Molecular and genetic analyses of freezing tolerance in the Triticeae cereals

DISSERTATION

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By

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Abstract

The Triticeae cereals – wheat (*Triticum* spp.), barley (*Hordeum vulgare*), and rye (*Secale cereale*) are, in general, tolerant to freezing temperatures. However, differences in their ability to survive freezing are significant enough to affect differences in their growing season and area of cultivation. Freezing tolerance is genetically regulated by two loci – *FROST RESISTANCE-1 (FR-1)* and *FR-2*. Genes underlying these loci have been known or speculated – *FR-1* is thought to be tightly linked to or the same as the flowering regulator, *Vernalization-1 (VRN-1)*, while *FR-2* is coincident with a cluster of about 13 *C-repeat Binding Factor (CBF)* genes that regulate low-temperature tolerance. However, the molecular mechanisms underlying the effects of these loci on freezing tolerance have been relatively unclear. We present data that enhances our understanding of the freezing tolerance mechanisms in the Triticeae.

The connection between *FR-1* and *VRN-1* is pleiotropy, not linkage. This conclusion derives from freezing experiments conducted using wheat *VRN-1* deletion mutants. Plants homozygous for *VRN-1* deletion are significantly more freezing-tolerant than plants carrying one or two copies of *VRN-1*. Expression analyses indicate that the lower freezing
tolerance of plants carrying VRN-1 is due to its repressive effects on the CBF genes at FR-2.

The effect of FR-2 is likely due to quantitative differences in CBF gene copy numbers. Sequencing FR-H2 in barley indicates that the more freezing-tolerant winter genotypes harbor greater copy numbers of the CBF genes compared to the relatively less freezing-tolerant spring genotypes. Because wild barleys are of winter growth habit, fewer CBF copy numbers in spring barleys appear to be the domesticated state. Using pedigree analysis of a spring barley, we show that the some of the spring CBF alleles arose about 100 years ago, at a time that coincides with the beginning of modern plant breeding.

We show that copy numbers of a 22 kb fragment encompassing CBF2A and CBF4B, which were previously found to be higher in winter barleys than in spring genotypes, also differ across winter barleys. Copy numbers of this fragment are associated with higher transcript levels of the encompassed CBFs, and of linked genes – CBF12 and CBF16. Chromatin immunoprecipitation (ChIP) experiments reveal that this association may be due to the ability of CBF2 to bind to the promoters of CBF12 and CBF16.

CBF copy number variations also occur in wheat. Winter genotypes of red wheats harbor more copies of CBF14 than spring genotypes. Additionally, CBF14 copy numbers vary across the red winter wheats such that genotypes grown in the more severe winters of the Great Plains harbor more CBF14 copies than those grown in the relatively less severe
winters of the Eastern US. Our data reveal that these CNVs of *CBF14* pre-existed in the diploid ancestors of tetraploid and hexaploid wheats.

Overall, we show that the likely molecular basis of *FR-1* is *VRN-1* and of *FR-2* is *CBF* copy number.
Dedication

Dedicated to my parents
Acknowledgments

I couldn’t have come this far if it was not for all the people around me. I have been very fortunate to be guided by knowledgeable faculty including my advisor, Dr. Eric J. Stockinger, and my committee members Drs. Esther van der Knaap, David M. Mackey, and Guo-Liang Wang; and additionally, Dr. David F. Francis. Designing the experiments and getting through the tough ones would not have been possible without their expert advice, and constant encouragement and support every step of the way. In addition to the great work environment, I’ve also cherished the all the Thanksgiving and Christmas Eve dinners, and bonfire potlucks. Thank you, Eric, Esther, and David for all the invitations and for creating a wonderful, warm, and friendly environment here at OARDC, Wooster.

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I thank all my friends who I’ve shared office with. Special thanks to my friends – Sukhbir, Audrey, Kristen, and Fulya for all the fun times over the long run.

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Figure 5.2. \( CBF2 \) and \( CBF4 \) cross-hybridizing patterns and relative signal intensities of (A and C) \( CBF2 \) 3’ probe and (B) \( CBF4B \) 5’ probe with ‘Dicktoo’, ‘Nure’, and ‘Admire’ DNAs. Copy number estimates of \( CBF2A \) and \( CBF4B \) are indicated below individual lanes, are based on hybridization signal intensity ratios of \( CBF2A/CBF2B \) (A) and \( CBF4B/CBF2 \) (B and C). The MW of the cross-hybridizing bands indicated are based on nucleotide sequence of ‘Dicktoo’ and ‘Nure’. The fragment of unknown MW intermediate between \( CBF2A \) and \( CBF2B \) is not yet accounted for at the structural level (A). Note: ‘Dicktoo’ DNAs are overloaded relative to ‘Nure’ and ‘Admire’. \( BgII \) digest does not discriminate between \( CBF2A \) and \( CBF2B \) as the two comigrate (C).
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Figure 5.4. Estimation of CBF2A and CBF4B copy numbers in an unstructured barley population using DNA blot hybridization. BamHI- or BglII-digested DNAs were hybridized to CBF2 3´ probe (A and C) or CBF4B 5´ probe (B). CBF2 3´ cross-hybridizing BglII fragments producing weak signal intensity are indicated by arrowheads in MO B699 and by arrows in MO B1252 and MO B1385. Relative copy numbers of CBF2A and CBF4B as estimated from CBF2A/CBF2B ratios (A) and CBF4B/CBF2 ratios (C), respectively, are provided below the individual DNA lanes (n.a. = not applicable). AD = ‘Admire’; MEB = ‘Missouri Early Beardless’. AD-1 and AD-2 were obtained from two different sources (Table E.1). The pedigrees of all genotypes are provided in Table E.1.

Figure 5.5. Relative CBF transcript levels in Groups I and II of MO B lines, ‘Missouri Early Beardless’ (‘MEB’), and ‘Admire’. Three-week-old plants entrained under short...
days and 18°C temperature. *CBF* transcript levels were examined in crown tissues harvested at 6 h into the subjective day. Log-transformed values of *CBF* expression relative to *actin* are plotted. Copy numbers of the *CBF2A–CBF4B* genomic region in groups I and II of MO B lines, and in ‘MEB’ and ‘Admire’ are above the X-axis. Error bars represent S.E.M. of three biological replicates of seedlings grown in the same experiment. *P*-values outlined above were calculated using ANOVA and indicate the significance of association between *CBF2A–CBF4B* copy numbers with expression levels of the *CBFs*. 

Figure 6.1. *CBF14* hybridization patterns in wheat lines nullisomic/tetrasomic for group 5 chromosome homoeologs. *Sac* I (A) and *Bgl* II (B) digested DNAs were hybridized with a probe derived from the *T. monococcum* *CBF14* promoter. The cross-hybridizing homoeologs (A, B, and D) are labeled. Absence of a cross-hybridizing fragment in a lane identifies the homoeolog for which the lines are nullisomic. *Sac* I restricts the B *CBF14* homoeolog into two cross-hybridizing bands; the lower MW of the two is indicated using an arrowhead. *Bgl* II restricts the A *CBF14* homoeolog into two cross-hybridizing bands. Insufficient quantity of DNA precluded the N5D/T5A line from the *Bgl* II digest.

Figure 6.2. *CBF14* copy number estimates in hexaploid wheats (*T. aestivum* subsp. *aestivum*). Genotypes are group into five market classes – white spring (WS), white winter (WW), hard red spring (HRS), soft red winter (SRW), and hard red winter (HRW). A) Total *CBF14* copy numbers relative to *Pinb*; B) Within-class comparisons of *CBF14/Pinb* ratios for each genome; C) Between-class comparisons of *CBF14/Pinb* ratios for each genome; D) Between-class comparisons of *CBF14* copy numbers in the A
genome relative to those in the D genome (CBF14-A/CBF14-D). WS, WW, HRS, SRW, and HRW classes include 9, 5, 7, 5, and 8 genotypes, respectively (identified in Fig. F.2 and Table F.2). Error bars represent standard error of mean (S.E.M.). Statistically significant differences are indicated by different letters above the error bars ($P < 0.05$).

Figure 6.3. Average CBF14/Pinb ratios in diploid wild (T. urartu, Ae. speltoides, and Ae. taushii) and cultivated (T. monococcum) wheats. Number of genotypes within each species (n) is listed on the X-axis. Error bars represent S.E.M. Statistically significant differences are indicated by different letters above the error bars ($P < 0.05$).

Figure 6.4. Stable inheritance of CBF14 in wheat chromosome 5 substitution lines. A) Substitution lines K-R5A and W-R5A carrying 5A homoeolog of ‘Rescue’ in ‘Kharkov MC22’ and ‘Winalta’ backgrounds, respectively, B) CS-CNN substitution lines carrying chromosome 5 homoeologs of ‘Cheyenne’ in ‘Chinese Spring’ background, and (C) WI-CNN substitution lines of chromosome 5 homoeologs of ‘Cheyenne’ in ‘Wichita’ background, and the reciprocal CNN-WI substitution lines. Sources of ‘Chinese Spring’, ‘Cheyenne’, and ‘Wichita’ accessions are listed in Table F.1. Note: Signal intensity differences between ‘Wichita’ accessions, WI-KS-1, WI-KS-2, and WI-NE-1 are attributed to differences in the quantity of DNA loaded on the gel. CS = Chinese Spring; CNN = Cheyenne; WI = Wichita. Digests and DNA blot hybridizations were carried out similarly as for Fig. 6.1A.

Figure A.1. RNA blot analyses of VRN-H1 transcripts in the facultative barley ‘Dicktoo’ (a) and the spring barley ‘Morex’ (b). Plants were grown at 18°C under short days (SD; 8
h light/16 h dark) and long days (LD; 16 h light/8 h dark). No samples were harvested in the first week of growth. Plants transferred from LD to SD (LD → SD) were grown under LD for 5 days. From week 2 onwards, crown tissues samples were harvested at 6 h under SD and 14 under LD on day 5 each week at 18°C. On day 6, temperature was decreased to 10°C at daybreak and samples were harvested at the same time points as under 18°C. Each sample comprised of five crowns. About 7 μg total RNA of each sample was used for RNA blot hybridizations to the coding sequence probes of VRN-H1 and actin. 

Figure A.2. RNA blot analyses of VRN-H1 transcripts in ‘Dicktoo’, ‘Morex’, and 15 D x M doubled haploid lines harboring the ‘Dicktoo’ VRN-H1 allele. Crown tissues of three-week-old plants grown under short days (a) and long days (b) at 18°C were used for VRN-H1 expression analysis. Samples were harvested on day 21 at 6 h under SD and 14 h under LD. RNA blot hybridizations were conducted the same as described in Fig. 1.1. Allelic status of PPD-H1, PPD-H2, and VRN-H3 of the genotypes are presented in (c).

Figure A.3. RNA blot analyses of VRN-H1 transcripts in ‘Dicktoo’, ‘Morex’, and 37 D x M doubled haploid lines harboring the ‘Dicktoo’ VRN-H1 allele. Transcript and genotyping data were collected in the same way as for Fig. 1.2.

Figure B.1. Dominant VRN-1 molecular marker used to classify segregating plants into homozygous mutants (mvp/mvp) and heterozygous plus homozygous wild type (Mvp/-) classes. The borders of the deletion are currently unknown.

Figure B.2. Apices of Mvp-2/- and mvp-2/mvp-2 plants before and after cold acclimation in the freezing tolerance test described in Fig. B.3 (-12°C). Apices were photographed
after 35 days at 20°C and again after additional 18 days of cold hardening at 4°C before
the freezing experiment. The apices from the mvp-2/mvp-2 mutant plants were at the
vegetative stage whereas those from Mvp-2/- plants were at the double ridge stage (dr).

Figure B.3. A) Average freezing scores using a scale from 0= dead plants to 5=
undamaged plants. The -8°C experiment included 22 mvp-1/mvp-1 homozygous mutants
(black bars) and 35 Mvp-1/- plants (gray bars) carrying at least one functional VRN-1
copy. The -12°C experiment used 24 mvp-2/mvp-2 homozygous mutants and 65 Mvp-2/-
plants. Plants were 35 days old before the cold acclimation treatment. P values
correspond to ANOVAS comparing mvp/mvp (black bars) and Mvp/- (gray bars) plants.

B) Average relative conductivity (ion leakage) of leaf segments from mvp-2/mvp-2 and
Mvp-2/- plants. Error bars represent standard errors of the means (SE) based on 9
replications per genotype / temperature combination.

Figure B.4. Quantitative RT-PCR analysis of transcript levels of the CBF genes present at
the FR-2 locus. Values are expressed relative to the TEF1 endogenous control (COR14b
transcript levels are included as reference). Leaf samples were collected from 8-weeks
old plants 8 h after transferring plants from 20°C to 4°C. Values in the Y axes were
normalized and calibrated using the \(2^{-\Delta\Delta CT}\) method (Livak and Schmittgen 2001). The
same calibrator was used for all genes to ensure scales are comparable across genes.
Homozygous mvp-1/ mvp-1 plants are indicated in black and those of Mvp-1/- plants
carrying 1 or 2 functional VRN-1 copies in gray. Values are averages of 10 biological
replications ± SE of the means. The inset shows the CBF genes with relatively lower
transcript levels using a different scale. \( P \) values were calculated using ANOVA of log(n+1)transformed values *: \( P<0.05 \) and **: \( P<0.01 \)........................................262

Figure D.1. \( CBF2, \ COR14B, \ DHN5, \) and \( DHN8 \) transcript levels in the early transformant generations as compared to non-transformed ‘Golden Promise’ (GP) and ‘Dicktoo’ (Dt). Lines 2, 6, and 10 were \( T_2 \), and line 13 was \( T_3 \) generation, respectively. Transcript levels were measured using qRT-PCR. Barley plants were grown under short day photoperiod (8 h light/16 h dark) and harvested after 0, 8, and 96 hours at 3°C ± 1°C. A minimum of four independent plants per transgenic line were used for qRT-PCR. Error bars represent standard error. Note different scales are used for each gene. ........................................270

Figure E.1. Time course analyses of \( CBF2 \) in ‘Golden Promise’ (GP) and ‘Admire’.

Plants were grown under short day (SD; 8 h light/16 h dark) for three weeks at 18°C. Warm samples were collected on day 21 at 18°C (A) and on day 22 following a temperature decrease to 6°C (B) at daybreak. Harvesting was carried out at two-hour intervals starting at daybreak until the 10 h time point in the subjective day. Each sample comprised of crown tissues of five seedlings. Seven \( \mu \)g of total RNA was loaded per lane. RNA blot was hybridized with \( CBF2 \) and \( actin \) CDS probes........................................275

Figure E.2. DNA blot analyses of two-row parents of MO B lines alongside ‘MEB’.

\( BamHI \)- or \( BglII \)-digested DNAs were hybridized to \( CBF2 \) 3’ probe (A and C) or \( CBF4B \) 5’ probe (B). \( BglII \) digest did not discriminate between the two \( CBF2 \) paralogs. Relative copy numbers of \( CBF2A \) and \( CBF4B \) as estimated from \( CBF2A/CBF2B \) ratios (A) and \( CBF4B/CBF2 \) ratios (C), respectively, are provided below the individual DNA lanes.
MEB = ‘Missouri Early Beardless’. All MO B accessions are identified as ‘Tschermak’ selections (Table D.1).

Figure F.1. CBF14 and Pinb hybridization patterns in different market classes of hexaploid wheat (*T. aestivum* subsp. *aestivum*). *Sac*I-digested DNAs were hybridized with *CBF14* 5’ (A) and *Pinb* CDS (B) probes. The A, B, and D genomes of CBF14 are labeled. A second weakly cross-hybridizing fragment of the B genome is indicated with an arrowhead. Copy numbers of CBF14 in each homoeolog relative to Pinb are provided in Table F.2. Differences in signal intensity of Pinb cross-hybridizing bands across the lanes are attributed to differences in the quantity of DNA loaded on the gel despite the normalizations using PicoGreen. WS = White spring; WW = White winter; HRS = Hard red spring; SRW = Soft red winter; HRW = Hard red winter.

Figure F.2. CBF14 and Pinb hybridizations in 16 unclassified hexaploid wheats (*T. aestivum*). Digests, hybridizations, and labeling of the cross-hybridizing bands is the same as described in Fig. F.1. This collection of hexaploid wheats includes genotypes whose growth habit and market class are ambiguous, or are annotated as facultative by the NPGS (Table F.1). Also included are those that exhibit a different CBF14 banding pattern.

Figure F.3. CBF14 and Pinb hybridizations of diploid wheats. *Sac*I-digested DNAs were hybridized with *CBF14* 5’ (A) and *Pinb* CDS (B) probes.

Figure F.4. CBF14 hybridization patterns in tetraploid wheats (*T. turgidum* subsp. *durum*). *Sac*I-digested DNAs were hybridized with *CBF14* 5’ probe. A and B genomes of CBF14 are labeled based on their similarity in MW with hexaploid wheat accessions.
(Fig. F.1 and F.2). A second weakly cross-hybridizing fragment of the BB genome is indicated using an arrowhead.

Figure F.5. *CBF14* hybridizations to *Bgl*II-digested DNAs of wheat chromosome 5 substitution lines. CNN = ‘Cheyenne’, WI = ‘Wichita’, and CS = ‘Chinese Spring’. The CS-CNN substitution lines and their parents are presented as evidence to support that the four fragments of the A homoeolog in CNN, WI, and their substitution lines reflect the *Bgl*II restriction pattern and are not the result from a partial digest.
Chapter 1: Introduction

Triticeae is a tribe of the Pooideae sub-family of Poaceae or Gramineae, generally known as the grass family. Two of the most important agronomic crops of the Triticeae include wheat (*Triticum spp.*)) and barley (*Hordeum vulgare*). They are ranked second and fourth, respectively, in world consumption in 2011-2012 with maize (*Zea mays*) being the most highly consumed and rice (*Oryza sativa*) being the third most highly consumed grain crop (http://www.fas.usda.gov/grain/Current/). Wheat and barley are cool season annuals grown around the world mostly between the latitudes 30°–60°N and 27°–40°S (Curtis et al., 2002). Whereas wheat can survive as low as -22°C, the lowest temperature that can be survived by barley is comparatively warmer, about -17°C (Wilen et al., 1996, Fowler and Limin, 2004). In addition to the difference in freezing tolerance levels between species, differences also occur within species. For example, a wheat or barley cultivar that survives -6°C temperature will most likely not survive -14°C. This difference in the ability to tolerate freezing stress is controlled by several genetic factors that interact in a complex network. Much research has been done to understand the molecular genetic basis of freezing tolerance. In the last five years, we have taken additional steps forward in this direction.
Scientific Background

Freezing tolerance is not a constitutive trait, but an acquired one. To acquire freezing tolerance, a plant must be exposed to gradually decreasing temperatures, a process called cold acclimation (Thomashow, 1998). Cold acclimation induces molecular pathways that increase the plant’s freezing tolerance (Guy et al., 1985; Thomashow, 1999).

In the Triticeae cereals, freezing tolerance of winter genotypes is greater than that of spring genotypes. Winter genotypes are sown in the fall, overwinter, and flower in spring of the following year. By contrast, spring genotypes are sown in the spring and are harvested in late summer – early fall of the same year. This difference in growing seasons is primarily because winter genotypes require vernalization, a prolonged exposure to low temperatures (0-10°C) in order to transition from vegetative to reproductive growth. In comparison, spring genotypes are inherently reproductively competent and do not require vernalization to flower. Winter types also respond to changes in day length, whereas spring genotypes have no or reduced sensitivity to photoperiod. A third growth habit is facultative. Facultative genotypes are of intermediate freezing tolerance, do not require vernalization to flower, and can be photoperiod-sensitive or insensitive (Skinner et al., 2006; von Zitzewitz et al., 2005, 2011).

In sum, growth habits mainly differ in their level of freezing tolerance, response to vernalization, and sensitivity to photoperiod. These three factors together define the winter hardiness of a plant i.e. its ability to survive through the temperate climate winter
(Levitt, 1980; Sakai and Larcher, 1987; Skinner et al., 2006). The number of genes regulating each factor and the interactions between them make winter hardiness a very complex trait. The interactions of these loci amongst each other and with environmental factors are depicted in Fig. 1.1 and are explained in the sections below.

**Genetics underlying vernalization and photoperiod sensitivity**

Three *VRN* loci affecting vernalization requirement and two *PPD* loci affecting photoperiod sensitivity are identified in wheat and barley (Laurie et al., 1995; Yan et al., 2003, 2004, 2006). These loci and their interactions are described in the sections below. Table 1.1 lists their chromosomal location, underlying genes, encoded proteins, Arabidopsis orthologs, and molecular basis of allelic differences.

*VRN-1*

*VRN-1* is the key locus determining the vernalization requirement of a Triticeae cereal. Winter and spring genotypes harbor contrasting alleles of *VRN-1* (Fu et al., 2005; Yan et al., 2003; von Zitzewitz et al., 2005). The molecular basis of *VRN-1* allelic variation is not its coding sequence, but the first of its seven introns (Fu et al., 2005; Szűcs et al., 2007; von Zitzewitz et al., 2005; Yan et al., 2003). Intron 1 of *VRN-1* usually exists as approximately 10-11 kb sequence in winter genotypes of wheat and barley (Fu et al., 2005; von Zitzewitz et al., 2005). However, in spring genotypes *VRN-1* intron 1 harbors deletions spanning several kilobases. The result is a differential expression pattern of winter and spring *VRN-1* alleles (Yan et al., 2003; Danyluk et al., 2003; Trevaskis et al.,
Expression of the recessive winter VRN-1 allele is restricted until the completion of vernalization. By contrast, the dominant spring VRN-1 allele is expressed constitutively. Because VRN-1 expression is required for flowering (Shitsukawa et al., 2007), winter genotypes flower in the spring after their vernalization is completed over the winter, whereas spring genotypes flower as soon as VRN-1 transcripts reach a certain threshold (Loukoianov et al., 2005; Trevaskis et al., 2003).

Alignments of VRN-1 intron 1 sequence of winter and spring genotypes of wheat and barley have led to the identification of a 2.8 kb minimal sequence that is deleted in spring types but is present in winter types (von Zitzewitz et al., 2005). This 2.8 kb sequence is termed vernalization critical region, as its deletion results in the loss of vernalization requirement and a constitutively expressed spring allele. The 2.8 kb region is therefore thought to contain cis regulatory elements necessary for the repression of VRN-1 expression; however no such motifs have yet been discovered. A notable exception to the spring VRN-1 allele reported till date is of ‘Tremois’ (von Zitzewitz et al., 2005). ‘Tremois’ harbors the 2.8 kb critical region much like a winter barley. Moreover, the first 440 bp of this 2.8 kb sequence, which is highly conserved between winter wheat and barley genotypes is also invariant in ‘Tremois’ (von Zitzewitz et al., 2005). Yet, ‘Tremois’ is a spring cultivar (Francia et al., 2004). The one deletion present in its intron 1 spans 38 bp and lies just upstream of the critical region. Of these 38 bp, 12 bp are unique to spring barleys and lie within a MITE (miniature inverted terminal repeat
element) sequence that is present only in barley. It is possible that in ‘Tremois’, this 38 bp deletion is the cause of its spring growth habit, but this is not yet confirmed.

Mutations in VRN-1 promoter are also associated with spring growth habit in wheat and barley. In barley, at least two insertion-deletion (InDel) polymorphisms and a single nucleotide polymorphism (SNP) are diagnostic of growth habit, although no known regulatory motifs overlap these sequence variations (von Zitzewitz et al., 2005). In diploid spring wheats, VRN-1 promoter harbors insertions, point mutations within a CArG-box that is a binding site for MADS-box transcription factors (Riechmann et al., 1996), and deletions encompassing a CArG-box (Dubcovsky et al., 2006; Pidal et al., 2009; Yan et al., 2003). VRN-1 promoter of spring genotypes of tetraploid and hexaploid wheats harbors insertions of fold-back elements or deletions of sequence previously contained within direct repeats (Fu et al., 2005; Yan et al., 2004a). However, these deletions are only present in the A genome of polyploid wheats (VRN-A1), but not in the B and D genomes (VRN-B1 and VRN-D1) (Yan et al., 2004a). Although these mutations are described in the VRN-H1 promoter as spring Triticeae cereals, they do not occur in all spring genotypes. Thus, promoter mutations are less strongly associated with growth habit than intron 1 mutations.

Additionally, epigenetics plays a role in determining the vernalization response. In barley, vernalization results in a decrease in histone 3 lysine 27 trimethylation (H3K27me3) and an increase in histone 3 lysine 4 trimethylation (H3K4me3) (Oliver et al., 2009). These
changes in methylation patterns are associated with increase in \textit{VRN-H1} expression levels. The increase in H3K4me3 levels is particularly high at the exon 1 and 5’ of intron 1 post-vernalization. Spring genotypes also possess methylation of exon 1 and 5’ intron 1 associated with the repressed state (H3K27me3) in winter genotypes, however the level of this methylation is lower compared to winter genotypes, suggesting that the sequence within the critical region of intron 1 may be involved in determining the methylation levels (Oliver et al., 2009).

In addition to nucleotide sequence variation and epigenetics, another mechanism controlling expression of \textit{VRN-1} and vernalization response is copy number variations (CNVs). Winter genotypes of hexaploid wheats harboring greater copy numbers of \textit{VRN-A1} recessive allele (winter allele) require longer periods of vernalization in order to flower compared to winter genotypes harboring fewer \textit{VRN-A1} copies (Diaz et al., 2012).

Overall, a complex array of factors determines allelic variation of \textit{VRN-1} and flowering time. These factors along with additional genetic factors (described below) allow for a wide geographical distribution of wheat and barley genotypes and their adaptation to variable environmental conditions.

\textit{VRN-2 and VRN-3}

Two additional loci affecting growth habit or flowering time are \textit{VRN-2} and \textit{VRN-3} (Yan et al., 2004, 2006). \textit{VRN-2} represses flowering by (indirectly) repressing \textit{VRN-1}
transcription, whereas \textit{VRN}-3 promotes flowering by upregulating the transcription of \textit{VRN}-1 (Fig. 1.1) (Distelfeld et al., 2009; Hemming et al., 2008; Trevaskis et al., 2006; Yan et al., 2004, 2006). All three \textit{VRN} loci exhibit epistatic interactions, such that spring alleles at all loci are epistatic to their winter alleles (Dubcovsky et al., 2005; Szücs et al., 2007; Takahashi and Yasuda, 1971; Tranquilli and Dubcovsky, 2000). Like \textit{VRN}-1, \textit{VRN}-3 is dominant for spring growth habit and recessive for winter habit. In contrast, \textit{VRN}-2 is dominant for winter growth habit and recessive for spring habit. Only the allelic combination \textit{vrn}-1/\textit{Vrn}-2/\textit{vrn}-3 results in winter growth habit, whereas all other combinations result in spring or facultative growth habit.

Facultative genotypes typically harbor a winter allele at \textit{VRN}-1 and a spring allele at \textit{VRN}-2 (\textit{vrn}-1/\textit{vrn}-2). Recessive spring \textit{vrn}-2 allele represents loss-of-function mutations or physical deletion of the locus (Dubcovsky et al., 2005, 2006; Karsai et al., 2005; Yan et al., 2004). This means that in the absence of the \textit{VRN}-2 repressor, expression of \textit{VRN}-1 increases with plant development or with the change in day length without vernalization. Allelic status of \textit{VRN}-3 can affect flowering time in facultative genotypes (\textit{vrn}-1/\textit{vrn}-2). Because the dominant spring allele at \textit{VRN}-3 is expressed to higher levels than the recessive winter allele (Yan et al., 2003), facultative genotypes harboring the spring \textit{Vrn}-3 allele (\textit{vrn}-1/\textit{vrn}-2/\textit{Vrn}-3) flower earlier than those harboring the winter \textit{vrn}-3 allele (\textit{vrn}-1/\textit{vrn}-2/\textit{vrn}-3). However, allelic variation at \textit{VRN}-3 is mostly limited to germplasm growing at very high altitudes. \textit{VRN}-3 is, therefore, sometimes excluded from the
epistatic growth habit-determining $VRN$ model (Szücs et al., 2007; Takahashi and Yasuda, 1971; von Zitzewitz et al., 2005).

**PPD-1 and PPD-2**

$PPD-1$ affects flowering time under long days (Laurie et al., 1995). A dominant allele at $PPD-1$ ($Ppd-1$) promotes flowering under long days by upregulating the expression of $VRN-3$, which consequently upregulates $VRN-1$ expression (Fig. 1.1) (Turner et al., 2005). In barley, a recessive allele of $PPD-H1$, resulting from a loss-of-function mutation in its coding sequence has reduced or no sensitivity to photoperiod and results in delayed flowering under long days (Turner et al., 2005). Thus, facultative barley genotypes harboring the long day responsive $Ppd-H1$ allele ($vrn-1/vrn-2/Ppd-1$) flower earlier than those harboring the photoperiod-insensitive allele ($vrn-1/vrn-2/ppd-1$) (Hemming et al., 2008). Compared to barley, the photoperiod-insensitive allele in wheat results from a 2 kb deletion in $PPD-D1$ promoter and is associated with early flowering under short days and long days due to altered expression pattern of $VRN-3$ (Beales et al., 2007). Copy number variations of $PPD-1$ in the B genome ($PPD-B1$) of hexaploid wheat also underlie phenotypic variation in flowering time (Diaz et al., 2012). Greater copy numbers of the photoperiod-insensitive allele of $PPD-B1$ are associated with higher expression levels and early flowering (Diaz et al., 2012).

The photoperiod locus, $PPD-2$ affects flowering time under short days (Laurie et al., 1995). Under short days, a dominant allele of $PPD-2$ promotes flowering, whereas a
recessive allele, resulting from physical deletion of the gene, delays flowering (Karsai et al., 2008; Kikuchi et al., 2009).

Overall, \( VRN-3 \) is a key integrator of the vernalization and photoperiod responses (Fig. 1.1). Vernalization of winter genotypes initiates a positive feedback loop where upregulation of \( VRN-1 \) downregulates \( VRN-2 \) releasing \( VRN-3 \) from repression, which reinforces \( VRN-1 \) expression ultimately causing flowering (Distelfeld et al., 2009).

**Response of \( VRN-H1 \) to photoperiod**

\( VRN-H1 \) expression in ‘Dicktoo’, a facultative barley genotype, exhibits a striking response to photoperiod. \( VRN-H1 \) in ‘Dicktoo’ is strongly induced under long days (16 h light/8 h dark), but is repressed under short days (8 h light/16 h dark) (Fig. A.1a). This repression under short days continues for at least 8 weeks (after which the expression was not monitored) (Fig. A.1a). These data are consistent with Danyluk et al. (2003).

‘Dicktoo’ harbors the LD-responsive \( \textit{Ppd-H1} \) allele (Karsai et al., 2008), which possibly explains its high level of \( VRN-H1 \) expression under LDs. However the factor(s) that repress \( VRN-H1 \) expression under short days are unknown.

To test whether upregulation of \( VRN-H1 \) under LDs in ‘Dicktoo’ is associated with its \( Ppd-H1 \) allele, and to identify the factors affecting \( VRN-H1 \) expression under SDs, we utilized a doubled haploid mapping population developed from a cross between ‘Dicktoo’ and ‘Morex’ (Hayes et al., 1997). ‘Morex’ is a spring barley (\( Vrn-H1/vrn-H2/Vrn-H3 \)....)
that harbors a photoperiod-insensitive \textit{ppd-H1} allele (Karsai et al., 2008). Transcripts of \textit{VRN-H1} in ‘Morex’ start to accumulate in the first week and are abundant under both photoperiods (Fig. A.1b). The strategy was to grow the D x M individuals harboring the ‘Dicktoo’ allele at \textit{VRN-H1} (N = 37 out of 94; genotyped by Pat Hayes laboratory, OSU, Corvallis) under short days (8 h light/16 h dark) and long days (16 h light/ 8 h dark) for up to 9 weeks, and examine their \textit{VRN-H1} expression. Because \textit{PPD-H1, PPD-H2}, and \textit{VRN-H3} are known to affect \textit{VRN-H1} expression, we genotyped these loci in the 37 D x M lines using PCR-based markers (Table A.1).

Initially, due to limited availability of the D x M lines, only 15 of the 37 lines were monitored for \textit{VRN-H1} expression at 3, 6, and 9 weeks after sowing. The 3-week data are presented in Fig. A.2. In this experiment, all six D x M individuals expressing \textit{VRN-H1} under LDs harbored the ‘Dicktoo’ allele of \textit{PPD-H1}, i.e. LD-responsive \textit{Ppd-H1} allele. However, no correlation was observed between \textit{VRN-H1} expression and allelic states of \textit{PPD-H1, PPD-H2}, and \textit{VRN-H3} under SDs. Upon availability of all 37 D x M lines this experiment was repeated at week 3 only. These data are presented in Fig. A.3. Except for the differences in the relative expression levels of \textit{VRN-H1}, the results of this experiment were consistent with the first experiment, i.e. genotypes expressing \textit{VRN-H1} (to high levels) harbored a LD-sensitive allele of \textit{PPD-H1} (\textit{Ppd-H1}). However, again, under SDs none of the three loci were associated with \textit{VRN-H1} expression. This suggests that novel loci may be involved in the repression of \textit{VRN-H1} expression under SDs.
To identify these novel loci, we are currently in the process of developing mapping populations from crosses of ‘Dicktoo’ as the female parent to 18 facultative genotypes. The strategy is to grow the populations under SD, rogue out the plants that flower (i.e. phenotypically select the plants that do not express VRN-H1 under SDs), and genotype the selected plants using the Illumina GoldenGate genotyping technology. ‘Dicktoo’ and 12 of the 18 parents have been previously genotyped for approx. 3000 markers using the Illumina GoldenGate assay (Close et al., 2009). This marker set may allow us to map the SD-specific repressor(s) of VRN-H1.

**Genetics of low-temperature response**

Low-temperature tolerance is regulated primarily by the *C-repeat Binding Factor* (*CBF*) genes. An understanding of the function of *CBF* s was first gained from their discovery in Arabidopsis. *CBFs* are transcription factors belonging to the APETALA 2 (AP2) domain-containing family of proteins. Arabidopsis harbors three tandemly arranged *CBFs* spanning a distance of 8.7 kb on chromosome 4 (Gilmour et al., 1998; Shinwari et al., 1998). Expression of these *CBFs* is induced by low-temperature (Gilmour et al., 1998). The CBFs bind to C-repeat/Dehydration Responsive Element (CRT/DRE) motifs of the core sequence CCGAC present in promoters of genes such as the *Cold Regulated* (*COR*) and *Dehydrin* (*DHN*) genes (Baker et al., 1994; Stockinger et al., 1997; Yamaguchi-Shinozaki and Shinozaki, 1994). COR and DHN are structural proteins that function to stabilize lipid bilayers in cellular membranes against freezing injury (Artus et al., 1996; Steponkus et al., 1998). *CBFs* also play a role in increasing the production of proline, by
upregulating the proline biosynthetic enzyme \( P5CS \), and of soluble sugars such as sucrose, raffinose, glucose, and fructose (Gilmour et al., 2000). Increase in solute concentration in the cell cytoplasm protects the cellular membranes from freezing injury by preventing osmotic flow of intracellular water into extracellular spaces (Thomashow, 1998, 1999). Genes under the regulatory control of the \( CBF \)s comprise the \( CBF \) regulon. About 18% of the Arabidopsis genome induced by low temperatures is regulated by the \( CBF \)s (Vogel et al., 2005). The \( CBF \) regulon functions to increase the freezing tolerance of the plant (Jaglo et al., 2001; Jaglo-Ottosen et al., 1998).

The \( CBF \) gene family is much larger in the Triticeae cereals compared to Arabidopsis. A cluster of at least 13 \( CBF \) genes occurs on the long arm of the homoeologous group 5 chromosomes spanning a physical distance of about 850 kb and a genetic distance of about 0.8 cM (Knox et al., 2008; Miller et al., 2006; Skinner et al., 2005). In these cereal crops, the \( CBF \)s on chromosome 5 are coincident with one of the two major quantitative trait loci (QTL), \( FROST \) \( RESISTANCE-2 \) (\( FR-2 \)) identified in wheat and barley populations derived from crosses between winter and spring genotypes (Båga et al. 2007; Francia et al., 2004, 2007; Galiba et al., 1995; Hayes et al., 1993; Vágújfalvi et al., 2000). The \( FR-2 \) \( CBF \)s belong to two phylogenetic clades or subgroups – HvCBFIII and HvCBFIV (Badawi et al., 2007; Skinner et al., 2005). HvCBFIII-subgroup includes \( CBF12 \), \( CBF15 \), \( CBF16 \), \( CBF3 \), \( CBF6 \), \( CBF10 \), and \( CBF13 \). HvCBFIV-subgroup genes include \( CBF2 \), \( CBF4 \), \( CBF9 \), and \( CBF14 \). Except for \( CBF3 \), \( CBF6 \), \( CBF10 \), and \( CBF13 \), all the \( FR-2 \) \( CBF \)s are cold responsive. Additional \( CBF \)s are also present dispersed on
other chromosomes, which included *CBF1, CBF5, CBF7*, and *CBF11* (Skinner et al., 2006). These *CBF* s belong to HvCBF1-subgroup and are non-responsive to cold (Badawi et al., 2007; Skinner et al., 2005).

