (Figure 5B). The first 0.44 kb of this 2.8 kb common region has the highest shared sequence identity between barley and wheat, suggesting it as the most likely region to harbor a *Triticeae*-shared vernalization critical motif.

We analyzed this region in additional barley genotypes via size-based PCR screens (Figure 5C; data not shown). We further refined the vernalization critical region by cloning and sequencing the region from additional genotypes displaying size polymorphisms indicative of internal deletions based on the PCR screen (Table 1, Figure 5C). In winter growth habit barley accessions, the proposed 0.44 kb vernalization critical region is retained, but the remainder of the larger 2.8 kb wheat:barley shared region can be eliminated from consideration based on the two Spanish winter growth habit accessions Pané and Albacete (Figure 5C). Also, this 0.44 kb vernalization critical region can be divided into three subregions based on the two ancestral *H. vulgare* subsp. *spontaneum* winter accessions OSU6 and OSU11. Directly upstream of this 0.44 kb vernalization region is a barley MITE element that is absent from wheat, but is a source of variation between a subset of spring and winter growth habit barley genotypes (Figure 5C). While the spring growth habit Tremois appears to contain a winter *HvBM5A* allele in the 0.44 kb vernalization critical region, a small deletion is present within the barley MITE element. While the absence of this region from both winter and spring wheat accessions implies it is not involved in a conserved *Triticeae* vernalization mechanism, it cannot currently be excluded as a potential barley-specific component resulting in the spring growth habit phenotype of Tremois. Sequence analysis of the 0.44 kb critical region indicates a number of *cis* regulatory motifs are predicted, including hormone- and light-responsive motifs, a cold-responsive CRT/DRE motif, and four Dof protein binding sites (Table S1).

The presence/absence of VRN-H2 *correlates with vernalization requirement and may define the facultative growth habit*

The *ZCCT-H* gene cluster (Yan *et al*., 2004a) map position coincides with, and is a candidate for, the *VRN-H2* vernalization response QTL (Figure 1). We characterized the *VRN-H2* locus in the 11 genotypes examined in this study by multiplex amplification of a conserved region of the *VRN-H2* candidate genes *ZCCT-Ha* and-*Hb* (referred hereafter as *ZCCT-H*), as well as the tightly linked *VRN-H2* locus-proximal *HvSnf2* gene (Yan *et al*., 2004a). The *ZCCT-H* genes are present in each winter accession, but deleted from the facultative and spring accessions (Figure 6). In contrast, the *VRN-H2*-proximal *HvSnf2* locus amplified from all varieties, verifying that the lack of *ZCCT-H* amplification in the facultative and spring genotypes is likely due to a physical

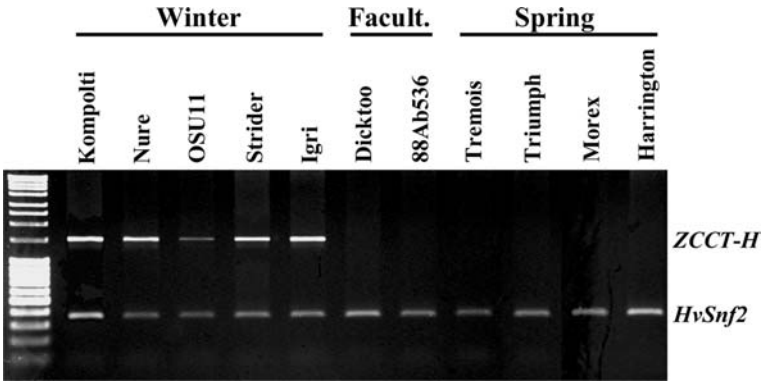

Figure 6. Multiplex PCR amplification of the *VRN-H2* loci markers *ZCCT-H* and *HvSnf2* in the primary barley genotype panel assessed in this study. A representative gel run is shown with genotypes and growth habit denoted. *ZCCT-H* and *HvSnf2* product positions are indicated.

deletion of these genes and not a PCR amplification failure. The physical deletion of the *VRN-H2* candidate gene from genotypes harboring winter *vrn-H1* alleles may be a basis of the vernalization requirement-lacking facultative growth habit (see Discussion).

