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Mapping of barley homologs to genes that regulate low temperature tolerance in Arabidopsis

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Abstract We investigated the allelic nature and map locations of *Hordeum vulgare* (barley) homologs to three classes of Arabidopsis low temperature (LT) regulatory genes—*CBF*s, *ICE1*, and *ZAT12*—to determine if there were any candidates for winterhardiness-related quantitative trait loci (QTL). We phenotyped the Dicktoo × Morex (D×M) mapping population under controlled freezing conditions and in addition to the previously reported 5H-L *Fr-H1* QTL, observed three additional LT tolerance QTLs on 1H-L, 4H-S, and 4H-L. We identified and assigned either linkage map or chromosome locations to 1 *ICE1* homolog, 2 *ZAT12* homologs, and 17 of 20 *CBF* homologs. Twelve of the *CBF* genes were located on 5H-L and the 11 with assigned linkage map positions formed 2 tandem clusters on 5H-L. A subset of these *CBF* genes was confirmed to be physically linked, validating the map position clustering. The tandem *CBF* clusters are not candidates for the D×M LT tolerance *Fr-H1* QTL, as they are ~30 cM distal to the QTL peak. No LT tolerance QTL was detected in conjunction with the *CBF* gene clusters in Dicktoo × Morex. However, comparative mapping using common markers and BIN positions established the *CBF* clusters are coincident with reported Triticeae LT tolerance and *COR* gene accumulation QTLs and suggest one or more of the *CBF* genes may be candidates for *Fr-H2* in some germplasm combinations. These results suggest members of the *CBF* gene family may function as components of winterhardiness in the *Triticeae* and underscore both the importance of extending results from model systems to economically important crop species and in viewing QTL mapping results in the context of multiple germplasm combinations.

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Introduction

In temperate cereal crops (e.g., wheat, barley, rye), winterhardiness is a trait of adaptive and economic importance (Thomashow 1999; Cattivelli et al. 2002). Winterhardiness has three principal components—low temperature (LT) tolerance, vernalization (VRN) response, and photoperiod (PPD) sensitivity—and within the Triticeae, a broad range of phenotypic variation occurs for all three components at the genus, species, and accession levels. VRN and PPD are adaptive traits amenable to phenotypic selection and recent advancements make them tractable targets for both marker-assisted selection (MAS) and genetic engineering. While LT tolerance is a trait of tremendous adaptive and economic significance due to the risk of winter injury faced by fall-sown cereal crops, the genetic basis of LT tolerance in the Triticeae, currently a focus of intensive research, is not as advanced as the VRN components.

The cereal tribe forms a homogeneous genetic system, and therefore insights into the components of winterhardiness are typically applicable between members of the cereal tribe (Dubcovsky et al. 1998; Mahfoozi et al. 2000). Barley (*Hordeum vulgare* subsp. *vulgare*) can function as a model for dissecting the trait components of winterhardiness in the Triticeae, including LT tolerance. Barley is a self-pollinated diploid, the primary gene pool contains abundant genetic variation for the winterhardiness components, and there is an ever-expanding set of genetic and molecular analysis tools (reviewed in Hayes et al. 2003), including multiple mapping populations, arrayed BAC clones, a large EST database, and a microarray chip (Close et al. 2004). Relative to the three winterhardiness traits, barley germplasm can be classified as winter, facultative, or spring. In general, winter varieties are LT tolerant, PPD sensitive, and highly responsive to VRN. Spring varieties typically have minimal LT tolerance capacity, do not respond to VRN, and are insensitive to short day PPD. The term "facultative" has lacked a rigorous definition, but is generally used to describe genotypes that are LT tolerant, do not respond to VRN, and may or may not be PPD sensitive. We recently found that a representative sample of facultative varieties have a complete deletion of the *VRN-H2* locus on 4H, accounting for their lack of a strong VRN response (Karsai et al. 2005; von Zitzewitz et al. 2005).

In the Triticeae genome, the long arm of chromosome 5 has been the region most frequently reported to be associated with LT tolerance and VRN response (Hayes et al. 1997; Cattivelli et al. 2002; Vágújfalvi et al. 2003; Francia et al. 2004). Until recently, these genes have been reported as quantitative trait locus (QTL) effects because they show complex, rather than Mendelian, inheritance. Most recently, two LT tolerance QTLs, approximately 25 cM apart, were reported in the Nure × Tremois (N×T) barley population (Francia et al. 2004). The N×T LT tolerance *Fr-H1* QTL corresponds to the Dicktoo × Morex (D×M) barley population LT tolerance QTL of Hayes et al. (1993), which in turn is coincident with *VRN* and flowering time (heading date) QTLs (Hayes et al. 1993, 1997; Laurie et al. 1995). The N×T LT tolerance *Fr-H2* QTL is syntenous with a LT tolerance QTL in diploid wheat (Vágújfalvi et al. 2003); *COR* gene product accumulation QTLs also map to this position in both species.

Recently, candidate genes have been mapped to these QTL positions. Barley *HvBM5A* maps to the *VRN-H1* QTL position (von Zitzewitz et al. 2005), in agreement with the position of *TmAP1*, the candidate *VRN-A^{m}1* gene of diploid wheat (Yan et al. 2003). It is possible that *HvBM5A* has pleiotropic effects on VRN response, flowering time, and/or LT tolerance. However, Karsai et al. (2001) have found that these three traits occur in all possible combinations in barley, suggesting linkage, rather than pleiotropy, may be responsible. Beales et al. (2005) recently mapped the photoreceptor *PhyC*—a potential candidate for the day length-influenced flowering time QTL—to this region in hexaploid wheat. We recently mapped a *CBF* gene coincident with LT tolerance and *COR* gene product accumulation QTLs distal to the *VRN-H1* locus/QTL cluster (Francia et al. 2004), and Choi et al. (2002) mapped *HvCBF3* to this same region. The presence of a *CBF* gene at the homoeologous region was also observed in diploid wheat (Vágújfalvi et al. 2003).