*Structural variation at FR-2 between winter and spring genotypes*

Like *VRN-1*, sequence variation at *FR-2* also discriminates winter from spring genotypes. Sequencing of lambda genomic phage clones of *FR-H2* in two winter hardy barleys ‘Nure’ and ‘Dicktoo’, and two spring barleys ‘Morex’ and ‘Tremois’ reveals that winter hardy barleys harbor greater copies of functional *CBF* genes than spring barleys (Knox et al., 2010). For example, ‘Nure’ and ‘Dicktoo’ harbor two paralogs of *CBF2* (*CBF2A* and *CBF2B*) and *CBF10* (*CBF10A* and *CBF10B*), but ‘Morex’ and ‘Tremois’ harbor only a single copy of *CBF2* and *CBF10* (Knox et al., 2010). Moreover, *CBF13* that exists a functional coding sequence in the winter hardy barleys, exists as a pseudogene in the spring barleys. Additionally, winter genotypes also harbor segmental duplications of the *CBFs*, which are absent from spring barleys (Knox et al., 2010). A 22 kb genomic fragment encompassing *CBF2A* and *CBF4B* is tandemly duplicated in ‘Dicktoo’ and ‘Nure’, resulting in two or three copies. However this fragment exists as a single copy in ‘Morex’ and ‘Tremois’. The duplicated copies are 100% identical in the *CBF* coding sequences, but can be discriminated using sequence polymorphisms occurring in the intergenic regions. The increased copies of *CBF2A–CBF4B* are confirmed by DNA blot hybridizations with *CBF2* and *CBF4* gene-specific probes. In wheat, DNA blot hybridizations suggest higher copy numbers of *CBF14* in winter wheats than in spring
wheats (Knox et al., 2010). Overall, greater copy numbers of CBFs occur in winter genotypes than in spring types, suggesting that total copy numbers of CBFs rather than a particular CBF may underlie the effect of FR-2 in the Triticeae.

**Interactions between the CBFs and VRN-1**

Expression of the CBF genes at FR-2 is negatively affected by VRN-1 (Stockinger et al., 2007). Upregulation of VRN-H1 expression in barley seedlings upon vernalization is accompanied by a downregulation of CBF expression. These changes in VRN-1 and CBF transcript levels are consistent with reduced levels of freezing tolerance post-vernialization (Fowler et al., 1996, 1999; Limin and Fowler, 2006). Interestingly, VRN-1 maps to the region that spans a second major QTL affecting freezing tolerance, which is FR-1 (Dubcovsky et al., 1998; Francia et al., 2004; Sutka, 1999). FR-1 is identified together with FR-2 in wheat and barley mapping populations. Conflicting data have emerged in the past concerning the molecular basis of FR-1. Some studies indicate that FR-1 and VRN-1 are two distinct but tightly linked genes (Galiba et al., 1995; Sutka, 1999), while others suggest that FR-1 and VRN-1 are pleiotropic effects of the same gene (Sutka and Snape, 1989). Our recent work supports the latter (below).

**Key Findings**

During my Ph.D. research, we have advanced the existing knowledge of freezing tolerance in the Triticeae. The sections below summarize the research results described in the subsequent chapters.
**VRN-1 underlies FR-1**

We have shown that *VRN-1* has pleiotropic effects on flowering and freezing tolerance. Due to its effects on freezing tolerance and its presence in the same genomic region as *FR-1*, we suggest that *VRN-1* and *FR-1* are the same gene (Chapter 2). This work was done in collaboration with the groups of Jorge Dubcovsky at UC, Davis, California and Gabor Galiba at Hungary.

My contributions in this study were to examine the freezing tolerance levels of the *VRN-A*m deletion mutants of diploid einkorn wheat (*T. monococcum*), called *maintained vegetative phase* (*mvp*) mutants. These mutants were originally generated by a Japanese group (Shitsukawa et al., 2007). The *mvp* mutants derive their name from their loss of flowering phenotype, which demonstrates that *VRN-1* is essential for flowering. Because the *mvp* mutants must be maintained in a heterozygous state in order to increase seed, it was necessary to genotype the segregating population to determine their *VRN-1* allelic status. I genotyped about 400 *mvp* mutants to discriminate the homozygous deletion mutants (*m/m*) from the remaining genotypes, i.e. heterozygous (*M/m*) and homozygous wild type (*M/M*) (PCR assay developed by Assaf Distelfeld, Dubcovsky lab). About 200 plants were grown under LDs at 23°C temperature until the appearance of the double ridge, which is a morphological marker at the shoot apical meristem indicative of the transition from vegetative to reproductive growth phase (Danyluk et al., 2003). Plants were then cold acclimated and subjected to -7°C and -9°C in the first set of experiments,
and to -9°C and -11°C in the second set. The effect of freezing on plant survival was measured using multiple assays, including re-growth assays, Fv/Fm ratios, and electrolyte leakage. At -9°C, homozygous mvp mutants exhibited a 87% survival rate determined using regrowth assays compared to 0% in the other class (M/-). These data were obtained with the second set of plants, which was developmentally advanced relative to the first set, determined using the developmental stage of the double ridge. The Fv/Fm ratios and electrolyte leakage of mvp mutants (m/m) were also significantly superior to the M/- plants. Similar data were also obtained at -11°C, although at this temperature the survival of homozygous deletion mutants (m/m) was also affected. Overall, these data suggest that the effect of FR-1 on freezing tolerance is due to VRN-1 i.e. FR-1 is the pleiotropic effect of VRN-1. Additionally, the mvp mutants (m/m) accumulated CBF and COR transcripts to significantly higher levels than M/- class, indicating that the VRN-1 affects freezing tolerance by repressing CBF transcription. We predict that connection between FR-1 and VRN-1 also holds true in barley based on the negative correlation between VRN-H1 and CBF transcript levels (Stockinger et al., 2007). However, the deletion in wheat spans additional genes including AGAMOUS-LIKE GENE 1 (AGLG1), CYSTEINE PROTEINASE (CYS), and PHYTOCHROME C (PHYC) (Distelfeld and Dubcovsky, 2010). Because PHYB and PHYD are known to repress CBF transcription in Arabidopsis, the possibility that PHYC affects freezing tolerance cannot be ruled out.

The repression of CBFs by VRN-1 has important consequences. In spring genotypes, where the growing season does not require tolerance to freezing temperatures, the
repression of CBFs occurs throughout the season due to the constitutive expression of VRN-1. This reduces the unnecessary transcriptional load on the cell. In winter genotypes, the CBFs are expressed when the temperatures are sub-zero, as the expression of the recessive VRN-1 allele is minimal prior to vernalization. During this time CBF expression helps the plants to survive the winter. In spring, however, when the temperatures are no longer freezing and CBF transcription is unnecessary, upregulation of VRN-1 expression represses CBFs in favor of the plant’s cellular economy.

**FR-H2 structural variation among spring barleys**

While sequencing of FR-H2 revealed structural variation between winter and spring barleys, it also revealed data indicating variation across barleys of the same growth habit (Knox et al., 2010). For example, in the case of spring barleys, CBF2 sequence of ‘Morex’ is identical to that of CBF2A in winter barleys. In comparison, CBF2 in ‘Tremois’ is similar to the CBF2B paralog in its 5’ region and similar to the CBF2A paralog in its 3’ region, suggesting that ‘Tremois’ CBF2 allele is a fusion of CBF2B and CBF2A winter alleles. Additionally, the non-sense mutations that make CBF13 a pseudogene in ‘Morex’ are different from those in ‘Tremois’ suggesting that the two spring alleles of CBF13 may have different lineages. Although a minor difference, CBF4 allele of ‘Tremois’ can also be discriminated from that of ‘Morex’ based on SNPs in its 3’ region.
Because ‘Tremois’ differs from ‘Morex’ at CBF2, CBF4, and CBF13, and the VRN-1 promoter and intron 1 mutations in ‘Tremois’ are also different from that of ‘Morex’ (von Zitzewitz et al., 2005), we conducted a pedigree analysis of ‘Tremois’ to study the origin of these alleles (Chapter 3). We identified the ancestors of ‘Tremois’ and developed markers for genotyping CBF2, CBF4, CBF13, VRN-H1 promoter and intron 1. We also phenotyped the ‘Tremois’ ancestors for flowering time under long days (LDs). Because VRN-2 and PPD-1 affect flowering time under LDs, ‘Tremois’ ancestors were also genotyped for these loci to determine their association and that of VRN-1 with flowering time. Data revealed that spring growth habit was prevalent in the ‘Tremois’ ancestors due to the ‘Morex’ VRN-1 promoter and intron 1 alleles. ‘Tremois’ allele of VRN-1 occurred in one genotype, which was a recent ancestor that was released as a cultivar in 1974. On the contrary, ‘Tremois’ CBF alleles were found in some of the oldest accessions that date back to 1914. These data suggest that the ‘Tremois’ CBF alleles are about 100 years old. A strong association was detected between the CBF2 and CBF13 alleles; a ‘Tremois’ CBF13 always co-occurred with a ‘Tremois’ CBF2 allele. Additionally, CBF2 and CBF13 co-harbored either spring alleles or winter alleles. Phenotyping for flowering time revealed that genotypes harboring a spring VRN-1 intron 1 (‘Morex’ or ‘Tremois’) were always of spring growth habit, whereas flowering time of accessions harboring winter VRN-1 intron 1 alleles was largely affected by the allelic states at VRN-H2 and PPD-H1. Overall, this study indicates that VRN-1 allele at FR-1 and CBF alleles at FR-2 are co-selected.
Overexpression of CBF2A in barley increases freezing tolerance and expression levels of other CBF genes

The group of Tony H.H. Chen at Oregon State University developed transgenic barley lines overexpressing Hv-CBF2A. They found that these lines were more freezing tolerant than the wild type plants. Our role came in to characterize the six CBF2A overexpressor lines. Initially, my contribution to this study was to confirm that each of the six transgenic lines was an independent transformation event using DNA blot hybridizations, and to examine expression levels of the CBFs in warm (18°C) and cold (6°C) (Chapter 4). However, this work became an integral part of my dissertation because we noticed an upregulation of other CBFs, especially CBF12 and CBF16 in the CBF2A overexpressor lines. This was of interest to us as it supported data that we were generating simultaneously in our lab suggesting an association between CBF2A copy numbers and expression levels of other CBFs (described below).

Copy number variations of CBF2A–CBF4B among winter barleys and its role in regulating expression of other CBF genes

We have discovered that copy numbers of the CBF2A–CBF4B genomic region that discriminate winter from spring barleys also vary among the winter barleys (Chapter 5). The idea that copy number variations (CNVs) of CBF2A–CBF4B may be occurring among winter barleys originated from the results of CBF expression analyses in the winter barleys ‘Admire’ and ‘Nure’. Transcript levels of several CBF genes in ‘Admire’ were greater than that of ‘Nure’ regardless of the time point in the subjective day and the
photoperiod under which the plants were grown. DNA blot analyses reveal that ‘Admire’ harbors about seven to eight copies of this genomic region. However, hybridizations with other CBF probes did not support CNVs of these other CBFs. Because some of the other CBFs harbor CRT/DRE motifs in their promoter regions (Knox et al., 2010), we hypothesized that higher expression levels of other CBFs may be associated with greater copy numbers of the CBF2A–CBF4B genomic region. We have generated data supporting our hypothesis using three independent approaches – 1) overexpression of CBF2A in the spring barley ‘Golden Promise’, 2) chromatin immunoprecipitation (ChIP) of CBF targets using α-CBF2 antibody in ‘Admire’ and ‘Golden Promise’, and 3) testing association of CBF expression levels with CBF2A–CBF4B copy numbers in an unstructured population derived from ‘Admire’. Approach 1) is described in Chapter 4 and approaches 2) and 3) in Chapter 5.

Chromatin immunoprecipitation (ChIP) experiments using α-CBF2 antibody followed by quantitative PCR (qPCR) revealed that CBF2 is present at CBF12 and CBF16 promoters similar to the positive control gene, DHN8. This was consistent across two independent replicates of warm-grown and cold-treated seedlings of ‘Golden Promise’ and ‘Admire’. To test the association of CBF12 and CBF16 expression with CBF2A–CBF4B copy numbers, we utilized the Missouri Barley (MO B) lines that trace back to ‘Admire’. DNA blot hybridizations reveal that CBF2A and CBF4B copy numbers in the MO B lines vary between zero and eight. Lines at either end of the copy number range were utilized for CBF expression analyses to maximize the power of detecting association between copy
numbers and CBF expression levels. RNA blot analyses reveal that expression levels of CBF2, CBF12 and CBF16 are significantly associated with CBF2A–CBF4B copy numbers in the warm, but not in the cold.

Overall, these data indicate a strong association of CBF2A–CBF4B copy numbers with CBF12 and CBF16 expression, and also suggest a mechanism underlying this association.

CBF14 copy number variations in wheat

CNVs of CBFs appear to be a recurring theme in the Triticeae. Previously, association of the winter 5A chromosome of hexaploid wheat with higher expression levels of CBF14, CBF15, and CBF16 compared to the spring 5A chromosome (Vágújfalvi et al., 2005) prompted us to examine copy numbers of these genes in spring and winter wheats. Among these CBFs, analysis of CBF14 via DNA blot hybridizations to a small collection of hexaploid wheats clearly indicated greater signal intensities of cross-hybridizing bands in winter wheats compared to spring wheats, and in hard red winter wheats grown in the Great Plains compared to soft red winter wheats grown in the Eastern US (Knox et al., 2010).

To determine that the identity of the cross-hybridizing bands and to determine whether differences in their signal intensities between genotypes reflect differences in CBF14 copy numbers, we conducted a much more comprehensive analysis (Chapter 6).
nullisomic-tetrasomic lines of wheat, the A, B, and D homoeologs of CBF14 were identified. Next, we examined copy numbers of CBF14 on each homoeolog were quantified relative to Puroindoline b (Pinb) that is reported to be a single copy gene in the hexaploid genome (Chantret et al., 2005). This was done using a large collection of winter and spring wheats belonging to five market classes – white spring (WS), white winter (WW), hard red spring (HRS), soft red winter (SRW), and hard red winter (HRW). Quantifications reveal that HRW wheats possess the highest copy numbers of CBF14, consistent with our hypothesis. Copy numbers of CBF14 were higher in winter genotypes than in spring genotypes of red wheats. The B genome harbors the least number of CBF14 copies in all hexaploid wheats. Copy numbers in the B genome were also lower than the A and D genomes in diploid and tetraploid wheats. These data also imply that CBF14 CNVs existed in the ancestors of polyploid wheats. Finally, analysis of wheat chromosome substitution lines suggests that the CBF14 copy numbers are stable in hexaploid wheats.

**Brief summary**

Overall, we have established that VRN-1 is most likely the underlying molecular basis of FR-1 and that it represses CBFs at FR-2. Copy numbers of the CBFs belonging to the HvCBF4-subgroup vary between genotypes of contrasting growth habits and also of similar growth habits in both wheat (CBF14) and barley (CBF2A and CBF4B). Additionally, we show that CBF2A–CBF4B copy numbers in barley are associated with expression levels of CBF12 and CBF16, and suggest that physical binding of CBF2A to
the CRT/DRE motifs in CBF12 and CBF16 promoters may be responsible for this association. We also present correlative evidence to suggest that copy numbers of CBFs are likely the molecular basis underlying FR-2. The testing of this hypothesis awaits further experimentation.

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Figure 1.1. Simplified schematic illustrating the interactions among the loci regulating winter hardness. FR-1 = Frost Resistance-1; FR-2 = Frost Resistance-2; CBFs = C-repeat Binding Factors; VRN = Vernalization; PPD = Photoperiod.
Table 1.1. Description of VRN-1, VRN-2, VRN-3, and PPD-1 loci in barley and wheat

<table>
<thead>
<tr>
<th></th>
<th>VRN-1</th>
<th>VRN-2</th>
<th>VRN-3</th>
<th>PPD-1</th>
<th>PPD-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomal location</td>
<td>Long arm of chr. 5</td>
<td>Chr. 4 in barley Chr. 5 (translocated from Chr. 4) in wheat</td>
<td>Chr. 7</td>
<td>Chr. 2</td>
<td>Chr. 1</td>
</tr>
<tr>
<td>Underlying gene</td>
<td>BM5A in barley WAP1 in wheat</td>
<td>ZCCT-Ha, -Hb, -Hc in barley ZCCT-1, ZCCT-2 in wheat</td>
<td>FT1 in barley FT in wheat</td>
<td>PRR</td>
<td>FT3</td>
</tr>
<tr>
<td>Arabidopsis ortholog</td>
<td>AP1, FUL, CAL</td>
<td>ZCCT transcription factor none</td>
<td>FT</td>
<td>PRR7</td>
<td>FT</td>
</tr>
</tbody>
</table>

Abbreviations: VRN = Vernalization; BM5A = Barley MADS-box 5A; WAP1 = Wheat Apetala1; FUL = Fruitful; CAL = Cauliflower; ZCCT = Zinc finger CCT (CO, CO-like, TOC1) domain [CO = Constans, TOC1 = Timing of CAB expression 1; CAB = Chlorophyll a/b binding protein]; FT = Flowering locus T; PEBP = Phosphatidylethanolamine binding protein; PPD = Photoperiod; PRR = Pseudo Response Regulator.
Chapter 2: Freezing tolerance and vernalization in cereals: the VRN-I connection

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Abstract

In winter wheat and barley varieties, long exposures to non-freezing cold temperatures accelerate flowering time (vernalization) and improve freezing tolerance (cold acclimation). However, when plants initiate their reproductive development, freezing
tolerance decreases suggesting a connection between the two processes. To better understand this connection we used two diploid wheat mutants “maintained vegetative phase” (mvp) that carry deletions encompassing the major vernalization gene, VRN-1. Homozygous mvp/mvp plants never flower, whereas plants carrying at least one functional VRN-1 copy (Mvp/-) exhibit normal flowering and high transcript levels of VRN-1 under long days. The Mvp/- plants showed reduced freezing tolerance and reduced transcript levels of several cold induced CBF transcription factors and COLD REGULATED genes (COR) relative to the mvp/mvp plants. In diploid wheat accessions with mutations in the VRN-1 promoter resulting in high transcript levels under both long and short days, a significant down-regulation of COR14b was observed under long but not under short days. Taken together these studies suggest that VRN-1 is required for the initiation of the regulatory cascade that down-regulates the cold acclimation pathway, but that additional genes regulated by long days are required for the down-regulation of the COR genes. In addition, our results show that allelic variation in VRN-1 is sufficient to determine differences in freezing tolerance suggesting that QTL for freezing tolerance previously mapped on this chromosome region are likely a pleiotropic effect of VRN-1 rather than the effect of a separate closely linked locus (FROST RESISTANCE-1) as proposed in early freezing tolerance studies.

Introduction

Exposure to low non-freezing temperatures, a process known as cold acclimation, increases a plant’s freezing tolerance (Thomashow, 1990; Thomashow, 1999). Freezing
tolerant plants that have not been cold acclimated are generally killed at approximately -3 to -5 °C while cold acclimated plants can survive much colder freezing temperatures. In addition, increasing the length of the cold acclimation period, up to a point, can also increase freezing tolerance. These two observations suggest that cold acclimation is an active process.

Freezing tolerance is essential for fall-planted temperate cereals (wheat, barley, and rye) to survive freezing temperatures during the winter. In contrast, spring-sown genotypes do not require high levels of freezing tolerance since they are not exposed to the freezing temperatures of winter. One feature that distinguishes winter and spring genotypes is the requirement of the former for a long period (several weeks) at cold temperature to accelerate the transition from the vegetative growth phase to the reproductive growth phase, a process called vernalization. Spring genotypes do not have a vernalization requirement and flower in the absence of the extended low temperature treatment (reviewed in Trevaskis et al., 2007; Distelfeld et al., 2009).

The requirement for exposures to non-freezing cold temperatures is common to both cold-acclimation and vernalization, suggesting a potential connection between these two processes. Winter genotypes maintained under continuous cold, after an initial increase in freezing tolerance, exhibit a progressive decrease in their cold acclimation ability (Fowler et al., 1996a; Fowler et al., 1996b; Fowler and Limin, 2004). This progressive decrease
inversely parallels the fulfillment of the vernalization requirement. A clear decrease in freezing tolerance occurs after the shoot apical meristem (SAM) advances to the double ridge stage (Fowler et al., 1996a, 1999; Limin and Fowler, 2006). These studies suggest that a regulatory component of freezing tolerance is linked to a developmental shift between the vegetative and reproductive stages. Limin and Fowler (2006) suggested that the main vernalization gene, *VRN-1* that is induced during vernalization, plays an important role in the decrease of the ability to cold acclimate with development.

Early genetic studies also revealed a correlation between growth habit and freezing tolerance; wheat genotypes having a spring growth habit were less freezing tolerant than genotypes having a winter growth habit (Hayes and Aamodt, 1927). Subsequent studies carried out using wheat chromosome substitution lines revealed that homologous group 5 chromosomes, where *VRN-1* is located, have the largest effect (Roberts, 1986). The first major locus affecting freezing tolerance and winter hardiness on homoeologous group 5 was designated *FROST RESISTANCE-1 (FR-1)* (Sutka and Snape, 1989). However, since *FR-1* co-segregates with *VRN-1* in most genetic studies, it is still not clear if *FR-1* is an independent gene or just a pleiotropic effect of *VRN-1* (Brule-Babel and Fowler, 1988; Sutka and Snape, 1989; Roberts, 1990; Hayes et al., 1993; Francia et al., 2004; Galiba et al., 2009).
More recently, a second locus associated with natural variation in freezing tolerance in wheat and barley was mapped on the long arm of homoeologous group 5. This locus, designated *FROST RESISTANCE-2 (FR-2)*, is approximately 30 cM proximal to *VRN-1* and includes a cluster of 11 (or more) *C-repeat Binding Factor (CBF)* genes (Vágújfalvi et al., 2003; Francia et al., 2004; Miller et al., 2006; Skinner et al., 2006; Francia et al., 2007; Knox et al., 2010). The *FR-2 CBF* gene cluster has surfaced as a major QTL affecting freezing tolerance in a number of wheat and barley mapping populations (Vágújfalvi et al., 2003; Francia et al., 2004; Båga et al., 2007; Francia et al., 2007).

The role of the *CBF* genes in freezing tolerance has been studied in detail in *Arabidopsis thaliana*. The CBFs are transcriptional activators that promote expression of genes whose upstream regulatory sequences harbor the CRT/DRE low temperature cis-acting DNA regulatory element (Stockinger et al., 1997). Approximately 20% of the Arabidopsis genes whose expression is altered during cold acclimation are directly or indirectly controlled by the CBF transcription factors (Vogel et al., 2005). Direct targets of the CBFs in Arabidopsis include the robustly-induced *Cold Regulated (COR)* genes (Jaglo-Ottosen et al., 1998). Similar candidate *CBF* target genes in the cereals, which also harbor CRT/DRE motifs in their upstream regulatory region, include *COR14b*, *DHN5*, and *DHN8* (Dal Bosco et al., 2003; Choi et al., 1999). Many of these *COR* genes are induced to higher levels in genotypes exhibiting greater freezing tolerance than in those having lesser freezing tolerance (Houde et al., 1992; Danyluk et al., 1994; Crosatti et al., 1996; Fowler et al., 1996b; Limin et al., 1997; Danyluk et al., 1998; Grossi et al., 1998;
The use of COR14b as an expression QTL to map loci affecting COR expression levels revealed two major loci; one of which is coincident with VRN-1 and the second one with FR-2 (Vágújfalvi et al., 2000; Francia et al., 2004).

Notably, genotypes carrying the vrn-1 allele for winter growth habit express certain CBF genes at higher levels than genotypes carrying the Vrn-1 allele for spring growth habit (Stockinger et al., 2007). Moreover, once the winter genotypes carrying the vrn-1 allele are vernalized, CBF transcript levels are dampened relative to levels detected in non-vernalyzed plants (Stockinger et al., 2007). This suggests that VRN-1 somehow acts to repress expression of the CBFs at FR-2, and in turn decrease freezing tolerance.

The molecular isolation of VRN-1 revealed that this gene encodes a MADS-box protein similar to the Arabidopsis meristem identity gene APETALA1 (API) (Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003). The characterization of VRN-1 alleles associated with winter and spring genotypes showed that the primary differences were insertions and deletions in regulatory regions located in the promoter and first intron (Yan et al., 2004; Fu et al., 2005; Pidal et al., 2009). Deletions in the VRN-1 promoter affecting a small region tentatively designated the ‘VRN-box’ (Pidal et al., 2009) or large deletions / insertions in the VRN-1 first intron are both associated with spring growth habit (Pidal et al., 2009; Fu et al., 2005). Genotypes with a winter growth habit (vrn-1 allele) show very low VRN-1 transcript levels until plants are vernalized. In contrast,
spring genotypes (Vrn-1 allele) constitutively express VRN-I to high levels. Flowering is initiated once VRN-I transcripts reach a critical threshold level (Loukoianov et al., 2005).

In addition to vernalization, photoperiod also plays a role in VRN-I regulation. In photoperiod sensitive genetic backgrounds long day photoperiods enhance VRN-I transcript accumulation while short day photoperiods delay transcript accumulation. In both wheat and barley the delay in the transition to floral initiation in plants grown under SD photoperiods is associated with increased freezing tolerance (Limin and Fowler, 2006; Fowler et al., 2001). One of the cis-elements responsible for the lack of VRN-I expression under short days in diploid wheat is thought to reside within the VRN-I promoter CArG motif, a binding site for MADS-box transcription factors located 3′ of the VRN-box (Dubcovsky et al., 2006; Pidal et al., 2009). Under short days, diploid wheat (Triticum monococcum L.) plants carrying deletions in this CArG motif (Vrn-1f and Vrn-1g alleles) show accumulation of VRN-I transcripts and a slow transition of the shoot apical meristem to the reproductive stage, whereas plants with intact CArG motifs (e.g. Vrn-1h and vrn-1) show no VRN-I expression in SD and remain in the vegetative phase (Dubcovsky et al., 2006). Under long days, all accessions with VRN-I alleles for spring growth habit show accumulation of VRN-I transcripts and a rapid initiation of the transition to the reproductive stage.
Materials and methods

Maintained vegetative phase (mvp) mutants

Two independent *T. monococcum* mutants (*mvp*-1 and *mvp*-2) that remain indefinitely in the vegetative state (Shitsukawa et al., 2007a) were used in this study. Since the two mutants carry similar deletions (Distelfeld and Dubcovsky, 2010), they were alternated among experiments depending on seed supply. Seeds from these mutants were kindly provided by K. Murai. These mutants were generated by ion-beam radiation and both have large deletions that include *VRN*-1 (Shitsukawa et al., 2007a) and several flanking genes (Distelfeld and Dubcovsky, 2010). The *mvp*-1 mutation was generated in the KU104-2 background, and the *mvp*-2 mutation in the KU104-1 background (Shitsukawa et al., 2007a). When grown in the greenhouse under long day conditions (16 h photoperiods) KU104-2 flowered 10 weeks after planting while KU104-1 flowered three weeks later.

Homozygous *mvp* individuals do not flower and must be maintained in a heterozygous state. Genotyping was carried out using a dominant *VRN*-1 molecular marker based on a set of three primers that are described in the supporting online materials (Fig. B.1). Using this assay the lines carrying one or two functional *VRN*-1 copies are detected as a single genotypic class, referred throughout the text as “Mvp/-”.
**VRN-If, VRN-1g and VRN-1h alleles**

*Triticum monococcum* lines with four different *VRN-1* alleles were used to test the effect of their differential regulation under short days on *COR14b* transcript levels. *Triticum monococcum* lines PI355546, PI427927, and PI237659 carry the “wild type” *vrn-1* allele and a recessive *vrn-2* allele that confers spring growth habit. Lines with the *vrn-1* allele showed no expression under short days in previous studies (Dubcovsky et al., 2006). *T. monococcum* lines PI191097, PI192063, and PI393496 carry the *Vrn-1f* allele, which has a 1-bp deletion in a CArG box located in the promoter, plus an insertion of a repetitive element in the first intron. *T. monococcum* lines PI326317, PI418582, and PI349049 carry the *Vrn-1g* allele, which has a 34-bp deletion including the promoter CArG box. Both the *Vrn-1f* and *Vrn-1g* alleles confer spring growth habit and show high levels of *VRN-1* transcripts under short days (Dubcovsky et al., 2006). *T. monococcum* accession PI306540 has the *VRN-1h* allele that has the same intron one insertion as *Vrn-1f* but lacks the CArG box mutation. This allele is not expressed under short days and confers a spring phenotype but with later flowering than the *Vrn-1f* and *Vrn-1g* alleles (Dubcovsky et al., 2006). Although the apex transitions to the double ridge stage, spike development progresses very slowly and spikes fail to elongate if these plants are left under short days (Dubcovsky et al., 2006).
Growth, cold acclimation, and freezing assays

Freezing experiments with the \textit{mvp} mutants were all carried out using long day conditions (LD, 16 h light / 8 h dark). Experiments with the \textit{T. monococcum} lines with different \textit{VRN-1} alleles were carried out using both long days and short day (SD, 8 h light / 16 h dark) photoperiod cycles.

The RNA blot analysis and the freezing experiment of the \textit{mvp}-2 mutants were done in parallel. Seed collected from \textit{Mvp}-2/- heterozygotes were grown under cool white fluorescent lamps in the laboratory at room temperature for 13 days using a light intensity of 50 \(\mu\text{mol m}^{-2} \text{s}^{-1}\). Genotyped seedlings were transplanted to wooden boxes having internal dimensions measuring 42 cm (l) x 22 cm (w) x 14 cm (h) and having 9.5 cm soil depth. The boxes were placed into a Conviron growth chamber (model PGW36, Controlled Environments Inc., ND) for an additional 47 d under cool white fluorescent and incandescent bulbs, using a light intensity of 130 \(\mu\text{mol m}^{-2} \text{s}^{-1}\), and a constant temperature of 23 °C. The growth chamber temperature was decreased to 10 °C and held at this temperature for 19 days.

Wooden boxes were then transferred to Percival growth chambers (model CU-36L2X, Geneva Scientific, Fontana, WI) where the seedlings were held at 4 °C for 12 d (the PGW36 growth chamber can only cool to +10°C). After cold acclimation the CU-36L2X growth chamber temperatures were decreased to -2 °C, and held for 12 h. Ice nucleation
was induced by spraying the leaves with ice water. Following 12 h at -2 °C, the temperature was then decreased 1 °C per h to the target temperatures of -9°C and -11 °C and held at these temperatures for 24 h. Afterwards the temperature was returned to +2 °C for 12 h. During this time the plants were kept in the dark. The chamber temperature was then raised to 20 °C and returned to a 16 h photoperiod.

**Chlorophyll fluorescence (Fv/Fm)**

Chlorophyll fluorescence measurements were made using a hand-held portable pulse amplitude modulated fluorometer (model OS-30p, Opti Sciences, Hudson, NH). $F_v/F_m$ measurements were taken 2-4 h after the plants were returned to normal growth conditions. Leaves were dark adapted for approximately 10 minutes prior to taking the measurements.

**Relative conductivity**

Conductivity measurements were taken on crown tissue consisting of a 1 to 1.5 cm segment of the white, nonphotosynthetic tissue between the upper photosynthetic green shoot and the primary root. Because the use of the crowns required the destruction of the plant the number of replications in the first experiment was limited to three $mvp/mvp$ homozygotes and six $Mvp/-$ plants. The second experiment used leaves instead of crown regions and nine plants from temperature / genotype combination. The electrolyte leakage assay methods are described in detail (in section ‘Methods used for Figure B.3’). Tubes
were shaken for 1 h at 300 rpm before reading the conductivity with an Accumet Basic AB30 electrical conductivity meter (Fisher Scientific). Tubes were then autoclaved for 20 minutes, cooled to room temperature and shaken for 1 h at 300 rpm before measuring the total potential conductivity. Values were adjusted by subtracting the conductivity of the deionized water. Relative conductivity represents the adjusted mean ion leakage as a percentage of the total adjusted leakage from frozen-killed samples (see formula in SOM).

**CBF qRT-PCR experiment**

Ten \( mvp-1/mvp-1 \) and ten \( Mvp-1/- \) plants were selected using the \( VRN-1 \) molecular marker and were grown in the greenhouse for eight weeks (20-25 °C, long days). At eight weeks plants were transferred to a growth chamber at 4°C for 8 hours. RNA samples were collected from leaves from 10 plants per genotype.

**COR14b qRT-PCR time course experiment**

Eight \( mvp-2 \) and eight \( Mvp-2/- \) plants were selected using the \( VRN-1 \) molecular markers and grown in the greenhouse under the same conditions described above. After four weeks, when the \( Mvp-2/- \) plants were still at the vegetative stage, plants were transferred to 4 °C and were kept at this temperature for 12 days at the same light intensity indicated above (long days). Leaf samples for RNA analysis were collected one day before the cold treatment and after 8 h, 32 h, 4 days and 12 days at 4 °C. Samples were always collected
at 2:00 pm (8 h after the subjective daybreak) to avoid potential differences at different times of the day.

**VRN-1/COR14b qRT-PCR experiment**

Five plants with each *VRN-1* allele were grown for either two or six weeks under short or long day conditions before transferring to 4 °C. RNA samples were collected from leaves after 32 hours of cold treatment.

For all qRT-PCR experiments, RNA was extracted using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Saint Louis, MO). First strand cDNA was synthesized from 1 µg of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). Primers for qRT-PCR expression analyses are presented in Table B.1. The *TRANSLATION ELONGATION FACTOR 1 (TEFI)* and *ACTIN* genes were used as endogenous controls (Distlefeld and Dubcovsky 2010).

**RNA blot analyses**

Tissue samples were collected 13-14 h after the subjective daybreak from both cold-acclimating plants and non-acclimated plants. Total RNA was isolated using RNeasy Plant Mini kits (Qiagen, Valencia, CA). Seven µg of total RNA was loaded per lane. RNA samples consisted of RNAs pooled from the crown tissue of ten (*mvp-2/- experiment) or five (*mvp-1* and *VRN-1* promoter deletion mutant experiments) plants.
Fragments used as probes were generated by PCR amplification of cloned cDNA inserts. Radiolabeled probes were generated by random priming (Feinberg and Vogelstein, 1983). Overnight hybridizations were at 42°C in 50% formamide, 5X SSC, 20 mM Na-phosphate buffer pH 6.8, 1X Denhardt’s solution, 0.1% SDS, 10% dextran sulfate and containing 100 µg/ml herring sperm DNA. Three to four 1 h moderate-stringency washes were performed at 62-65°C in 0.2X SSC, 0.05% SDS and 0.01% Na-pyrophosphate. Images were generated using the Molecular Dynamics Storm840 PhosphorImager (GE Healthcare) and phosphor autoradiography.

Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permission will be the responsibility of the requestor.

*Triticum monococcum* mutants with deletions of the *VRN-1* gene fail to flower indicating that this gene is indispensable for the transition to the reproductive phase (Shitsukawa et al., 2007a). Two independently induced nitrogen-ion-beam mutants, designated maintained vegetative phase 1 and 2 (*mvp-1* and *mvp-2*) were generated in different *T. monococcum* genetic backgrounds. The deletions in these two mutants encompass the complete *VRN-1* gene and several closely linked genes (Distelfeld and Dubcovsky, 2010).
To investigate the role of VRN-1 in freezing tolerance we made use of the mvp mutants and of natural T. monococcum accessions that differ in their ability to express VRN-1 under short days. We found that freezing tolerance and transcript levels of several CBF and COR genes were higher in the mvp mutants relative to the plants carrying at least one functional VRN-1 copy. However, the expression of VRN-1 under short days was not as effective as under long days to down-regulate COR14b gene transcription. Taken together these results suggest that VRN-1 transcription is necessary but not sufficient to down-regulate the COR genes.

Results

Effect of the mvp mutations on freezing tolerance

The homozygous mvp-2 mutants (mvp-2/mvp-2) and the plants carrying at least one functional copy of VRN-1 (Mvp-2/-) were identified using a dominant molecular marker for VRN-1 as described (in section for ‘Methods for Figure B.1’). Just before the freezing experiments, there were clear differences in apical development between plants from each group. The apices from the mvp-2/mvp-2 mutant were at the vegetative stage whereas those from the Mvp-2/- plants were already at the double ridge stage (Fig. B.2).