Discussion

The VRN-H2 *locus is deleted in spring and facultative growth habits*

Winter and spring growth habits are easily distinguished based on the presence vs. absence of a vernalization requirement. The two-locus *VRN-2*/*VRN-1* epistatic interaction model of Yan *et al.* (2004a) provides a genetic mechanism to explain these opposite phenotypes and is supported by the results of this work and others (Danyluk *et al.*, 2003; Murai *et al.*, 2003; Trevaskis *et al.*, 2003). We found complete association of the deleted *vrn-H2* candidate *ZCCT-H* genes with a loss of vernalization requirement in the important North American and European genotypes used to generate key winter hardiness mapping populations. Only winter growth habit genotypes contained the *Vrn-H2* locus, while it had been deleted from both the spring and facultative growth habits (Table 1). The agronomic growth habit term 'facultative' has lacked a rigorous definition, and is loosely used to define genotypes that, while lacking a vernalization requirement, have retained a low temperature tolerance capacity equivalent to winter habits that allows fall-sowing and over-winter survival in environments lethal to spring growth habits. Interestingly, both the facultative genotypes examined also harbor a winter *HvBM5A* (recessive *vrn-H1*) allele, suggesting the deleted *vrn-H2* locus *ZCCT-H* gene set is responsible for converting an otherwise winter genotype into a form, a facultative, that lacks a vernalization requirement. In agreement with facultative genotypes being a subclass of winter genotypes, we have noted in a large allelic characterization of multiple *CBF* genes, key components of the winter hardiness trait of low temperature tolerance, that like the *HvBM5A* alleles, the facultative and winter *CBF* alleles are typically the most similar (and are frequently identical) relative to alleles from spring growth habits (Skinner *et al.*, 2005). The physical deletion of the *vrn-H2* candidate gene results in loss of the *trans*-acting repressor of the winter *vrn-H1* allele, yielding the 'pseudo-spring' facultative phenotype, from which a vernalization requirement has essentially been eliminated. We propose that the term facultative growth habit may be appropriate to describe genotypes that are low temperature-tolerant and have 'winter' alleles at *VRN-H1* (e.g., recessive *vrn-H1*), but lack the repressor encoded by *VRN-H2* (e.g., recessive *vrn-H2*). Additional confirmation in a broader range of facultative genotypes is required to support this as a general explanation. As spring genotypes typically flower more quickly than facultative genotypes (Karsai *et al.*, 2004), additional regulatory inputs such as photoperiod are likely influencing the expression of the winter

vrn-H1 allele (relative to a spring *Vrn-H1* allele) present in facultative genotypes and may account in part for the maturation difference.