We found the occurrence of a *CBF* gene coincident with LT tolerance and *COR* gene product accumulation QTLs most intriguing because in Arabidopsis *CBF* genes encode LT-induced transcription factors that regulate an important branch of the cold acclimation pathway (Fowler and Thomashow 2002). Within this branch are many of the characterized *COR* genes that contribute to LT tolerance capacity and the *COR* genes harbor CRT motif(s) in their promoters that the CBFs bind to induce expression. The LT-induced expression of the *AtCBF* genes is regulated via MYC *cis* elements bound by ICE transcription factors belonging to the bHLH domain family and ICE1 (*AtICE1*) directly regulates *AtCBF3* (Chinnusamy et al. 2003). In Arabidopsis, the majority of the most highly induced cold-regulated genes can be assigned to the CBF and ZAT12 regulons (Fowler and Thomashow 2002; Vogel et al. 2005). ZAT12 (At-ZAT12) is a C2H2 Zn-finger domain factor containing similar sequences in its promoter to the ICE-controlled MYC elements of *CBF* genes, suggesting *AtICE1*, or a close relative, also regulates *AtZAT12* expression. Importantly, the combined *AtCBF* and *AtZAT12* regulons account for a majority of the LT-upregulated genes (Vogel et al. 2005) and leads to the questions "is there a comparable LT tolerance pathway in the Triticeae?" and "do cereal regulatory factor homologs account for LT tolerance QTL?"

The availability of the extensive barley EST sequence collection (~420 K ESTs, representing multiple genotypes, as of the 07/08/05 release) makes the routine identification and analysis of barley homologs to Arabidopsis genes feasible. As a first step in this comparative mapping exercise, we cloned nearly twenty *CBF* genes from the barley genotype Dicktoo (Skinner et al. 2005). Our objectives in the current study were to (1) determine the level of allelic variation at these *CBF* loci in representative accessions, (2) assign their linkage map positions using the Dicktoo × Morex mapping population (a standard reference for winterhardiness trait mapping) and (3) determine the positional relationships of the *CBF* genes with LT tolerance QTL in Dicktoo × Morex and other Triticeae mapping resources. In addition to the *CBF* genes, we investigated the linkage map positions, and thus QTL candidacy, of barley homologs to *AtICE1* and *AtZAT12*.

Materials and methods

Plant material

Barley genotypes Dicktoo, Morex, Strider, and 88Ab536 were used as mapping and allele sequencing resources.

The Dicktoo × Morex (D×M) facultative × spring population is a standard for winterhardiness physiology and genetics research and the linkage map based on this population has sufficient markers in common with other mapping populations to allow for comparison and alignment (Pan et al. 1994; van Zee et al. 1995; Hayes et al. 1997; Karsai et al. 1999; Fowler et al. 2001; Mahfoozi et al. 2000). The Strider × 88Ab536 (STAB) winter × facultative population was developed as a complimentary mapping tool segregating for VRN requirement (Filichkin et al. 2005). The D×M population was phenotyped for LT tolerance under controlled freezing conditions in the Agricultural Research Institute of the Hungarian Academy of Sciences (Martonvásár, Hungary) phytotron facility as in Szűcs (2003), to determine the relationship of observed LT tolerance QTL with candidate genes and QTL reported in other Triticeae populations. Briefly, five randomly arranged replications of 15 rows of 10 plants each were grown in a chamber for 6 weeks where temperature, light intensity, and illumination length were gradually reduced at weekly intervals to a final temperature of 3°C to simulate natural fall/winter conditions before exposure to two subsequent hardening phases. In hardening phase 1, daily temperature fluctuated between +3°C and −3°C for 1 week, with 21 h day length and 190 μmol/m^2 s illumination. In hardening phase 2, the temperature was reduced to −4°C and kept constant for 4 days without illumination. Following phase 2, chamber temperature was reduced 1°C/h to −12.5°C and the plants maintained at −12.5°C for 24 h. The temperature was then raised 1°C/h to a final temperature of 17°C. After 2 days, plants were cut back to a height of 3 cm, grown for a further 3 weeks with a day/night temperature of 17/16°C, 14 h day length, and 130 μmol/m^2 s illumination. Following the third week, growing plants that had survived freezing were clearly distinguished from those that had died. Surviving plant number was expressed as a percentage of the plant number before freezing.

Allele isolation

The allele isolation method (EST, PCR, etc.) for each Arabidopsis LT tolerance regulon barley homolog is indicated in Table 1 and GenBank accession numbers for novel alleles are listed in Table S1. Full cDNA insert sequences for each EST clone were determined by direct sequencing. For PCR amplicon cloning, total gDNA was extracted from a single plant of each genotype using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and used as template. PCR was performed under standard conditions using a Taq DNA polymerase kit (Qiagen) and the products cloned using a pGEM-T-Easy Kit (Promega, Madison, WI, USA). Primer sets and sequences utilized for allelic PCR amplicon cloning are listed in Table S2. For each GenBank-reported gene/allele isolated via PCR, cloned amplicons of at least two or more independent PCR reactions were sequenced and verified as identical to confirm PCR-based nucleotide substitutions had not occurred. Only the non-primer portion of each consensus amplicon was reported. *CBF*-positive Morex BAC clones listed in Choi et al. (2002) were obtained from Andris Kleinhofs (Washington State University).

Linkage mapping and QTL analysis

Linkage map positions for *HvCBF* genes, *HvICE2*, *HvZFP16-1*, and *HvZFP16-2* were assigned by (1) mapping allelic polymorphisms in the D×M or STAB populations ($n = 92$ and 91 doubled haploid (DH) lines, respectively) or, when no polymorphisms were detected, by (2) assignment of loci to chromosome arms via the barley-wheat disomic addition lines (Islam et al. 1981). The mapping strategy employed for each locus (population vs. addition line) is indicated in Table 1; mapping primer pairs and corresponding sequences are provided in Table S3. JoinMap 3.0 (Van Ooijen and Voorrips 2001) was used for linkage map construction using default parameters. QTL Cartographer Version 2.5 (Wang et al. 2005a) was used for QTL analyses employing the standard Composite Interval Mapping model and the forward and backward regression method with seven control markers and a 10 cM window size. Linkage map positions (Table 1) were related to all winterhardiness QTL reported in the literature using both common markers and the BIN map concept of Kleinhofs and Graner (2001).