Significant differences in survival rates were detected between the mvp-2/mvp-2 and Mvp-2/- plants in controlled freezing experiments. Differences between the two
genotypic classes were detected at both -9°C and -11°C freezing temperatures (Table 2.1).
In the group frozen to -9°C none of the Mvp-2/- plants survived, whereas 87% of the
mvp-2/mvp-2 mutants survived (Table 2.1). In the group frozen to -11°C none of the Mvp-
2/- plants survived, whereas approximately half (46%) of the mvp-2/mvp-2 mutants
survived (Table 2.1). In a second freezing experiment performed under slightly different
acclimation and freezing conditions (SOM) approximately 70% of the mvp-2/mvp-2
mutants survived -12°C freezing temperatures whereas only 40% of the Mvp-2/- plants
survived the same treatment (P=0.009, Fig. B.3). In this second experiment, all plants
from both genotypic classes were killed at -13°C (Fig. B.2).

To evaluate the effect of freezing on the functionality of the Photosystem II (PSII), the
maximum quantum yield of the PSII photochemistry was measured by the ratio of
variable (Fv) to maximal (Fm) fluorescence in a dark-adapted state, Fv/Fm (Butler and
Kitajima 1975). Fv/Fm ratios taken 2-4 hours after returning the plants to 20°C paralleled
the survival results. Homozygous mvp-2/mvp-2 mutant plants showed significantly higher
Fv/Fm values than the Mvp-2/- plants (Table 2.1, P<0.0001), in agreement with the
greater freezing tolerance of the mvp-2/mvp-2 plants.

Along with the Fv/Fm measurements, samples of the crown and adjoining tissues were
collected to measure relative conductivity. This measurement estimates the cellular
electrolytes leached from the freeze-damaged tissue as a proportion of the total cellular
electrolytes and is based on the principle that the greater the damage to cells from freezing injury, the greater the exosmosis of cellular electrolytes into a water solvent (Dexter, 1956). The Mvp-2/- plants showed higher relative conductivity values than those from the homozygous mvp-2/mvp-2 mutants, both at -9°C (19% increase) and -11°C (45% increase). However, the differences between genotypes were only marginally significant (Table 2.1, P = 0.05), likely due to the limited number of mutant plants sacrificed for relative conductivity measures in this first experiment (three mvp-2/mvp-2 and six Mvp-2/- plants per temperature). A second experiment using leaves from 9 plants per genotype-temperature combination confirmed the higher relative conductivity of the Mvp-2/- plants compared with the mvp-2/mvp-2 (76% average increase over the three temperatures, P=0.003, Fig. B.3).

Taken together these three sets of data indicate that the presence of the VRN-1 gene in the Mvp-2/- lines is associated with a decrease in freezing tolerance relative to the mvp mutants.

**Effect of the mvp mutations on CBF transcript levels**

Quantitative RT-PCR was used to compare transcript levels of 11 CBF genes in 4 week-old Mvp-1/- and mvp-1/mvp-1 plants both before (20 °C) and after 8 h of 4 °C cold treatment (Fig. 2.1). With the exception of CBF2, which showed very low levels of expression both at 20 °C and 4 °C, the other 10 CBF genes showed very low transcript levels at 20 °C and were significantly up-regulated after 8 h of cold treatment (P<0.001).
Five CBF genes (CBF2, CBF4, CBF9, CBF12, CBF17) showed significantly higher expression levels ($P<0.01$) in the $mvp-1/mvp-1$ homozygous mutant plants than in the $Mvp-1/-$ plants (Fig. 2.1). The same difference was marginally significant for CBF14 ($P=0.03$) and nonsignificant for the other CBF genes.

To validate the previous differences, the experiment was repeated using 8-week old plants and measurement of CBF transcript levels after 8 h cold treatment (but not prior to cold treatment, Fig. B.4). In the $Mvp-1/-$ plants the shoot apical meristems were between the double ridge and terminal spikelet stages, whereas in the $mvp-1/mvp-1$ mutants the shoot apical meristems were in the vegetative stage. As in the 4-week old plants, the older 8-week plants showed significantly lower transcript levels of CBF2, CBF4, CBF9, CBF12, and CBF17 in the $Mvp-1/-$ plants relative to the homozygous $mvp-1/mvp-1$ mutants after the cold treatment. Whereas CBF14 transcript level differences were marginally significant at 4 weeks in this second experiment the differences in CBF14 transcript levels were not significant at 8-weeks. Curiously, transcript levels of CBF12 and CBF16 were much lower relative to the other genes in the 8-week old plants in comparison to the 4-week old plants.

Quantitative PCR measurements of VRN-1 in both experiments revealed high levels of VRN-1 transcripts in the $Mvp-1/-$ plants and, as expected, no VRN-1 transcripts in the
homozygous \( mvp/mvp \) mutants. Taken together, these results suggest that the presence of \( VRN-1 \) modulates the response of several \( CBF \) genes to cold.

**Effect of the \( mvp \) mutations on \( COR \) gene transcript levels**

Eight hours after transferring 8-week old plants from 20 °C to 4 °C, \( COR14b \) transcripts were two-fold higher \((P<0.05)\) in the homozygous \( mvp-1/mvp-1 \) plants than in the \( Mvp-1/- \) plants (Fig. B.4). Two additional experiments, in which plants were exposed to longer periods of cold temperatures, were performed to further characterize the differences in \( COR14b \) transcript levels between mutants and non-mutants.

In the first experiment, four-week old \( mvp-2/mvp-2 \) and \( Mvp-2/- \) plants were transferred from room temperature to 4 °C and kept at that temperature for 12 days. Leaf samples for RNA analysis were collected the day prior to the cold treatment and 8 h, 32 h, 4 days, and 12 days after transferring the plants to 4 °C. All samples were collected at 2 pm to avoid differences that might be caused by circadian effects. In this qRT-PCR experiment, transcript levels from both genotypes peaked at 32 h and then decayed slowly during the next 11 days. At each of the last three sampling points the \( COR14b \) transcript levels were significantly higher \((P<0.001)\) in the \( mvp-2/mvp-2 \) homozygous plants than in \( Mvp-2/- \) plants (Fig. 2.2).
In the second experiment, steady state transcript levels of \textit{COR14b} and two additional \textit{COR} genes (\textit{DHN5} and \textit{DHN8}) were evaluated by RNA blot analysis using a more gradual decrease in temperatures and longer time exposures to the inductive temperatures (19 d at 10 °C followed by 12 d at 4 °C, all under long days) (Fig. 2.3). At the beginning of the cold induction the \textit{Mvp-2/-} plants were already induced to flower and showed high levels of \textit{VRN-1} transcripts whereas the \textit{mvp-2/mvp-2} plants were in the vegetative stage and, as expected from the homozygous deletion, showed no \textit{VRN-1} transcripts (Fig. 2.3).

At all time points after the cold induction, the transcript levels of \textit{COR14b} and \textit{DHN5} were higher in the \textit{mvp-2/mvp-2} plants (no \textit{VRN-1} transcripts) than in the \textit{Mvp-2/-} (high \textit{VRN-1} transcripts). The \textit{DHN8} gene did not show this alternate pattern between genotypes (Fig. 2.3). In \textit{mvp-2/mvp-2} homozygous mutants \textit{COR14b} and \textit{DHN5} levels remained high throughout the sampling time course (Fig. 2.3).

Taken together, the expression data from these experiments showed that in plants with high \textit{VRN-1} transcript levels several \textit{CBF} and \textit{COR} genes are down-regulated. To test if the down-regulation of these genes was a direct effect of the increase in \textit{VRN-1} transcript levels or a result of the transition to the reproductive phase, the relationship between \textit{VRN-1} and \textit{COR14b} transcript levels was studied in \textit{T. monococcum} lines that differ in the expression of \textit{VRN-1} under short day, but that suffer a similar delay in the progression to the reproductive phase.
Effect of VRN-1 transcription on COR14b transcript levels under short days

*Triticum monococcum* lines carrying a “wild-type” *vrn-A1* allele and recessive *vrn-A2* alleles have a spring growth habit and show no expression of *VRN-1* under short days (Dubcovsky et al., 2006). However, *T. monococcum* lines carrying a 1-bp deletion in the *VRN-1* promoter CArG box and an insertion in intron one (*Vrn-1f* allele), or a 34-bp deletion encompassing the complete CArG box (*Vrn-1g* allele), show high *VRN-1* transcript levels under short days (Dubcovsky et al., 2006). These plants show a transition of the shoot apical meristem to the double ridge stage under short days, but further development of the spike is delayed until the plants are transferred to long days (Dubcovsky et al., 2006).

Expression profiling of three independent accessions of *T. monococcum* lines carrying each of the three genotypes confirmed previously published results (Dubcovsky et al., 2006). Under short days, lines having the *Vrn-1f* or *Vrn-1g* alleles showed high *VRN-1* transcript levels whereas those having the wild-type *vrn-1* allele showed no *VRN-1* transcripts (Fig. 2.4A). As expected, transcript levels of *VRN-1* were higher at 6 weeks than at 2 weeks, and *COR14b* transcripts were high after cold temperatures and nearly absent in plants maintained at warm temperature (Fig. 2.4A).

In the six-week old *Vrn-1f* and *Vrn-1g* lines grown under short days, *COR14b* was highly responsive to cold temperatures, despite relatively high *VRN-1* transcript levels in these
lines (Fig. 2.4A, arrows). In contrast, when 6-week old plants of these same genotypes were grown under long day conditions, the same cold treatment failed to induce COR14b to high levels (Fig. 2.4A). This result indicates that under short days the expression of VRN-1 was not sufficient to down-regulate COR14b.

Analyses of the two-week old plants (Fig. 2.4A) further confirmed the inverse correlation between VRN-1 and COR14b. Under short days, the cold treatments resulted in a strong up-regulation of COR14b (low VRN-1 transcript levels in all genotypes), but under long days the down-regulation of COR14b was not as strong as in the 6-week old plants, likely because the VRN-1 transcript levels in two-week-old plants was not as high as in the 6-week old plants (Fig. 2.4A). To confirm these results this experiment was repeated using the same genotypes at the same developmental stage, in which VRN-1 and COR14b transcript levels were compared using qRT-PCR (Fig. 2.4B). In plants grown under short days and exposed for 32 h to 4°C, COR14b transcript levels showed no-significant differences ($P = 0.95$) between wild-type and Vrn-1 mutant lines (Vrn-1f and Vrn-1g), despite significant differences in VRN-1 transcript levels. In contrast, highly significant differences in COR14b transcript levels ($P = 0.006$) were detected under long days, in which COR14b transcript levels in the Vrn-1f and Vrn-1g mutant lines were lower than in the wild-type (Fig. 2.4B).
An additional experiment was carried out to compare \textit{COR14b} expression levels in \textit{T. monococcum} lines carrying the \textit{Vrn-1f} and \textit{Vrn-1g} alleles (early spring growth habit) to those in a \textit{T. monococcum} accession carrying the \textit{Vrn-1h} allele (late spring growth habit). The \textit{Vrn-1h} allele has the identical repetitive element insertion in the first intron as \textit{Vrn-1f}, but lacks the CArG box mutation (Dubcovsky et al. 2006). As previously reported the line with the \textit{Vrn-1h} allele showed low \textit{VRN-1} transcripts under short days and maintained low \textit{VRN-1} transcript levels even when plants were grown under long days for six weeks. The results presented in Fig. 2.4C confirmed that high \textit{VRN-1} transcript levels under short days (\textit{Vrn-1f} and \textit{Vrn-1g}) were not sufficient to suppress the induction of \textit{COR14b}. In contrast, when plants were grown under long days, the \textit{Vrn-1h} line (low \textit{VRN-1} transcript levels) showed a significantly stronger ($P=0.006$) induction of \textit{COR14b} than the \textit{Vrn-1f} and \textit{Vrn-1g} mutants (Fig. 2.4C). As in the previous experiments, both high \textit{VRN-1} transcript levels and long days were necessary for the down-regulation of \textit{COR14b}.

Taken together, the results from this and previous experiments suggest that \textit{VRN-1} expression is required to initiate the developmental processes that reduce the ability of \textit{COR14b} to respond to cold temperatures, but that \textit{VRN-1} transcription alone is not sufficient to produce this effect.
Discussion

*The decreased freezing tolerance of the Mvp/- plants is associated with changes in both leaves and apical meristems*

When the shoot apical meristem of the *Mvp/-* plants transitions to the reproductive phase, it stops producing new leaves. Therefore, *Mvp/-* plants are expected to exhibit a reduced ability to re-grow as they transition to the reproductive phase. In contrast the shoot apical meristem of the *mvp/mvp* homozygous mutants never transitions to the reproductive phase and thus, their ability to re-grow after freezing should not be drastically altered with time.

While changes in the susceptibility of the shoot apical meristem to freezing damage may account for the observed differences in regrowth, the *mvp/mvp* homozygous mutants also exhibited greater freezing tolerance in the existing leaves after acclimation than the *Mvp/-* plants as suggested by higher $F_v/F_m$ values and lower relative conductivity after the freezing treatment. This suggests that the presence of *VRN-1* transcript levels and the concomitant dampening of *COR14b* induction by cold contributed to a reduction of freezing tolerance in the existing leaves of the *Mvp/-* plants.

In Arabidopsis, increasing the levels of COR15 (which like barley COR14b is a hydrophilic protein targeted to the chloroplast stromal compartment (Crosatti et al. 1995)), increases freezing tolerance (Artus et al. 1996). COR15 appears to stabilize
membranes from freeze-induced injury, which would account for the reduced electrolyte leakage and higher $F_v/F_m$ values (Artus et al. 1996). In addition, the higher transcript levels of dehydrin genes such as $DHN5$ (Fig. 2.3) in the leaves of the $mvp/mvp$ homozygous mutants relative to the $Mvp/-$ plants likely contributes to their improved freezing tolerance. Dehydrins have a highly conserved 15 amino acid segment (the “K-segment”) which interacts with acidic phospholipids in lipid vesicles. This interaction results in a conformational change of the protein structure that is hypothesized to stabilize membrane integrity (Koag et al. 2003, 2009).

*The increased freezing tolerance of the mvp mutants is likely caused by the VRN-1 deletion*

The MADS-box meristem identity gene $VRN-1$ plays an essential role in the regulation of the transition between vegetative and reproductive phases, and its deletion results in plants that fail to flower. In Arabidopsis, gene duplications of the $VRN-1$ homolog that occurred after the monocot-dicot divergence, resulted in three paralogous genes $APETALA1$ (*AP1*), $CAULIFLOWER$ (*CAL*), and $FRUITFULL$ (*FRU*) that have retained partial ability to promote the transition of the vegetative shoot apical meristem (SAM) to the reproductive phase. Simultaneous deletions of all three genes are required to generate non-flowering Arabidopsis plants (Ferrandiz et al., 2000).

An additional difference between the temperate cereals and Arabidopsis meristem identity genes is their spatial expression profile. $VRN-1$ transcripts are detected at high
levels in the leaves of wheat (Danyluk et al., 2003; Yan et al., 2003; Li and Dubcovsky, 2008), barley (Schmitz et al., 2000; Trevaskis et al., 2003), *Lolium* (Petersen et al., 2004) and oat (Preston and Kellogg, 2008), suggesting a similar profile among the temperate cereals. In contrast, the Arabidopsis meristem identity homologs are expressed primarily in the apical meristem and reproductive tissues. *AP1* and *CAL* transcripts are abundant in the induced SAM and floral primordia in Arabidopsis, but are undetectable or are present at much lower levels in some vegetative tissues such as the vascular tissues of cotyledons (Abe et al., 2005). *FUL* is also expressed primarily in the meristem and floral tissue, but it is also detected in cauline leaves (Teper-Bammolker and Samach, 2005). In the winter cereals, the expression of *VRN-1* in the leaves (and apices) occurs only after vernalization providing a potentially useful regulatory signal to down-regulate the cold acclimation pathway in this tissue in the spring.

Winter wheat lines exposed to continuous cold temperatures improve their freezing tolerance during the first 3-4 weeks of the treatment but then gradually start losing those gains. The inflexion point in this freezing tolerance curve coincides with the transition of the shoot apical meristem to the double ridge stage and high *VRN-1* transcript levels in the leaves (Danyluk et al., 2003). Limin and Fowler (2006) found that wheat near-isogenic lines for *VRN-1* carrying the allele for winter growth habit tolerate freezing temperatures 11°C lower than lines carrying the *VRN-1* allele for spring growth habit. The authors also showed that when the same near-isogenic spring lines were grown under short days, which are less promotive of *VRN-1* expression than long days, the plants
tolerated temperatures 8.5°C colder than the same lines grown under long days (Limin and Fowler, 2006). Based on these results these authors hypothesized that the expression of $VRN-I$ might be an important signal to regulate the freezing tolerance pathway.

Previous studies using near-isogenic lines (Limin and Fowler, 2006), and others using QTL mapping for freezing tolerance, all point to the $VRN-I$ region as important in the regulation of freezing tolerance (Sutka and Snape, 1989; Roberts, 1990; Hayes et al., 1993; Francia et al., 2004; Galiba et al., 2009). However, the recombination points flanking the $VRN-I$ gene in the lines used in these studies are not known and can encompass large chromosome regions including a large number of genes. The use of deletion mutants in this study provides a more precise delimitation of the chromosome region responsible for the differences in freezing tolerance.

Nonetheless, some caution is still required in the interpretation of the mvp results, since the deletions present in these lines include other genes flanking $VRN-I$. A screening with probes for multiple genes in the $VRN-I$ region showed that the mvp deletions include the $AGLG1$, $CYS$, and $PHYC$ genes but exclude flanking genes $CYB5$ and $ADA2$ (Distelfeld and Dubcovsky, 2010). The exclusion of $ADA2$ from the deleted regions is relevant because this gene is critical for $CBF$ mediated trans-activation (Stockinger et al., 2001). Based on colinearity with Brachypodium the mvp deletion is also predicted to include two additional genes, an oligopeptide transporter ($Bradi1g08420$), and a proteinase inhibitor.
I9 (Bradi1g08450) (Distelfeld and Dubcovsky, 2010). Most of these additional genes are unlikely candidates for the improved freezing tolerance observed in the mvp mutants, with the exception of phytochrome PHYC, since phytochromes B and D have been shown to affect the CBF regulon in Arabidopsis (Franklin and Whitelam, 2007). Thus, we cannot rule out the possibility of the presence of additional genes with an effect on freezing tolerance in the mvp deleted region until it is completely sequenced.

However, the expression studies provided an independent source of evidence pointing to VRN-1 as the best candidate gene for the down-regulation of the cold acclimation response. In all the T. monococcum accessions carrying the VRN-1f or VRN-1g alleles, the higher transcript levels of VRN-1 were always associated with a significant down-regulation of COR14b when plants were grown in long day conditions (Fig. 2.4B and 2.4C, LD). In addition, larger differences in VRN-1 transcript levels during development were correlated with larger differences in COR14b transcript levels (Fig. 2.4B and 2.4C, LD). All the expression results presented here support the hypothesis that VRN-1 is the best candidate for the dampening of the cold acclimation response among the genes present within the mvp deletions. We are currently developing TILLING mutants of the VRN-A1 and VRN-B1 genes in tetraploid wheat (Uauy et al. 2009) to provide an independent validation of this hypothesis.
Homozygous mvp mutants exhibit higher transcript levels of several CBF and COR genes after a short cold treatment

A negative association between VRN-1 and COR genes COR14b and DHN5 (=WCS120) transcript levels has also been reported in previous wheat and barley studies (Vágújfalvi et al., 2000; Danyluk et al., 2003; Knox et al., 2008). In a doubled-haploid barley population segregating for VRN-H1, the lines carrying the recessive vrn-H1 allele showed higher transcript levels of CBF and COR genes than those carrying the dominant Vrn-H1 allele (Stockinger et al., 2007). In addition, lines grown under short days (reduced VRN-H1 levels) showed higher CBF and COR transcript levels than lines grown under long days when plants were transferred to the cold (Stockinger et al., 2007). The reductions in the transcript levels of multiple CBF genes and their downstream COR gene targets in plants with high VRN-1 transcript levels provide a simple explanation for the gradual decrease in freezing tolerance observed after the initiation of the reproductive phase.

The mvp mutants characterized in this study exhibited a similar negative association between VRN-1 and both CBF and COR transcription profiles. Five of the eleven CBF genes tested by quantitative RT-PCR and the COR14b gene showed significantly higher transcript levels 8h after moving the plants to 4°C in the mvp deletion homozygotes than in those carrying at least one functional copy of VRN-1 (Fig. 2.1 and Fig. B.4). In plants maintained at 4°C for 12 days, the COR14b transcript levels were still 10-fold higher in the plants homozygous for the mvp deletion (Fig. 2.2). However, analyses of two additional COR genes, DHN5 and DHN8 showed that not all COR genes respond in the same way. Whereas DHN5 showed the same negative correlation with VRN-1 as
The DHN8 gene was not significantly affected by the change in the level of VRN-1 transcripts, which indicates that not all COR genes are down-regulated by VRN-1.

Similarly, for half of the 11 CBF genes present in the FR-2 cluster, no significant differences in transcript levels were detected between Mvp/- and mvp/mvp lines (Fig. 2.1 and Fig. B.4), suggesting that cold activation of these genes is not regulated by VRN-1. Thus it appears that this VRN-1-mediated mechanism may play a role in the regulation of a specific subset of cold responsive genes.

**Allelic differences in VRN-1 are likely sufficient to explain differences in freezing tolerances previously assigned to a separate FR-1 locus**

In earlier studies, differences in freezing tolerance mapped to the VRN-1 region were considered to be the result of a closely linked gene designated FR-1. However, only two studies have reported recombination between FR-1 and VRN-1, and they differ in the relative position of these two genes, with FR-1 distal to VRN-1 in the initial mapping studies (Galiba et al. 1995, 1997) and proximal to VRN-1 in a later mapping study using deletion lines (Sutka et al., 1999). Although the differences in freezing tolerance across the deletion lines used in the latter study were clear, it is still possible that the reduced freezing tolerance observed in the larger deletion used to map FR-1 to a proximal deletion bin than VRN-1, was the result of the loss of a larger number of genes and an overall reduction in plant vigor rather than the effect of a single FR-1 gene. It is also possible that simultaneous segregation at the linked FR-2 locus, which was not known at the time of these two studies, affected the mapping results.
The improved freezing tolerance and higher transcript levels of *CBF* and *COR* genes in the *mvp* mutants suggests that *VRN-1* allelic differences are likely sufficient to explain differences in freezing tolerance previously considered to be the result of a separate *FR-1* gene. Therefore, our results support the hypothesis that *FR-1* is a pleiotropic effect of *VRN-1* rather than a separate gene. This hypothesis is also supported by experiments showing that the repression of *VRN-1* by short days in spring wheat genotypes is associated with increased freezing tolerance (Limin et al., 2006), and that *VRN-1* transcript levels in the different Triple Dirk near-isogenic lines are inversely correlated with freezing tolerance (Koemel et al., 2004).

**VRN-1 transcription is not sufficient to promote the down-regulation of COR genes**

The experiment using the *T. monococcum* lines carrying the *VRN-1f* and *VRN-1g* alleles (Fig. 2.4) shows that up-regulation of *VRN-1* transcript levels under short days is insufficient to produce a significant down-regulation of *COR14b* as that observed under long days. Under short days, six-week old plants carrying these alleles show high transcript levels of *VRN-1* and a transition of the vegetative apex to the double ridge stage. However, under continuous short days spike development proceeds slowly and stems fail to elongate. Once plants are transferred to long days, genotypes having the *VRN-1f* and *VRN-1g* alleles complete their spike development faster and head earlier than genotypes with the wild-type *VRN-1* allele due to their more advanced developmental state (Dubcovsky et al., 2006).
When grown under long days, plants carrying the \textit{VRN-1f} and \textit{VRN-1g} alleles showed significantly lower levels of \textit{COR14b} than lines carrying the \textit{vrn-1} or \textit{Vrn-1h} alleles. These results were consistent across three independent accessions for each of the \textit{VRN-1} alleles supporting the hypothesis that the differences in \textit{COR14b} were associated to the differences in the \textit{VRN-1} alleles. However, no differences in \textit{COR14b} were observed among the same genotypes under short days despite large differences in \textit{VRN-1} transcript levels. These results suggest that the down-regulation of the \textit{COR14b} requires the presence of additional factors that are activated under long days and that require the expression of \textit{VRN-1}. Taken together, the \textit{mvp} mutant and \textit{VRN-1f} and \textit{VRN-1g} experiments suggest that \textit{VRN-1} expression is necessary but not sufficient to down-regulate several \textit{COR} genes and reduce freezing tolerance in the leaves of wheat plants.

A similar phenomenon has recently been described in Arabidopsis where the floral activator MADS-box gene \textit{SOC1} functions as a negative regulator of the cold response pathway through the direct repression of the \textit{CBF} genes (Seo et al., 2009). In Columbia wild type, \textit{SOC1} was expressed most strongly in leaves, but was also detected in vegetative apices, inflorescence, stems of flowering plants, and roots (Lee et al., 2000). A microarray experiment comparing 7-day-old seedlings of a \textit{soc1} knockout mutant and a \textit{SOC1} over-expressing line with wild type plants revealed that six \textit{COR} genes were amongst the 20 genes most negatively regulated in the \textit{SOC1} over-expressing line. In addition, the expression level of the three Arabidopsis \textit{CBF} genes increased in the \textit{soc1}
mutants and decreased in *SOC1* over-expression lines, without affecting the transcript levels of the *CBF* regulatory genes *ICE1, HOS1* or *ZAT12*. A chromatin immunoprecipitation (ChIP) experiment using a SOC1 antibody revealed that the CArG-box regions in the *CBF* promoters were enriched in the SOC1 over-expressing line relative to the *soc1* knockout, which suggests that SOC1 negatively regulates cold response through direct repression of the transcription of the *CBF* genes (Seo et al., 2009). It is interesting to point out here that the expression of the wheat homolog of Arabidopsis *SOC1, WSO1*, is not affected by vernalization or photoperiod, suggesting different functions in these two species (Shitsukawa et al. 2007b).

Although both *SOC1* in Arabidopsis and *VRN-1* in the temperate cereals seem to be associated with the down-regulation of the *CBF* and *COR* genes in the leaves, the effect of *VRN-1* on the *CBF* genes does not seem to be as direct as the effect of *SOC1* in Arabidopsis. The results from the experiments using *T. monococcum* accessions with differential expression of *VRN-1* under short days suggest that additional genes operating downstream of *VRN-1* and that are regulated by long-days are required to mediate the negative effect of *VRN-1* on freezing tolerance. The identification of these downstream genes and the understanding of their regulatory mechanisms could potentially lead to novel strategies to prevent the premature dampening of the cold acclimation pathway in environments where the premature activation of *VRN-1* may increase the risk of freezing damage.
Do temperate cereals respond differently to the same cool temperatures in the fall and the spring?

The system described above provides the temperate cereals with the ability to differentiate the same cool temperature in the fall and the spring. A cool temperature in the fall, when plants have low VRN-1 transcript levels, results in the induction of the CBF and the downstream COR genes initiating the acclimation of the plants to cold temperatures. This is essential in the fall, when cool temperatures are an indication of the approaching freezing temperatures of the winter. The same cool temperature in the spring, when VRN-1 transcript levels in the leaves increase significantly in response to lengthening photoperiod, would result in a significantly lower up-regulation of several CBF and COR genes. Since cool temperatures in spring are generally not a prelude of coming freezing temperatures, a robust up-regulation of the CBF pathway response in the spring would likely not be advantageous for plant survival.

A similar system seems to be operating in Arabidopsis. Arabidopsis soc1 null mutants show increased responsiveness of the CBF genes to cold and improved freezing tolerance suggesting that low levels of SOC1 transcripts during the fall may favor plant acclimation to cold temperatures (Seo et al., 2009). SOC1 transcript levels increase significantly by the time of the initiation of Arabidopsis flowering (Lee et al., 2000), indicating that high SOC1 transcript levels will be present in the leaves in the spring, down-regulating the CBF genes and their downstream COR targets.
The activation of the CBF regulon has a potentially high energetic cost to plants since numerous COR genes are up-regulated in the leaves by these transcription factors (Fowler and Thomashow, 2002). In addition, CBF genes have also been shown to repress plant growth (Achard et al. 2008). Therefore, the down-regulation of the CBF genes during the spring has a potential adaptative value, ensuring the plant’s rapid development under optimal conditions and may explain the presence of related systems in Arabidopsis and the temperate cereals.

Acknowledgments

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Table 2.1. Survival, relative conductivity and Fv/Fm in mutant plants (\textit{mvp-2/mvp-2= m/m}) and plants carrying at least one wild type \textit{VRN-1} allele (\textit{Mvp-2/- = M/-})

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Freezing Temperature</th>
<th></th>
<th>Factorial ANOVA (\textit{P} between Genotypes)</th>
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<tbody>
<tr>
<td></td>
<td>-9 °C</td>
<td>-11 °C</td>
<td></td>
</tr>
<tr>
<td>F$_v$/F$_m$</td>
<td>\textit{M/-}</td>
<td>\textit{m/m}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.56</td>
<td>0.78</td>
<td></td>
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<tr>
<td>Relative conductivity (%)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>\textit{M/-}</td>
<td>\textit{m/m}</td>
<td></td>
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<tr>
<td></td>
<td>12.0</td>
<td>10.1</td>
<td></td>
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<tr>
<td>No. of plants that regrew per total (%)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>\textit{M/-}</td>
<td>\textit{m/m}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0/50</td>
<td>26/30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0%)</td>
<td>(87%)</td>
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<tr>
<td></td>
<td>\textit{M/-}</td>
<td>\textit{m/m}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0/59</td>
<td>11/24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0%)</td>
<td>(46%)</td>
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</tr>
</tbody>
</table>

* All \textit{Mvp-2/-} plants failed to re-grow, therefore there is no variance within this class to perform an ANOVA. The differences between \textit{mvp-2/mvp-2} and \textit{Mvp-2/-} were obvious.

Sixty-day-old plants were acclimated at 10°C for 19 days, 4°C for 12 days, and -2 °C for 12 h before the freezing treatment. \textit{P} values correspond to factorial ANOVAs including temperature, genotype, and their interactions in the model. Experimental conditions are described in “Materials and Methods”.
Figure 2.1. qRT-PCR analysis of transcript levels of the CBF genes present at the FR-2 locus relative to ACTIN endogenous control. Samples were collected from leaves of 4-week old mvp-2/mvp-2 and Mvp-2/- plants (20°C) and again 1 d later at the same time following an 8-h cold treatment at 4°C. Values in the y axis were normalized and calibrated using the 2-ΔΔCT method (Livak and Schmittgen 2001). The same calibrator was used for all genes, so scales are comparable across genes. Values are averages of eight biological replications ± SE. The inset shows CBF14 transcript levels, which were significantly higher than the other genes at this locus. P values for the differences between mvp/mvp and Mvp/- after the cold treatment were calculated using ANOVA and are indicated by asterisks: * P < 0.05, **: P < 0.01.
Figure 2.2. qRT-PCR transcript levels of COR14b relative to TEF1 endogenous control. Plants were 4 weeks old at the beginning of the experiment and were exposed to 4 °C for 12 d. Values on the y axis were normalized and calibrated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Homozygous mvp-2/mvp-2 plants (null VRN-1) are indicated by black squares, and Mvp-2/- plants (one or two VRN-1 copies) by gray triangles and lines. Values are averages of eight biological replications in the untransformed scale ± SE. $P$ values were calculated using ANOVA of log(n+1)-transformed values for each time point: ** $P < 0.01$. 
Figure 2.3. Time courses of VRN-1, and COR genes COR14b, DHN5, and DHN8 transcripts in mvp-2/ mvp-2 plants homozygous for a VRN-1 deletion (m) and Mvp-2/- plants with one or two functional VRN-1 copies (M). The presence of a faint VRN-1 hybridization in the 87 days mutant sample is suspected to be due to cross-contamination.
Figure 2.4. *VRN-1* and *COR14b* transcript levels in a set of *T. monococcum* lines differing in *VRN-1* expression under short days. Abbreviations are as follows: W, warm conditions; C, decrease from 18°C to 6°C (occurring at daybreak); 2W, 2 weeks old; 6W, 6 weeks old; SD, short day; LD, long day; *vrn-1*, wild type; *Vrn-A1f*, allele with 1-bp deletion in the CArG box coupled with *VRN-1* intron 1 insertion; *Vrn-A1g*, allele with a 34-bp deletion encompassing the CArG box, *Vrn-A1h*, allele with an insertion in *VRN-1* intron 1. The three accessions with the *Vrn-1f* allele carry the dominant *Vrn-A2* allele, whereas all the other accessions carry nonfunctional *vrn-2* alleles. All the accessions have a spring growth habit. A, mRNA-blot analyses of three genotypes per promoter class (indicated by different PI numbers). Arrows and arrowheads identify the presence and absence, respectively, of *COR14b* transcripts in 6-week-old plants in the *Vrn-1f* and *Vrn-1g* natural mutants under short days and long days. B and C, qRT-PCR validation of *VRN-1* and *COR14b* transcript levels relative to *ACTIN* endogenous control at 4°C. Values in the y axes were normalized and calibrated using the 2^ΔΔCT method (Livak and Schmittgen 2001). Lines carrying the wild-type allele (*vrn-1*) or the *Vrn-1h* (spring, not induced in short days) are indicated by black bars, and lines carrying the *Vrn-1f* and *Vrn-1g* alleles (spring, induced in short days) are indicated by gray bars. Values are averages of five biological replications ± SE. *P* values were calculated using contrasts between either *vrn-1* or *Vrn-1h* and the average of the lines carrying the *Vrn-1f* and *Vrn-1g* alleles. Samples were collected when the plants were 2 weeks old (B) and 6 weeks old (C).
Chapter 3: Pedigree analysis of the spring barley ‘Tremois’ reveals a historical origin of its $CBF2$, $CBF4$, and $CBF13$ alleles and of spring growth habit

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Abstract

$FROST\ RESISTANCE-H1$ ($FR-H1$) and $FR-H2$ loci affect winter hardiness in barley ($Hordeum\ vulgare$). The gene underlying $FR-H1$ is thought to be $VRN-H1$ that affects freezing tolerance and flowering time of the Triticeae cereals. $FR-H2$ is coincident with a cluster of $C-repeat\ Binding\ Factor$ ($CBF$) genes affecting low-temperature tolerance. While $FR-H1$ and $FR-H2$ loci are identified in crosses between winter and spring genotypes, sequence variation at these loci also occurs among spring barleys. At $FR-H2$, the spring barley ‘Tremois’ harbors sequence polymorphisms in $CBF2$, $CBF4$, and $CBF13$ genes relative to the spring barley ‘Morex’. At $FR-H1$, while large deletions are frequent in the $VRN-H1$ intron 1 of spring genotypes such as the 5.2 kb deletion in
‘Morex’, ‘Tremois’ harbors only a 38 bp deletion. *VRN-H1* promoter also harbors sequence variation that differentiates the two spring barleys. To determine the origin of the ‘Tremois’ *CBF* and *VRN-H1* alleles, we genotyped these genes in 67 accessions ancestral to ‘Tremois’. Results indicate that the ‘Tremois’ *CBF2, CBF4*, and *CBF13* alleles existed in some of the accessions that are about 100 years old. ‘Tremois’ *VRN-H1* promoter and intron 1 sequence occurred in a recent ancestor of ‘Tremois’. Phenotyping for flowering time reveals that although the spring *VRN-H1* intron 1 allele is perfectly correlated with flowering time, this is not the case in 45% of the accessions harboring the winter *VRN-H1* intron 1 allele. Overall, inheritance patterns in the ‘Tremois’ pedigree suggest that the *CBF*s and *VRN-H1* may have been co-selected.

**Introduction**

Barley cultivation began about 8,500 years ago with its domestication in the Fertile Crescent and another domestication about 1,500-3,000 km east of this region (Morrell and Clegg, 2007). Barley accessions that were domesticated in the Fertile Crescent contribute to the European and American germplasm, while those domesticated in the east contribute towards the Central Asian and Eastern germplasm (Morrell and Clegg, 2007). All cultivated barleys (*H. vulgare* subsp. *vulgare*) have arisen from their wild ancestors (*H. vulgare* subsp. *spontaneum*) (Badr et al., 2000). Wild barleys are of winter growth habit, whereas cultivated barleys are of winter or spring habit (Takahashi et al., 1963; von Bothmer et al., 2003). Due to its prevalence in wild barleys, winter habit is
believed to be the ancestral state (Flood and Halloran, 1986; Halloran, 1967; Kihara, 1958).