HvBM5A *is probably* VRN-H1

We found that two closely related *HvBM5* genes, *HvBM5A* and *HvBM5B*, are present in barley. Sequence data, RT-PCR expression results, and the lack of *HvBM5B*-like EST sequences in GenBank, all suggest *HvBM5B* is a pseudogene that is not expressed in any of the three representative growth habit genotypes we studied. While *HvBM5B* is not a candidate for the *VRN-H1* QTL based on its map location, it is in the same genetic proximity of that predicted for *VRN-H3* (*Sh3*) (Takahashi and Yasuda, 1971). In contrast, orthology, map location, and expression pattern relative to vernalization and the floral state transition provide very strong support for the hypothesis that *HvBM5A* is *VRN-H1*. HvBM5A protein sequences show highest identity with and is orthologous to the wheat *VRN-1* candidates *TmAP1* (Yan *et al*., 2003), *TaVRT-1* (Danyluk *et al*., 2003) and *WAP1* (Murai *et al*., 1998). *HvBM5A* maps to Bin 11 of chromosome 5H, the position of vernalization response QTL in the Nure × Tremois (Francia *et al*., 2004) and Igri × Triumph (Laurie *et al*., 1995) mapping populations. Barley 5H Bin 11 is syntenous to the reported position of *VRN-A^m1* and flanking markers in *T. monococcum*, and based on *Triticeae* homoeology, the position of *VRN-1* in the B and D genomes. The expression patterns of *HvBM5A* relative to genotype growth habit – in terms of both transcript level between genotypes, and the correlation of transcript appearance and level relative to meristem differentiation – agree with previous reports in the literature for the putative *Triticum* spp. orthologs of *VRN-1* (Danyluk *et al*., 2003; Murai *et al*., 2003; Trevaskis *et al*., 2003; Yan *et al*. 2003). Expression of the *HvBM5* gene of Schmitz *et al*. (2000), which we establish is *HvBM5A*, was also examined by Danyluk *et al*. (2003) in Dicktoo and by Trevaskis *et al*. (2003) in a number of spring and winter barley genotypes, and the results of these two groups also agreed with the respective growth habits, further supporting the *VRN-H1* candidacy. Danyluk *et al*. (2003) investigated *HvBM5A* expression under strict SD and LD conditions and found *HvBM5A* expression was strongly repressed under the initial weeks of SD-vernalizing conditions. Our detection of *HvBM5A* transcript via RT-PCR and the formation of a double-ridged meristem at the beginning of the vernalization trial suggest that even the brief germination and seedling growth under the longer greenhouse day length conditions prior to cold transfer was sufficient to derepress *HvBM5A*. This highlights the complexity of multiple input signals (e.g., temperature and photoperiod) that influence the resultant growth habit of a specific haplotype (Danyluk *et al*., 2003), including those of the facultative habit. Conserved with the *AP1* role in Arabidopsis (Henderson *et al*., 2003), *HvBM5A* and its wheat orthologs appear to specify a key floral meristem identity gene. In cereals, this developmental gene has evolved so that its expression is under the direct control of the two major environmental inputs – vernalization and photoperiod – determining growth habit, and allelic variation of the vernalization regulatory region results in the *VRN1* QTL effect (this work; Danyluk *et al*., 2003; Murai *et al*., 2003; Trevaskis *et al*., 2003; Yan *et al*. 2003). Definitive proof that *HvBM5A* is *VRN-H1* will require forward and/or reverse genetic strategies.

Functional allelic variation at the VRN-H1 *locus is regulatory, not structural*

Given that all the data support the hypothesis that *HvBM5A* is *VRN-H1*, we analyzed whether differences in vernalization requirement are due to structural and/or regulatory differences between *HvBM5A* alleles. Our data provide very strong evidence that differences in vernalization requirement are due to gene regulation, not to differences in the encoded polypeptide. The coding regions of all 11 genotypes we surveyed – which represent an ancestral form and cultivated genotypes from the major germplasm groups (e.g. spring, facultative, and winter habit; two-row and six-row inflorescence) of North America and Europe – are identical. Integrating these results with those from *Triticum* spp. (Danyluk *et al*., 2003; Yan *et al*., 2004a) reveals a high degree of *VRN-1* gene sequence conservation, and presumably function, in the *Triticeae*.

Based on the lack of polypeptide variation and the report of Yan *et al*. (2003) that promoter mutations in a CArG-like motif correlated with

differences in *T. monococcum* growth habit, we searched for promoter differences in the eleven representative barley genotypes. We found the CArG-like motif of each accession was invariant. Yan *et al*. (2004a, b) also found dominant alleles of *VRN-1* that do not have CArG-like motif mutations, and these findings prompted a more comprehensive analysis of the promoter and intron 1 regions in the A, B, D, and H genomes (Fu *et al*., 2005). There was, however, no likely promoter polymorphism separating winter from spring *VRN-H1* alleles that appeared to account for the difference in vernalization requirement. Combined with the lack of coding region variation, this implied the predominant vernalization critical regulatory region may be intron-based. It is possible, however, that in barley, as in wheat (Yan *et al*., 2004b), the promoter may control barley vernalization requirement in some accessions. This could be the case for Tremois (see below).

Intron-based regulation of MADS-box gene expression related to flowering and vernalization occurs in Arabidopsis (Sieburth and Meyerowitz, 1997; He *et al*., 2003; Bastow *et al*., 2004). We found that the 5.2-kb intron 1 region deleted from the *HvBM5A*-Mx spring allele was retained in each accession harboring a winter *vrn-H1* allele among our winter hardiness population parental genotypes. Likewise, the spring growth habit varieties, with one exception, had lost this region. Only the spring growth habit Tremois failed to fit the predominate intron 1 deletion vs. growth habit pattern and may represent an alternate allelic mode of generating a spring genotype in barley (below).