Phylogenetic analysis

Genes and accession numbers used for phylogenetic analysis are listed in Table S1. Protein sequences were aligned using ClustalW, and refined by hand using GeneDoc Version 2.6 (http://www.psc.edu/biomed/genedoc). Phylogenetic analyses on refined alignments were conducted using MEGA Version 2.1 (http://www.megasoftware.net/) and trees generated using neighbor joining and minimum evolution default methodologies on 1,000 bootstrap replications.

Results

Barley homolog identification strategy

Our identification strategy for barley homologs to Arabidopsis regulatory genes via analysis of the barley EST database revealed the presence of a large *HvCBF* gene family, as well as *AtICE1* and *AtZAT12* homologs. We narrowed our searches by also using the rice genome to verify that searches using the closest rice homolog to a queried Arabidopsis gene identified the same barley EST candidate. Following EST identification via database analyses, the respective clones were obtained, the cDNA insert sequenced, and alleles amplified from Dicktoo and

Table 1 Allele isolation and mapping summary

Gene	HvCBF-subgroup	Cloned alleles[a]	Mapping genotype(s)[b]	Chromosome[c]	BIN
HvICE2	NA	Dt[4], Mx[1,4]	D×M	3H-L[1]	13/14
HvZFP16-1	NA	Dt[4], Mx[4], Op[1]	D×M	1H-S[1]	4
HvZFP16-2	NA	Dt[4], Mx[4], Op[1]	Betzes	1H[3]	ND
HvCBF1	1	Dt[4], Mx[1,4], St[4], Ab[4], Hn[6,d]	STAB	6H-L[1,3]	7
HvCBF2A	4	Dt[4], Mx[4], St[4], Ab[4]	Dicktoo	5H-L[4]	10
HvCBF2B	4	Dt[4], St[4], Hn[6,d]	Betzes	5H-L[3]	ND
HvCBF3	3	Dt[4], Mx[4,6,d], St[4], Ab[4]	D×M, STAB	5H-L[1]	10
HvCBF4A	4	Dt[2], Mx[6], St[4,e]	D×M	5H-L[1,f]	10
HvCBF4B	4	Dt[2], Mx[1], CI[1]	D×M	5H-L[1,f]	10
HvCBF4D	4	Ab[4]	ND	ND	ND
HvCBF5	1	Dt[4], Mx[4], St[4], Ab[4], Op[1]	D×M	7H-S[1]	2
HvCBF6	3	Dt[4], Mx[1], St[4], Ab[4]	D×M, STAB	5H-L[1,2]	10
HvCBF7	1	Dt[4], Mx[1], St[4], Ab[4]	Betzes	6H-L[3]	ND
HvCBF8A	3	Dt[4], Mx[5], Ab[4]	ND	ND	ND
HvCBF8B	3	Dt[4], Mx[4], St[4], Ab[4]	ND	ND	ND
HvCBF8C	3	Dt[4], Mx[4]	D×M	2H-S[1]	8
HvCBF9	4	Dt[4], Mx[1,2], St[4], Ab[4]	D×M	5H-L[1]	10
HvCBF10A	3	Dt[4], Mx[4], St[4], Ab[4]	D×M	5H-L[1]	10
HvCBF10B	3	Dt[4], St[4], Ab[4], Op[1]	Dicktoo	5H-L[4]	10
HvCBF11	1	Dt[4], Mx[4], St[4], Ab[4], Op[1]	Betzes	2H-L[3]	ND
HvCBF12	3	Dt[3], Mx[3]	D×M	5H-L[1]	10
HvCBF13	3	Dt[3]	D×M	5H-L[4,5]	10
HvCBF14	4	Dt[4], Mx[4]	D×M	5H-L[1]	10

NA not applicable, *ND* not determined

[a]Isolation method and genotype allele codes: *1* EST, *2* cDNA library, *3* gDNA library, *4* PCR, *5* BAC, *6* Reported in Genbank; Genotypes: Ab: 88Ab536, CI: CI16151, Dt: Dicktoo, Hn: Halcyon, Mx: Morex, Op: Optic, St: Strider

[b]Barley genotype used to determine/infer linkage map or chromosome location: Dicktoo × Morex population (D×M), Strider × 88Ab536 population (STAB), barley–wheat addition lines (Betzes), Dicktoo gDNA phage clone (Dicktoo)

[c]Chromosome location or map position determined by: *1* CAPs assay, *2* InDel, *3* barley–wheat addition lines, *4* inferred via phage clone gene linkage, *5* presence/absence

[d]Barley CBF alleles first reported in Choi et al. (2002), Xue (2002), or Xue (2003)

[e]It is currently unclear if the Strider *HvCBF4* allele represents a *4A*, *4B*, or novel *HvCBF4* form

[f]*HvCBF4A* and *HvCBF4B* coamplify and map as a single locus in the extended ($n = 236$) D×M mapping population

Morex; in many cases, we additionally amplified alleles from Strider and 88Ab536. These amplified alleles were used to (1) investigate the degree of allelic polymorphism between genotypes contrasting for winterhardiness traits, and to (2) identify suitable mapping polymorphisms to determine whether a gene was a LT tolerance QTL candidate.

Identification and allelic variation of barley *CBF* genes

We recently reported that barley contains a large *CBF* family of at least 20 genes (designated *HvCBF*s), focusing on alleles from Dicktoo (Skinner et al. 2005). In this report, we expand on these findings with information on allelic variation at these loci. We cloned Morex alleles to each *HvCBF*, where possible, in order to determine the amount of allelic variation relative to Dicktoo and allow placement in the D×M population. Alleles at a subset of the *HvCBF* genes were also isolated from Strider and 88Ab536, and alleles from additional genotypes were obtained via sequencing of EST clones derived from these varieties (Table 1). Based on coding sequence, the barley *CBF* genes can be grouped into three phylogenetic clades, which we have designated the HvCBF1-, HvCBF3-, and HvCBF4-subgroups (Fig. 1); these clades are supported by the *CBF* gene families of rice and wheat (Skinner et al. 2005).