The loss of vernalization requirement and a decrease in the freezing tolerance levels in spring genotypes suggests a co-selection of these phenotypes during domestication. Vernalization refers to a prolonged exposure to low temperatures required for flowering, and is primarily under the control of _Vernalization-1 (VRN-1)_ (Yan et al., 2003). Sequence variation in the _VRN-1_ intron 1 usually discriminates winter and spring growth habits. _VRN-1_ intron 1 in winter genotypes is usually an approximately 10-11 kb sequence, whereas spring genotypes mostly carry intron 1 deletions spanning several kilobases in length (Fu et al., 2005; von Zitzewitz et al., 2005). Alignments of _VRN-1_ intron 1 sequences from winter and spring genotypes of barley and wheat reveal a minimal 2.8 kb “vernalization critical region” that is present in winter genotypes, but is deleted from spring genotypes (von Zitzewitz et al., 2005). This sequence variation affects the expression pattern of _VRN-1_ such that the expression of winter _VRN-1_ allele is repressed until the plants are vernalized, but that of the spring allele (intron 1 deletions) is constitutive. Sequence variation in the _VRN-1_ promoter in the form of single nucleotide polymorphisms (SNPs), insertions-deletions (InDels), and simple sequence repeats (SSRs) is also associated with growth habit, although no known regulatory motifs are identified within this variable sequence (Fu et al., 2005; von Zitzewitz et al., 2005; Yan et al., 2003).
In addition to the effect of vernalization on expression of winter VRN-1 allele, its expression is also affected by VRN-2, VRN-3, and Photoperiod-1 (PPD-1) (Turner et al., 2005; Laurie et al., 1995; Yan et al., 2004). The genes underlying VRN-H2 in barley are three ZCCT (zinc-finger CCT domain) transcription factors – ZCCT-Ha, ZCCT-Hb, and ZCCT-Hc (Dubcovsky et al., 2005), while the genes underlying VRN-H3 and PPD-H1 are the FLOWERING LOCUS T (FT-1) and the pseudo-response regulator (PRR) orthologous to PRR7 of Arabidopsis, respectively (Faure et al., 2007; Turner et al., 2005; Yan et al., 2006). Under LDs, VRN-H2 indirectly represses the expression of VRN-H1, whereas VRN-H3 and PPD-H1 upregulate VRN-H1 expression (Distelfeld et al., 2009). Thus, VRN-H2 represses flowering whereas VRN-H3 and PPD-H1 promote flowering.

VRN-1 is thought to underlie the freezing tolerance locus, FROST RESISTANCE-1 (FR-1) on the long arm of group 5 chromosome homoeologs in wheat and barley (Francia et al., 2004; Hayes et al., 1993; Vágújfalvi et al., 2003). The pleiotropic effects of FR-1/VRN-1 on freezing tolerance are due to the repression of VRN-1 on the genes at FR-2, located ~30 cM distal to FR-1 (Dhillon et al., 2010; Stockinger et al., 2007). FR-2 is coincident with a cluster of about 13 C-repeat Binding Factor (CBF) genes. CBFs act as transcriptional activators of a battery of downstream target genes comprising the CBF regulon involved in enhancing the freezing tolerance of the plant (Stockinger et al., 1997; Thomashow 1999). These CBFs span a genetic distance of approximately 0.8 cM and a physical distance of approximately 1 Mb (Francia et al., 2004, 2007; Knox et al., 2008, 2010; Miller et al., 2006; Skinner et al., 2005). Seven CBF genes are positioned
genetically such that $CBF2B$ is about 0.2 cM proximal to the $CBF2A$–$CBF4B$ genomic region and $CBF13$ is about 0.35 cM distal to the $CBF2A$–$CBF4B$ region (Francia et al., 2007). $CBF12$ lies 0.03 cM proximal to $CBF13$, and $CBF3A$, $CBF10B$, and $CBF6$ are linearly arranged at 0.08–1.0 cM intervals distal to $CBF13$ (Francia et al., 2007).

Sequencing collinear regions of $FR-H2$ in two winter hardy barleys ‘Nure’ and ‘Dicktoo’, and two spring barleys ‘Morex’ and ‘Tremois’ reveal greater copy numbers of functional $CBF$ genes in winter barleys than in spring barleys (Knox et al., 2010). Increases in $CBF$ gene copy numbers in winter barleys result from the presence of two sequence paralogs of certain $CBFs$ such as $CBF2A$ and $CBF2B$, $CBF10A$ and $CBF10B$, $CBF12A$ and $CBF12B$, and $CBF15A$ and $CBF15B$, one of which is absent in spring genotypes (Knox et al., 2010). Greater $CBF$ copy numbers in winter genotypes also result from tandem duplications of the $CBF$-containing genomic regions that are absent in spring barleys (Knox et al., 2010). For example, winter genotypes harbor at least two in-tandem repeats of a 22 kb genomic segment encompassing $CBF2A$ and $CBF4B$, whereas a single copy of this genomic region exists in the spring genotypes (Knox et al., 2010). Another notable difference is the presence of a functional $CBF13$ gene in the winter genotypes that exists as a pseudogene in the two spring genotypes (Knox et al., 2010). Overall, winter genotypes have greater number of functional $CBF$ genes than spring genotypes.

In addition to the differences between winter and spring genotypes, differences at $FR-H2$ also exist between the spring barley genotypes, ‘Morex’ and ‘Tremois’. The $CBF2$ in
‘Morex’ is 98% similar to the $CBF2A$ paralog of winter barleys, whereas the $CBF2$ in ‘Tremois’ is similar to $CBF2B$ in its 5’ region and to $CBF2A$ in its 3’ region suggesting that it may be a fusion between the $CBF2B$ and $CBF2A$ paralogs (Knox et al., 2010). The $CBF13$ pseudogenes in ‘Morex’ and ‘Tremois’ are substantially different from each other, suggesting that mutations in the $CBF13$ gene sequence occurred independently in the lineages leading to the two spring genotypes. Although a minor difference, $CBF4$ in ‘Tremois’ is also distinct from that in ‘Morex’ due to single nucleotide polymorphisms (SNPs) in its 3’ region (Knox et al., 2010).

‘Tremois’ also differs from ‘Morex’ in its $VRN-H1$ intron 1 and promoter sequence (von Zitzewitz et al., 2005). While the $VRN-H1$ intron 1 in ‘Morex’ is deleted for 5.2 kb spanning the vernalization critical region, ‘Tremois’ carries no deletion in this region. Moreover ‘Tremois’ is 100% identical in its nucleotide sequence in the first 0.44 kb of this vernalization critical region that is conserved across the winter genotypes of wheat and barley. The one deletion identified in ‘Tremois’ $VRN-H1$ intron 1 is that of 38 bp located just upstream of the critical region (von Zitzewitz et al., 2005). Of this 38 bp, 12 bp are unique to spring barleys and lie within a MITE (Miniature Inverted repeat Terminal Element) sequence that is present only in barley genotypes. In the $VRN-H1$ promoter, some of the polymorphisms in ‘Tremois’ are shared with spring barleys and others with winter barleys (von Zitzewitz et al., 2005). This $VRN-H1$ promoter–intron 1 haplotype of ‘Tremois’ is unique among a collection of ten other winter and spring
barleys that have been frequently used as parents of mapping populations (von Zitzewitz et al., 2005).

The existence of ‘Tremois’ CBF2, CBF4, CBF13, and VRN-H1 promoter and intron 1 alleles raises several questions. When in time did these alleles appear in the ‘Tremois’ lineage? Did they appear as a result of introgressions or accumulation of mutations? Did they emerge together or separately? Did the ‘Tremois’ VRN-H1 promoter–intron 1 haplotype cosegregate with spring growth habit in its pedigree? To answer these questions, we conducted a pedigree analysis of ‘Tremois’. Ancestors of ‘Tremois’ were genotyped for the CBF, VRN, and PPD-H1 loci and phenotyped for flowering time. Results reveal that the ‘Tremois’ CBF alleles existed in some of the earliest accessions in its pedigree. The ‘Tremois’ VRN-H1 promoter–intron 1 haplotype occurred in only one ancestral genotype. Phenotyping for flowering time revealed that spring VRN-H1 promoter–intron 1 haplotypes (‘Tremois’ and ‘Morex’) are always associated with spring growth habit. However, flowering time in nine of 20 accessions harboring winter VRN-H1 intron 1 allele deviated from expectations based on their VRN-H2–PPD-H1 haplotype.

Materials and methods

Plant Material

A list of the accessions used in this study (N=71; 67 ‘Tremois’ ancestors and 4 known standards) is provided in Table C.1 along with their horticultural classification, row-type,
growth habit, pedigree, and location of collection or development. All accessions and
relevant information were obtained from the National Plant Germplasm System (NPGS,
http://www.ars-grin.gov/npgs/index.html) unless otherwise noted. NPGS records indicate
the oldest accessions to be about a 100 years old. Seed increased by single seed descent
was used for all analyses.

‘Tremois’ pedigree construction
To construct a pedigree of ‘Tremois’, genotypes in its pedigree were identified beginning
with its parents Dram / Aramir // Berac (Francia et al., 2004; correction from ‘Berar’ to
and ‘Berac’ were obtained from the NPGS, which were then used to identify accessions
in the previous generation. This process was reiterated to the earliest generation for which
written records were available in the NPGS. Seventy-four genotypes were identified in
the ‘Tremois’ pedigree, which were assembled in a tree to display the relation of the
individual genotypes with each other and with ‘Tremois’ (Fig. 3.1).

PCRs
Genomic DNAs were isolated from leaf tissue using DNeasy Plant Mini kit (QIAGEN,
www.qiagen.com). Primers were designed using Primer 3 (http://frodo.wi.mit.edu) (Table
C.2). PCRs were carried out in a 25 µl volume using 10-30 ng DNA, Taq polymerase, 1X
Taq buffer, 2.5 mM dNTPs, 0.2 µM forward and reverse primers, and 1.25% DMSO, or
in a 12.5 µl volume using Go Taq® Green Master Mix (Promega, www.promega.com).
PCR conditions included an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 56°C for 30 seconds, and extension at 72°C for 30 s/500 bp, followed by a final extension at 72°C for 5 minutes. ‘Dicktoo’, ‘Nure’, ‘Morex’, and ‘Tremois’ alleles of all the genes were used as known variants for the PCRs and genotyping assays.

**Genotyping CBFs, VRN-H1, VRN-H2, and PPD-H1**

The *CBF* genes were genotyped using cleaved amplified polymorphic sequence (CAPS) markers that were designed using the CAPS Designer tool (http://solgenomics.net/tools/caps_designer/caps_input.pl) (Table C.2). A *SacII* CAPS marker was used to determine if the two *CBF2* paralogs (*CBF2A* and *CBF2B*) occurred as independent sequences or as the *CBF2B/A* fusion, while an *MluI* CAPS assay was used to further discriminate between *CBF2A* and *CBF2B*. *CBF2* allele state was assigned as ‘Dicktoo’-‘Nure’ (DN) if *CBF2A* and *CBF2B* were both present as independent sequences, as ‘Tremois’ (Tr) if they existed as the *CBF2B/A* fusion, and as ‘Morex’ (Mx) if only *CBF2A* was present. *CBF4* was genotyped using a *HindIII* CAPS assay that discriminated a winter (DN) allele from a spring (Mx/Tr) allele and a *BglII* CAPS assay to differentiate the ‘Mx’ *CBF4* from ‘Tr’ *CBF4*. Lastly, *CBF13* alleles were genotyped using a *SalI* CAPS marker that discriminated between winter (DN) and spring (Mx/Tr) alleles, and a *BglII* CAPS that distinguished the ‘Tr’ *CBF13* from all other alleles. Digested PCR products were resolved on 1X TBE-3% agarose gels.
The *VRN-H1* promoter and intron 1 alleles were differentiated using differences in PCR product sizes (Table C.2). *VRN-H1* promoter was assigned a winter (DN) or spring allele using a 5 bp indel diagnostic for growth habit (von Zitzewitz et al., 2005). The spring promoter allele was further differentiated as ‘Mx’ or ‘Tr’ using a *Bgl*I CAPS marker. *VRN-H1* intron 1 was genotyped using PCR primers spanning the deletion within the barley-specific MITE (Miniature Inverted-repeat Transposable Element) (von Zitzewitz et al., 2005). This region was selected because it allowed us to resolve the ‘Tremois’ allele as being distinct from other known spring alleles and also enabled us to differentiate between winter and facultative alleles (‘Nure’ vs. ‘Dicktoo’; von Zitzewitz et al., 2005). The intron 1 allele was assigned as ‘Dt’ (no deletion; 336 bp), ‘Nu’ (218 bp), ‘Tr’ (298 bp), or ‘Mx’ (null; because the primer sites reside within a larger deletion) (Table C.2). *VRN-H1* promoter and intron 1 PCR products were resolved on 1X TBE-24% polyacrylamide gels.

*VRN-H2* was genotyped for the presence or absence of three genes, *ZCCT-Ha*, *ZCCT-Hb*, and *ZCCT-Hc* (Table C.2) (Dubcovsky et al., 2005). PCR products were resolved on 1X TBE-2% agarose gels.

*PPD-H1* was genotyped using a *BstUI* CAPS marker developed for SNP22, a Gly-to-Trp change in the CCT domain spanning exon 8 (Table C.2) (Turner et al., 2005).
**Growth habit determination**

Of the 74 genotypes identified in the ‘Tremois’ pedigree, seed of 67 were available from the NPGS, some of which (25 total) shared common names but had distinct Plant Introduction (PI) or Cereal Investigations Hordeum (CIho) numbers. All 67 genotypes were sown on May 27, 2012 and grown in the greenhouse under natural long days (day length on May 27: 14 h 50 mins) using supplemental lighting. ‘Dicktoo’, ‘Morex’, ‘Nure’, and ‘Tremois’ were included as standards for growth habit. Time of heading was recorded for all genotypes until 120 days after sowing.

**Results**

*‘Tremois’ pedigree tree*

Seventy-four accessions ancestral to ‘Tremois’ were identified using reiterative process of identifying the parents of a genotype using its pedigree information available at the NPGS. The pedigree depicts the relationship of the accessions with each other and with ‘Tremois’ (Fig. 3.1). Majority of the accessions are European, while fewer are from USA, Africa, Australia, and Asia (Table C.1). Archer-1 is the oldest accession among all accessions identified in the ‘Tremois’ pedigree; its seed was received from the National Plant Germplasm System (NPGS) in 1914. Other accessions including Algerian-1, Abacus-1, and Imperial-1 were received by the NPGS in 1915, 1919, and 1924, respectively. All of these accessions were received landraces or of unknown horticultural classification (Table C.1). Archer and Algerian are documented to be the first landraces used for cultivar improvement (Fischbeck, 2003). Among the most recent ancestors,
‘Dram’ was released as a cultivar in 1974 (Russell et al., 2000), ‘Aramir’ and ‘Berac’ were developed in 1972 and 1969, respectively (NPGS). Thus, the traceable ancestry of ‘Tremois’ spans a period of about 60 years.

‘Tremois’ CBF2, CBF4, and CBF13 alleles arose early in its pedigree

To score CBF2, CBF4, and CBF13 alleles in accessions of the ‘Tremois’ pedigree, we developed CAPS assays (Table C.2). A schematic of known allelic variants used as standards for genotyping ‘Tremois’ ancestors is presented in Fig. 3.2 (a-c).

Of the available 67 accessions in the pedigree, 17 harbored ‘Tremois’ (T) alleles at all three CBFs (Table 3.1; Fig. 3.1). These included some of the most ancestral accessions such as the three Archer accessions, ‘Heils Franken’, and Australische Fruhe, as well as some of the most recent ancestors including ‘Dram’ and ‘Berac’ (Table 3.1; Fig. 3.1). ‘Berac’ however may be a seed mix-up, as its CBF alleles do not resemble either one of its parents (Fig. 3.1). Eight additional accessions carried ‘Tremois’ alleles at one or two CBFs (Table 3.1; Fig. 3.1). For example, Laevigatum and Algerian-1 harbored ‘Tremois’ alleles at CBF2 and CBF13, Algerian-2, Algerian-3, and Algerian-4 carried ‘Tremois’ alleles only at CBF2, while Imperial-1, ‘Volla’, and ‘Aramir’ carried ‘Tremois’ alleles only at CBF4 (Table 3.1; Fig. 3.1).

Among the 42 accessions that did not possess a ‘Tremois’ allele at any of the three CBFs assayed, 35 harbored ‘Morex’ (M) alleles at CBF2 and CBF13, and a ‘Dicktoo’–‘Nure’
(DN) allele at $CBF4$ (Table 3.1). The remaining 7 accessions might harbor novel alleles at $CBF4$ or at $CBF13$ as they could not be PCR amplified with the primer pairs used. None of the accessions harbored the ‘Morex’ allele at $CBF4$. All accessions harbored one of the three known allelic variants at $CBF2$ (DN, M, or T); no genotype harbored $CBF2B$ alone.

Overall, the existence of ‘Tremois’ $CBF2$, $CBF4$, and $CBF13$ alleles in some of the earliest accessions such as Archer-1 (dates back to 1914), Algerian-1 (1915), and Imperial-1 (1924) suggests that these alleles existed in the germplasm pool at least 100 years ago. Because of these accessions were from diverse geographical locations and did not appear to be related-by-descent, it could not be ascertained whether the ‘Tremois’ alleles of all three $CBF$s appeared together or separately.

‘Tremois’ $VRN\text{-}H1$ promoter–intron 1 haplotype occurred in a single and recent ancestor of ‘Tremois’

To determine the frequency with which the ‘Tremois’ $VRN\text{-}H1$ promoter–intron 1 haplotype occurred in the lineage leading to ‘Tremois’, we genotyped accessions in its pedigree using CAPS and size-based markers (Table C.2). The alleles used as standards for genotyping $VRN\text{-}H1$ promoter and intron 1 in ‘Tremois’ ancestors are listed in Table 3.2 and depicted in Fig. 3.2d-e.
Of the 67 accessions in the pedigree, ‘Tremois’ VRN-H1 promoter–intron 1 haplotype occurred only in ‘Dram’, an immediate ancestor of ‘Tremois’ (Table 3.1; Fig. 3.1).

‘Morex’ VRN-H1 promoter–intron 1 haplotype occurred in 45 accessions including the most ancestral accessions (Table 3.1; Fig. 3.1). Of the remaining 21 accessions, 7 carried a winter VRN-H1 promoter–intron 1 haplotype, while 14 possessed a winter VRN-H1 intron 1 together with a ‘Morex’ allele at VRN-H1 promoter (Table 3.1; Fig. 3.1).

Taken together, these data indicate ‘Dram’ as the source of ‘Tremois’ VRN-H1 promoter–intron 1 haplotype.

*Spring VRN-H1 promoter–intron 1 haplotype is always associated with spring growth habit*

Deletions in the intron 1 of VRN-H1 and mutations in its promoter are associated with spring growth habit (Fu et al., 2005; von Zitzewitz et al., 2005). Accessions of the ‘Tremois’ pedigree were scored for time to flowering under natural long day conditions to test the association between the VRN-H1 promoter–intron 1 haplotype and growth habit. Of the 61 accessions that were phenotyped for flowering time, 52 flowered in less than 60 days, 6 flowered in 60-90 days, and 3 did not flower by the time the experiment was terminated at 120 days (Table 3.3).

Flowering time of various accessions was then compared to their VRN-H1 promoter–intron 1 haplotype. Of the 52 accessions flowering in <60 days, 40 harbored the ‘Morex’
VRN-H1 promoter–intron 1 haplotype and one (‘Dram’) harbored the ‘Tremois’ VRN-H1 haplotype (Table 3.3). Of the remaining 11 accessions, 3 were of winter VRN-H1 promoter–intron 1 haplotype and 8 possessed a ‘Morex’ promoter with a winter VRN-H1 intron 1 allele (Table 3.3). The three accessions that did not flower by 120 days, ‘Lignee 125’, ‘Bordia’, and ‘Lyallpur’, harbored the winter VRN-H1 promoter–intron 1 haplotype (Table 3.3). One of the six accessions that flowered within 60-90 days after sowing, Abacus-1, harbored the winter VRN-H1 promoter–winter intron 1 haplotype (Table 3.3). The other five accessions were of the ‘Morex’ VRN-H1 promoter–winter VRN-H1 intron 1 (Table 3.3).

In sum, all accessions of the ‘Tremois’ or ‘Morex’ VRN-H1 haplotype behaved as spring genotypes (flowering time <60 days). However, early flowering (< 90 days) in 17 of 20 accessions harboring winter VRN-H1 intron 1 was unexpected.

**Flowering time of winter VRN-H1 genotypes is largely explained by VRN-H2 and PPD-H1 alleles**

Of the 20 accessions harboring the winter VRN-H1 intron 1 allele (D or N), only three behaved as true winter types (no flowering until 120 days). As allelic variation at VRN-H2, VRN-H3, and PPD-H1 is known to affect flowering time, we genotyped all accessions for these loci. However, the marker used for genotyping VRN-H3 did not discriminate between the known winter and spring alleles, because of this it was excluded from the analysis. Data on the allelic status of VRN-H2 and PPD-H1 in all ancestors of
‘Tremois’ is shown in Table 3.1. VRN-H2 and PPD-H1 alleles are identified based on the known standards (Table 3.2; Fig. 3.2). Table 3.4 parses out the VRN-H2 and PPD-H1 allele data for the 17 accessions harboring the winter VRN-H1 intron 1 allele that flowered in <90 days after sowing.

VRN-H2 is comprised of three linked ZCCT genes that are essential for winter growth habit (Fig. 3.2) (Dubcovsky et al., 2005). In all accessions of the ‘Tremois’ ancestral tree, all three ZCCT genes were either present or absent (Table 3.1). Among the 17 accessions harboring the winter vrn-H1 intron 1 allele, the ZCCT genes were deleted in ten (vrn-H2), and present in the remaining seven (Vrn-H2) (Table 3.4). At PPD-H1, the LD-responsive allele (Ppd-H1) was present in 9 of 17 accessions, and the photoperiod-insensitive allele (ppd-H1) occurred in the remaining 8 accessions (Table 3.4).

Overall, the combination of VRN-H2 and PPD-H1 alleles in winter VRN-H1 background explained early flowering in 8 of these 17 accessions. However, early flowering in the nine accessions deviated from expectations based on their VRN-H1–VRN-H2–PPD-H1 haplotype.

Discussion

‘Tremois’ alleles of CBF2, CBF4, and CBF13 are distinct from those of ‘Morex’ (Knox et al., 2010). Additionally, its VRN-H1 promoter and intron 1 sequences are more similar to those of winter barleys than spring barleys (von Zitzewitz et al., 2005). We
investigated the origin of the ‘Tremois’ CBF and VRN-H1 alleles in the ‘Tremois’ ancestry and determined the association of VRN-H1 allele with flowering time.

The ‘Tremois’ alleles of CBF2, CBF4, and CBF13 existed together or separately in 37% of the accessions in the ‘Tremois’ pedigree. These included some of the most ancestral accessions such as all four accessions of Algerian, three accessions of Archer, Imperial-1, ‘Heils Franken’, and Australische Fruhe (Fig. 3.1; Fischbeck, 2003). NPGS records indicate that Archer-1, Algerian-1, and Imperial-1 exist since early the 1900s, suggesting that the ‘Tremois’ CBF alleles are at least about a hundred years old. The CBF haplotype of Archer accessions perpetuated in the ‘Tremois’ pedigree via ‘Prentice’ and that of ‘Heils Franken’ and Australische Fruhe via Morgenrot-2, whereas the CBF haplotypes of Algerian accessions, Imperial-1 and Laevigatum were not inherited by their progenies (Fig. 3.1).

Among the CBFs, a strong allelic association was apparent between CBF2 and CBF13. ‘Tremois’ CBF13 always co-occurred with a ‘Tremois’ CBF2, and not in combination with a ‘Morex’ or a ‘Dicktoo’–‘Nure’ (DN) CBF2 (Table 3.1). Additionally, alleles of both CBF2 and CBF13 were either winter or spring; a winter allele of one gene did not co-exist with a spring allele of the other (Table 3.1). However, no such allelic association was observed for CBF4. This may be because the allelic variation between spring and winter alleles of CBF4 is not biologically relevant. In contrast, the allele types of CBF2 and potentially CBF13 may be associated with their relative transcript levels, although
CBF13 transcripts have not been detected (Badawi et al., 2007; Knox et al., 2010; Stockinger et al., 2007).

‘Tremois’ alleles of CBF2, CBF4, and CBF13 occurred in its two immediate ancestors, ‘Dram’ and ‘Berac’ (Fig. 3.1). ‘Dram’ may have inherited these CBF alleles from Morgenrot-2 via ‘Tern’ (Fig. 3.1). However, the CBF alleles of ‘Berac’ are completely dissimilar to its immediate parents, ‘Balder’ and ‘Erika’, and to its ancestors in several previous generations until ‘Zephyr’ (Fig. 3.1). This suggests that the accession of ‘Berac’ genotyped in this study may not be the same identical line developed by the breeder, but a single plant selection from its population that may have been segregating at the time.

‘Tremois’ alleles of VRN-H1 promoter and intron 1 occurred only in ‘Dram’, which was released as a spring cultivar in 1974 (Russell et al., 2000). ‘Dram’ is the progeny of Tern/RMGH 61229 (Table C.1). It is possible that the ‘Tremois’ VRN-H1 promoter–intron 1 haplotype pre-existed in RMGH 61229 and its ancestry. The other possibility is that ‘Dram’ derived its VRN-H1 haplotype from ‘Tern’ as a result of simultaneous mutations in its promoter (a SNP) and intron 1 (a 38 bp deletion). This ambiguity can be resolved if the VRN-H1 genotype of RMGH 61229 is known. However RMGH 61229 is unavailable from the NPGS and other European barley catalogues and its pedigree is also unknown. Because of these reasons the origin of the ‘Tremois’ VRN-H1 is currently uncertain. Nonetheless, ‘Tremois’ most likely inherited its CBF and VRN-H1 alleles from ‘Dram’. The presence of VRN-H2 in ‘Dram’ but not in ‘Tremois’ suggests that either ‘Tremois’
inherited the \textit{VRN-H2} deletion from ‘Aramir’ or ‘Berac’ or was deleted for \textit{VRN-H2} after its inheritance from ‘Dram’. The allelic status of \textit{PPD-H1} in ‘Tremois’ and its immediate ancestors was invariable.

In general, the three \textit{CBF}s and \textit{VRN-H1} appeared to be inherited as one haplotype (Fig. 3.1). Although recombination between these genes may seem to be absent in cases where the alleles were similar in both parents, it was clearly absent in the pedigrees of ‘Carlsberg’ (Prentice / Maja), ‘Pirol’ (WMI / Morgenrot), and ‘Vada’ (Laevigatum / Gull) (Fig. 3.1; Table 3.1). This was unexpected given the previous reports of recombination between these three \textit{CBF}s and between the \textit{CBF}s and \textit{VRN-H1} (Francia et al., 2004, 2007). It is likely that absence of recombination signifies a selection against the recombinants harboring alleles of contrasting growth habits at the \textit{CBF}s and \textit{VRN-H1} intron 1. This would also explain the loss of the \textit{CBF–VRN-H1} haplotype of all four Algerian accessions and of Laevigatum from the ‘Tremois’ ancestry.

Because winter growth habit is suggested to be ancestral to spring habit (Flood and Halloran, 1986; von Zitzewitz et al., 2005), the allelic combination occurring at \textit{CBF2}, \textit{CBF13}, and the \textit{VRN-H1} promoter and intron 1 suggest a temporal history in the conversion of winter to spring alleles. Spring alleles of \textit{CBF2} and \textit{CBF13} co-occurred with winter alleles at both \textit{VRN-H1} promoter and intron 1 in three accessions and with winter \textit{VRN-H1} intron 1 alone in 13 accessions (Table 3.1). In contrast, winter alleles of \textit{CBF2} and \textit{CBF13} or of \textit{VRN-H1} promoter did not co-occur with the spring \textit{VRN-H1}
intron 1 allele (Table 3.1). This pattern is consistent with the conversion of winter to spring alleles first for the CBFs, followed by VRN-H1 promoter and later by VRN-H1 intron 1. In this scenario, the ‘Trefois’ CBF2B/A allele may have arisen via non-allelic homologous recombination between the CBF2B and CBF2A paralogs of a winter barley. This hypothesis may be tested by carrying out sequence alignments of the region spanning CBF2B/A of ‘Trefois’ and its ancestors against the region spanning the CBF2B–CBF2A of winter genotypes beginning from the RAPT-I sequence upstream of CBF2B until the Grx-like sequence downstream of CBF2A (Knox et al., 2010). RAPT-I and Grx-like are collinear between winter and spring barleys (Knox et al., 2010). Microhomologies within this sequence would be indicative of breakpoints supporting NAHR. This methodology is frequently used to identify the molecular mechanisms underlying genomic disorders in humans (Liu et al., 2011).

Flowering time was affected by VRN-H2 and PPD-H1 in 20 genotypes harboring the winter VRN-H1 intron 1 allele. Among these 20 accessions, flowering time of 11 was consistent with their VRN-H2–PPD-H1 haplotype and with published reports (Table 3.4) (Hemming et al., 2008; Turner et al., 2005). However, in the remaining nine accessions flowering occurred much earlier than predicted by the markers used to genotype these individuals. It is less likely that this phenotype is due to the early flowering allele at VRN-H3, as its effect would be masked by VRN-H2 (Vrn-H2 allele) or not be realized in genotypes that are deleted for VRN-H2 and harbor the photoperiod-insensitive allele of PPD-H1 (vrn-H2–ppd-H1) (Distelfeld et al., 2009; Hemming et al., 2008; Trevaskis et
al., 2007; Yan et al., 2006). It is possible that these accessions whose \textit{VRN-H1} intron 1 is ‘Dicktoo’-like in the MITE sequence (no deletion) harbor deletions in the 2.8 kb critical region. Although such a haplotype of intron 1 is reported, it is not associated with spring growth habit (von Zitzewitz et al., 2005). Whether there is additional untapped genetic variation controlling flowering time may be worth exploring using the nine unexpectedly early flowering accessions.

This study also underscores the importance of providing the specific ‘Plant Introduction’ (PI) or ‘Cereal Investigations Hordeum’ (CIho) numbers of accessions in scientific communications. Accessions identified with the same name but different PI or CIho numbers were genetically and phenotypically distinct (Table 3.1 and Table C.1). In some cases such as ‘Berac’, progenies did not resemble either parent at one or more loci. Other similar examples include ‘Drost’ (Maja / Kenia), ‘Proctor’ (Kenia / Plumage Archer), and ‘Armelle’ (Ceres / Clermont) (Fig. 3.1; Table 3.1). These examples possibly represent cases where the single plant selections actually used in making the cross were not the ones genotyped in this study. Thus, PI or CIho numbers may be a more reliable identifier for scientific use.

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Table 3.1. Allelic states of CBFs, VRN-H1, VRN-H2, and PPD-H1 in known allelic variants and ‘Tremois’ ancestors.

<table>
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<th>Genotype name</th>
<th>Accession number</th>
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<th>CBF4</th>
<th>CBF13</th>
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<th>VRN-H1 intron 1</th>
<th>VRN-H2</th>
<th>PPD-H1</th>
<th>Growth habit</th>
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<tr>
<th>Genotype name</th>
<th>Accession number</th>
<th>CBF2</th>
<th>CBF4</th>
<th>CBF13</th>
<th>VRN-H1 promoter</th>
<th>VRN-H1 intron I</th>
<th>VRN-H2</th>
<th>PPD-H1</th>
<th>Growth habit&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyra</td>
<td>PI 410863</td>
<td>M DN</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>–</td>
<td>NMT</td>
<td>S A S</td>
</tr>
<tr>
<td>Vada</td>
<td>Clho 11765</td>
<td>M DN</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>–</td>
<td>NMT</td>
<td>S A S</td>
</tr>
<tr>
<td>Volla</td>
<td>PI 280423</td>
<td>M T</td>
<td>Novel</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>–</td>
<td>NMT</td>
<td>S A S</td>
</tr>
<tr>
<td>WMI</td>
<td>Clho 11842</td>
<td>M DN</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>–</td>
<td>NMT</td>
<td>S A S</td>
</tr>
<tr>
<td>Zephyr</td>
<td>PI 339815</td>
<td>T T</td>
<td>T</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>–</td>
<td>NMT</td>
<td>S A S</td>
</tr>
</tbody>
</table>

<sup>a</sup> CBF, VRN-H1, and PPD-H1 alleles were assigned one of four known classes, ‘Dicktoo’ (D), ‘Nure’ (N), ‘Morex’ (M), and ‘Tremois’ (T), or as DN, MT, or NMT when the alleles of these genotypes were indistinguishable. CBF alleles were designated as novel if no PCR product was amplified upon multiple attempts. VRN-H2 was genotyped as ‘+’ or ‘−’ when all three linked ZCCT genes were present or absent, respectively.

<sup>b</sup> Shortened accession names are described in Table C.1.

<sup>c</sup> Predicted growth habit is based on VRN-H1–VRN-H2 haplotype. Observed growth habit represents flowering time categories based on the number of days elapsed between sowing and flowering: A) < 60 days; B) 60-90 days; C) did not occur until 120 days. Genotypes unavailable at the time of phenotyping are labeled n.a. Growth habit as annotated by NPGS is also listed for reference.
Table 3.2. Description of *VRN-H1*, *VRN-H2*, and *PPD-H1* alleles used to genotype 'Tremois' ancestors.

<table>
<thead>
<tr>
<th>Alleles</th>
<th><strong>VRN-H1</strong> promoter</th>
<th><strong>VRN-H1</strong> intron 1</th>
<th><strong>VRN-H2</strong></th>
<th><strong>PPD-H1</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Facultative</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dicktoo</td>
<td>No mutation (<em>vrn-H1</em>)</td>
<td>No deletion (<em>vrn-H1</em>)</td>
<td>Deleted (<em>vrn-H2</em>)</td>
<td>No mutation (<em>Ppd-H1</em>)</td>
</tr>
<tr>
<td><strong>Winter</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nure</td>
<td>No mutation (<em>vrn-H1</em>)</td>
<td>118 bp deletion within the MITE upstream of the critical region (<em>vrn-H1</em>)</td>
<td>Present (<em>Vrn-H2</em>)</td>
<td>Loss-of-function point mutation in exon 8 (<em>ppd-H1</em>)</td>
</tr>
<tr>
<td><strong>Spring</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morex</td>
<td>A 5 bp InDel and a SNP consistent with spring growth habit (<em>Vrn-H1</em>)</td>
<td>5.2 kb deletion spanning the critical region (<em>Vrn-H1</em>)</td>
<td>Deleted (<em>vrn-H2</em>)</td>
<td>Loss-of-function point mutation in exon 8 (<em>ppd-H1</em>)</td>
</tr>
<tr>
<td>Tremois</td>
<td>A 5 bp InDel consistent with spring growth habit and a SNP consistent with winter growth habit (<em>Vrn-H1</em>)</td>
<td>38 bp deletion within the MITE upstream of the critical region (<em>Vrn-H1</em>)</td>
<td>Deleted (<em>vrn-H2</em>)</td>
<td>Loss-of-function point mutation in exon 8 (<em>ppd-H1</em>)</td>
</tr>
</tbody>
</table>
Table 3.3. Grouping of ‘Tremois’ ancestors according to their flowering time and $VRN$-$H1$ promoter–intron 1 haplotype.

<table>
<thead>
<tr>
<th>Flowering time category (days after sowing)</th>
<th>Tremois</th>
<th>$VRN$-$H1$ promoter–intron 1 haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (&lt;60)</td>
<td>Dram</td>
<td>A. Bavaria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A. Fruhe</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A. Isaria-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A. Isaria-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Abacus-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Agio</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arabische-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arabische-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aramir</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Archer-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Balder</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bavaria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Berac</td>
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<tr>
<td></td>
<td></td>
<td>Bruens Wisa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carlsberg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CarlsbergII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ceres</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Claret</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clermont</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Danubia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Delta-2</td>
</tr>
<tr>
<td></td>
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<td>Drost</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Emir</td>
</tr>
<tr>
<td></td>
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<td>Erika</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gull</td>
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<tr>
<td></td>
<td></td>
<td>H. Franken</td>
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<td></td>
<td></td>
<td>Imperial-1</td>
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<tr>
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<td></td>
<td>Isaria-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kenia-1</td>
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<tr>
<td></td>
<td></td>
<td>Kenia-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maja</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pflugs</td>
</tr>
<tr>
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<td>Prentice</td>
</tr>
<tr>
<td></td>
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<td>Prector</td>
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<td></td>
<td></td>
<td>Rika</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tyra</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vada</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Volla</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WMI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zephyr</td>
</tr>
<tr>
<td>B (60-90)</td>
<td></td>
<td>Abacus-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Algerian-1</td>
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<tr>
<td></td>
<td></td>
<td>Algerian-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Algerian-3</td>
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<tr>
<td></td>
<td></td>
<td>Plumage</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P. Archer</td>
</tr>
</tbody>
</table>

Continued
Table 3.3 continued

<table>
<thead>
<tr>
<th>Flowering time category (days after sowing)</th>
<th>VRN-H1 promoter–intron 1 haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tremois</td>
</tr>
<tr>
<td>C (&gt;120)</td>
<td>Bordia</td>
</tr>
</tbody>
</table>
Table 3.4. *VRN-H2–PPD-H1* haplotype of 17 early flowering accessions harboring winter *VRN-H1* allele (*vrn-H1*).