Since winter habit is the ancestral form in the *Triticeae*, it was likely that deletion of this intron 1 region could be of functional and evolutionary significance, and is therefore a candidate for the source of *VRN-H1* QTL effects in all barley mapping populations, except Nure × Tremois. In separate work, were able to determine that the analogous intron 1 region of *TmAP1*/*TaVRT-1* was affected in a similar pattern relative to spring vs. winter growth habits of wheat (Fu *et al*., 2005), further supporting the importance of this region. By comparing multiple accessions from barley and wheat, we were able to delimit a 2.8 kb minimal vernalization critical region that was retained in all vernalization responsive (i.e., winter) growth habit accessions, of which the first 0.44 kb was the most highly conserved. A PCR survey of the 2.8 kb vernalization critical region in additional barley accessions reduced this region to the 0.44 kb region displaying the highest barley:wheat conservation. This was typified by the two winter growth habit Spanish landrace accessions Pané and Albacete, which have an identical deletion of the remainder of the 2.8-kb barley:wheat-shared critical region 3′ to this highly conserved 0.44-kb segment. Additionally, the 0.44-kb minimal region is divided into three subdomains in the two ancestral winter *spontaneum* accessions OSU6 and OSU11, indicating two insertion events have occurred in domesticated winter barley that are independent of vernalization response. While multiple motifs are predicted within this putative vernalization critical region, currently the most interesting are the four predicted Dof sites, which are conserved between the barley and wheat alleles, and can function as repressor motifs in plants (Yanagisawa and Sheen, 1998). The mechanisms of *VRN-H1* regulation have yet to be demonstrated, and the question also remains if the *VRN-H2* candidate ZCCT-H is the sole *trans*-acting regulatory factor of *VRN-H1* repression or is a cofactor in a complex that affects binding to a *VRN-H1* repression motif. Actual delimitation of the particular motif and factor(s) responsible for pre-vernalization repression of *HvBM5A* expression in winter growth habits will require biochemical and/or transgenic experiments.

Tremois has a unique haplotype relative to the vernalization critical region versus corresponding growth habit. Tremois would appear to be a facultative genotype based on the winter-like size of *HvBM5A* intron 1 (Figure 5A) and the deletion of the *VRN-H2* candidate gene loci (Figure 6). However, a *VRN-H1* QTL effect is absent in winter × facultative crosses (Table 2), and therefore, Tremois must harbor a dominant, or spring, *HvBM5A* allele. While Tremois contains, and is invariant in, the vernalization critical 0.44-kb region relative to winter wheat and barley genotypes, it is variable directly outside of this 0.44-kb region, where a 38-bp deletion, 12 bp of which is unique to spring habit alleles, has occurred in a barley MITE element (Figure 5C). This MITE is not observed in wheat accessions, suggesting it is barley-specific and not involved in a conserved *Triticeae*-based vernalization response. Two scenarios are possible at present in which the Tremois

Table 2. Relationship between *VRN-H1* and *VRN-H2* allele types and reported vernalization requirement QTL.

Mapping population	Parents	Growth habit	Chromosome 4H		Chromosome 5H		
			VRN-H2[a]	QTL[b]	*VRN-H1* Promoter	*VRN-H1* Intron 1	QTL[b]
D×M	Dicktoo	Facultative	–	–	Winter	Winter	–
	Morex	Spring	–		Spring	Spring	
D×K	Dicktoo	Facultative	–	+	Winter	Winter	–
	Kompolti	Winter	+		Winter	Winter	
StAb	Strider	Winter	+	+	Winter	Winter	–
	88Ab536	Facultative	–		Winter	Winter	
N×T	Nure	Winter	+	+	Winter	Winter	+
	Tremois	Spring	–		Spring	Undetermined	
I×T	Igri	Winter	+	+	Winter	Winter	+
	Triumph	Spring	–		Spring	Spring	

[a] *VRN-H2* candidate *ZCCT-H* gene cluster deleted (–) or present (+).
[b] Respective significant vernalization QTL effects are detected (+) or not present (–) in population.