In general, alleles at each of the *HvCBF* loci were highly conserved. A detailed summary of allelic variation features for each locus is presented in the Online Supplement; a general summary follows. Single nucleotide polymorphisms (SNPs) were the most common variant, and when present, occur throughout the coding and untranslated regions (UTRs). Most coding region-localized SNPs either did not generate an amino acid substitution or resulted in a conservative substitution. In the latter case, the majority of amino acid substitutions occurred in the C-terminal region, although these changes did not alter the overall domain acidity or side chain character of amino acid residues (Table S1) critical for trans-activation (Wang et al. 2005b). Insertion/deletion (InDel) events were rare, but present, while no simple sequence repeat (SSR) polymorphisms were observed. Probable gene deletions were observed: *HvCBF2B* and *HvCBF10B* appear to be absent from Morex, while *HvCBF4A*/*B* appear to be absent from 88Ab536, and *HvCBF4D* is absent from Dicktoo, Morex, and Strider. While the differences were minimal relative to the variation observed, the HvCBF3-subgroup members displayed the most polymorphisms, HvCBF1-subgroup members were the

most conserved, and HvCBF4-subgroup members were intermediate.

Identification of barley *ICE* gene

BLAST searches were conducted against the rice genome and barley EST collection to identify monocot homologs to *AtICE1*. A rice gene designated *ICE1*-like (AP004332), termed *OsICE1*-like hereafter, is the closest rice homolog. BLAST searches with both AtICE1 and OsICE1-like identified the same 5′-truncated Morex EST clone (BE602161); subsequent analysis identified a second truncated Morex EST clone (BI947346) from the same gene containing additional 5′ sequence. We obtained and sequenced both ESTs (Table S1), designating the gene *HvICE2*; a closely related but distinct gene has already been designated *HvICE1* (Tondelli et al. 2006). We amplified and cloned Morex and Dicktoo *HvICE2* alleles from gDNA (Table S1). *HvICE2* contains three introns and three SNPs were present between the alleles—one conservative SNP in the coding region and two in the 3′ UTR; the alleles are predicted to specify identical polypeptides (Fig. S1). The large amino terminal extension of OsICE1-like is unrelated to that of AtICE1 (Fig. S1).

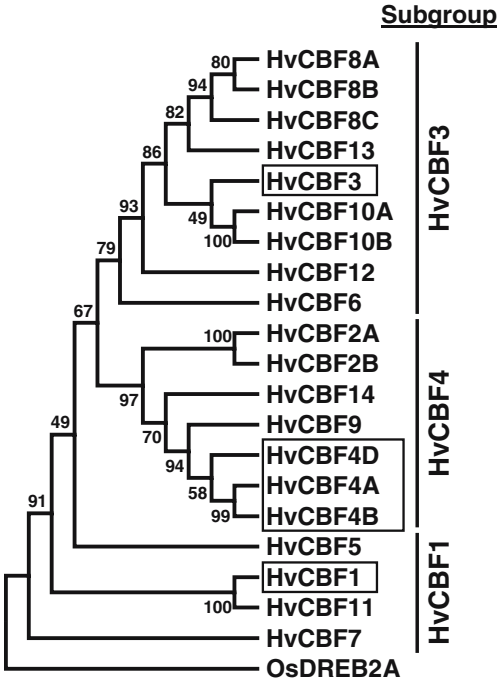

Fig. 1 Barley CBFs form three phylogenetic clades. A minimum evolution phylogenetic tree was derived from an alignment of the barley CBF polypeptides; an analagous tree topology was obtained utilizing the neighbor-joining function on the same alignment (not shown). For HvCBF8 members, theoretical polypeptide sequences were generated that adjusted the coding sequences to insure the best fit accounting for the pseudogene-based frame shifts (see Online Supplement). Dicktoo alleles were used for all polypeptides except HvCBF4D, for which the 88Ab536 allele was utilized (Table S1). OsDREB2A (AF300971), a closely related monocot non-CBF AP2 domain family member, was used as a CBF-related outlier. *Vertical bars* denote the respective HvCBF-subgroups and members, while each subgroup-defining member(s) is *boxed*

Identification of barley *ZAT12* homologs

BLAST searches were conducted against the barley EST collection (http://www.ncbi.nlm.nih.gov) and rice genome to identify monocot homologs to AtZAT12, which revealed two barley ESTs (BQ761311, BI777789) from the genotype Optic and the rice gene *ZFP16* (AY305865). The two barley ESTs represent distinct but closely related genes which we designate *HvZFP16-1* and *HvZFP16-2* (see Online Supplement) and refer to the rice gene as OsZFP16 (Table S1). We amplified and cloned alleles to both genes from Morex and Dicktoo (Table S1). Dicktoo and Morex *HvZFP16-2* alleles are identical while the *HvZFP16-1* alleles differ by five SNPs—three are in the coding region, leading to one amino acid substitution, and two are in the 3′ UTR (Fig. S2).

Freezing test and LT tolerance QTL analyses results

The Martonvásár phytotron LT tolerance phenotyping under controlled experimental freezing conditions support previous reports for the D×M population (Hayes et al. 1993, 1997; Pan et al. 1994). At −12.5°C, survival values for the Dicktoo and Morex parental genotypes were 85.3 and 0.0%, respectively, while the population average was 26.2% (Fig. S3). Using this new data and a LOD 2.5 threshold, novel LT tolerance QTLs are apparent on 1H (LOD 5.7), 4H-S (LOD 3.7), and 4H-L (LOD 3.0), in addition to the previously reported large-effect *Fr-H1* QTL on 5H (LOD 23.9) whose position is now better-resolved (Fig. 2, Table 2); the position of the 1H LT tolerance QTL appears to be syntenous with the *Ppd-H2* PPD QTL (Laurie et al. 1995).