<table>
<thead>
<tr>
<th>Flowering time category (days after sowing)</th>
<th><em>VRN-H2–PPD-H1</em> haplotype</th>
<th><em>vrn-H2–Ppd-H1</em></th>
<th><em>Vrn-H2–Ppd-H1</em></th>
<th><em>Vrn-H2–ppd-H1</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>A (&lt;60)</td>
<td>Morgenrot-1</td>
<td>Armelle</td>
<td>Algerian-4</td>
<td>Frisia</td>
</tr>
<tr>
<td></td>
<td>Morgenrot-2</td>
<td>Imperial-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Imperial-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pirol</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Piroline</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tern</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B (60-90)</td>
<td>Plumage</td>
<td>Algerian-1</td>
<td>Algerian-2</td>
<td>Algerian-3</td>
</tr>
<tr>
<td></td>
<td>P. Archer</td>
<td></td>
<td></td>
<td>Abacus-1</td>
</tr>
</tbody>
</table>

*a* Allele designations: *Vrn-H2* = all three *ZCCT* genes present; *vrn-H2* = all three *ZCCT* genes deleted; *Ppd-H1* = LD-responsive allele; *ppd-H1* = photoperiod-insensitive allele

Note: Accessions in bold flowered earlier than expected based on their *VRN-H1–VRN-H2–PPD-H1* haplotype. Complete names of accessions are provided in Table C.1.
Figure 3.1. ‘Tremois’ pedigree tree illustrating $CBF2$, $CBF4$, $CBF13$, $VRN-H1$ promoter, $VRN-H1$ intron 1, $VRN-H2$, and $PPD-H1$ alleles in ‘Tremois’ ancestors. Pedigree of ‘Tremois’ is depicted bottom-up with the most ancestral accessions shown at the top. Pedigrees in Purdy format are provided in Table C.1. Symbols below each accession represent allelic states of the genotyped loci as summarized in the ‘KEY’ (top left). Allele abbreviations: ‘Tr’ = ‘Tremois’; ‘Mx’ = ‘Morex’; ‘Dt’ = ‘Dicktoo’; ‘Nu’ = ‘Nure’; ‘DN’ = ‘Dicktoo’-‘Nure’; ‘MT’ = ‘Morex’-‘Tremois’. Accessions unavailable from NPGS were not genotyped. The symbol depicts a cross where the accessions above the horizontal line on the left are the female parent and the male parent, respectively, and the accession below the horizontal line is the progeny. A single vertical line connects an accession (below the line) that was selected from a population of another accession (above the line). Abbreviated names in parentheses represent progeny genotypes that were not registered with the NPGS. Complete names of accessions are described in Table C.1. Genotypes that flowered in 60-90 days after sowing are depicted in italics, those that did not flower until 120 days are in bold, while all others flowered within 60 days.
Figure 3.2. Schematic depicting CBF, VRN, and PPD-H1 alleles and the genotyping assay used to genotype ‘Tremois’ ancestors. These alleles are based on their sequence in ‘Dicktoo’, ‘Nure’, ‘Morex’ and ‘Tremois’. Filled rectangles represent exons. Horizontal arrowheads indicate primer-binding sites. Vertical arrowheads indicate restriction sites of endonucleases used in the CAPS assays or other sequence variation utilized in genotyping assays. CBF2 (a), CBF4B (b), and CBF13 (c) are intron-less genes. Arrows above their exons point towards their 3’ end. Note: the size of CBF13 coding sequence (CDS) differs across genotypes. VRN-H1 contains 8 exons; the first two exons (E1 and E2) relative to its promoter (d) and intron 1 (e) are indicated. SNF2 gene and Xucw24 marker flank the ZCCT genes underlying VRN-H2 (f). The partial CDS of ZCCT-Hc is shown in gray. PPD-H1 contains eight exons (E1-E8) (f). Drawings are not to scale, except for VRN-H1 intron 1.
Chapter 4: Increasing CBF2A levels in barley acts in combination with cold temperatures to accelerate accumulation of COR gene transcripts and the acquisition of freezing tolerance

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Abstract

C-Repeat Binding Factors (CBFs) are DNA-binding transcriptional activators of genes imparting freezing tolerance. Poaceae contain three CBF subfamilies, two of which, HvCBF3/CBFIII and HvCBF4/CBFIV, are unique to this taxon. To gain mechanistic insight into HvCBF4/CBFIV CBFs we overexpressed Hv-CBF2A in spring barley (Hordeum vulgare) cultivar ‘Golden Promise’. The Hv-CBF2A overexpressing lines exhibited stunted growth, poor yield, and greater freezing tolerance compared to non-transformed ‘Golden Promise’. Differences in freezing tolerance were apparent only upon cold acclimation however. Freezing tolerance of the Hv-CBF2A overexpressing lines increased more rapidly during cold acclimation than in ‘Golden Promise’, and paralleled those of the winter hardy barley ‘Dicktoo’. Transcript levels of candidate CBF target genes, COR14B and DHN5 were increased in the overexpressor lines at warm temperatures and at cold temperatures they continued to accumulate to much higher levels in the Hv-CBF2A overexpressors than in ‘Golden Promise’. Hv-CBF2A overexpression also increased transcript levels of HvCBF3/CBFIII subgroup genes CBF12 and CBF16, suggesting these are CBF2 targets. CBF12 and CBF16 transcript levels exhibited a relatively constant incremental increase above levels in ‘Golden Promise’ at both warm temperatures and during exposure to cold temperatures. These data indicate that Hv-CBF2A activates target genes at warm temperatures and that
transcript accumulation for some of these targets is greatly enhanced by cold temperatures.

**Introduction**

Many plants increase in freezing tolerance in response to low non-freezing temperatures, a phenomenon known as cold acclimation (Thomashow, 1999). During cold acclimation biochemical and physiological changes occur in the plant conditioning it to survive cellular dehydration caused by freezing temperatures (Thomashow, 1999). Playing a key structural role in conferring some of these adaptive changes are the Cold Regulated (COR) and Dehydrin (DHN) proteins that function to stabilize membrane structure (Thomashow, 1999, Koag et al., 2009). Transcripts for the COR and DHN genes accumulate to high levels at low temperatures and during dehydrating conditions and are generally not present under non-stress conditions (Thomashow, 1999, Cattivelli et al., 2002). One of the key regulators affecting cold and dehydration induced expression of the COR and DHN genes is the family of C-Repeat Binding Factor/Dehydration Responsive Element Binding factors (CBF/DREB), which bind to the C-repeat/Dehydration Responsive Element (CRT/DRE) present in the COR and DHN gene upstream regions and activate their expression (Stockinger et al., 1997, Liu et al., 1998, van Buskirk and Thomashow, 2006). Transcripts for the CBFs are also themselves induced by cold temperatures and in a time frame that precedes that of COR and DHN transcript accumulation (Gilmour et al., 1998). In *Arabidopsis thaliana* many of the biochemical and physiological changes and the increases in freezing tolerance that occur during cold
acclimation can be mimicked at normal warm growth temperatures through constitutive high level overexpression of the \textit{CBF}s (Jaglo-Ottosen et al., 1998, Liu et al., 1998, Kasuga et al., 1999, Gilmour et al., 2000, Gilmour et al., 2004, Vogel et al., 2005).

The \textit{CBF} gene family of the temperate climate Triticeae cereals (barley, \textit{Hordeum vulgare}; wheat, \textit{Triticum} spp.; and rye, \textit{Secale cereale}), is much larger than that of Arabidopsis, other dicots, and tropical cereals such as maize (\textit{Zea mays}) and rice (\textit{Oryza sativa}). The barley genome harbors more than 20 \textit{CBF} coding sequences in a single genotype (Skinner et al., 2005). At least 13 of these colocalize in a cluster on the long arm of chromosome 5 (Skinner et al., 2005, Miller et al., 2006, Skinner et al., 2006, Badawi et al., 2007, Francia et al., 2007, Knox et al., 2010). These \textit{CBF}s are grouped into two phylogenetic subgroups – HvCBF3/CBFIII and HvCBF4/CBFIV, both of which are unique to the grasses (Skinner et al., 2005; Badawi et al., 2007). Additional \textit{CBF}s, belonging to the HvCBF1/CBFI-CBFII subgroup, occur dispersed on four other chromosomes (Skinner et al., 2005; Badawi et al., 2007). To date the chromosome 5 cluster has received the greatest attention because genes of this cluster are implicated in the molecular basis of \textit{FROST RESISTANCE-2 (FR-2)}, one of two major quantitative trait loci affecting freezing tolerance and winter hardiness of Triticeae cereals (Vágújfalvi et al., 2003, Francia et al., 2004, Båga et al., 2007, Knox et al., 2008, Knox et al., 2010).

In vitro experiments and overexpression strategies suggest a complex relationship between the \textit{CBF}s, their targets, and temperature. In vitro experiments using recombinant
CBF proteins indicate HvCBF4/CBFIV members bind to oligonucleotides having CRT/DRE motifs if binding reactions are carried out at cold temperatures, but not at warm temperatures (Xue, 2003, Skinner et al., 2005). In contrast, recombinant proteins of the HvCBF3/CBFIII and HvCBF1/CBF1-CBFII subgroups are not affected by temperature (Xue, 2003, Skinner et al., 2005). Overexpression of HvCBF3/CBFIII CBFs in Arabidopsis also induces COR gene expression at normal growth temperatures and increases in freezing tolerance, whereas overexpression of HvCBF4/CBFIV CBFs does not induce COR gene expression at warm temperatures, nor does it have an effect upon freezing tolerance (Skinner et al., 2005). The reasons for the differences between HvCBF4/CBFIV and HvCBF3/CBFIII subgroup CBFs in Arabidopsis are not clear but it was considered that the low temperature-dependence observed in vitro might be indicative of a low temperature-dependence for activity in vivo, or simply, that the HvCBF4/CBFIV subgroup CBFs are unable to activate genes in Arabidopsis (Skinner et al., 2005). In rice, overexpression of barley CBF4 increases tolerance to drought, salinity, and chilling temperatures and induces stress-related genes in the absence of stress (Oh et al., 2007, Lourenco et al., 2011), indicating that in a monocot, the overexpressed transgene is functionally active.

To further our mechanistic understanding of HvCBF4/CBFIV CBFs, we generated transgenic barley plants overexpressing Hv-CBF2A under control of the CaMV 35S RNA promoter in the spring cultivar ‘Golden Promise’. To date, ectopic expression of CBFs in Triticeae cereals has been carried out using wheat orthologs of barley HvCBF1/CBFI-
CBFII subgroup CBFs, CBF5 and CBF7, Ta-DREB3 and Ta-DREB2, respectively (Pellegrineschi et al., 2004, Morran et al., 2011) and Arabidopsis CBF3/DREB1A but not with members of the either the HvCBF3/CBFIII or HvCBF4/CBFIV subgroups. The Hv-CBF2A overexpressing plants, alongside non-transformed ‘Golden Promise’ and the winter-hardy facultative barley cultivar ‘Dicktoo’ were evaluated for freezing tolerance and for candidate target gene expression. The data from these experiments indicated that the Hv-CBF2A overexpressors had greater levels of freezing tolerance than ‘Golden Promise’ when plants were exposed to cold temperatures, and that the Hv-CBF2A overexpressors activated predicted target genes at normal growth temperatures but that cold temperatures had a pronounced stabilizing effect on the accumulation of certain of these target genes.

Materials and methods

Plasmid construction

*Hordeum vulgare* cv. ‘Dicktoo’ CBF2A clone (AY785841) encompassing the coding sequence and flanking untranslated regions was PCR amplified using primers 5′-CCACAACGCACCTCTCGACGC-3′ and 5′-GCATATTCATGGTTGAGATTG-3′ by *Pfu* DNA polymerase (Promega Corporation; [www.promega.com](http://www.promega.com)). As *Pfu* produces blunt-ended PCR products, A-overhangs were added using a *Taq* polymerase incubation, and these were then cloned into a T-overhang *XcmI* site between the Cauliflower Mosaic Virus 35S promoter (CaMV 35S) and 7’T-5’T terminator elements of pGA643 assembled in the pLITMUS28 vector (New England Biolabs, [www.neb.com](http://www.neb.com)) yielding a CaMV
35S:CBF2A:7’T-5’T expression cassette. This construct was sequenced to confirm errors were not introduced. The CaMV 35S:CBF2A:7’T-5’T cassette was excised from the pLITMUS28 vector with AgeI and BsiWI, subcloned into identically-cut pGEM/HAB, and then sequenced. (pGEM/HAB was constructed by subcloning a synthetic HindIII-AgeI-BsiWI (HAB) adapter having A-overhangs into pGEM-T-Easy.) The CaMV 35S:CBF2A:7’T-5’T cassette in pGEM/HAB was excised using NotI and subcloned into NotI-cut binary vector pWBVec10a (Wang et al., 1998). (pWBVec10a contains hygromycin resistance (hygromycin phosphotransferase, hph) and GUS (β-glucuronidase, uidA) marker genes internal to the T-DNA border sequences and flanking the CaMV 35S:CBF2A:7’T-5’T cassette; both hph and uidA expression cassettes contain introns so that their expression occurs only in the transformed eukaryotic cells (Wang et al., 1998)). The final construct, pWBVec10a/CaMV 35S-CBF2A, was transformed into A. tumefaciens AGL1 (Lazo et al., 1991) according to the procedure described (Walkerpeach and Velten, 1994).

**Plant material and transformation**

*Hordeum vulgare* L. cv. ‘Golden Promise’ plant material, spike collection, sterilization and excision of immature embryos were carried out using established methods (Chang et al., 2003).

Transformation of ‘Golden Promise’ was carried out using established methods (Horvath et al., 2002) with the following modifications. *A. tumefaciens* AGL1 harboring
pWBVec10a/CaMV 35S -CBF2A binary vector was grown in YEP medium containing spectinomycin and carbenicillin (100 μg ml\(^{-1}\) each) at 28\(^\circ\)C overnight. Bacterial cultures were centrifuged and the harvested bacterial pellets were resuspended in two volumes (relative to the culture medium volume) of infection medium (IM; MS, 30 g L\(^{-1}\) maltose, pH 5.2) containing 100 μM acetosyringone. 1 ml aliquots were then transferred to 1.5 ml tubes. After excision, each immature embryo was immediately transferred into a 1.5 ml tube containing the \textit{A. tumefaciens} suspension. Immature embryos from a single spike were collected into the same tube. The tube contents were gently mixed by inverting 4-5 times. All immature embryos were then allowed to settle to the bottom of the tube. After collecting immature embryos from 3-5 spikes per round of transformations, tubes were again mixed by inverting for 1 min and then incubated at room temperature. After 15 min the IM containing the majority of \textit{A. tumefaciens} cells was removed by pipetting. The immature embryos were then placed scutellum-side down onto co-cultivation medium: callus induction medium (CIM) containing 100 μM acetosyringone, pH5.2. The immature embryos from each tube were plated on their own plates where they were co-cultured for 2–3 days at 25\(^\circ\)C in complete darkness. After co-cultivation, immature embryos were collected and washed 3-5 times with liquid MS medium containing 30 g L\(^{-1}\) maltose and 250 mg L\(^{-1}\) Timentin (pH5.8).

Selection on CIM, regeneration on shoot generation medium, and rooting on root generation medium were carried out using established methods (Horvath et al., 2002). The three media contained 50, 20, and 50 mg L\(^{-1}\) hygromycin, respectively, and 150 mg
L Timentin. Ten seeds from each T₀ plant were then germinated in individual square (5.7 cm) bio-degradable plant containers (Jiffypots, www.jiffypot.com) filled with vermiculite. Each seedling was screened for β-glucuronidase activity (Jefferson, 1987). The β-glucuronidase-positive seedlings were transplanted to soil in 15 cm-diameter pots and grown to maturity. Spikes were harvested from each plant separately and stored in paper bags until further use. Each line was carried forward by single seed descent through each generation.

**Evaluation of freezing tolerance**

Freezing tolerance was determined using two independent tests previously established to assay freezing tolerance levels of Triticeae cereals (Veisz and Sutka, 1989, Gusta et al., 2001, Limin and Fowler, 2006). One method consisted of isolating the crown region from plants and then subjecting the excised crowns to the target freezing temperatures. The second method involved subjecting the whole plants in soil to target freezing temperatures. Both methods utilized regrowth assays, providing a robust means of assaying freezing tolerance (Olien, 1964).

For freezing experiments using isolated crowns, barley seeds were sown in 10 cm square plastic pots, 15 seeds/pot, in Sunshine SB40 mix (Sun Gro Horticulture Inc., www.sungro.com), and grown in a growth chamber using a 16 h/8 h photoperiod, 100 µmol m⁻² s⁻¹ PPFD at 18°C for two weeks. To cold acclimate, the plants were transferred to a growth room maintained at 3 ± 1°C (8 h or 16 h photoperiod; Very High Output
Phillips CW/VHO fluorescents, 100 µmol m$^{-2}$ s$^{-1}$ PPFD) for the defined periods. The crowns of the barley plants were then harvested for the freezing experiments. The crowns consisted of the region approximately 2 mm below and 5 cm above the apex. Five crowns per genotype at each sampling were placed on moistened paper that was then placed into 16×120 mm test tubes. Test tubes were placed into a low temperature Neslab bath (Model LT-50DD, www.thermo.com) set at -1°C, and incubated for 1 h. After 1 h, ice nucleation was initiated by adding an ice chip to each tube. Test tubes with the samples remained in the -1°C temperature bath overnight to ensure complete freezing of plant tissues. The following day the temperature was decreased at a rate of 2°C h$^{-1}$. Sample tubes were removed at either -3°C or -6°C for percent survival determination, or at 2°C intervals in the temperature range from -2°C to -12°C for LT$_{50}$ determination, and then placed at 4°C, at which they were allowed to thaw overnight. Three replicates were performed per genotype, per treatment. After thawing, crowns were planted into soil in pots for regrowth under normal growth conditions. Freezing tolerance was expressed as either LT$_{50}$ values (temperature at which 50% of the crowns failed to regrow determined 3 weeks after replanting them in soil) or percent survival (number of plants surviving/total number of plants tested) at -3°C or -6°C.

Freezing whole plants in soil utilized plants grown in wooden boxes (38cm x 26cm x 11cm) in which there were nine rows of plants per box, 20 plants per row. Plants were grown in a Conviron PGR-15 climatic chamber (Conviron, www.conviron.com) for 3 weeks at a constant 18°C, 16 hours illumination, 300 µmol m$^{-2}$ s$^{-1}$ PPFD. Wooden boxes
were transferred to a Conviron C-812 climatic chamber and the temperature was lowered at the rate of 0.5°C h\(^{-1}\) to -3°C, or to -6°C. The minimum temperatures were maintained for 24 hours before being returned to 18°C at the rate of 0.5°C h\(^{-1}\). Plants were allowed to recover at normal growth conditions for three weeks before determining survival.

**DNA blot hybridization**

High MW DNAs from barley were isolated using established methods (Stockinger et al., 1996). Approximately 10 µg DNA was digested separately with each *Bam*HI, *Bgl*II, and *Xho*I restriction endonucleases. Enzyme-digested DNAs were electrophoresed on 0.8% TAE-agarose gels, transferred to Hybond N (GE Healthcare, www.gehealthcare.com), and UV crosslinked to Hybond N membranes using standard procedures (Ausubel et al., 1993). Hybridization and washes were carried out as described (Knox et al., 2010). The same filter was sequentially hybridized starting with a *CBF2A* gene specific probe encompassing the *CBF2A 3' region*, followed by coding sequence (CDS) probes of *hph*, *CBF2A*, and *uidA*. After each round of hybridization the probe was stripped from the filter in 0.1% SDS at 65°C.

**RNA blot hybridization**

Five seeds each of ‘Dicktoo’, ‘Golden Promise’, and the CaMV 35S-*CBF2A* lines were sown in 9 cm x 9 cm pots (Kord Products, www.kord.ca) in eight replicates and transferred to Conviron growth chambers after germination. Seedlings were grown at 18°C on a 8 h light/16 h dark photoperiod for three weeks, then the growth chamber
temperature was decreased to 6°C at daybreak of day 21. Samples were harvested at 2 h intervals starting at daybreak through the 12 h time point. The temperature was maintained at 6°C for two additional days and samples were harvested at the 6 h time point each day. The 6 h time point was chosen to avoid circadian clock effects and on unpublished data showing peak expression of CBF genes occurs at approximately 6-8 hours during the subjective day. At each harvest time point, crown tissue of five seedlings was pooled and flash frozen in liquid nitrogen. Total RNA was isolated using RNeasy Plant Mini Kits (Qiagen, www.qiagen.com). 7 µg was electrophoresed on a 1.2% MOPS-agarose gel and transferred to Hybond N membrane (GE Healthcare, www.gehealthcare.com) using standard procedures (Ausubel et al., 1993). All subsequent steps were as described under ‘DNA blot hybridization’ above, except Denhardt’s solution concentration in the hybridization buffer was 2X instead of 5X. The same RNA blot was sequentially hybridized with barley CDS probes of radiolabeled CBF2A, COR14B, DHN5, and DHN8, and subsequently with each of the CBF gene probes.

**Real-time quantitative PCR (RT-qPCR)**

‘housekeeping’ gene *cyclophilin* was carried out using 1 µl of a 10-fold dilution of cDNA template as described (Doblin et al., 2009). RT-qPCR analysis of the later generation plants utilized RNA extracted using the RNeasy Plant Mini Kits (Qiagen, [www.qiagen.com](http://www.qiagen.com)). These cDNA populations were generated using the QuantiTect Reverse Transcription kit (Qiagen, [www.qiagen.com](http://www.qiagen.com)) following the manufacturer’s instructions, including removal of genomic DNA.

RT-qPCR analysis of *CBF2, COR14b, DHN8,* and *DHN5* transcript levels was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, [www.appliedbiosystems.com](http://www.appliedbiosystems.com)) as described (Burton et al., 2004). Values were normalized to *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*). The PCR product sizes, and forward (F) and reverse (R) primer sequences were: *CBF2* (128 bp. F: 5’- CGGATCAAGTTGCAGGAGACGC -3’; R: 5’- GTGCCGAGCCAGAGCCTGGAGTA -3’), *COR14B* (103 bp. F: 5’- TACATCGTCAATGACGAGACC -3’; R: 5’- TTGAGGATGTGAGCAAATGAG -3’), *DHN5* (106 bp. F: 5’- TGGCGAAGTTCCACCGTATGC -3’; R: 5’- ACGAAAACTGTTGCCACACTG -3’), *DHN8* (144 bp. F: 5’- GCTCCAGCTCCAGCTCGTCTA -3’; R: 5’- CAGCCTCGTTTCTCTTGTGGCCCG -3’), *GAPDH* (198 bp. F: 5’- GTGAGGCTGGTGTGCTGATTCA -3’; R: 5’- CGTGGTGCAGCTAGCATTTGAGAC -3’).

The forward (F) and reverse (R) primer sequences and PCR product sizes used for analyses of *CBF2* and *DHN8* in the early generation plants differed from those use for the later generation plants and are as follows: *CBF2* (274 bp. F: 5’-
CCATCACCTCAAGCGACCTATCG-3’; R:5’-GCCTGACGCTGGTGGAAGAAC-3’), DHN8 (278 bp. F:5’-GCTCCAGCTCCAGCTCGTCTA-3’; R:5’-CTTCTCCTCCTCGGGCACTG-3’). Both sets of CBF2 primers were in regions 100% identical between the endogenous and transgene CBF2. For the later generation of plants, qRT-PCRs were carried out in a final volume of 20 µl using 10 ng of cDNA, 10 nM each primer, and 10 µl of “Power Master Mix”. Cycling was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, www.appliedbiosystems.com). The specificity of the PCR was confirmed by sequencing and product melt curves were analyzed using standard procedures. Data was analyzed using Sequence Detection Software (v 1.4) (Applied Biosystems, www.appliedbiosystems.com) and Microsoft Excel (Microsoft, www.microsoft.com). Standard curves indicated linear amplification of the products between 1 pg and 1 ng of template and all qPCR reactions were performed with template concentrations within this range.

Samples of PCR products were sequenced to confirm products consisted only of that expected. A melt curve was obtained from the product following cycling by heating from 70°C to 99°C, which was also used to detect the presence of any non-specific sequences. Data was analyzed using Sequence Detection Software (v 1.4) (Applied Biosystems, www.appliedbiosystems.com) and Microsoft Excel (Microsoft, www.microsoft.com). A 10-fold dilution series replicated three times indicated that COR14B amplification was linear (R = 0.9967) using between 1 pg and 10 ng of cDNA template per reaction. Amplification products of CBF2 increased linearly using 1 pg to 1 ng template, and
began to plateau at the higher concentration range. All qPCR reactions were carried out using this concentration range of template.

**Morphological analyses**

T$_3$ and T$_4$ generation plants were grown alongside ‘Golden Promise’ plants in a glasshouse with supplementary fluorescent lighting over an Australian spring season. Maximum/minimum temperatures were 28°C/15°C during the day/night. Plants were grown in 15 cm-diameter pots with one plant per pot.

Plants were photographed using an IXUS 70 digital camera (Canon, www.canon.com). Reported plant height is the distance from the soil to the tip of the tallest leaf. The 1000 grain weight was calculated by weighing 100 randomly selected seed that were threshed from multiple spikes and then multiplying by ten. For plant weight, each mature plant’s aerial portion (3 cm above soil surface) was harvested. Prior to weighing, the plant aerial tissue was dried at room temperature for four weeks and the spikes were dried at 37°C for one week. The reported total plant biomass is the combined dry weights from aerial tissue and spikes. Yield is the number of grain from each plant.

**Statistical analysis**

Analyses of variance (ANOVA)s were performed using the general linear model procedure implemented in SAS v9.1 (SAS Institute Inc, Cary, NC). Statistical significance of survival scores at a given time point were determined by two independent
mean separation tests using SAS v9.1: Duncan's multiple-range test and least square means separation after a False Discovery Rate (Benjamini and Hochberg, 1995) multi-test adjustment. In all cases identical results were obtained with both methods. Means followed by the same letter are not significantly different at $P<0.05$.

Results

Generator and characterization of the Hv-CBF2A overexpressor lines

Hv-CBF2A from the facultative barley, ‘Dicktoo’ was introduced into the spring barley ‘Golden Promise’ under control of the CaMV 35S promoter (Fig. 1a). Eighteen independent transgenic lines were recovered that were both hygromycin-resistant and $\beta$-glucuronidase (GUS) positive. Of these, six lines were selected for propagation to homozygosity and taken forward to the $T_4$ and $T_5$ generations. These lines were selected based on transgene expression and fecundity relative to the other lines.

To verify the six transgenic lines resulted from independent T-DNA insertion events, $XhoI$-digested DNAs of the transgenic lines, non-transgenic ‘Golden Promise’ and ‘Dicktoo’ were hybridized to the hygromycin phosphotransferase ($hph$) and $\beta$-glucuronidase ($uidA$) gene probes (Fig. 1b and 1c). The molecular weights of the fragments cross-hybridizing to the $hph$ and $uidA$ probes are expected to be different for each independent transformation event depending on the distance between the $XhoI$ site in the ‘Golden Promise’ genome and the $XhoI$ sites in the insert (Fig. 1a). Distinct hybridization banding patterns across the six transgenic lines confirmed that all six lines originated from independent transformation events (Fig. 1b and 1c). The banding pattern
of line 3 in the T₄ generation was identical to that in the T₂ generation, indicating that the transgene was stable (Fig. 1b and 1c). Multiple cross hybridizing fragments were detected with both probes, suggesting multiple inserts (Fig. 1b and 1c).

The same DNA blots were also hybridized with the CBF2 coding sequence (CDS) and the region immediately downstream of the CDS, which served as a gene specific probe (Fig. 1d and 1e). These hybridizations confirmed the presence of the CBF2A transgene in each line (Fig. 1d and 1e). The signal intensity of the band cross-hybridizing to the transgene was higher than that cross-hybridizing to the endogenous gene (Fig. 1d), consistent with multiple copies of the transgene in each line. Fragments of the expected size were observed in five of the six lines. However, the banding pattern obtained with line 2 using the CBF2 probes differed from that predicted (Fig. 1d and 1e). This unexpected banding pattern appeared to be due to a rearrangement because hybridization to BamHI restricted DNAs, which should produce a single fragment via BamHI sites internal to the construct, also differed from that predicted (not shown).

To verify the transgene was expressed and that expression was stably maintained through generational passages, Hv-CBF2A transformants were assayed for CBF2 expression in a subset of the early generation plants (T₂ and T₃) using qRT-PCR (Supplemental Fig. S1), and in the latter generation plants (T₄ and T₅) using RNA blot hybridization (Fig. 2a). In both generational sets, expression was assayed at normal warm growth temperatures, and at cold temperatures but using different time courses following temperature decreases.
With each set of plants CBF2 levels were elevated above levels in ‘Golden Promise’ and ‘Dicktoo’ at warm and cold temperatures (Fig. 2a, Supplemental Fig. S1). In the latter generation plants sampling at 2 h intervals over a 12 h time course and again at 30 and 54 h indicated that CBF2 transcript levels remained relatively constant throughout the time course (Fig. 2a).

Taken together these data indicate that the six transgenic lines result from independent transformation events and that each constitutively expresses the CBF2A transgene at levels higher than the non-transgenic ‘Golden Promise’ and ‘Dicktoo’ do in response to cold temperatures, and that transgene expression was stably maintained through these generational passages.

**Hv-CBF2A overexpression negatively affects plant development**

Overexpression of CBF genes is often associated with detrimental side effects (Liu et al., 1998, Gilmour et al., 2000, Jaglo et al., 2001, Gilmour et al., 2004, Ito et al., 2006). Such was the case with many of the primary ‘Golden Promise’ transformants. Numerous T0 lines were infertile while many of those that were fertile produced non-viable seed. To more thoroughly characterize the effect of CBF2A overexpression on several phenotypic characters and yield-associated traits, six of the Hv-CBF2A overexpressing lines were grown to maturity alongside ‘Golden Promise’, and a set of phenotypic characters were measured during their life cycle. Figure 3a shows the expression levels of the Hv-CBF2A transgene in seedlings of the same group of plants phenotyped (Fig. 3b). Relative to
‘Golden Promise’ the Hv-CBF2A overexpressors showed varying degrees of stunted growth (Fig. 3b and 3c) and delayed maturity (not shown). The final plant height of lines 2, 3, 6, and 15 was significantly less than ‘Golden Promise’ (p<0.0001) (Fig. 3b and 3c). Visually, lines 10 and 13 also exhibited shorter stature, although this difference was not significantly different from ‘Golden Promise’ (Fig. 3b and 3c). Total biomass and the total grain weight produced by each Hv-CBF2A overexpressor line was significantly less than that of ‘Golden Promise’ (p<0.0001) (Fig. 3d and 3e). Misshapen spikes appeared more frequently in the Hv-CBF2A overexpressors and the grains were less plump (not shown). Many of the spikes produced on the Hv-CBF2A overexpressors were shorter with fewer grains, resulting in lower seed yield (Fig. 3f). The Hv-CBF2A overexpressors also tended to have thinner and darker leaves, and produced fewer tillers (not shown).

**Overexpression of Hv-CBF2A enhances freezing tolerance upon cold acclimation**

Given the role of CBFs in affecting freezing tolerance, a central question was whether the Hv-CBF2A overexpressors were altered in the capacity to survive freezing temperatures. To address this question we assayed the freezing tolerance levels of several Hv-CBF2A overexpressor lines alongside non-transformed ‘Golden Promise’ and ‘Dicktoo’. Freezing tolerance levels were assayed by quantifying survival percentages of both whole plants and excised crowns subjected to -3°C and -6°C, and by measuring LT50 values of excised crowns from cold acclimated and non-acclimated plants.
Using the whole plant freezing assays, none of the plants regrew following freezing to -6°C, whereas freezing to -3°C produced differences in regrowth (Fig. 4). The percentages of plants resuming growth after freezing to -3°C were 14% for ‘Golden Promise’, 95% for ‘Dicktoo’, and 50–75% for the four Hv-CBF2A-overexpressing lines 2, 10, 13, and 15 (Fig. 4).

In separate experiments using excised crowns, survival of lines 2 and 10 alongside ‘Golden Promise’ and ‘Dicktoo’ was assayed following freezing to -3°C (Fig. 5a) and -6°C (Fig. 5b). In these experiments none of the excised crowns from non-acclimated plants (0 h time point) resumed growth following freezing to -3°C or -6°C (Fig. 5a and 5b). In comparisons, excised crowns from the cold acclimated plants exhibited increases in regrowth, and with increasing lengths of cold acclimation (Fig. 5a and 5b). Following freezing to -3°C, the regrowth percentage of excised crowns from plants cold-acclimated for 4 days was about 65% for ‘Golden Promise’ and 85–100% for the two Hv-CBF2A-overexpressing lines and ‘Dicktoo’ (Fig. 5a). Regrowth percentages of excised crowns from all genotypes including ‘Golden Promise’ approached 100% when the plants had been cold acclimated for one week (Fig. 5a). Following freezing to -6°C the regrowth percentage of excised crowns from plants cold-acclimated for one week was approximately 80% for lines 2 and 10, and ‘Dicktoo’, whereas excised crowns from ‘Golden Promise’ did not resume growth (Fig. 5b). After two weeks of cold acclimation these percentages increased to approximately 100% for the two Hv-CBF2A overexpressors and ‘Dicktoo’, while ‘Golden Promise’ increased to approximately 30%
(Fig. 5b). After four weeks cold acclimation 100% of ‘Golden Promise’ excised crowns also exhibited regrowth (Fig. 5b).

Determining the temperature for which 50% of the excised crowns failed to regrow indicated that at one week of cold acclimation the LT$_{50}$ of lines 2 and 10, and ‘Dicktoo’ were about -7°C, and the LT$_{50}$ of ‘Golden Promise’ was about -4°C (Fig. 5c). The LT$_{50}$ values remained relatively unchanged for line 2, line 10, and ‘Dicktoo’ after two weeks of cold acclimation while ‘Golden Promise’ increased to about -5°C (Fig 5c). After four weeks of cold acclimation the LT$_{50}$ of line 2 was -7°C while that of line 10, ‘Golden Promise’, and ‘Dicktoo’ were all approximately -8°C (Fig 5c).

These experiments indicate that overexpression of Hv-CBF2A in the spring barley ‘Golden Promise’ increased the freezing tolerance of ‘Golden Promise’ to levels similar to those of the facultative barley ‘Dicktoo’. These data also indicate that a period of cold temperatures was necessary to impart freezing tolerance.

*Effect of Hv-CBF2A overexpression and cold temperatures on COR and DHN transcript levels*

In the absence of cold temperatures CBF overexpression leads to the activation of genes harboring CRT/DRE motifs in their upstream regions that are normally activated only in response to cold temperatures or other osmotic stresses (Jaglo-Ottosen et al., 1998, Liu et al., 1998, Kasuga et al., 1999, Gilmour et al., 2000, Gilmour et al., 2004, Vogel et al.,
2005). To test how CBF target genes in barley would respond to Hv-\textit{CBF2A} overexpression, expression analyses of candidate target genes \textit{COR14B}, \textit{DHN5}, and \textit{DHN8} were carried out. Early generation plants were assayed using qRT-PCR (Supplemental Fig. S1) and later generation plants were assayed using both RNA blot hybridization and RT-PCR (Fig. 2a). A comparative measure of transcript levels was made by quantifying and normalizing RNA blot hybridization signals to \textit{actin} and RT-PCR values to \textit{GAPDH}.