HvBM5A allele yields a dominant spring allele. The first is that the deleted MITE region of Tremois comprises part of a barley-specific vernalization component. The *VRN-H2* gene product may be part of a multi-component repressor complex where this 'alternate' site is important for stable interaction of *VRN-H2* with the adjacent critical region of intron 1. The second scenario is that the Tremois *HvBM5A* promoter is responsible for the spring habit, which would be dominant over the intron 1 critical region. In wheat, in addition to the intron 1 architecture that typically predicts vernalization requirement (Fu *et al.*, 2005), we noted the promoter as a basis for differential vernalization response of some spring accessions (Yan *et al.*, 2004b). Tremois may represent a barley example of this promoter-based alternate wheat class. Tremois has a unique promoter haplotype that is only shared with Triumph (Figure S3), the other European spring habit accession. Triumph has the intron 1 deletion typical of spring alleles, so a promoter-based dominant vernalization effect would be masked in Triumph. Functional domain swapping comparison – Tremois promoter plus winter intron 1 vs. winter promoter plus Tremois intron 1 – will be needed to determine which region is responsible for the Tremois spring growth habit. Thus, in barley, like wheat, two regions of *HvBM5A* need to be considered as potential sites regulating vernalization response, with the intron 1 critical region appearing to be the predominate location of winter vs. spring variation.

The VRN-2/VRN-1 *two-locus model provides a genetic explanation for reported vernalization requirement QTL*

Vernalization requirement in barley is not absolute, as Karsai *et al.* (2001) found in a study of a diverse barley germplasm array that when unvernalized, each genotype would eventually flower. However, the heading date of some unvernalized genotypes was so delayed compared to vernalized (e.g., up to 141 days) that these genotypes can be said to have a vernalization requirement. Accordingly, vernalization requirement/response in barley is quantitatively measured as the difference in unvernalized vs. vernalized heading date under a specified photoperiod, and these phenotypic data are analyzed using QTL rather than Mendelian tools. Vernalization requirement QTL are reported from a number of barley mapping populations, and *VRN* loci were hypothesized to be candidate genes for these QTL effects (Hayes *et al.*, 1997). Our characterization of the *VRN-H1* and *VRN-H2* loci genotypes in mapping population parents explains QTL effects in all reported mapping populations (Table 2). The Igri × Triumph and Nure × Tremois winter × spring crosses are variable for both *VRN-H1* and *VRN-H2* loci, so QTLs to each are expected and observed in these populations (Laurie *et al.*, 1995; Francia *et al.*, 2004). A *VRN-H2* QTL was not reported on 4H in the initial characterization of the Nure × Tremois population due to an incomplete genetic linkage map for this region (Francia *et al.*, 2004), but

reexamination of the population data with the scored additional loci in this work expands the coverage of 4H, and the *VRN-H2* QTL coincident with the *ZCCT-H* locus (LOD = 5.4, $R^2 = 0.2$) is present as predicted (Online Supplemental Section S2). These winter × spring population crosses confirm that a vernalization requirement is not observed in backgrounds with a physical deletion of the *VRN-H2* locus, as well as in those harboring *VRN-H2* but lacking the target binding site for the repressor in the *HvBM5A* gene (i.e., dominant *Vrn-H1*). Both parents of a facultative by spring cross (Dicktoo × Morex) lack the *VRN-H2* candidate *ZCCT-H* repressor gene, so neither a *VRN-H1* nor *VRN-H2* QTL will be detected (Hayes *et al.*, 1997), despite the presence of a repressible *vrn-H1* allele in the facultative parent. In contrast, the Strider × 88Ab536 and Dicktoo × Kompolti korai winter × facultative crosses contrast for *VRN-H2* alleles, where both parents harbor winter *vrn-H1* alleles while only the winter parent contains the *ZCCT-H* genes, and thus only the *VRN-H2* QTL is detected (Karsai *et al.*, 2005). The finding that *VRN-H1* and *VRN-H2* allele data are sufficient to predict and explain all reported vernalization requirement QTL data from domesticated barley crosses is additional evidence that *VRN-H3* is a determinant of vernalization response only in a limited range of exotic barley germplasm, as reported by Takahashi and Yasuda (1971). In summary, the correlation of molecular haplotypes with the corresponding phenotypes of these key winter hardiness mapping population parents strongly supports the respective *VRN-H2* and *VRN-H1* candidacies of the *ZCCT-H* and *HvBM5A* genes. The molecular basis of these haplotypes we define in the current study will allow design of functional assays to definitively prove the vernalization-based role of these genes.

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