Map locations of barley LT regulatory factor homologs

HvICE2 mapped to 3H, and while *HvZFP16-1* mapped to 1H, it was not coincident with the LT tolerance QTL (Figs. 2, 3). The Dicktoo and Morex *HvZFP16-2* alleles were not polymorphic, but could be assigned to 1H via the barley-wheat addition lines; its position relative to the QTL is unknown. Of the 20 *HvCBF*s, 11 were mapped in the D×M population, 3 in the STAB population, and 3 assigned to chromosome arms via the barley-wheat addition lines (Table. 1, S3). The barley *CBF* genes are located on four chromosomes, with a majority located in two tandem clusters on the long arm of chromosome 5H (Table 1, Figs. 2, 3). Five *HvCBF* genes were mapped or localized to chromosomes other than 5H. The *HvCBF8C* pseudogene (see Online Supplement) was mapped to chromosome 2H-S, *HvCBF5* to

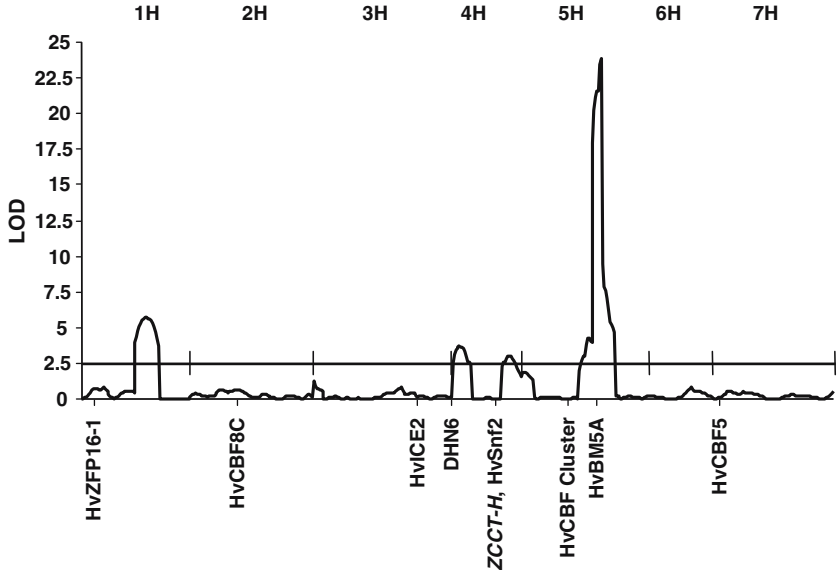

Fig. 2 Four barley chromosome regions influence LT tolerance capacity at −12.5°C in the Dicktoo × Morex population. The LT tolerance QTL-controlled freeze profile at −12.5°C for the D×M population was plotted relative to chromosomes and a LOD 2.5 cutoff score used. The LOD 2.5 value is indicated by the *horizontal line*; *hatch marks* denote segments corresponding to the above indicated chromosome. The positions of key genes are indicated relative to the QTL profile and the large cluster of *HvCBF* genes on 5H (see Fig. 3) is indicated as "HvCBF Cluster". *HvBM5A*, the *VRN-H1* candidate gene, and *HvSnf2* positions are from von Zitzewitz et al. (2005), while the *HvSnf2*-linked *VRN-H2* candidate *ZCCT-H* gene loci (*italics*) is from Karsai et al. (2005)

7H-S and *HvCBF1* was mapped to 6H-L. Due to a lack of allelic variation at *HvCBF7*, *HvCBF8A*, *HvCBF8B*, and *HvCBF11* in the four genotypes, we used the barley-wheat addition lines to assign linkage map positions. We were able to assign chromosome arm locations for *HvCBF7* (6H-L) and *HvCBF11* (2H-L) (Table 1); wheat cross-amplification currently precludes *HvCBF8A* and *HvCBF8B* assignment.

Using both the D×M and STAB populations, 11 *HvCBF*s (*2A*, *3*, *4A*, *4B*, *6*, *9*, *10A*, *10B*, *12*, *13*, and *14*) map to a localized region on the long arm of chromosome 5H (5H-L) (Figs. 3, 4), the region where major effect LT tolerance QTL, are reported in the *Triticeae* (Hayes et al. 1997; Vágújfalvi et al. 2003; Francia et al. 2004). However, these genes are approximately 30 cM distal to the D×M LT tolerance *Fr-H1* QTL peak (Figs. 2, 4). Comparative mapping using common markers and BIN assignments established these *CBF* genes are coincident with the 5H-L LT tolerance *Fr-H2* QTL reported in the barley N×T population (Fig. 4) (see Discussion). *HvCBF*s *3*, *4A*, *4B*, *6*, *9*, *10A*, *12*, *13*, and *14* were mapped in D×M (Table 1). *HvCBF3* and *HvCBF6* were also mapped in the STAB population to a corresponding linkage map position (Table 1; not shown). The map positions of *HvCBF2A*, *HvCBF10B*, and *HvCBF13* were inferred via linkage to physically mapped 5H-L *HvCBF* genes present on Dicktoo gDNA phage clones (Table 1). *HvCBF2A* and *HvCBF4B* reside on the same bacteriophage λ clone, as do *HvCBF10A* and *HvCBF10B*, and *HvCBF3* and *HvCBF13* (Stockinger et al. 2005). *HvCBF2B* was assigned to 5H-L using the barley–wheat addition lines.

The *HvCBF* genes mapping to 5H-L are all members of the HvCBF3- and HvCBF4-subgroups (Fig. 1, Table 1). *HvCBF*s *2A*, *4A*, *4B*, and *9* form one linked cluster, while *HvCBF*s *3*, *6*, *10A*, *10B*, *12*, *13*, and *14* form a second linked cluster one cM distant. In addition, *HvCBF3*, *HvCBF10A*, and *HvCBF6* are present on the single BAC clone 804E19 (Table S4), which is consistent with the mapping data indicating that the cosegregating map positions accurately reflect the physical arrangement of *HvCBF* genes at this multigene locus.