Expression analyses of the early generation Hv-\textit{CBF2A} overexpressing plants indicated transcripts for all three candidate target genes were elevated in the non-cold acclimated Hv-\textit{CBF2A} overexpressors relative to non-cold acclimated ‘Golden Promise’ (Supplemental Fig. S1). RNA blot hybridization was unable to detect \textit{COR14B} in both the non-cold acclimated Hv-\textit{CBF2A} overexpressors and ‘Golden Promise’, but RT-PCR with the same RNAs indicated that \textit{COR14B} transcript levels were elevated nearly 100-fold in the Hv-\textit{CBF2A} overexpressors relative to ‘Golden Promise’ (Fig. 2a, 2c inset). \textit{DHN5} was also not detectable by RNA blot hybridization in non-cold acclimated ‘Golden Promise’ and only a weak signal was detected in the Hv-\textit{CBF2A} overexpressors but RT-PCR with the same RNAs indicated that \textit{DHN5} transcript levels were approximately 20-fold higher in the Hv-\textit{CBF2A} overexpressors relative to ‘Golden Promise’ (Fig. 2a-c). \textit{DHN8} transcript signals were detectable by RNA blot hybridization
in both the Hv-\textit{CBF2A} overexpressors and ‘Golden Promise’ and were 1.4-fold higher in the Hv-\textit{CBF2A} overexpressors (Fig. 2a-c).

As the time course proceeded \textit{COR14B}, \textit{DHN5}, and \textit{DHN8} transcripts were detected at measurably higher levels in the Hv-\textit{CBF2A} overexpressors relative to ‘Golden Promise’ (Fig. 2a-c). In the Hv-\textit{CBF2A} overexpressors \textit{COR14B}, \textit{DHN5} and \textit{DHN8} transcript levels were approximately 35-fold, 5-fold, and 2.5-fold higher, respectively at the 54 h time point relative to their levels at the 6 h time point. In comparison, \textit{COR14B}, \textit{DHN5} and \textit{DHN8} transcript levels were about 14-fold, 2.3-fold, and 1.4-fold higher, respectively in ‘Golden Promise’ relative to their levels at the 6 h time point. This greater magnitude of increase at the 54 hour time point in the Hv-\textit{CBF2A} overexpressors appeared to be due to continued increases in \textit{COR14} and \textit{DHN} levels combined with a reduction of their levels in ‘Golden Promise’; i.e., in ‘Golden Promise’ \textit{COR14B}, \textit{DHN5}, and \textit{DHN8} transcript levels relative to \textit{actin} exhibited a peak at 30 h that tapered off at the 54 h time point whereas these transcript continued to increase in the Hv-\textit{CBF2} overexpressors at the 54 h time point (Fig. 2c). \textit{COR14B}, \textit{DHN5}, and \textit{DHN8} transcript levels were also higher in the Hv-\textit{CBF2A} overexpressing lines than in ‘Dicktoo’ (Fig. 2b). Taken together these data suggest Hv-\textit{CBF2A} overexpression induces expression of \textit{COR14B}, \textit{DHN5}, and \textit{DHN8} at normal growth temperatures and that cold temperature has a pronounced stabilizing effect upon their transcript levels.
**Overexpression of Hv-CBF2A upregulates other FR-H2 CBF genes**

Sequencing the genomic regions encompassing the CBF genes at FR-H2 revealed many of the CBF promoter regions have CRT/DRE motifs (Miller et al., 2006, Knox et al., 2010), and (Table I). Those data suggested CBF genes at FR-H2 harboring CRT/DRE motifs might also be targets of the CBFs. To test for this possibility, relative levels of CBF4, CBF9, CBF12, CBF14, CBF15, and CBF16 were also assayed in the Hv-CBF2A overexpressors. Carrying out RNA blot hybridizations with this set of probes indicated that in each Hv-CBF2A overexpressors line CBF12, CBF15, and CBF16 transcripts were elevated above levels in ‘Golden Promise’ at all of the assayed time points (Fig. 2d and 2e). This was most pronounced for CBF12, followed by CBF16 and CBF15. Over the time course the expression pattern of these three CBFs in the overexpressor lines was similar to that in ‘Golden Promise’ in that CBF transcript levels rapidly increased following the temperature decrease, peaked at 8–12 h, and then decreased at the 30 h time point (Fig. 2d and 2e). Over the time course CBF12, CBF15, and CBF16 averaged 3.0-, 1.5-, and 2.2-fold higher, respectively, in the Hv-CBF2A overexpressors over ‘Golden Promise’ (Fig. 2e). Relative levels of CBF9 and CBF14 were also slightly increased over that of ‘Golden Promise’, but this was less striking (Fig. 2d). Transcript levels of CBF4 over the time course were about the same in the Hv-CBF2A overexpressors as in ‘Golden Promise’. Among all the overexpressor lines, line 2 was the most consistent in having higher transcript levels of all CBFs at the different time points (Fig. 2d).
Discussion

An objective of this study was to further our mechanistic understanding of HvCBF4/CBFIV CBFs and the role they play in cold acclimation and freezing tolerance. Results from our experiments with barley plants transformed with Hv-CBF2A indicated that the freezing tolerance of the Hv-CBF2A overexpressors was increased to levels that approximated ‘Dicktoo’ and that freezing tolerance was not constitutive in the Hv-CBF2A overexpressors. Excised crowns from cold acclimated and non-cold acclimated plants indicated freezing tolerance of the Hv-CBF2A overexpressors and ‘Dicktoo’ essentially paralleled each other during the course of cold acclimation. Both the Hv-CBF2A overexpressors and ‘Dicktoo’ exhibited significantly greater regrowth percentages following freezing to -3°C than ‘Golden Promise’ at one, two, and four days of cold acclimation in the one week cold acclimation time course. Similarly in the four week cold acclimation time course followed by freezing to -6°C the Hv-CBF2A overexpressors and ‘Dicktoo’ also exhibited significantly greater regrowth percentages than ‘Golden Promise’ at one and two weeks of cold acclimation. At the conclusion of both of these cold acclimation periods, regrowth percentages of ‘Golden Promise’ approached that of the Hv-CBF2A overexpressors and ‘Dicktoo’. The target freezing temperatures of -3°C and -6°C were likely not low enough to discriminate the Hv-CBF2A overexpressors and ‘Dicktoo’ from ‘Golden Promise’ at these time points while the -8°C LT₅₀ exhibited by ‘Dicktoo’ is about the maximum this genotype attains when grown and cold acclimated under long days, whereas under short days ‘Dicktoo’ is capable of greater freezing tolerance (Limin et al., 2007). And although no defined period of cold
acclimation treatment was applied in the whole plant freezing assays, the relatively longer period of time these plants were at cool temperatures as a result of the 36 h time period used to cool to the target freezing temperatures may have allowed for Hv-CBF2A-induced changes to occur resulting in a greater level of freezing tolerance in the Hv-CBF2A overexpressors.

Hv-CBF2A overexpression induced expression of COR and DHN genes harboring CRT/DRE motifs in their upstream promoter regions, but at normal growth temperatures the RNAs for some of these genes including COR14B and DHN5 did not accumulate to levels detectable by RNA blot hybridization. A temperature decrease and continued exposure to cold temperature led to substantial increases in their transcript levels however. DHN8, which was detectable in the non-acclimated plants and whose transcripts were not as dramatically increased by cold temperatures, appears to be less affected by this temperature effect.

Transcript levels of other CBF genes at FR-H2 were also increased in the Hv-CBF2A overexpressors. CBF12 transcripts were about three-fold higher in the Hv-CBF2A overexpressors compared to ‘Golden Promise’ and this differential increase remained nearly constant throughout the entire low temperature time course. The CBFs harboring fewer CRT/DRE motifs, including CBF9, CBF15, and CBF16 exhibited more variable transcript levels, but which were nonetheless elevated above those of ‘Golden Promise’. CBF4 and CBF14, which do not harbor CRT/DRE motifs in their upstream regions, did
not exhibit increased levels in the Hv-\textit{CBF2A} overexpressors. Similar observations were made by Morran et al. (2011), who observed that overexpression of HvCBF1/CBF1-CBFII subgroup genes \textit{DREB2} and \textit{DREB3}, orthologs of barley \textit{CBF7} and \textit{CBF5}, respectively, increased transcript levels of certain \textit{CBF} genes. Despite the increased levels of these other \textit{FR-H2 CBF} transcripts in the Hv-\textit{CBF2A} overexpressors, these genes still exhibited a low temperature responsive expression pattern that is characteristic of the \textit{CBFs} i.e., \textit{CBF12}, \textit{CBF16}, and \textit{CBF15} transcript levels increased and then decreased over the 12 h time course in both ‘Golden Promise’ and the Hv-\textit{CBF2A} overexpressors. In the Hv-\textit{CBF2A} overexpressors these \textit{CBFs} – and \textit{CBF12} in particular – were incrementally increased above levels in ‘Golden Promise’. This data indicates the cold response pathway is still active in these plants, and that it is independent of mechanisms driving the down-regulation of the \textit{CBFs} during the transition from vegetative to reproductive growth (Stockinger et al., 2007, Dhillon et al., 2010).

In essence these expression data also suggest the DNA binding and transcriptional activation activity of the overexpressed Hv-\textit{CBF2A} is as active at the normal growth temperatures as at cold temperatures and that the increase in DNA binding that occurs in the recombinant HvCBF4/CBFIV subgroup proteins in vitro (Xue, 2003, Skinner et al., 2005) is probably not occurring in the barley plant. Nonetheless, directly addressing whether CBF2 protein binding to target sites in vivo is altered by temperature requires a means to assay bound and unbound protein in the cell. Such experimentation may be possible using a robust anti-CBF2 antibody and chromatin immunoprecipitation. The
increase in the activity of the *E. coli*-produced recombinant proteins detected in vitro (Xue, 2003, Skinner et al., 2005) could be due to increased solubility, reduced misfolding, and reduced aggregation, all of which occur because the hydrophobic interactions contributing to these phenomena are weakened at colder temperatures (Baneyx and Mujacic, 2004, Vera et al., 2007).

While levels of *CBF2* transcript in the Hv-CBF2A overexpressors were very high there was also disconnect between their levels and those of the presumed CBF protein target genes. Transcript levels of *CBF2* were more than 1000-fold higher in the Hv-CBF2 overexpressors than they were in either ‘Golden Promise’ or ‘Dicktoo’ (Supplemental Fig. S1). The most consistent of the candidate CBF2 targets in terms of expression levels, *CBF12*, exhibited approximately three-fold higher transcript levels in the Hv-CBF2 overexpressors. And while the cold temperature-induced increase in transcript levels of *COR* and *DHN* genes was greater in the Hv-CBF2A overexpressors than in either ‘Golden Promise’ or ‘Dicktoo’, the relative increased levels did not parallel the 1000-fold higher *CBF2* levels in these plants. This disconnect may be due to an upper limit on the amount of CBF protein in the cell controlled through post-transcriptional and post-translational mechanisms. Again a robust anti-CBF2 antibody would aid addressing protein levels.

While there were also growth and development problems resulting from high level Hv-CBF2A overexpression, as is typical when the CBFs are overexpressed to high levels (Liu
et al., 1998, Gilmour et al., 2000) these growth defects and other undesirable phenotypes may be independent of the CBF regulon and the genes directly targeted by the CBF proteins because other, and very different types of transcriptional activators will also cause stunted growth phenotypes when overexpressed (Tamagnone et al., 1998, Kang and Singh, 2000). Overexpression of a fusion construct between the yeast GAL4 DNA binding domain and the CBF1 COOH activation domain also causes severe growth stunting (Wang et al., 2005). COR genes are not induced in the GAL4DBD/CBF1AD overexpressing plants, presumably because the GAL4DBD does not tether the CBF1 activating region to the CRT/DRE (Wang et al., 2005). Because the growth stunting phenotype is alleviated when clusters of hydrophobic residues in the CBF activation domain are altered to alanine and the activating capacity of the fusion construct is abolished, the activating function appears to be responsible for the growth stunting (Wang et al., 2005). Nonetheless while the upper limit of CBF levels in the Hv-CBF2A overexpressors may have been attained, the greater copy numbers of the HvCBF4/CBFIV subgroup CBF genes in winter genotypes over their spring genotype counterparts suggests there is a mechanism in place to accommodate increased levels of CBF in the plant.

Acknowledgements

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Marcos was supported by a postdoctoral fellowship from the Spanish Ministerio de Ciencia e Innovación (MICINN). Salaries and research support in the Stockinger lab provided by state and federal funds appropriated to The Ohio State University, Ohio Agricultural Research and Development Center, the Ohio Plant Biotechnology Consortium, and USDA-CSREES subaward CO396A-F.

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References


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Table 4.1. Number of CRT/DRE motifs in a 1,250 bp region upstream of the ATG in barley *CBF*, *COR*, and *DHN* genes*.

<table>
<thead>
<tr>
<th>CBF</th>
<th>Number of CRT/DRE motifs</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBF2A</td>
<td>0</td>
</tr>
<tr>
<td>CBF2B</td>
<td>0</td>
</tr>
<tr>
<td>CBF3</td>
<td>0</td>
</tr>
<tr>
<td>CBF4B</td>
<td>0</td>
</tr>
<tr>
<td>CBF6</td>
<td>1</td>
</tr>
<tr>
<td>CBF9</td>
<td>1</td>
</tr>
<tr>
<td>CBF10A</td>
<td>1</td>
</tr>
<tr>
<td>CBF10B</td>
<td>7</td>
</tr>
<tr>
<td>CBF12A</td>
<td>5</td>
</tr>
<tr>
<td>CBF12B</td>
<td>5</td>
</tr>
<tr>
<td>CBF13</td>
<td>0</td>
</tr>
<tr>
<td>CBF14</td>
<td>0</td>
</tr>
<tr>
<td>CBF15A</td>
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</tr>
<tr>
<td>CBF15B</td>
<td>1</td>
</tr>
<tr>
<td>CBF16</td>
<td>3</td>
</tr>
<tr>
<td>COR14B</td>
<td>3</td>
</tr>
<tr>
<td>DHN5</td>
<td>3</td>
</tr>
<tr>
<td>DHN8</td>
<td>10</td>
</tr>
</tbody>
</table>

*Numbers are based on cultivar ‘Dicktoo’ (Choi et al. 1999; Knox et al. 2010) except *COR14B*, which is from barley cv. ‘Aurea’ (Dal Bosco et al. 2003).
Figure 4.1. DNA blot hybridization indicates $CBF2A$ overexpressing lines result from independent transformation events. (a) Map of the construct used for transformation shows the region between the right and left borders of pWBVec10a binary vector. $XhoI$ sites, X, are identified above the map. (b) $hph$ (hygromycin phosphotransferase), (c) $\beta$-glucuronidase (GUS), (d) $CBF2A$ coding sequence (CDS), and (e) $CBF2A$ 3' gene-specific probe (GSP) radiolabeled DNA probes were hybridized to $XhoI$-digested DNAs of ‘Dicktoo’ (Dt), ‘Golden Promise’ (GP), and $CBF2A$-transformed lines in the ‘Golden Promise’ background. Arrows marked by E identify the endogenous $CBF2$, arrowheads marked by T identify the predicted $CBF2A$ transgene cross-hybridizing fragments, and arrows marked by T identify the unexpected $CBF2A$ transgene cross-hybridizing fragments in line 2. Transgenic lines 2, 3, 6, 10, and 13 were T$_4$ generation plants, Line 15 was T$_3$, and Line 3-T$_2$ was T$_2$. 
Figure 4.2. COR and CBF transcript levels in the Hv-CBF2A overexpressing lines following a temperature decrease. (a) RNA blot hybridization analyses of the six CBF2 transgenic lines, ‘Golden Promise’, and ‘Dicktoo’ (*seed numbers of line 3 were limiting precluding assaying at the 2, 6, 10, 30, and 54 h time points) (b) Quantitation of COR14B, DHN5, and DHN8 transcript signals normalized to actin for Hv-CBF2A overexpressing lines 2, 10, and 13. (c) Relative mean of normalized COR14B, DHN5, and DHN8 levels in Hv-CBF2A overexpressor lines 2, 6, 10, 13, and 15 to normalized levels in ‘Golden Promise’. Inserts show RT-qPCR data for COR14B and DHN5 transcript levels for a subset of time points in Hv-CBF2A overexpressing lines 2, 10, and 13. COR14B and DHN5 threshold cycle, C_T were normalized relative to the C_T values of GAPDH using the Δ C_T method. The same RNA samples used for RNA blot hybridization were used for generating the cDNA (1 µg total RNA per cDNA synthesis reaction). (d) FR-H2 CBF transcript signals normalized to actin for Hv-CBF2A overexpressing lines 2, 10, and 13. (e) Relative mean of normalized CBF12, CBF15, and CBF16 levels in Hv-CBF2A overexpressor lines 2, 6, 10, 13, and 15 to levels in ‘Golden Promise’. Each probe was hybridized in succession to the same filter shown in (a). Phosphorimage signals were then quantified and are expressed relative to actin.
(a) Harvest time (h) 0 2 4 6 8 10 12 30 54
<table>
<thead>
<tr>
<th>CBF2</th>
<th>Actin</th>
<th>CBF14B</th>
<th>DHN5</th>
<th>DHN8</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBF2A</td>
<td></td>
<td></td>
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</tbody>
</table>

(b) COR14B/Actin
- Dicktoo
- GP
- Line 2
- Line 10
- Line 13

(c) COR14B/Actin
- GP
- CBF2ox

(d) CBF4/Actin
- Dicktoo
- GP
- Line 2
- Line 10
- Line 13

(e) CBF12/Actin
- GP
- CBF2ox
Figure 4.3. Survival percentages of whole plants following freezing. Seedlings of ‘Dicktoo’ (Dt), ‘Golden Promise’ (GP) and five Hv-\textit{CBF2A} overexpressing lines (L) in the ‘Golden Promise’ background were grown in wooden boxes at a density of approximately 180 plants per ~1000 cm$^2$. The growth chamber was cooled from the normal growth temperature of 18°C to the target freezing temperature of -3°C at the rate of 0.5°C h$^{-1}$. Target temperature was held for 24 h before allowing the growth chamber to return to normal temperatures. Error bars denote standard error. Bars with the same letter are not significantly different between genotypes at $P < 0.05$ by Duncan’s multiple range test.
Figure 4.4. Survival percentages and LT$_{50}$ values of excised crowns following freezing in relation to different lengths of cold acclimation. (a) Survival after freezing to -3°C for plants cold-acclimated one to seven days. (b) Survival after freezing to -6°C for plants cold-acclimated one to four weeks. (c) LT$_{50}$ values for plants cold-acclimated one to four weeks. Plants were grown under LD. Cold acclimation was at 3°C (+1°C). Excised crowns from cold acclimated and noncold-acclimated plants were frozen to the target temperatures at the rate of 2.0°C h$^{-1}$. Values represent the mean ±standard error from three independent experiments. At a given day of cold acclimation, means marked with the same letter are not significantly different between genotypes at $P<0.05$ by Duncan’s multiple range test.
Figure 4.5. CBF2A expression levels and trait comparison between ‘Golden Promise’ (GP) and the Hv-CBF2A overexpressing barley lines. Plants were grown at 28°C day/15°C night in a glasshouse with supplementary fluorescent lighting over an Australian spring season. (a) CBF2A transcript levels of five plants per line, as determined by RNA gel blot hybridization. Asterisks denote plants photographed and shown in part (b). (b) Side view showing a representative plant of each Hv-CBF2A overexpressing line nearing maturity; (c) Average values of plant height (to tallest extended leaf). (d) Biomass (aerial portion including spikes). (e) 1000 grain weight (calculated from 100 grain weight). F, grain yield. T₄ generation plants were used for lines 2, 3, 6, 10, and 15 and T₅ for line 13. Bars with the same letter are not significantly different between genotypes at $P<0.05$ by Duncan’s multiple range test.
Chapter 5: CBF gene copy number variation and the role it plays in regulating expression of FR-2 CBF genes

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Abstract

Frost Resistance-2 (FR-2) is one of the two major loci affecting freezing tolerance in the Triticeae. FR-2 coincides with a cluster of at least twelve C-repeat binding factor (CBF) genes. CBFs are transcriptional activators of genes harboring C-repeat/Dehydration Responsive Element (CRT/DRE) in their promoters; expression of these targets enhances a plant’s freezing tolerance. In barley (Hordeum vulgare), CBF transcript levels vary between winter and spring genotypes. This difference is at least partly associated with copy numbers of a 22 kb CBF2A–CBF4B genomic region. Winter genotypes usually
harbor two to three copies of this genomic region and consequently accumulate CBF2 and CBF4 transcripts to higher levels than spring genotypes that harbor only a single copy of the 22 kb fragment. CBF transcript levels also vary among the winter barleys. Here we show that this difference in CBF transcript levels among winter barleys are also partly associated with copy numbers of the 22 kb CBF2A–CBF4B genomic region. DNA blot hybridizations reveal that copy numbers of this genomic region vary between one and eight among winter barley genotypes. Genetic analyses using a collection of winter barleys related-by-descent reveals that copy numbers of this 22 kb genomic region are associated with the expression levels of CBF2, and those of CBF12 and CBF16. Chromatin immunoprecipitation experiments indicate that this association is likely due to direct binding of CBF2 to CRT/DRE motifs present in the promoters of CBF12 and CBF16. Overall, this study reveals that CBF2A, a member of the HvCBF4/CBFIV subgroup can regulate the expression of some HvCBF3/CBFIII-subgroup genes.

Introduction

The Triticeae cereals – wheat (Triticum spp.), barley (Hordeum vulgare), and rye (Secale cereale) are temperate-climate crops. These cereals are broadly described as having a winter or a spring growth habit. Spring genotypes complete their life cycle between spring and fall of the same year. In comparison, winter genotypes are sown in the fall, overwinter, and flower in the following spring. Between sowing in fall and flowering in spring, winter genotypes must establish a level of freezing tolerance adequate to cope with freezing temperatures that occur during winter.
Freezing tolerance is regulated by two major loci – *FROST RESISTANCE-1 (FR-1)* and *FR-2* that localize on the long arm of chromosome 5 homoeologs in the Triticeae cereals. Current evidence indicates that the gene underlying *FR-1* is *Vernalization-1 (VRN-1)* (Dhillon et al., 2010; Sutka and Snape, 1989). *FR-2* is approximately 30 cM proximal to *FR-1* (Vágújfalvi et al., 2003; Francia et al., 2004; Båga et al., 2007) and is coincident with a cluster of *C-Repeat Binding Factor (CBF)* genes (Skinner et al., 2005; Miller et al., 2006; Knox et al., 2010). CBFs are transcriptional activator proteins that bind to the CRT/DRE low temperature DNA regulatory element (Stockinger et al., 1997). CBFs are induced by low temperatures, typically reaching a peak in transcript levels followed by a decline (Skinner et al., 2005; Stockinger et al., 2007). There are at least 12 distinct CBF genes at *FR-2* that occur in a complex array (Francia et al., 2007; Knox et al., 2008; Knox et al., 2010). In barley, several of the *FR-H2 CBF* genes occur as highly similar but distinct gene paralogs. For example, individual barley genotypes harbor CBF sequence paralogs that are ~98% or greater in nucleotide similarity, for example, *CBF2A* and *CBF2B*, *CBF10A* and *CBF10B*, *CBF12A* and *CBF12B*, and *CBF15A* and *CBF15B* (Knox et al., 2010). Sequencing and DNA blot hybridizations indicate that one of the two paralogs is absent in spring genotypes, while both are present in winter genotypes (Knox et al., 2010).

In addition to the CBF paralogs that can be discriminated using nucleotide variation in their coding sequences (CDSs), another form of CBF duplications exists where the
duplicated copies cannot be discriminated. This form of duplication occurs for a 22 kb fragment, encompassing CBF2A and CBF4B, of which two-three copies exist in the winter barleys ‘Nure’ and ‘Dicktoo’, but a single copy exists in the spring barleys ‘Morex’ and ‘Tremois’ (Knox et al., 2010). The CBF2A and CBF4B CDSs in one 22 kb fragment are 100% identical in sequence to their copies in the adjacent 22 kb fragment, although the intergenic regions in the duplicated copies are 97-98% identical (Knox et al., 2010). A CBF2 gene-specific probe that recognizes the genomic region immediately 3’ of its CDS produces two strongly cross-hybridizing BamHI fragments in winter genotypes, one of which corresponds to CBF2A and the other to CBF2B. The constancy in signal intensity of the CBF2B cross-hybridizing fragment across the winter genotypes alongside genomic sequence data indicates that the CBF2B fragment is present as a single copy of the CBF2B gene in the genome of these winter genotypes. Signal intensity of the CBF2A cross-hybridizing fragment is frequently two to three fold greater than that of the CBF2B fragment. In spring genotypes a single CBF2 cross-hybridizing fragment is detected that is comparable in signal intensity to the CBF2B fragment detected in winter genotypes. Similarly, in hexaploid wheat (T. aestivum), CBF14 varies in copy numbers; greater copy numbers occur in winter wheats than in spring wheats (Knox et al., 2010). Recently Laurie and colleagues reported that the VRN-A1 and PPD-B1 of wheat vary in copy numbers (Diaz et al., 2012). Thus, copy number variations (CNVs) appear to be a recurring theme among all loci affecting winter hardiness in the Triticeae – CBFs, VRN-1, and PPD-1.
A CNV is defined as a DNA segment ranging in size from 1 kb to several megabases (Mb) whose copy numbers are variable between the genomes of two or more individuals within a species (Cook and Scherer, 2008; Stankiewicz and Lupski, 2010). In the human genome, the frequency of CNVs is two-four orders of magnitude greater than that of single nucleotide polymorphisms (SNPs) (Lupski, 2007). When the genes within the copy number variable region are dosage-sensitive or have regulatory roles, the variability in copy numbers can have gross overall phenotypic consequences (Stankiewicz and Lupski, 2010). As such CNVs are often associated with several genomic disorders and other abnormal conditions in humans (Stankiewicz and Lupski, 2010; Lupski, 2007). In addition to affecting the genes within the structurally variable region, CNVs also affect changes in transcript levels of genes residing up to a megabase distal to the CNV region (Henrichsen et al., 2009a; Henrichsen et al., 2009b; Chaignat et al., 2011).

Previous expression analyses indicate that the winter barley cultivar ‘Nure’ expresses CBF2 and CBF4 to higher levels than the spring cultivar ‘Tremois’ (Stockinger et al., 2007). This difference in expression levels in part cosegregates with the allelic state at FR-H2 in which CBF2A and CBF4B copy numbers in ‘Nure’ are increased over those in ‘Tremois’ as a consequence of the 22 kb segmental duplication through the CBF2A–CBF4B genomic region in ‘Nure’ (Stockinger et al., 2007; Knox et al., 2010). These data raise the question whether other barley cultivars that express CBF transcripts to even higher levels than ‘Nure’ might have even greater copy numbers of the CBF genes. An objective of the experiments carried out here was to gain greater mechanistic
understanding of the higher CBF expression levels in ‘Admire’, a winter genotype expressing all CBFs to higher levels than other winter barleys (Stockinger et al., 2007). A key finding of this study is that seven to eight copies of the CBF2A and CBF4B paralogs occur for every copy of the CBF2B paralog in ‘Admire’ suggesting that the entire CBF2A–CBF4B genomic region is tandemly amplified seven to eight times in this genotype. Additional results presented here suggest that multiple mechanisms may be contributing towards higher transcript levels of the other CBF genes at FR-H2 as a consequence of the amplified CBF2A–CBF4B genomic segment in ‘Admire’.

Materials and methods

Plant Material

The genotypes used in this study along with information on their seed source, pedigree, growth habit, row-type, and origin are described in Table E.1. The Missouri Barley (MO B) lines trace back to the cross between ‘Admire’ and another winter barley ‘Missouri Early Beardless’ (‘MEB’) cross, however the pedigrees of most MO B lines include additional winter barley genotypes (see Table E.1 for pedigrees of the MO B lines). The MO B lines were used in this study, as ‘Admire’ is not a parent of a biparental mapping population. These lines, originally developed by Dr. J.M. Poehlman at University of Missouri (1936-1980) (Lambert, 1958; Poehlman, 1973; Poehlman and Cloninger, 1955), were obtained from the National Plant Germplasm System (NPGS) (Germplasm Resource Information Network, GRIN, http://www.ars-grin.gov/). Approximately half of these MO B lines are six-row types that were developed with the objective of breeding
winter hardy six-row feed barley; the other half are two-row types resulting from a redirected focus on developing winter hardy, two-row-malting quality barley (Duelos, 1971). All MO B lines obtained from the NPGS were increased via single seed descent and the seed harvested from these plants was used for all DNA and RNA blot hybridization experiments. The one exception was MO B595, in which case RNA blot hybridizations were conducted using NPGS seed that was not increased by single seed descent.

**RNA blot hybridization**

RNA blot hybridizations were used to examine CBF expression over a time course in ‘Admire’, ‘Nure’, and ‘Golden Promise’, and to test for association between CBF2A–CBF4B copy numbers and expression levels of other CBF genes in the MO B lines. Per genotype per harvest, one 9 cm x 9 cm pot (Kord Products, www.kord.ca) was used for the time course experiments, and 3 pots were used for the association tests. Each pot contained five seeds of a genotype sown in Bacto high porosity soil mix. Pots were transferred to Conviron (BDR-16) growth chambers (Conviron, www.conviron.com). Seedlings were grown for 3 weeks under short day (SD; 8 h light/16 h dark) and long day (LD; 16 h light/8 h dark) photoperiods, and a constant temperature of 18°C. Warm (18°C) samples were harvested on day 21. At the daybreak of day 22, temperature was decreased to 6°C and held constant for 24 h for harvesting the cold samples. For the time course analyses, sampling was done at two-hour intervals, whereas for association experiment samples were harvested at 6 h and 12 h into the subjective day. Each sample
was comprised of crown tissues of five seedlings. Samples were flash frozen in liquid nitrogen immediately after harvest and stored at -80°C until RNA isolation.

Total RNA was isolated using RNeasy Plant Mini Kit (QIAGEN, www.qiagen.com) and quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop, www.nanodrop.com). Seven micrograms total RNA per sample was electrophoresed on a 1.2% MOPS-agarose gel, transferred to Hybond N membrane (GE Healthcare, www.gelifesciences.com), and UV-crosslinked to the membrane using standard procedures (Ausubel et al., 2003). CBF coding sequences were radiolabeled with ω-dCTP 32P by random hexamer primer labeling method using Megaprime DNA labeling kit (GE Healthcare, www.gelifesciences.com). RNA blot hybridizations and washes were carried out as described (Knox et al., 2010) with the modification that the concentration of Denhardt’s solution in the hybridization buffer was 2X instead of 5X. Following each hybridization RNA blots were exposed to phosphorscreens and images were scanned using a Molecular Dynamics Storm840 PhosphorImager (GE Healthcare, www.gelifesciences.com). After each hybridization the probe was stripped off using 0.1% SDS at 65°C. Stripped blots were imaged to verify there was no residual signal.

**DNA blot hybridization**

About fifty seeds of each genotype were sown in Bacto high porosity soil mix in 9 cm x 9 cm pots (Kord Products, www.kord.ca) and placed in the greenhouse. When
approximately 10 cm tall, leaf tissue was harvested about 2 cm above the soil, flash-frozen in liquid N, and lyophilized prior to DNA isolation.

High MW barley DNAs were isolated as described (Stockinger et al., 1996). Approximately 10 µg DNA was digested each with BamHI and BgII restriction endonucleases (NEB, www.neb.com), ethanol precipitated, resuspended in 10 mM Tris (pH 8.5), and quantified using the Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen, www.invitrogen.com). Normalized quantities of each sample were then electrophoresed on 0.8% TAE-agarose gel(s), transferred to Hybond N membrane (GE Healthcare, www.gelifesciences.com), and UV-crosslinked to the membrane using standard procedures (Ausubel et al., 1993). The DNA blots were hybridized with radiolabeled CBF gene-specific probes that recognize sequences immediately upstream or downstream of the coding sequences. Primer sequences used to amplify the probe templates are provided in Table E.2. Hybridization and washes were carried out as described (Knox et al., 2010). After each hybridization the probe was stripped off the membrane using 0.1% SDS at 65°C and membranes were imaged prior to the next hybridization.

**CBF2A and CBF4B copy number estimation**

Copy numbers of CBF2A and CBF4B were estimated using DNA blot hybridizations with gene-specific probes and quantifications of signal intensities of the cross-hybridizing fragments. CBF2A copy numbers were quantified as described previously (Knox et al., 2010). Briefly, genomic DNAs digested with BamHI, which discriminates between
CBF2A and CBF2B paralogs, were hybridized to CBF2 3’ probe and CBF2A/CBF2B signal intensity ratios were quantified to estimate CBF2A copy numbers. Copy numbers of CBF4B were estimated relative to CBF2 based on CBF4B 5’ and CBF2 3’ probe hybridizations to BgII-digested DNAs. CBF2 was used to quantify CBF4B copy numbers because no second and distinct single-copy paralog has been identified for CBF4. Although BgII does not discriminate between the CBF2A and CBF2B cross-hybridizing fragments, it was preferred over BamHI for quantifying CBF4B copy numbers because BamHI results in a high MW CBF4B cross-hybridizing fragment that complicated analyses.

**Statistical analyses**

Association between CBF2A–CBF4B copy numbers and CBF transcript levels was tested using R ([http://www.r-project.org](http://www.r-project.org)). Analysis of variance (ANOVA) was performed on log-transformed values of CBF/Actin signal intensity ratios using the linear model for fixed effects. Statistical significance of differences between CBF2, CBF12, and CBF16 expression within a genotype was tested using paired t-test.

**α-CBF2 antibody**

Affinity-purified, peptide-directed antibodies recognizing HvCBF2 were generated by Rockland Immunochemicals ([http://www.rockland-inc.com/](http://www.rockland-inc.com/)). Antigens consisted of peptides QDYMTVWPEEQEYRT and SFRLAPAQEIKDAV, which are amino (N) and carboxy (C) terminal, respectively, to the CBF signature sequences flanking the AP2
domain. The N- and C-terminal peptides were separately conjugated to keyhole limpet hemocyanin (KLH) for immunization.

Pre-immune sera of four rabbits were screened for cross-reactivity to plant protein extracts by immunodetection. Two rabbits showing little cross-reactivity were selected for antibody production. KHL-conjugated N- and C-terminal peptides were coinjected into these two rabbits. Immunoblot analyses using test bleeds from both rabbits, translational fusions of CBF2, CBF12, CBF15, and CBF16 to Histidine (HIS) and Glutathione-S-transferase (GST) tags, and KLH-conjugated and unconjugated N- and C-terminal CBF2 peptides demonstrated that the immune sera specifically recognized only CBF2. When no further increase in cross-reactivity titers of the test bleeds to CBF2 antigen was observed, antisera of rabbits were harvested and pooled with previous bleeds. For affinity purification, antiserum of one of the two rabbits was used. Antiserum was divided into two halves. One half was affinity purified over a resin bound with the N-terminal CBF2 peptide. The second half was purified against the C-terminal peptide. These two purified antibodies were designated as the \( \alpha - \text{CBF2-1} \) and \( \alpha - \text{CBF2-2} \) antibodies, respectively. The specificity of the affinity purified \( \alpha - \text{CBF2} \) antibodies for the CBF2 protein was confirmed by immunodetection. In this study, only the results using \( \alpha - \text{CBF2-2} \) antibody are shown, which will simply be referred to as \( \alpha - \text{CBF2} \) antibody from here onwards.
Chromatin immunoprecipitation (ChIP)

Crown tissues of 3-week-old ‘Golden Promise’ and ‘Admire’ seedlings maintained under short day photoperiod (8 h light/16 h dark) at a constant warm temperature (18°C) or cold-treated (6°C) for 8 h prior to harvest were used for the ChIP experiments. Tissues were fixed, ground in liquid N (60 mg per immunoprecipitation), followed by preparation of nuclear extracts, and sonication to an average fragment length of 300 bp (Morohashi et al., 2012). Prior to preparation of extracts, Dynabeads Protein A (Invitrogen, www.invitrogen.com) were incubated in blocking buffer (0.1 mg/ml BSA and 0.2 mg/ml yeast tRNA in lysis buffer) on a rotator with or without α-CBF2 antibody, overnight at 4°C. After preclearing with blocking buffer-treated Dynabeads for 4 h at 4°C, nuclear extracts were used for overnight immunoprecipitations using α-CBF2 antibody-treated Dynabeads at 4°C. Aliquots of the precleared extracts not used for ChIP were used as input (pre-IP) in quantitative real time PCR (below). Washing of Dynabeads, elution of ChIPed DNAs, reverse-crosslinking of immunoprecipitated and input DNAs, and subsequent purification was conducted as described (Morohashi et al., 2009). Immunoprecipitations were carried out using crown tissues harvested from warm and cold-treated seedlings grown in two independent growth chamber experiments.