Discussion

Our strategy for using the large *H. vulgare* EST collection to identify barley homologs to key regulatory genes controlling Arabidopsis LT tolerance regulons was successful. We identified 20 barley *CBF*s, 1 barley

Table 2 D×M LT tolerance QTL peak summary

Chromosome	BIN	Position (cM)	LOD score	Closest marker	Additive effect	R^2
1H-L	11	82	5.7	saflp164	7.6	0.08
4H-S	5	8	3.7	saflp176	−5.4	0.04
4H-L	12	68	3.0	Bmy1	5.1	0.04
5H-L	11	98	23.9	HvBM5A	20.8	0.62

homolog to *AtICE1*, and 2 barley homologs to *AtZAT12*. Per the *HvCBF* genes, this multigene family is clearly larger in barley, a monocot, relative to two dicots where the genome has been sequenced: Arabidopsis and poplar each contain only six *CBF* genes (Benedict et al. 2005). While we did not fully explore the *ICE* and *ZAT* gene families in barley, the identified genes represented the closest homologs present in the current EST collection. Given the large size of the gene families in Arabidopsis (Reichmann and Ratcliffe 2000) to which *ICE* and *ZAT12* belong, it is likely that there are more homologs in barley functioning in a similar capacity.

Our aim to map barley homologs of key Arabidposis LT tolerance regulatory factors and identify candidates for Triticeae LT tolerance QTL was also successful, although some key questions remain. A first step in aligning candidate genes relative to QTL was to re-score the LT tolerance phenotype in the D×M population using controlled freeze tests. This experiment corroborated previous reports, in which the chromosome 5H *Fr-H1* QTL has a very large effect, explaining 62% of the phenotypic variation, and identified three additional smaller effect QTL. Additionally, it established the D×M population does not segregate for the nearby *Fr-H2* effect observed in the N×T population (Francia et al. 2004). At the chromosome 4H-S QTL, the LT tolerance susceptible parent contributed the favorable allele; in many QTL mapping populations both parents are found to contribute favorable alleles, a hypothesized basis for transgressive segregation (Hayes et al. 2003). Of the four QTL identified in D×M, none are coincident with the linkage map positions of the currently known barley homologs to key genes in the Arabidopsis cold acclimation pathway (Fig. 2). As none of the candidate genes mapped colocalize to the D×M LT tolerance QTL positions and we mapped homologs to multiple locations in the barley genome, three scenarios are possible: (1) the homologs map to LT tolerance QTL present in germplasm other than D×M, (2) the homologs map to positions where there are no LT tolerance QTL effects, and (3) the LT tolerance QTL are at positions where candidates have not yet been identified.

Relative to the first scenario, we mapped genes in the D×M population to complement our Dicktoo allele isolation efforts (Skinner et al. 2005) and place the genes in a population that had numerous common markers to aid in the comparative mapping analysis with other Triticeae populations. The most significant finding of this research is the presence of 10 of the 17 mapped *HvCBF* genes coincident with the position of a LT tolerance QTL we reported in another barley population (Francia et al. 2004) and which is coincident with a QTL in diploid wheat (Vágújfalvi et al. 2003) (Fig. 4). We had earlier reported one *HvCBF4* form at this position

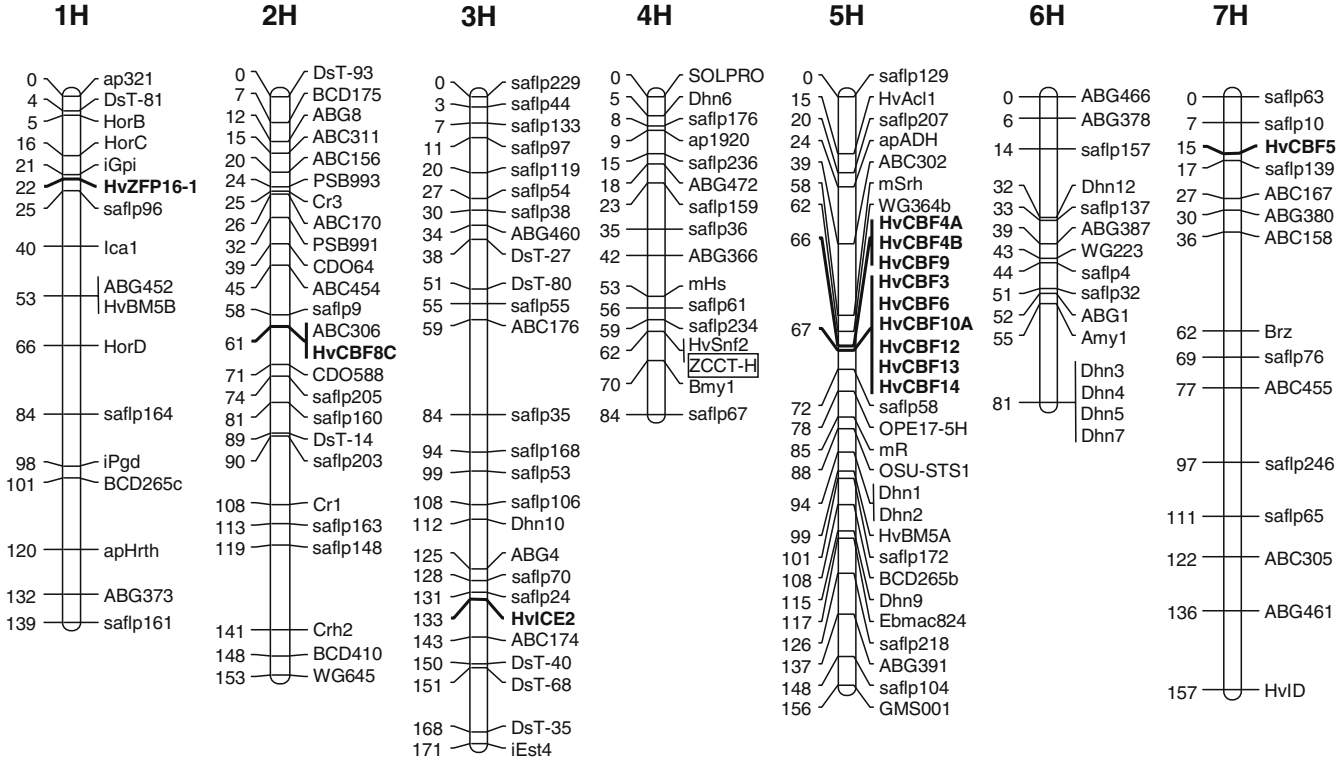

Fig. 3 Chromosome linkage maps of the Dicktoo × Morex barley mapping population. Chromosomes are indicated and presented with the short arm at the *top*. LT tolerance regulatory genes assigned in this study are in *bold face*. All markers were assigned in the D×M mapping population except for *ZCCT-H* (*boxed*). *ZCCT-H*, the *VRN-H2* candidate gene loci, is absent from both the Dicktoo and Morex parents, but is inseparable from the linked *HvSnf2* marker in both the Dicktoo × Kompolti (Karsai et al. 2005) and Nure × Tremois (von Zitzewitz et al. 2005) populations

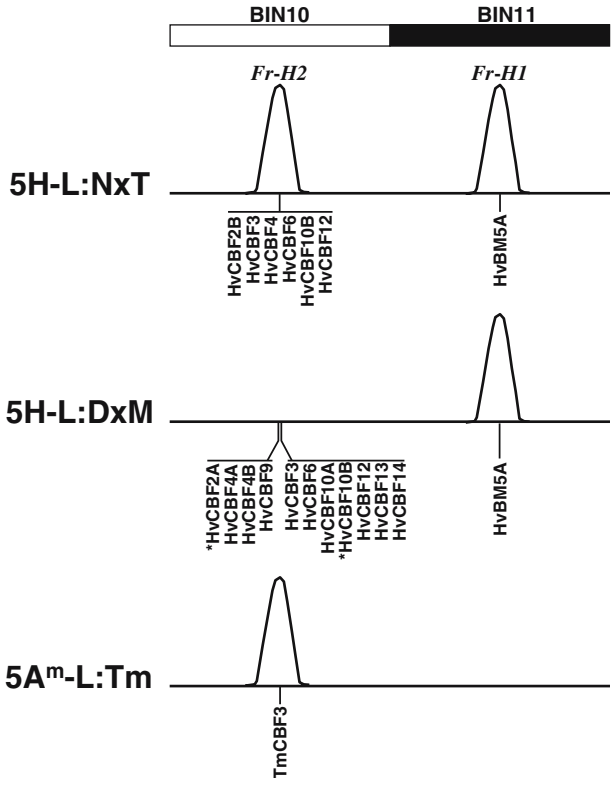

Fig. 4 Comparative positional relationships and BIN map assignments of candidate genes for the *Fr-H1* and *Fr-H2* LT tolerance QTLs in the Triticeae. Candidate regulatory genes falling into BINs 10 and 11 using the BIN map concept of Kleinhofs and Graner (2001) are indicated relative to the *Fr-H1* and *Fr-H2* QTLs of the Nure × Tremois (N×T) population (Francia et al. 2004). The syntenous BIN 10 or 11 LT tolerance QTLs from the Dicktoo × Morex (D×M) (this study) and the winter × spring *T. monococcum* (*Tm*) population of Vágújfalvi et al. (2003) are indicated. All D×M genes were assigned by linkage mapping (Fig. 3) except *HvCBF2A* and *HvCBF10B* (noted by an *asterisk*), which are assigned based on physical linkage via gDNA clones to *HvCBF4B* and *HvCBF10A*, respectively. Mapped orthologous candidate genes from the N×T population (Tondelli et al. 2006) and the Tm population (Vágújfalvi et al. 2003) are shown

(*Fr-H2*, BIN10) (Francia et al. 2004), confirming the syntenous D×M *HvCBF4* loci and co-clustered genes mapped in this work lie directly under the *Fr-H2* QTL. In a recent collaborative effort, we established five additional *CBF* genes, including *HvCBF3*, are also present at this locus underlying the *Fr-H2* QTL (Fig. 4) (Tondelli et al. 2006). The barley *HvCBF3* gene was used as a probe to map the wheat homolog to the homoeologous position in *T. monococcum* under the syntenous wheat *Fr-2* QTL position (Vágújfalvi et al. 2003), and independently confirmed at this position in the D×M population (Choi et al. 2002). The co-mapping of *HvCBF3* and its homoeolog to the same location in the three populations confirms the syntenous location of this second cluster relative to the reported QTL at this position. Therefore, while genotypic variation leading to an *Fr-H2* QTL effect is not observed in the "facultative" × "spring" D×M population (Fig. 2), the two tandem *HvCBF* gene clusters map to this syntenous locus. Whether the *Fr-H2* QTL is the result of a single *HvCBF* gene, the combined effect of a subset (or all) of the *HvCBF* genes, or independent of the *HvCBF* genes remains to be determined. Another likely functional role for the CBFs is the finding that QTLs for the accumulation of two *COR* gene products are coincident in the N×T population with these *HvCBF* clusters (Francia et al. 2004). One of these is *COR14b*, which contains a single CRT motif controlling the LT-responsiveness of the gene (Dal Bosco et al. 2003). CBFs bind CRT elements and we have found that the *COR14b* CRT motif is specifically bound by a number of the HvCBF products at this locus (Skinner et al. 2005), implicating a direct interaction between the HvCBFs and *COR14b* gene regulation. Allele cloning from one winter, two facultative, and one spring genotype demonstrated that each contains alleles encoding for essentially identical polypeptides (see Online Supplement). This suggests that if *CBF* alleles are differentially affecting LT tolerance, it is more likely due to differences in expression than the encoded polypeptide form. The possibility of a critical amino acid substitution cannot be ruled out, and gene deletion may be of importance. However, in the three likely cases of gene deletion, the missing gene is a member of a multigene family and the genotype contains a closely related subfamily member that could function in a compensatory fashion.