Quantitative PCR (qPCR)

Purified immunoprecipitated and input DNA samples were analyzed by real time qPCR using iQ5 thermal cycler (Bio-Rad, www.bio-rad.com). Nucleotide sequence 900 bp upstream of the start codon of CBF12, CBF16, DHN8, and Plastocyanin (Pc) contains 4,
3, 7, and zero CRT/DRE motifs, respectively. Primers were designed using Primer3 (http://frodo.wi.mit.edu/) to amplify 80-100 nt of CBF12, CBF16, DHN8, and Plastocyanin (Pc) promoter regions within 100-500 nt upstream of the start codon (Table E.2). qPCR was carried out in a 10 µl volume containing 1X SYBR green (Bio-Rad, www.bio-rad.com), 2.5 µM forward primer, 2.5 µM reverse primer, and 1 µl DNA. PCR conditions included a 10 min initial denaturation at 95°C followed by 40 repeats of two-step cycling comprised of 15 s at 95°C and 1 min at 60°C. For data normalization, all values were normalized to input DNA and expressed relative to Pc.

Results
‘Admire’ accumulates CBF transcripts to higher levels than other winter barleys

Previously, we observed that CBF transcript levels were higher in the winter barley ‘Admire’ than in other barley cultivars (Stockinger et al., 2007). The higher transcript levels in ‘Admire’ were detected under both short day and long day photoperiods following a 6 h cold treatment (Stockinger et al., 2007). To test whether the higher transcript levels detected in ‘Admire’ might be due to a temporal shift in peak levels following the temperature decrease, CBF expression was tracked in ‘Nure’ and ‘Admire’ plants over a period of 24 h following cold exposure. The data from this experiment revealed that ‘Admire’ accumulated CBF transcripts to substantially higher levels than ‘Nure’ regardless of the time of the day and the photoperiod under which the plants were grown (Fig. 5.1). Additionally, CBF transcript levels peak 8-10 h following a temperature decrease under long days (LD), and 4-6 h under short days (SD) (Fig. 5.1). Thus, LD
photoperiod produces a 2-4 h delay in the peak transcript levels of the CBFs compared to SD conditions.

‘Admire’ harbors at least seven copies of CBF2A and CBF4B

As differences in CBF2 and CBF4 copy numbers are associated with differences in CBF expression levels (Knox et al., 2010; Stockinger et al., 2007), we tested the possibility that ‘Admire’ might harbor additional increases in CBF gene copy number over ‘Nure’.

Hybridization of a CBF2 gene-specific probe to BamHI-digested DNAs produced similar MW bands in ‘Admire’ as in ‘Nure’ and ‘Dicktoo’ (Fig. 5.2A). Comparable loading of ‘Nure’ and ‘Admire’ DNAs indicated that similar copy numbers of CBF2B occurred in ‘Admire’ and ‘Nure’ (Fig. 5.2A). Relative to the signal intensity of CBF2B, signal intensity of the CBF2A cross-hybridizing band was seven- to eight-fold greater in ‘Admire’, and two- to three-fold greater in ‘Nure’ and ‘Dicktoo’ (Fig. 5.2A). This data suggests that for every copy of CBF2B, ‘Admire’ harbors about seven to eight copies of CBF2A, while ‘Nure’ and ‘Dicktoo’ harbor two to three copies (Fig. 5.2A).

Copy numbers of CBF4B are increased along with those of CBF2A in ‘Nure’ and ‘Dicktoo’ as part of the 22 kb genomic fragment (Knox et al., 2010). To test if the same 22 kb genomic region is duplicated in ‘Admire’, we carried out DNA blot hybridizations using a CBF4B gene-specific probe and quantified CBF4B copy numbers relative to CBF2. These DNA blot hybridizations were carried out multiple times using
independently isolated genomic DNAs and independently-sourced ‘Admire’ seed. In one experiment the CBF4B to CBF2 normalized values for ‘Dicktoo’, ‘Nure’, and ‘Admire’ were 0.8, 0.7, and 0.6, respectively (Fig. 5.2C). A second experiment using an independent prep of ‘Admire’ DNA from the same seed source and from a second seed source (AD-1 and AD-2; Table E.1) produced CBF4B/CBF2 ratios of 0.9 and 1.0, respectively (Fig. 5.4C). The values from this second experiment are consistent with the ‘Admire’ genome having similar copy numbers of CBF4B as of CBF2 while the lower value of 0.6 is consistent with fewer copies of CBF4B relative to CBF2. The variability in CBF4B/CBF2 ratios between experiments is attributed to variability in the specific activity of the probes used in the separate hybridizations. In contrast, the CBF2A/CBF2B ratios are determined using single probe hybridization. Nonetheless, CBF4B/CBF2 ratios for ‘Dicktoo’, ‘Nure’, and ‘Admire’ approximated one (Fig. 5.2C and Fig. 5.4C) supporting the notion that CBF4B copy numbers parallel CBF2A copy numbers in all three genotypes.

Taken together, these data suggest that ‘Admire’ harbors greater copy numbers of the CBF2A–CBF4B genomic region than ‘Nure’ and ‘Dicktoo’. These increased copy numbers are likely in part responsible for the higher transcript levels of CBF2 and CBF4 in ‘Admire’ than ‘Nure’. However, hybridization with other CBF probes did not provide clear evidence to suggest their copy numbers were increased in ‘Admire’.
**CBF2 physically binds CBF12 and CBF16 promoters**

Separate experiments in which the Hv-CBF2A is overexpressed in barley indicate that the overexpressor lines may have increased transcript levels of *CBF12* and *CBF16* (Jeknic et al., submitted). *CBF12* and *CBF16* promoters harbor multiple CRT/DRE motifs, which suggests that CBF2A may be increasing the expression of *CBF12* and *CBF16* by directly binding to these motifs. Chromatin immunoprecipitation (ChIP) experiments were carried out using antiserum raised against HvCBF2 to test for the enrichment of *CBF12* and *CBF16* promoter DNAs. *DHN8*, a putative target of the CBFs was used as a positive control.

Extracts prepared from ‘Admire’ and ‘Golden Promise’ seedlings that were incubated with α-CBF2 antibody exhibited enrichment of *CBF12*, *CBF16*, and *DHN8* promoter DNAs (Fig. 5.3). This was the case for extracts prepared from both warm and cold-treated plants. In Golden Promise’, enrichment of *CBF12* and *CBF16* promoters from the cold-treated plants was greater than that in the warm-grown plants by 2.7-fold and 2.2-fold, respectively (*P* < 0.05). Enrichment of the *DHN8* promoter regions from cold-treated plants was higher, although not significantly, compared to that of the warm-grown plants (Fig. 5.3). Compared to ‘Golden Promise’, ‘Admire’ seedlings exhibited a more pronounced enrichment of *CBF12*, *CBF16*, and *DHN8* both in the warm and cold (Fig. 5.3). Greater immunoprecipitation in the warm in ‘Admire’ is consistent with its higher transcript levels of *CBF2* in the warm than in ‘Golden Promise’ (Fig. E.1). Cold
treatment increased the amount of immunoprecipitates of CBFs and DHN8 on an average by 1.4-fold or less, which was not significant (Fig. 5.3).

Overall, enrichment of CBF12, CBF16, and DHN8 in ‘Golden Promise’ and ‘Admire’ was greater than that of Plastocyanin (Pc) suggesting that CBF2 is specifically present at the CRT/DRE motifs in these gene promoters. CBF2 protein is present at the promoters of CBF12 and CBF16 in the absence of a cold stimulus in both genotypes. These data imply that CBF2 is capable of upregulating the expression levels of CBF12 and CBF16 in vivo.

Transcript levels of CBF12 and CBF16 are associated with copy numbers of the CBF2A–CBF4B genomic region

To independently test whether the higher transcript levels of CBF12 and CBF16 detected in ‘Admire’ are associated with CBF2A–CBF4B genomic region copy numbers, we utilized a set of 29 barley lines that have ‘Admire’ in their pedigree. These lines (Missouri Barley; MO B) were first scored for CBF2A–CBF4B copy numbers and then a subset was assayed for the expression levels of other CBF genes at FR-H2.

Copy numbers of CBF2A and CBF4B were estimated from hybridization signals of BamHI- and BglII-digested DNAs, respectively cross-hybridizing to CBF2 and CBF4 probes. Data from these hybridizations indicated that the MWs of CBF2 and CBF4 cross-hybridizing bands in 26 of 29 MO B lines, ‘Missouri Early Beardless’ (‘MEB’), and other
parents of MO B lines were similar to those in ‘Admire’, suggesting that the structure of 
\textit{CBF2A} genomic region is similar across these genotypes (Fig. 5.4 and Fig. E.2). Among 
the 26 lines, copy numbers of \textit{CBF2A} varied in the range of 1–8 with 5–6 being the most 
frequent (Fig. 5.4A). Ten MO B lines harbored 5–6 copy numbers of \textit{CBF2A} (Fig. 5.4A). 
Copy numbers of 1–3 occurred in 4 and of 6–8 occurred in 6 lines (Fig. 5.4A). No MO B 
line harbored four copies of \textit{CBF2A} (Fig. 5.4A). ‘MEB’ harbored 5–6 copies of \textit{CBF2A}, 
and the numbers varied between 2–6 in the other parents (Fig. 5.4A and Fig. E.1). 
Quantifications of \textit{CBF4B}/\textit{CBF2} ratios approximated 1.0 for all 26 MO B lines as well as 
for ‘MEB’ (Fig. 5.4C) indicating that \textit{CBF4B} copy numbers paralleled those of \textit{CBF2A} in 
these genotypes.

To test for association between \textit{CBF2A–CBF4B} copy numbers and \textit{CBF} transcript levels, 
MO B lines harboring 1–3 copies (Group I) and 6–8 copies (Group II) were selected for 
RNA blot analyses alongside ‘Admire’ and ‘MEB’ (Table 5.1). \textit{CBF} transcript levels 
were measured in crown tissues of warm (18ºC) and cold-treated (6ºC) plants at 6 h and 
12 h in the subjective day and normalized relative to \textit{actin} (Fig. 5.5). Significant 
differences in \textit{CBF} expression levels were detected between the two groups at 6 h in the 
warm (Fig. 5.5). Transcript levels of \textit{CBF2} in group II MO B lines were significantly 
greater than that in the group I lines ($P < 0.001$). Transcript levels of \textit{CBF12} and \textit{CBF16} 
in group II MO B lines were also significantly higher than group I lines ($P < 0.001$). 
However, this association was not observed in the cold (data not shown). Variation in 
expression levels between replicates of a genotype was not significant for any of the three
CBFs. Although there was variability in expression levels of the three CBFs within a group, this variation was non-significant ($P > 0.05$). Regardless of the $CBF2A$–$CBF4B$ copy numbers, transcript levels of $CBF2$ were the highest followed by $CBF12$ and then $CBF16$; differences between three CBFs were significant as indicated by paired t-test ($P < 0.001$).

Together these data indicate that copy numbers of $CBF2A$ and $CBF4B$ roughly parallel each other and are associated with transcript levels of $CBF12$ and $CBF16$. This association was however observed only in the warm at 6 h, but not at 12 h and at either time point in the cold (data not shown).

**Discussion**

A primary objective of this study was to understand the molecular basis of differences in $CBF$ transcript levels among winter barleys. Characterization of the winter barley ‘Admire’ and MO B lines reveals that this phenotype is partly associated with copy numbers of the $CBF2A$–$CBF4B$ genomic region.

Compared to 2-3 copies of $CBF2A$ in ‘Dicktoo’ and ‘Nure’ (Knox et al., 2010), at least 7 copies occur in ‘Admire’, at least 5 in ‘Missouri Early Beardless’ (‘MEB’), and in the range 0-8 in MO B lines that are derived from ‘Admire’ and ‘MEB’ (Fig. 5.4A). The ‘MEB’ copy number is recovered in 15 of 29 MO B lines, whereas that of ‘Admire’ in only in 3 lines (MO B575, MO B2169, and MO B2171). It is unclear why none of the
genotypes examined in this study and previously (Knox et al., 2010) harbor four copies of CBF2A. Because copy numbers of CBF2A are quantified relative to CBF2B, the estimates reflect the haploid state. In the diploid context, these numbers equate to at least fourteen copies of CBF2A in ‘Admire’, at least 10 in ‘MEB’, and between 0-16 in the MO B lines. Copy numbers of CBF4B parallel those of CBF2A in these genotypes, except MO B699. Because the MWs of CBF2 and CBF4 cross-hybridizing bands in ‘Admire’, ‘MEB’, and 26 of 29 MO B lines are similar to those in ‘Nure’ and ‘Dicktoo’ (Fig. 5.4 and Fig. E.1), this implies that these genotypes harbor the ‘Dicktoo’–‘Nure’ (DN) allele of CBF2A–CBF4B and that the repeating unit in these genotypes is the 22 kb region similar to ‘Nure’ and ‘Dicktoo’.

In addition to the DN allele, three additional alleles of CBF2A–CBF4B were identified in the MO B lines, one of which (MO B1385) was previously detected (Knox et al., 2010), while two others (MO B699 and MO B1252) were novel. In MO B1385, the MWs of CBF2 cross-hybridizing bands and absence of cross-hybridization to the CBF4 probe (Fig. 5.4) are characteristic of the Korean landrace, Mumie Pori (MP allele; Knox et al., 2010). DNA blot hybridizations indicate that MO B1385 likely inherited its allele from MO B969 (data not shown and Table E.1), which itself is a selection from the Korean landrace, Chae-Rae-Chang (PI 157659; NPGS). Together these data suggest that the two Korean alleles may be identical. Similarity to the MP allele implies despite absence of CBF4 hybridization to the BglII-digest of MO B1385, it harbors a CBF4 gene but one with much diverged 5’ and 3’ sequences (Fig. 5.4C; Knox et al., 2010). Of the two novel
alleles, one occurs in MO B699 and the other in MO B1252 (Fig. 5.4). MO B699 harbors $CBF4B$ and $CBF2B$ but is clearly deleted for $CBF2A$ (Fig. 5.4A). In MO B1252, the MWs of the $CBF2$ and $CBF4$ cross-hybridizing bands are distinct from all other genotypes (Fig. 5.4A).

In an effort to develop a high-throughput means of quantifying $CBF2A$ and $CBF4B$ copy numbers and to independently verify the results of DNA blot analyses, we attempted real-time quantitative PCRs (qPCRs) using $CBF2B$ or the neighboring gene, *Related to AP2 Triticeae-1 (RAPT-1)* as the reference single copy sequence and ‘Golden Promise’ as the calibrator genotype. However, this was less straightforward than expected considering the limited availability of nucleotide sequence unique to $CBF2A$ and $CBF2B$ that may be used for designing primers. Moreover, the large number of technical replicates deemed necessary to statistically prove a unit difference in copy numbers may compromise the high-throughput aspect of the technique (Weaver et al., 2010). DNA blot hybridizations were therefore the most appropriate method and a “gold standard” to analyze $CBF2A$ and $CBF4B$ copy numbers. Estimates of $CBF2A$ and $CBF4B$ copy numbers were consistent between experiments for ‘Admire’ and ‘MEB’ (Fig. 5.2, 5.4, and E.1), and for ‘Nure’ and ‘Dicktoo’ (Fig. 5.2 and Knox et al., 2010) strongly supporting that this method of quantification is reproducible. Consistent copy numbers between experiments also suggest that copy numbers of $CBF2A–CBF4B$ fragment are stable. Moreover, DNA blot analysis revealed three additional allele structures of the $CBF2A–CBF4B$ region that would have been missed using qPCRs.
Because a common 22 kb CBF2A–CBF4B genomic appears to be involved in CNVs among the 26 MO B lines and all other winter barley genotypes examined in this study, it suggests that a mechanism such as non-allelic homologous recombination (NAHR) may be operative. NAHR can occur if the non-allelic copies have >95% sequence similarity (Hastings et al., 2009). Indeed, PCR amplification and end-sequencing of the regions flanking or within CBFIVb Y, BARE-1, and Inav retroelement, all of which are encompassed within the 22 kb duplicated region (Knox et al., 2010) did not reveal any polymorphisms in ‘Admire’, ‘MEB’, and other parental genotypes including ‘Carstens’, and the ‘Tschermak’ lines (data not shown). This suggests that all CBF2A–CBF4B repeats in these genotypes might be identical thus supporting NAHR as the possible underlying mechanism. However, in MO B699 presence of CBF4B, structural rearrangement of CBF2B, and absence of CBF2A together imply that the structure of the CBF2B – CBF4B region is different from that in other genotypes, suggesting that a mechanism other than NAHR such as non-homologous end joining (NHEJ) or fork stalling and template switching (FoSTeS) may be involved (Hastings et al., 2009).

While NAHR could result in a maximum of 26 haploid copies of CBF2A–CBF4B in ‘Admire’ x ‘MEB’ progeny, none of the 29 MO B lines harbored more than eight haploid copies of CBF2A–CBF4B (Fig. 5.4). Whether 7-8 copies is the maximum threshold and whether plants have an innate mechanism to abort embryos that have more than eight haploid copies of CBF2A–CBF4B is intriguing. Overexpression of CBFs is known to
have detrimental consequences on plant growth and vigor (Gilmour et al., 2000; Liu et al., 1998; Zhang et al., 2004; Morrán et al., 2011). *CBF2A* overexpression in the spring barley ‘Golden Promise’ also results in growth retardation (Jeknic et al., submitted). It is possible that progeny harboring greater than eight haploid copies of *CBF2A–CBF4B* do not complete their life cycle due to severe growth retardation.

The increase in *CBF2A–CBF4B* genomic region copy numbers appears biologically relevant. Greater copy numbers of *CBF2–CBF4B* are associated with elevated expression levels of *CBF2, CBF12* and *CBF16*, as indicated by the MO B lines (Fig. 5.5; expression levels of *CBF4* were not tested). We focused on the expression levels of *CBF2, CBF12*, and *CBF16* because overexpression of *CBF2A* in ‘Golden Promise’ results in constitutive upregulation of *CBF2, CBF12*, and *CBF16* expression (Jeknic et al., submitted). Transcript levels of *CBF2, CBF12*, and *CBF16* were significantly higher in the MO B lines harboring 6-8 copies of the *CBF2A–CBF4B* genomic region than in the lines harboring 1-3 copies, in the warm (Fig. 5.5). Chromatin immunoprecipitation experiments provide direct evidence that this upregulation of *CBF12* and *CBF16* is likely due to the physical binding of CBF2 to the CRT/DRE motifs in the promoters of *CBF12* and *CBF16* (Fig. 5.3). This is the first report supporting an HvCBF4-subgroup gene upregulating the expression of HvCBF3-subgroup genes. Previous reports of CBFs regulating other *CBFs* were based on overexpression of HvCBF1/CBF1-CBFII-subgroup CBFs of wheat in barley (Morrán et al., 2011). However, we provide direct evidence using ChIP to support the binding of a CBF to the promoter of other *CBFs*. Additionally,
the MO B expression analyses and ChIP data together strongly suggest that cold temperature is not necessary to activate CBF2 in vivo in contrast to previous reports based on in vitro experiments (Xue, 2003; Skinner et al., 2005).

Some differences were observed between expression analyses. Using the MO B lines, association of CBF2A–CBF4B copy numbers with expression levels of CBF12 and CBF16 was significant only at warm but not at cold temperatures. This was unlike the data presented in Fig. 5.1 where expression of the CBFs appeared to be correlated with the ‘Admire’ FR-H2 allele both at warm (0 h time point) and at cold (all other time points). These differences may be an effect of the genetic backgrounds. For example, MO B2188 and MO B2301 harbor approximately three copies of CBF2A–CBF4B, the expression of CBF2, CBF12, and CBF16 in MO B2188 is lower than that in MO B2301 at the 6 h time point in both warm and cold (Fig. 5.5 and data not shown). Similarly, CBF expression levels differ across MO B575, MO B2169, and ‘Admire’, all of which harbor about 7-8 copies of CBF2A–CBF4B (Fig. 5.5). To overcome these limitations, we are currently in the process of developing biparental mapping populations using ‘Admire’ as a parent.

No significant differences were observed in the quantity of CBF12 and CBF16 immunoprecipitates between ‘Golden Promise’ and ‘Admire’ in the cold (Fig. 5.3). This may be because even though copy numbers of CBF2A in ‘Admire’ are 7-fold greater than in ‘Golden Promise’, the number of CRT/DRE motifs available to CBF2 in CBF12 and
CBF16 promoters may not be variable as both genotypes harbor similar copy numbers of the two genes. Increase in the quantity of immunoprecipitates in the cold over the warm was significant for CBF12 and CBF16 in ‘Golden Promise’ but not in ‘Admire’. This was because compared to ‘Golden Promise’ the immunoprecipitate levels were higher in ‘Admire’ in the warm. Although the level of CBF2 protein in the plant is unknown, as the α-CBF2 antibody did not recognize the plant protein using immunoblotting, the higher immunoprecipitate levels of CBF12 and CBF16 in the warm are consistent with the higher CBF2 transcript levels in ‘Admire’ than in ‘Golden Promise’ (Fig. E.2). Overall, ChIP data indicate that α-CBF2 antibody specifically binds to CBF12 and CBF16 promoters. The strength of this binding is stronger than its binding to Plastocyanin (Pc) that harbors no CRT/DRE motifs in its promoter, and is similar to its binding to DHN8. This also provides evidence that DHN8 is a direct target of CBF2 in vivo.

CBF2A–CBF4B copy numbers appear to be correlated with the level of freezing tolerance. Spring genotypes harbor only single copies of CBF2 and CBF4 compared to multiple copies in winter genotypes (Knox et al., 2010). Freezing tests under controlled conditions suggested ‘Admire’ to be about two degrees more freezing-tolerant than ‘Dicktoo’ and ‘Nure’ (TD and EJS, unpublished data). Winter field survival of ‘Carstens’ (3 copies; Fig. E.1) was reported to be 59% compared to 80% in MO B475 (6 copies) (Poehlman 1973). These data suggest a greater level of freezing tolerance associated with greater copy numbers of CBF2A–CBF4B.
The elevated expression levels of *CBF9*, *CBF14*, and *CBF15* in ‘Admire’ did not appear to be associated with *CBF2A–CBF4B* copy numbers. It is possible that copy numbers of these genes are increased in ‘Admire’, however DNA blot analyses did not provide clear evidence. Copy numbers of *CBF14* are variable in wheat (TD and EJS, unpublished data). Thus it is possible that *CBF14* in barley is also variable in copy numbers, but was not apparent from *BamHI* and *BglII* digests.

CNVs of the *CBFs* in barley and wheat are not particularly unique phenomena. CNVs of the flowering pathway genes *PPD-B1* and *VRN-A1* have been recently reported in wheat (Diaz et al., 2012). Several CNVs have been discovered in other plant species including Arabidopsis, soybean, maize, sorghum, using array comparative genome hybridization (aCGH) (DeBolt, 2010; Haun et al., 2011; Springer et al., 2009; Zheng et al., 2011). Array CGH might soon become available in barley with the release of its complete genome sequence (The International Barley Genome Sequencing Consortium, 2012), which might help discover CNVs of many more genes in addition to confirming the CNVs of *CBF2A–CBF4B*. Now that a reference of barley genome sequence is available, sequencing *FR-H2* of ‘Admire’ may be feasible. Alternatively, techniques such as fiber-FISH (Fluorescence In Situ Hybridization) may also prove informative. If carried out using labeled *CBF2A* and *CBF4B* probes on BAC clones of ‘Admire’, fiber-FISH can help determine the extent of the repeating unit, the orientation of the various repeats, and the exact copy numbers of the encompassed *CBF* genes, thus providing a comprehensive view of the *FR-H2* locus in ‘Admire’. 

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Acknowledgements

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Table 5.1. Missouri Barley (MO B) lines used for *CBF* expression analyses.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Group I</th>
<th>1-3</th>
<th>MO B574</th>
<th>MO B1395</th>
<th>MO B2188</th>
<th>MO B2301</th>
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<td>Group II</td>
<td>6-8</td>
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<td>MO B475</td>
<td>MO B575</td>
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*a CBF2A–CBF4B genomic region copy numbers were estimated using CBF2A/CBF2B signal intensity ratios (Fig. 5.4).*
Figure 5.1. *CBF* transcript accumulation in ‘Nure’ and ‘Admire’ following a temperature decrease from 18°C to 6°C. Plants were grown under short day (SD; 8 h light/16 h dark) and long day (LD; 16 h light/8 h dark) photoperiods. The t = 0 sample was collected at daybreak (18°C) immediately prior to the temperature decrease to 6°C. Seven μg of total RNA was loaded per lane. Hybridizations to the same filter were carried out in the top-down sequence presented in the figure, with the exception of *actin*, which followed *CBF2* hybridization.
Figure 5.2. CBF2 and CBF4 cross-hybridizing patterns and relative signal intensities of (A and C) CBF2 3’ probe and (B) CBF4B 5’ probe with ‘Dicktoo’, ‘Nure’, and ‘Admire’ DNAs. Copy number estimates of CBF2A and CBF4B are indicated below individual lanes, are based on hybridization signal intensity ratios of CBF2A/ CBF2B (A) and CBF4B/CBF2 (B and C). The MW of the cross-hybridizing bands indicated are based on nucleotide sequence of ‘Dicktoo’ and ‘Nure’. The fragment of unknown MW intermediate between CBF2A and CBF2B is not yet accounted for at the structural level (A). Note: ‘Dicktoo’ DNAs are overloaded relative to ‘Nure’ and ‘Admire’. BglII digest does not discriminate between CBF2A and CBF2B as the two comigrate (C).
Figure 5.3. Physical association of CBF2 with *CBF12*, *CBF16*, and *DHN8* promoter regions. Nuclear extracts prepared from crown tissues were incubated with α-CBF2 antibody and analyzed and the immunoprecipitates were then assayed for the presence of *CBF12*, *CBF16*, and *DHN8* promoter regions using qPCR. qPCR values of extracts incubated with α-CBF2 antibody are normalized to qPCR values of extracts not incubated with the antibody (pre-IP) and are expressed relative to *Plastocyanin* (*Pc*). Error bars represent S.E.M. of two biological replicates of seedlings grown in two independent growth chamber experiments. Different letters above error bars indicate statistically significant differences between warm and cold samples for the same gene within that genotype using one-tailed Student’s t-test.
Figure 5.4. Estimation of CBF2A and CBF4B copy numbers in an unstructured barley population using DNA blot hybridization. *Bam*HI- or *Bgl*II-digested DNAs were hybridized to CBF2 3’ probe (A and C) or CBF4B 5’ probe (B). CBF2 3’ cross-hybridizing *Bgl*II fragments producing weak signal intensity are indicated by arrowheads in MO B699 and by arrows in MO B1252 and MO B1385. Relative copy numbers of CBF2A and CBF4B as estimated from CBF2A/CBF2B ratios (A) and CBF4B/CFB2 ratios (C), respectively, are provided below the individual DNA lanes (n.a. = not applicable). AD = ‘Admire’; MEB = ‘Missouri Early Beardless’. AD-1 and AD-2 were obtained from two different sources (Table E.1). The pedigrees of all genotypes are provided in Table E.1.
Figure 5.5. Relative $CBF$ transcript levels in Groups I and II of MO B lines, ‘Missouri Early Beardless’ (‘MEB’), and ‘Admire’. Three-week-old plants entrained under short days and 18°C temperature. $CBF$ transcript levels were examined in crown tissues harvested at 6 h into the subjective day. Log-transformed values of $CBF$ expression relative to $actin$ are plotted. Copy numbers of the $CBF2A$–$CBF4B$ genomic region in groups I and II of MO B lines, and in ‘MEB’ and ‘Admire’ are above the X-axis. Error bars represent S.E.M. of three biological replicates of seedlings grown in the same experiment. $P$-values outlined above were calculated using ANOVA and indicate the significance of association between $CBF2A$–$CBF4B$ copy numbers with expression levels of the $CBFs$. 
Chapter 6: Variation in CBF14 copy numbers in A, B, and D genomes of wheat at all ploidy levels

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Abstract

In the Triticeae, chromosome 5 homoeologs harbor two major loci affecting freezing tolerance. Here, we report that the effect of one of these loci, FROST RESISTANCE-2 (FR-2) may be in part due to copy number variations (CNVs) of the C-repeat Binding Factor gene, CBF14, one of the 13 known CBF genes that cluster at FR-2. We used DNA blot hybridizations and subsequent signal-intensity quantifications to examine copy numbers of CBF14 in the A, B, and D genomes of diploid, tetraploid, and hexaploid wheats. In diploid and hexaploid wheats, copy numbers of CBF14 were estimated in each homoeolog relative to the single copy gene Puroindoline b (Pinb). In tetraploid wheats, CBF14 copy number was estimated using the ratio of the A genome relative to the B
genome. Our data indicate that the 5B homoeolog harbors lower copy numbers of CBF14 compared to 5A and 5D homoeologs. This trend occurs at all ploidy levels, indicating that CBF14 CNVs existed in the diploid ancestors prior to polyploidization. Analysis of chromosome substitution lines suggests that CBF14 copy numbers are stable in present day hexaploid wheat. Among hexaploid wheats, hard red winter wheats harbor the most copies of CBF14 followed by soft red winter wheats and then hard red spring wheats. However, the difference in CBF14 copy number in winter vs. spring wheats does not exist in white wheats, suggesting another mechanism of cold adaptation independent of CBF14. In sum, CNVs of CBFs appear to be a recurring theme in the Triticeae.

Introduction

Wheat (Triticum aestivum) is an allohexaploid comprised of three different genomes, A, B, and D (Gill et al., 2004). Extant diploid relatives of hexaploid wheat include T. urartu (AA), an as-yet-unidentified relative of Ae. speltoides (BB), and Ae. tauschii (DD), all of which are wild species endogenous to the Fertile Crescent (Gill et al., 2004). The genomes of T. urartu and an Ae. speltoides relative came together through successful intergenic hybridization and non-reduction of gametes, giving rise to tetraploid emmer wheat, T. turgidum ssp. dicoccoides (BBAA) (Luo et al., 2007; Ozkan et al., 2011). Cultivated emmer (T. turgidum ssp. dicoccon) and its free-threshing form (T. turgidum ssp. durum) were then brought into close proximity of Ae. tauschii through migration by humans. Another cycle of intergenic hybridization and non-reduction of gametes resulted, ultimately producing hexaploid wheat (BBAADD) (Zohary et al., 1969; Dvořák et al.}

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Wild wheats are typically of the winter growth habit (Flood and Hallroan, 1986; Halloran, 1967; Kihara, 1958), while cultivated wheats are both winter and spring types, suggesting that domestication and selection by humans played an important role in the selection for the spring habit trait. Today wheat is cultivated throughout the world. It is most successfully grown in the temperate climate regions of the world at latitudes 30° – 60°N and 27° – 40°S, and is also grown in more tropical regions (Curtis, 2002).

In the temperate climate regions, wheat is either sown in autumn or in spring. Autumn-sown genotypes usually have a winter growth habit characterized by their requirement for vernalization, an 8-10 week exposure to low temperatures (0-10°C), in order to transition from the vegetative to the reproductive growth phase. After sowing in the fall, winter genotypes overwinter and flower in the following spring. In comparison, spring-sown genotypes are sown in the spring and harvested in late summer or early fall. They are inherently reproductively competent and do not require vernalization to flower. These differences in the vernalization requirement of winter and spring genotypes are controlled in large part by differential regulation of the vernalization gene, VRN-1 (Yan et al., 2003). Expression of VRN-1 is restricted in winter (vrn-1) genotypes until vernalization requirements are met, but is constitutive in spring (Vrn-1) genotypes (Distelfeld et al., 2009; Trevaskis, 2010). In addition to the differences in the vernalization requirement, winter and spring genotypes also differ in their ability to survive freezing temperatures. Spring genotypes are less freezing tolerant, generally capable of surviving to about -9°C,
whereas winter genotypes can survive down to -22°C (Wilen et al., 1996; Fowler and Limin, 2004).

Loci regulating vernalization requirement and freezing tolerance reside on the long arm of homoeologous group 5 chromosomes. One of these loci is FROST RESISTANCE-1 (FR-1) (Bága et al., 2007; Hayes et al., 1993; Francia et al., 2004; Skinner et al., 2006; Vágújfalvi et al., 2003). The gene underlying FR-1 is thought to be VRN-1 (Dhillon et al., 2010; Sutka and Snape, 1989). The second locus is FR-2 that is about 30 cM proximal to FR-1. FR-2 is coincident with a cluster of at least 13 genes encoding C-Repeat Binding Factors (CBFs), which are transcription factors that play a critical role in cold acclimation and freezing tolerance (Badawi et al., 2007; Knox et al., 2008; Miller et al., 2006; Skinner et al., 2005; Stockinger et al., 1997).

Sequencing the FR-H2 genomic region in barley reveals that differences in CBF gene copy numbers occur between winter and spring genotypes (Knox et al., 2010). The winter hardy barley genotypes ‘Nure’ and ‘Dicktoo’ harbor greater copy numbers of certain CBFs compared to the spring genotypes ‘Morex’ and ‘Tremois’ (Knox et al., 2010). These differences in copy numbers result from the presence of CBF sequence paralogs and additional segmental duplications of some of these paralogous sequences in winter barleys that are absent in spring barleys. For example, winter genotypes harbor two paralogs of CBF2 (CBF2A and CBF2B), whereas spring genotypes possess only a single copy of CBF2 (Knox et al., 2010). Additionally, winter barleys possess two to three
tandemly arranged copies of a 22 kb genomic region encompassing \textit{CBF2A} and \textit{CBF4B}, whereas only a single copy of this genomic region exists in spring barleys (Knox et al., 2010). Additional data indicate that copy numbers of CBF2A-CBF4B genomic region also vary among the winter barleys (TD and EJS, unpublished data).

In wheat, use of chromosome substitution lines have significantly advanced our understanding of freezing tolerance. In these lines a chromosome pair is replaced using the same pair from another variety (Law and Worland, 1996; Unrau et al., 1956). These studies have repeatedly indicated that 5A and 5D homoeologs have the largest effects on winter hardiness, irrespective of whether these chromosomes were swapped between spring and winter genotypes or between two winter genotypes (Cahalan and Law, 1979; Galiba et al., 1995; Prášil et al., 2005; Roberts, 1986, 1990; Snape, 1997; Sutka and Snape, 1989; Toth et al., 2003; Yazdi-Samadi et al., 2006). These physiological differences in the freezing tolerance levels are associated with differences in the expression levels of the \textit{CBFs}. For instance, substitution lines of the spring wheat ‘Chinese Spring’ carrying chromosome 5A of winter wheat ‘Cheyenne’ accumulate \textit{CBF14}, \textit{CBF15}, and \textit{CBF16} transcripts to significantly higher levels than the substitution lines carrying chromosome 5A of a spring genotype of \textit{T. spelta} (Vágújfalvi et al., 2005). This difference in the transcript levels of CBF14 is suggested to be due to copy number variations of CBF14 (Knox et al., 2010).
DNA blot hybridization of a small collection of spring and winter wheat genotypes using a CBF14 gene-specific probe indicate differences in the signal intensities of the cross-hybridizing bands suggestive of differences in the copy numbers of CBF14 (Knox et al., 2010). Of the four cross-hybridizing bands, hybridizing signal intensities are lower in spring wheats than in winter wheats (Knox et al., 2010). Moreover, among the winter wheats hard red winter wheats reveal greater signal intensities than soft red winter wheats (Knox et al, 2010). These data suggest potential CNVs of CBF14 between spring and winter wheats and among winter wheats.

CBF14 CNV data generated from a small collection of hexaploid wheats raised additional questions. Which chromosome 5 homoeologs (A, B, or D) harbor increased copy numbers of CBF14? What is the extent of CBF14 CNVs in hexaploid wheat? Did these copy number differences occur in their diploid and tetraploid ancestors? Are the copy numbers of CBF14 stable over generations? We first determined the identity of the CBF14 cross-hybridizing bands using nulli-tetrasomic lines of chromosome 5 homoeologs. These lines are deleted for a chromosome pair; the deletion is balanced using the addition of a pair of its homoeolog. Estimation of CBF14 copy numbers in each homoeolog relative to the single copy reference gene, Puroindoline b (Pinb) revealed that CBF14 copy numbers vary across the three homoeologs within a genotype and across genotypes of hexaploid wheats. These CNVs also occurred among diploid and tetraploid wheats, indicating their existence prior to polyploidization. Our data also suggest that CBF14 copy numbers are stable in present day hexaploid wheats.
Materials and methods

Plant Material

Wheat genotypes used in this study, along with their ploidy level, seed source, horticultural classification, growth habit or market class, pedigree, and location where the accession was collected or developed are described in Table F.1.

DNA blot hybridizations

About 50 seeds of each genotype were sown in 9 cm x 9 cm pots (Kord Products, www.kord.ca) filled with Bacto high porosity soil mix and placed in the greenhouse. At approximately 10 cm height, the seedlings were cut about 2 cm above the soil. Leaf tissue was flash frozen in liquid nitrogen, lyophilized, and used for the isolation of high MW DNAs as described (Stockinger et al., 1996).