Through both gDNA phage and BAC clones containing multiple *HvCBF* genes, we confirmed for a subset of the *HvCBF*s that cosegregation coincident with the *Fr-H2* LT tolerance QTL is representative of the genomic region's physical structure. A single recombination event has occurred within this cluster in the D×M population, dividing it into two (Fig. 3); additional mapping in the full D×M mapping population ($n = 236$) has not revealed any additional recombination events (not shown). QTL resolution is insufficient to identify one of the two groups as the most likely set of determinants of the LT tolerance phenotype. The gene sets of the two clusters are all members of the HvCBF3- and HvCBF4-subgroups, and with one exception, fall into distinct linkage clusters. The lone exception is *HvCBF14*, an HvCBF4-subgroup member (Fig. 1) that clusters with the HvCBF3-subgroup members. To date, all the cloned physically tandem *HvCBF* gene sets belong to the same subgroup. While purely speculative, if the subgroup members are all tandem (i.e., all HvCBF4-subgroup then all HvCBF3-subgroup genes), this would imply the D×M recombination breakpoint has occurred between *HvCBF14* and another of the HvCBF4-subgroup members (*2A*, *4A*, *4B*, or *9*). *HvCBF2B*, assigned to 5H-L using addition lines (Table 1), has been confirmed to lie at this locus in the N×T population (Fig. 4) (Tondelli et al. 2006). This physical clustering of the HvCBF3- and HvCBF4-subgroups is not unique to barley. In rice, a single scaffold clone contains three *CBF* genes (*OsDREB1*s) in the order *OsDREB1B-OsDREB1H-OsDREB1A*; *OsDREB1B* is an HvCBF4

subgroup member while *OsDREB1A* and *OsDREB1H* are HvCBF3-subgroup members (Skinner et al. 2005). The physical clustering of these groups in barley and rice suggests that this arrangement was set early in cereal evolution and the large number of *HvCBF* genes occurring at this 5H locus may be due to localized duplication and divergence events.

In terms of scenario two, we found numerous examples of barley homologs to Arabidopsis LT tolerance regulators mapping to positions where there are no reported Triticeae LT tolerance QTL. These include five *HvCBFs—HvCBF1*, *HvCBF5*, *HvCBF7*, *HvCBF8C*, and *HvCBF11*—and two additional regulatory genes: *HvICE2* and *HvZFP16-1*. Similarly, *HvICE1* maps to a region of 7H where no LT tolerance QTLs are present (Tondelli et al. 2006). Possible explanations for these finding include the limited sample of Triticeae germplasm in which LT tolerance QTL have been mapped and alternative functions for the regulatory genes. For example, *CBF* genes also confer tolerance to drought and salinity stresses. QTLs for drought and/or salt tolerance lie on these non-5H chromosomes (summarized in Cattivelli et al. 2002) and marginally significant QTLs accounting for a low percentage (< 10%) of LT tolerance have been reported on barley chromosomes 2H and 6H (Tuberosa et al. 1997); the positional relationship of the non-5H CBFs to these various abiotic stress QTL effects is currently under investigation. The *AtICE* and *AtZAT* genes belong to large gene families displaying multiple regulatory functions, one of which is LT tolerance, and EST analyses show that barley also contains many distinct expressed genes belonging to these families. Therefore, further characterization and mapping of additional members from these gene families will be necessary to establish which members are candidates for LT tolerance contributions in the Triticeae. Likewise the *Fr-H2* QTL effect absent in the D×M population (vs. presence in the N×T) emphasizes the need to characterize multiple germplasm crosses before concluding a region is not a source of phenotypic variation.

Finally there is the interesting third scenario: LT tolerance QTL with no current candidate. The smaller-effect LT tolerance QTLs on 1H and 4H need to be explored further and validated. *HvZFP16-2* was assigned to 1H via the addition lines, but its positional relationship to, and thus candidacy for, the 1H LT tolerance QTL is currently unknown. In terms of the large-effect D×M chromosome 5H LT tolerance QTL in BIN 11 (i.e., *Fr-H1*), it is possible that an as yet uncharacterized regulatory gene (e.g., a novel *HvICE* and/or *HvZFP* gene family member) will map to *Fr-H1*. We currently consider it more likely, however, that this QTL is a pleiotropic effect of the *HvBM5A* gene (Fig. 4), the candidate for the *VRN-H1* gene and QTL (von Zitzewitz et al. 2005). The maintenance of a vegetative state during the periods of lowest ambient and soil temperatures is associated with maximum LT tolerance, and concomitant with the transition to a reproductive fate via the VRN or PPD response pathway in cereals, there is a loss of LT tolerance capacity (Limin and Fowler 2002; Danyluk et al. 2003). Therefore, it is plausible that *Fr-H1* is reflecting the genotypic combinations in the mapping population for the retention of the vegetative growth state (higher LT tolerance capacity) versus how rapidly the floral fate transition (lower LT tolerance capacity) occurs.

In summary, our use of the extensive EST collection to identify barley homologs to key Arabidopsis LT regulatory genes was effective. The combination of EST database screens, allele cloning, and the barley-wheat addition lines yielded a high success rate (87%, or 20 of 23 genes) of gene placement on the barley linkage map. We established that a large cluster of *HvCBF* genes is not a candidate for the *Fr-H1* LT tolerance QTL effect in Dicktoo × Morex. However, by comparative analysis we found that the position of the *CBF* cluster (BIN10, 5H) is coincident with the reported position of *Fr-H2* in the Nure × Tremois barley mapping population and in a diploid wheat mapping population. These results suggest that in the *Triticeae*—as in Arabidopsis —members of the *CBF* gene family may function as determinants of winterhardiness in some germplasm. The functions of the other *CBF* and regulatory genes, relative to LT tolerance, remain to be determined. Our progress in establishing and understanding the components of winterhardiness in barley, based on the findings in Arabidopsis, simultaneously underscores the importance of model systems, as well as the need to extend comparative analyses to economically important crop species where the information can potentially be used to develop superior varieties.

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