Approximately 10 µg DNA was digested with SacI or BglII restriction endonucleases (NEB, www.neb.com), ethanol precipitated, resuspended in 10 mM Tris (pH 8.5), and quantified using the Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen, www.invitrogen.com). Equal quantities of each DNA sample were electrophoresed on 0.8% TAE agarose gels, transferred to Hybond N membrane (GE Healthcare, www.gelifescience.com) and UV-crosslinked using standard procedures (Ausubel et al., 1993). The DNA blots were hybridized with the promoter fragment of CBF14 and the coding sequence (CDS) fragment of Puroindoline b (Pinb). Gene-specific primers were
designed using Primer3 (http://frodo.wi.mit.edu). A 189 bp CBF14 5’ fragment was PCR amplified 368 bp upstream of the CBF14 start codon from the T. monococcum accession DV92 using forward primer: 5’-agcgagctgtccttgctcatt-3’ and reverse primer: 5’-gcatcttttgtggeaaat-3’. A 201 bp fragment of the Pinb CDS was PCR amplified from the nullisomic-tetrasomic line N5A/T5D of T. aestivum (forward: 5’-tgatggacgcgtttcaca-3’ and reverse: 5’-atacctcacctcgccaaat-3’). TmCBF14 5’ and TaPinb CDS templates were cloned into pGEM-T Easy (Promega, www.promega.com) and sequenced. The cloned CBF14 5’ and Pinb CDS fragments were PCR amplified using M13 primers, which were then labeled with α-dCTP 32P via random hexamer labeling using the MegaPrime DNA labeling kit (GE Healthcare, www.gelifescience.com). Hybridization and washes were carried out as described (Knox et al., 2010). Blots were exposed to phosphorscreens and images were scanned using a Molecular Dynamics Storm840 PhosphorImager (GE Healthcare, www.gelifesciences.com). After each hybridization the probe was stripped off using 0.1% SDS at 65°C and filters were reused.

**CBF14 copy number estimation**

Copy numbers of CBF14 in hexaploid and diploid wheats were estimated relative to the grain hardness gene, Pinb. Pinb is a 447 bp gene that exists as a single copy in diploid wheats, is completely absent from the tetraploid wheats, and is present on the D genome in hexaploid wheats (Chantret et al., 2005; Gautier et al., 2000). Cross-hybridizing signal intensities of CBF14 5’ and Pinb CDS probes were quantified using the ImageQuant 5.0 software (Molecular Dynamics) and CBF14/Pinb ratios were used to estimate haploid...
copy numbers of *CBF14* in diploid and hexaploid wheats. In some diploid wheats, more than one cross-hybridizing band was produced with both probes. In those instances cumulative signal intensities of the cross-hybridizing bands were used to estimate *CBF14/Pinb* ratios. In tetraploid wheats, copy numbers in the A genome were estimated relative to those in the B genome using *CBF14*-A/*CBF14*-B signal intensity ratios.

**Results**

*Identification of A, B, and D homoeologous CBF14 genes using nullisomic-tetrasomic lines*

Previously, hybridization of a promoter fragment from the *T. monococcum CBF14* gene to *SacI*-digested hexaploid wheat DNAs produced four cross-hybridizing bands (Knox et al., 2010). We hypothesized that the different bands represented the three homoeologous *CBF14* genes from the A, B, and D genomes. To test whether this was the case, the same *CBF14* 5’ probe was hybridized to *SacI*- and *BglII*-digested DNAs of wheat lines nullisomic-tetrasomic for group 5 homoeologous chromosomes (Sears, 1966). In these lines, deletion of a chromosome pair is compensated by the addition of one its other homoeologous pair. For example, the line N5A/T5B is nullisomic for chromosome 5A, but carries an extra copy of its homoeolog 5B pair. There are two nullisomic-tetrasomic lines for each chromosome; for example, lines nullisomic for 5A can be tetrasomic for 5B (N5A/T5B) or 5D (N5A/T5D). Thus, there are a total of six nullisomic-tetrasomic lines for a chromosome pair in hexaploid wheat.
DNA blot hybridizations of chromosome 5 nullisomic-tetrasomic lines produced two or three cross-hybridizing bands (Fig. 6.1). For example, using a SacI-digest, lines nullisomic for 5A (N5A/T5B and N5A/T5D) and 5D (N5D lines) revealed three cross-hybridizing bands, while 5B nullisomics (N5B lines) revealed two cross-hybridizing bands (Fig. 6.1A). In comparison, a BglII-digest produced three cross-hybridizing bands for 5B and 5D nullisomic lines, and two cross-hybridizing bands for 5A nullisomics (Fig. 6.1B). In both digests, the absent band(s) corresponded to the homoeolog for which the lines were nullisomic (Fig. 6.1A and 6.1B). Thus, absence of cross-hybridization revealed the identity of that fragment. This data also indicated that the 5B homoeologous fragment carries an extra SacI restriction site within the region recognizing the probe resulting in two bands of the B genome CBF14 (Fig. 6.1A). Similarly, an additional BglII site is present in the A genome CBF14 (Fig. 6.1B).

The signal intensity of the chromosome in tetrasomic configuration was greater than that of the chromosome in disomic configuration (Fig. 6.1A and 6.1B). For example, signal intensity of 5B homoeolog in N5A/T5B was approximately 2-fold greater than 5B in N5A/T5D (Fig. 6.1A and 6.1B). Similarly, signal intensity of 5D homoeolog in N5A/T5D was about 2-fold greater than 5B in N5A/T5B (Fig. 6.1A and 6.1B).
**CBF14 is copy number variable in hexaploid wheats with more copies in the A and D genomes relative to the B genome**

In a previous experiment, signal intensity differences exhibited by the CBF14 cross-hybridizing bands indicated that CBF14 copy numbers might be higher in winter wheats than in spring wheats (Knox et al., 2010). To further investigate the extent of CBF14 copy number variation in wheat, we surveyed a panel of 50 hexaploid wheats, 34 of which are assigned to one of five market classes – white spring (WS), white winter (WW), hard red spring (HRS), soft red winter (SRW), and hard red winter (HRW) (Cox et al., 1990; Zeven and Hintum, 1992; NPGS [http://www.ars-grin.gov/npgs/index.html](http://www.ars-grin.gov/npgs/index.html)). To estimate CBF14 copy numbers, we normalized CBF14 signal intensities to the single copy gene Puroindoline b (Pinb).

DNA blot hybridization data for wheats belonging to one of five market classes are presented in Fig. F.1. Data for the remaining 16 unclassified accessions are presented in Fig. F.2. In majority of the 50 accessions, the CBF14 cross-hybridizing bands were of similar MWs compared to each other and to the nullisomic-tetrasomic lines (Fig. F.1A and F.2A; Fig. 6.1). The MW of the D genome cross-hybridizing band was different in three accessions – ‘Blueboy’, ‘Mayview’, and ‘Odessa’ suggesting a different haplotype of CBF14 (Fig. F.2A). In these accessions, the D genome fragment ran just below that of the B genome (Fig. F.2A). In addition, five genotypes did not produce a B genome cross-hybridizing fragment including ‘Sonora’ and ‘Mandel Gehun’ (Fig. F.1A), and two genotypes of the subspecies *sphaerococcum*, ‘PI 70711’ and ‘PI 40941’ (Fig. F.2A). This
data suggests that either the CBF14 promoter sequence is deleted or that the entire CBF14 gene is deleted from the B genome in these accessions.

The same DNA blots were next hybridized with the Puroindoline b (Pinb) CDS probe (Fig. F.1B and F.2B). All wheats produced a single cross-hybridizing band. To estimate CBF14 copy numbers, signal intensity ratios of CBF14/Pinb were determined for each of the A, B, and D CBF14 cross-hybridizing fragments. Table F.2 presents copy numbers of CBF14 relative to Pinb in the A, B, and D genomes, and collectively in the hexaploid genome of individual genotypes. Averages of a market class are plotted in Fig. 6.2

Comparisons of total numbers of CBF14 copies in the A, B, and D genomes revealed the order – HRW > SRW > HRW = WW = WS (Fig. 6.2A). The differences in CBF14 copy numbers between HRW, SRW, and HRS wheats were significant (Fig. F.2A). In all genotypes, CBF14 copy numbers were lower in the B homoeolog compared to their A and D homoeologs (Fig. 6.2B; Table F.2). Within a market class, SRW and HRW wheats possessed significantly higher CBF14 copy numbers in their A genome relative to their D genome, however no significant differences occurred between A and D genome copy numbers in genotypes of the other three classes (Fig. 6.2B). Comparisons of genomes between market classes revealed that copy numbers in the A genome of HRW and SRW wheats were significantly higher than those in the other three classes (Fig. 6.2C). The B genome copy numbers were significantly different from each other for all market classes, with the HRW harboring the highest copy numbers (Fig. 6.2C). CBF14 copy numbers for the D genome of HRW wheats were significantly higher than those of WS and HRS.
wheats. However, the D genome copy numbers in the WW and SRW wheats were not significantly different from those of the other classes (Fig. 6.2C).

To independently test whether $CBF14/Pinb$ ratios were reflective of $CBF14$ copy number differences, $CBF14$ copy numbers were also estimated within a genotype using signal intensity ratios of the A genome relative to the D genome. The $CBF14$-A/$CBF14$-D normalizations followed a similar trend as that of the $Pinb$ normalizations (Fig. 6.2D). These ratios were approximately one for the WS, WW, and HRS wheats, suggesting similar copy numbers in their A and D genomes (Fig. 6.2; Table F.2). In comparison, the $CBF14$-A/$CBF14$-D ratios for SRW and HRW wheats were about 3:1, suggesting three copies of $CBF14$ in the A genome for every copy in the D genome (Fig. 6.2D; Table F.2). The one exception was the HRS wheat cultivar ‘Cadet’, which had about 3-fold greater copy numbers in the D genome compared to A genome (Table F.2). Overall, $CBF14$-A/$CBF14$-D ratios of SRW and HRW wheats were significantly greater than those of the WS, WW, and HRS wheats (Fig. 6.2D).

Taken together, these data indicate that HRW wheats have the highest $CBF14$ copy numbers. $CBF14/Pinb$ ratios imply that greater copy numbers of $CBF14$ in HRW wheats compared to SRW wheats occur due to copy number differences in their B genome, although more of the HRW wheats have higher copy numbers of $CBF14$ in their A genome than the SRW wheats (Table F.2). Regardless of the market class and growth habit, the B genome harbored the least copy numbers of $CBF14$. Additionally, significant
differences in \textit{CBF14} copy numbers occur between winter and spring genotypes of red wheats, primarily due to the A genome, however these differences do not occur between winter and spring genotypes of white wheats.

\textbf{CBF14 copy numbers vary between the A, B, and D genomes of diploid and tetraploid wheats}

Given that the winter growth habit is the ancestral state, the much higher \textit{CBF14} copy numbers in red winter wheats over the red spring wheats raised the question as to what was the state of \textit{CBF14} copy numbers in the ancestral diploid and tetraploid wheat genomes. To address this question we surveyed progenitors of modern hexaploid wheats including the diploid wheats, \textit{T. urartu} (AA), \textit{Aegilops speltoides} (BB), and \textit{Ae. tauschii} (DD), and the tetraploid wheats, \textit{T. turgidum} subsp. \textit{durum} (BBAA). We also examined winter and spring accessions of \textit{T. monococcum}, a cultivated relative of \textit{T. urartu}.

Using a \textit{SacI} digest and the same \textit{CBF14} promter fragment as a probe, a single MW cross-hybridizing fragment was detected in the cultivated einkorn wheat genotypes (\textit{T. monococcum}) (Fig. F.3A). The wild wheats including \textit{T. urartu} and \textit{Ae. tauschii} exhibited two different banding patterns (Fig. F.3A). The cross-hybridizing bands in the \textit{T. urartu} accessions, PI 427328 and PI 487272 and all \textit{T. monococcum} accessions (Fig. F.3A) ran at the same relative positions as the A genome bands of the hexaploid wheats. The MWs of the cross-hybridizing bands in \textit{T. urartu} accessions, PI 428183 and PI 428316 ran lower (Fig. F.3A). \textit{T. urartu} accession, G1812 did not cross-hybridize to the
CBF14 5’ probe, although it did to the Pinb CDS probe (Fig. F.3). CBF14 might be missing from this accession since genomic clones of CBF14 from the G1812 BAC library were not recovered (Akhunov et al., 2005). Accessions of Ae. Speltoides exhibited several different hybridization patterns (Fig. F.3A). This increase in polymorphism is consistent with Ae. speltoides being an out-crossing species indicating higher genetic diversity at the locus than in other relatives including the cultivated types.

The same blots were then hybridized to Pinb probe (Fig. F.3B). CBF14 copy numbers in diploid wheats were estimated in a manner similar to hexaploid wheats by determining the CBF14/Pinb ratios. Approximate CBF14 copy numbers varied between 3-10 in T. urartu accessions (excluding G1812), 8-12 in T. monococcum, 3-7 in Ae. speltoides, and 5-20 in Ae. tauschii (Table F.3). No significant differences in average CBF14 copy numbers were detected between the T. urartu, T. monococcum, and Ae. tauschii accessions (Fig. 6.3) or between winter and spring genotypes of T. monococcum (Fig. F.3A; Table F.3). However, CBF14 copy numbers were significantly lower in Ae. speltoides when compared to the other three diploid wheats (Fig. 6.3).

Among the tetraploid wheats, majority of the accessions produced two CBF14 cross-hybridizing bands (Fig. F.4), the upper band was at similar relative position as the B genome band in hexaploid wheats and the lower band was similar in MW to the A genome complement of hexaploid wheats. The B genome of six accessions failed to cross-hybridize to the CBF14 probe including Odessa#66, VA05WD-1, VA05WD-12,
VA05WD-16, XVAD99067-24, and XVAD99147-1 (Fig. F.4). For all other accessions, 
CBF14 copy numbers were estimated using signal intensity ratios of the A genome 
relative to the B genome, as Pinb is absent in tetraploid wheats (Chantret et al., 2005; 
Gautier et al., 2000). Hybridization of tetraploid wheats with the Pinb probe confirmed its 
absence in our collection of tetraploid wheats (data not shown). The CBF14-A/CBF14-B 
ratios varied between 5-25 (Table F.4). These ratios were highest in XVAD99069-18 and 
VA05WD-39, and lowest in XVAD99068-14 (Table F.4). However, these ratios are at 
best only a rough estimate of CBF14 copy numbers because the high MW of the B 
genome (Fig. F.4) and potentially variable transfer efficiency during blotting could result 
in inaccurate estimation of copy numbers in the A genome.

Overall, CBF14 copy numbers in diploid and tetraploid wheats were lower in the B 
genome compared to the A and D genomes, a result which paralleled our findings with 
the hexaploid genomes. No association was detected between growth habit and CBF14 
copy numbers in the T. monococcum accessions examined.

**CBF14 copy numbers are stably inherited**

Chromosome substitution lines have been extensively used for freezing experiments and 
are reported to vary in their level of freezing tolerance depending on the source of the 
substituted homoeolog (Limin et al., 1997; Roberts and McDonald, 1988; Yazdi-Samadi 
et al., 2006). We hypothesized that CBF14 copy numbers differed between donor and 
recipient genomes of the substitution lines and that the donor CBF14 copy numbers may
be stably inherited in the recipient background. To test this hypothesis, we examined four independent sets of disomic chromosome 5 substitution lines. These lines were increased by single seed descent. Seeds from individually bagged heads were used for conducting DNA blot hybridizations. *CBF14* hybridization patterns are shown in Fig. 6.4.

Signal intensity of the 5A homoeologous *CBF14* was much higher in the winter wheats ‘Kharkov MC22’ and ‘Winalta’ compared to that of the spring wheat ‘Rescue’ (Fig. 6.4A). Replacement of the 5A homoeolog of ‘Kharkov MC22’ and ‘Winalta’ using that of Rescue in the substitution lines K-R5A and W-R5A, respectively, reverted the signal intensities of the 5A bands to that of ‘Rescue’ (Fig. 6.4A).

Three additional sets of substitution lines developed using ‘Cheyenne’ (CNN) were examined. The substitution lines CS-CNN-5A, CS-CNN-5B, and CS-CNN-5D carry the 5A, 5B, and 5D homoeologs, respectively, of CNN in the ‘Chinese Spring’ (CS) background (Fig. 6.4B). Similarly, the WI-CNN substitution lines carry chromosome 5 homoeologs of CNN in the ‘Wichita’ (WI) background, whereas CNN-WI lines carry the reciprocal substitutions (Fig. 6.4C). Six CNN accessions were obtained from three sources (Table F.1). *CBF14* hybridization indicated variability in signal intensities of the cross-hybridizing bands across the CNN accessions (Fig. 6.4B). The banding pattern of CNN-NE-1 was unique; CNN-NE-4 was similar to CNN-KS, whereas the remaining three CNN accessions were similar to each other. No such variability was observed between the two CS or between the three WI accessions obtained from different sources.

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(Fig. 6.4B and 6.4C; Table F.1). In the CS-CNN substitution lines, signal intensity of the substituted homoeologous CBF14 was similar to its intensity in CNN, whereas the other homoeologs were similar in their signal intensity to those in CS (Fig. 6.4B). Among the reciprocal WI-CNN and CNN-WI substitution lines, WI-CNN-5B and CNN-WI-5B exhibited reciprocal signal intensities in the B genome (Fig. 6.4B and 6.4C), which was independently confirmed using BglII-digested DNAs (Fig. F.5).

Because these lines have gone through several cycles of selfing since their development (PS Baenziger, UNL, personal communication) and because the signal intensity of the substituted homoeolog was similar to that of its donor parent, it suggests that CBF14 copy numbers are stable over generations.

Discussion

Loci affecting winter survival, FR-1 and FR-2 separated by a physical distance of about 850 kb, appear to be co-selected. This is supported by data in barley (Knox et al., 2010) and in wheat (this study). In barley, greater copy numbers of the CBF genes at FR-H2 co-occur with the vernalization-requiring allele of VRN-1 at FR-1 in winter genotypes, and fewer CBF copies co-occur with the vernalization-insensitive allele in spring genotypes (Stockinger et al., 2007; Knox et al., 2010). In red wheats, greater copy numbers of CBF14 occur in winter genotypes than in spring genotypes (Fig. 6.2). Because winter growth habit is believed to be the ancestral form in the Triticeae (Flood and Halloran, 1986; Matus and Hayes, 2002; von Bothmer et al., 2003; Yan et al., 2003), it suggests
that greater \textit{CBF} copy numbers is also the ancestral state. As wheat and barley began to be cultivated in warmer climates with shorter growing season, mutations in \textit{VRN-1} resulting in spring growth habit (von Zitzewitz et al., 2005) may have been accompanied by the loss of excessive \textit{CBF14} copies that would otherwise be an unnecessary genetic load on the plant in warmer climates. These data suggest a possible role of \textit{CBF14} in environmental adaptation of wheat.

In addition to the environment, have humans shaped the \textit{FR-2} locus? The effect of human selection on \textit{CBF14} copy numbers can be determined from comparisons between wild and cultivated accessions. Among the diploid wheats, \textit{T. monococcum} or einkorn wheat, a relative of \textit{T. urartu}, is the only cultivated species, domesticated approximately 9,600 – 9,000 years before present (BP); cultivated relatives of \textit{Ae. speltoides} and \textit{Ae. tauschii} do not exist (Luo et al., 2007; Ozkan et al., 2011). Comparisons of \textit{CBF14} copy numbers between \textit{T. monococcum} and \textit{T. urartu} reveal no significant differences (Fig. 6.3), suggesting absence of human selection pressures. However, this conclusion may not be final given that only a small number of accessions were examined in this study. To gain confidence in this conclusion, more accessions of these species may need to be examined. Comparisons of wild and cultivated species of tetraploid wheats (\textit{T. turgidum}) may also provide helpful clues.

Within the hexaploid wheats copy numbers are significantly lower in the B homoeolog than in the A and D homoeologs (Fig. 6.2). Tetraploid durum wheats exhibit a similar
trend, as indicated by greater signal intensity of the A homoeolog than the B homoeolog (Fig. F.4). This trend is also exhibited by the diploid ancestors where *Ae. speltoides*, a relative of the B genome donor has lower *CBF14* copy numbers than the A genome of *T. urartu* and *T. monococcum*, and D genome of *Ae. tauschii* (Fig. 6.3 and F.3). These data indicate that differential copy numbers of *CBF14* between genomes pre-existed in the diploid ancestors, and did not emerge after polyploidization.

Greater *CBF14* copy numbers in the A and D genomes in hexaploid wheats are consistent with previous reports of the 5A and 5D homoeolog having the largest effects on freezing tolerance (Cahalan and Law, 1979; Roberts, 1986, 1990). Absence of *CBF14* hybridization to the B genome in some tetraploid and hexaploid wheats was striking (Fig. F.1, F.2, and F.4). This may be due to the complete deletion of *CBF14* from their B genome or due to divergence of the *CBF14* promoter sequence in these genotypes. Nonetheless, the B genome appears to be responsible for the significant difference in *CBF14* copy numbers between SRW and HRW wheats (Fig. 6.2).

All estimates of *CBF14* copy numbers are based on the hardness gene, *Puroindoline b* (*Pinb*). *Pinb* is one of the three tightly linked genes along with *Pina* and *Grain Softness Protein-1* (*GSP-1*) comprising the Hardness locus (*Ha*) that determines endosperm texture (Gautier et al., 1994; Morris et al., 2002). *Pinb* has also undergone copy number changes over the history of wheat evolution. It is present in all diploid ancestors, is deleted from the A and B genomes of tetraploid wheats, and has been restored in the D
genome of hexaploid wheats via *Ae. tauschii* (Chantret et al., 2005). Soft wheats carry functional coding sequences of both *Pina* and *Pinb*, whereas hard wheats possess either null mutations of *Pina* or Gly-to-Ser point mutation in *Pinb* CDS (Morris et al., 2001). Occasionally, *Pinb* deletion also occurs in hexaploid wheats (Li et al., 2008). However, *Pinb* more frequently occurs as a single copy in all wheats (Chantret et al., 2005) making it ideal for use as a single copy reference for *CBF14* copy number estimations.

Although copy numbers of *CBF14* are highly variable between and within genotypes, they appear to be stable in present day hexaploid wheat as indicated by the *CBF14* hybridizations to chromosome substitution lines (Fig. 6.4). In all substitution lines examined, signal intensity of the substituted homoeolog resembled that of its donor parent while the signal intensities of the other homoeologs resembled that of their recipient parent. Differences in cross-hybridizing signal intensities of 5B and 5D homoeologs across the multiple ‘Cheyenne’ accessions suggest that these accessions are genetically different lines (Fig. 6.4B). These data also suggest that different ‘Cheyenne’ accessions were used to develop the different CS-CNN, WI-CNN, and CNN-WI substitution lines (Fig. 6.4B and 6.4C). For example, while CNN-NE-1 may have been used to develop CS-CNN-5A, it was most likely not used to develop CS-CNN-5D.

Freezing tolerance data of the substitution lines reported by others and *CBF14* copy numbers reported in this study reveal an association between the two. Increased *CBF14* copy numbers occurring on all three homoeologs of ‘Cheyenne’ compared to ‘Chinese
Spring’ and on 5B of ‘Cheyenne’ compared to ‘Wichita’ (Fig. 6.4A and 6.4C) are consistent with greater freezing tolerance of CS-CNN-5A, CS-CNN-5B, CS-CNN-5D lines compared to ‘Chinese Spring’ (Limin et al., 1997; Veisz and Sutka, 1998) and of WI-CNN-5B than ‘Wichita’ (Yazdi-Samadi et al., 2006). Similarly, lower $CBF14$ copy numbers on 5A of ‘Rescue’ compared to ‘Kharkov MC22’ and ‘Winalta’ (Fig. 6.4A) are consistent with the lower freezing tolerance of K-R5A and W-R5A lines than that of ‘Kharkov MC22’ and ‘Winalta’, respectively (Roberts and McDonald, 1988). ‘Rescue’ substitution line harboring 5D homoeolog of ‘Cadet’ (R-C5D) is also more freezing tolerant than ‘Rescue’ (Roberts, 1986), which is consistent with the higher copy numbers of $CBF14$ on the 5D homoeolog of ‘Cadet’ compared to ‘Rescue’ (Table F.2).

Additionally, HRW wheats that harbor the greatest copy numbers of $CBF14$ (Fig. 6.2A; Table F.2) possess the highest level of freezing tolerance among all other wheats (Fowler et al., 1977).

Overall, $CBF14$ CNVs appear to be correlated with freezing tolerance levels of winter and spring genotypes. This is also the case for $CBF2A$–$CBF4B$ CNVs in barley (Knox et al., 2012). CNVs of $VRN-A1$ and $PPD-B1$ of wheat are reported to be associated with flowering time (Diaz et al., 2012). Thus, these data suggest that CNVs possibly play a much larger role in the adaptation of the Triticeae cereals to different environments than has been previously realized. In addition to wheat and barley, CNVs of genes are also identified in other plant species including Arabidopsis, maize, sorghum, and soybean as a result of genome sequencing and re-sequencing projects (DeBolt, 2010; Haun et al.,
2011; Springer et al., 2009; Zheng et al., 2011). More recently, it has been suggested that effects of many quantitative trait loci affecting biotic stress response may be due CNV of the NB-LRR genes in soybean (McHale et al., 2012).

To gain insight into the CBF14 locus structure, sequencing the genomic region would be the most direct approach. BAC libraries of each chromosome 5 homoeolog exist in diploid, tetraploid, and hexaploid wheats (Akhunov et al., 2005; Chantret et al., 2005; Lijavetzky et al., 1999). Sequencing of G1812 (T. urartu) BAC library may not be useful, as it does not harbor CBF14 (Fig. F.3 and data not shown). Sequencing the DV92 (T. monococcum) CBF14 clones may provide important clues. Even though DV92 is a spring genotype, CBF14/Pinb ratios indicate that it harbors about ten copies of CBF14 on one copy of chromosome 5A (Table F.3). Whether BAC sequencing of other wheats will be productive will require determining whether they harbor CBF14. Sequencing the locus will provide valuable information including the exact copy numbers of CBF14, orientation of the repeats, kilobase span of the duplicated sequence, whether all duplicated copies are functional coding sequences, and possibly also indicate a mechanism underlying the duplications such as non-allelic homologous recombination.

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state.edu/wgrc/Resources/resources_index.html), David F. Garvin at the University of Minnesota, P.S. Baenziger at the University of Nebraska-Lincoln, Carl A. Griffey at the Virginia Polytechnic Institute and State University. Kansas State University for providing wheat accessions and genetic stocks. We also thank Alexandra Schaffner for technical assistance. Salaries and research support in the Stockinger lab provided by state and federal funds appropriated to The Ohio State University, Ohio Agricultural Research and Development Center. This work was supported by grants from the Ohio Plant Biotechnology Consortium (2010-011), and the United States Barley Genome Project (USDA-CSREES subaward CO396A-F).

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CBF14 hybridization patterns in wheat lines nullisomic/tetrasomic for group 5 chromosome homoeologs. *Sac*I (A) and *Bgl*II (B) digested DNAs were hybridized with a probe derived from the *T. monococcum* CBF14 promoter. The cross-hybridizing homoeologs (A, B, and D) are labeled. Absence of a cross-hybridizing fragment in a lane identifies the homoeolog for which the lines are nullisomic. *Sac*I restricts the B CBF14 homoeolog into two cross-hybridizing bands; the lower MW of the two is indicated using an arrowhead. *Bgl*II restricts the A CBF14 homoeolog into two cross-hybridizing bands. Insufficient quantity of DNA precluded the N5D/T5A line from the *Bgl*II digest.
Figure 6.2. CBF14 copy number estimates in hexaploid wheats (*T. aestivum* subsp. *aestivum*). Genotypes are grouped into five market classes – white spring (WS), white winter (WW), hard red spring (HRS), soft red winter (SRW), and hard red winter (HRW). A) Total CBF14 copy numbers relative to Pinb; B) Within-class comparisons of CBF14/Pinb ratios for each genome; C) Between-class comparisons of CBF14/Pinb ratios for each genome; D) Between-class comparisons of CBF14 copy numbers in the A genome relative to those in the D genome (CBF14-A/CBF14-D). WS, WW, HRS, SRW, and HRW classes include 9, 5, 7, 5, and 8 genotypes, respectively (identified in Fig. F.2 and Table F.2). Error bars represent standard error of mean (S.E.M.). Statistically significant differences are indicated by different letters above the error bars (*P* < 0.05).
Figure 6.3. Average CBF14/Pinb ratios in diploid wild (*T. urartu*, *Ae. speltoides*, and *Ae. taushii*) and cultivated (*T. monococcum*) wheats. Number of genotypes within each species (n) is listed on the X-axis. Error bars represent S.E.M. Statistically significant differences are indicated by different letters above the error bars (*P* < 0.05).
Figure 6.4. Stable inheritance of *CBF14* in wheat chromosome 5 substitution lines. A) Substitution lines K-R5A and W-R5A carrying 5A homoeolog of ‘Rescue’ in ‘Kharkov MC22’ and ‘Winalta’ backgrounds, respectively, B) CS-CNN substitution lines carrying chromosome 5 homoeologs of ‘Cheyenne’ in ‘Chinese Spring’ background, and (C) WI-CNN substitution lines of chromosome 5 homoeologs of ‘Cheyenne’ in ‘Wichita’ background, and the reciprocal CNN-WI substitution lines. Sources of ‘Chinese Spring’, ‘Cheyenne’, and ‘Wichita’ accessions are listed in Table F.1. Note: Signal intensity differences between ‘Wichita’ accessions, WI-KS-1, WI-KS-2, and WI-NE-1 are attributed to differences in the quantity of DNA loaded on the gel. CS = Chinese Spring; CNN = Cheyenne; WI = Wichita. Digests and DNA blot hybridizations were carried out similarly as for Fig. 6.1A.
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Appendix A: Response of \textit{VRN-H1} to photoperiod
Table A.1. Primers used to genotype D x M lines.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5'→ 3')</th>
<th>Genotyping marker</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPD-H1</td>
<td>ATGCAGATGTGATGTCGGTTCGCG</td>
<td>BstUI CAPS</td>
<td>Turner et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>TATGCTGGTGGCGTTGCGCTGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPD-H2</td>
<td>CAAGGCTAGCGACTGTTAATTG</td>
<td>Dominant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GATTTCCGCTCAACAAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VRN-H3</td>
<td>CTTTTGCCCTCATACCCCTAG</td>
<td>BclI CAPS</td>
<td>Karsai et al. (2008), Yan et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>GCTTAATTCTGTGGCTTC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure A.1. RNA blot analyses of *VRN-H1* transcripts in the facultative barley ‘Dicktoo’ (a) and the spring barley ‘Morex’ (b). Plants were grown at 18°C under short days (SD; 8 h light/16 h dark) and long days (LD; 16 h light/8 h dark). No samples were harvested in the first week of growth. Plants transferred from LD to SD (LD → SD) were grown under LD for 5 days. From week 2 onwards, crown tissues samples were harvested at 6 h under SD and 14 under LD on day 5 each week at 18°C. On day 6, temperature was decreased to 10°C at daybreak and samples were harvested at the same time points as under 18°C. Each sample comprised of five crowns. About 7 µg total RNA of each sample was used for RNA blot hybridizations to the coding sequence probes of *VRN-H1* and *actin*. 
Figure A.2. RNA blot analyses of *VRN-H1* transcripts in ‘Dicktoo’, ‘Morex’, and 15 D x M doubled haploid lines harboring the ‘Dicktoo’ *VRN-H1* allele. Crown tissues of three-week-old plants grown under short days (a) and long days (b) at 18°C were used for *VRN-H1* expression analysis. Samples were harvested on day 21 at 6 h under SD and 14 h under LD. RNA blot hybridizations were conducted the same as described in Fig. 1.1. Allelic status of *PPD-H1*, *PPD-H2*, and *VRN-H3* of the genotypes are presented in (c).
Figure A.3. RNA blot analyses of \( VRN-H1 \) transcripts in ‘Dicktoo’, ‘Morex’, and 37 D x M doubled haploid lines harboring the ‘Dicktoo’ \( VRN-H1 \) allele. Transcript and genotyping data were collected in the same way as for Fig. 1.2.
Appendix B: Freezing tolerance and flowering regulation in cereals: the \textit{VRN-1} connection
Table B.1. Primers used for quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>Prod. bp</th>
<th>Eff. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBF12</td>
<td>GTCCCACTCCACCTCACAG</td>
<td>ACATGTGGTGGCCACAATGC</td>
<td>74</td>
<td>95</td>
</tr>
<tr>
<td>CBF14</td>
<td>CCACCAAAATATGGGAGGA</td>
<td>GCTTTCACAATGAACGGCA</td>
<td>73</td>
<td>92</td>
</tr>
<tr>
<td>CBF15</td>
<td>CATGTTCAAGCTGATATGCTCGGGG</td>
<td>GGGACACAGCTTCGGTTGTTCATGC</td>
<td>213</td>
<td>100</td>
</tr>
<tr>
<td>CBF16</td>
<td>GCGGCAAGCTCTCAACAGGCGAG</td>
<td>ACAGTCCAGGTGCCATCTCCCAG</td>
<td>210</td>
<td>93</td>
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<tr>
<td>CBF13</td>
<td>TGGACATCGACATGTTCAGGCTGGTTT</td>
<td>CAGAGCAGAATCAGATGGGGAATC</td>
<td>214</td>
<td>91</td>
</tr>
<tr>
<td>CBF10</td>
<td>TGTTCAGTAGGCTTGACCTGGCTCCCG</td>
<td>GCAGAATCAGCTACAGCTCCAG</td>
<td>180</td>
<td>86</td>
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<tr>
<td>CBF3</td>
<td>GCAGGTGGACACGGATATGTTTAGCCG</td>
<td>GCAGGGAAATTATCGACTGTAC</td>
<td>204</td>
<td>87</td>
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<tr>
<td>CBF17</td>
<td>GAGTTCGACTTGGAGCTGGA</td>
<td>CGAAATCAGGCTTAGGGAG</td>
<td>67</td>
<td>96</td>
</tr>
<tr>
<td>CBF9</td>
<td>CCAGCAGCAGCAGATCATT</td>
<td>CGCGGAAAAGCATGTAAAAC</td>
<td>65</td>
<td>92</td>
</tr>
<tr>
<td>CBF4</td>
<td>GTGTGTCTCCATGTCGTCAG</td>
<td>GTAGTACGACCCGGCAACC</td>
<td>82</td>
<td>94</td>
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<tr>
<td>CBF2</td>
<td>GTCCCATCCTCCCTCAACGACT</td>
<td>GCAGGAATTTTGCGACTGTA</td>
<td>89</td>
<td>86</td>
</tr>
<tr>
<td>COR14b</td>
<td>GAGGAGTCCCTGATACCGAC</td>
<td>CTACGGCTCCCTGTACTCTTG</td>
<td>135</td>
<td>96</td>
</tr>
<tr>
<td>ACTIN</td>
<td>ACCCTGAGTGCCCCACCAA</td>
<td>CAGAGTCCAGCACAATACCATGTTG</td>
<td>91</td>
<td>98</td>
</tr>
<tr>
<td>TEF1</td>
<td>GCCCTCCTTGGTTCATCTCTT</td>
<td>AACCGGCTTTGAGTACTTG</td>
<td>91</td>
<td>99</td>
</tr>
</tbody>
</table>
Methods for Fig. B1: This dominant marker uses three primers in the PCR reaction. Primers MVP_F-18 and MVP_R-22 amplify a 172-bp band in both the mutant and non-mutant lines that is used as an amplification control, whereas primers MVP_F-18 and MVP_R-23 amplify a 340-bp band that is only present in the non-mutant lines. PCRs were carried out in a 20 µl volume using 500 pmol each of MVP_R22 and MVP_R23, and 750 pmol of MVP_F18.

Primers:

MVP_F-18: 5′-AGCCACAAGAACCACGGACTA-3′
MVP_R-22: 5′-ATTCAAGCCCCAATGTTCTC-3′
MVP_R-23: 5′-CCCACAACCTTTGCAGGTATC-3′

PCR conditions:

40 cycles of: 94°C-20 s, 60°C-20 s, 72°C-15 s.
Figure B.2. Apices of Mvp-2/- and mvp-2/mvp-2 plants before and after cold acclimation in the freezing tolerance test described in Fig. B.3 (-12°C). Apices were photographed after 35 days at 20°C and again after additional 18 days of cold hardening at 4°C before the freezing experiment. The apices from the mvp-2/mvp-2 mutant plants were at the vegetative stage whereas those from Mvp-2/- plants were at the double ridge stage (dr).
Figure B.3. A) Average freezing scores using a scale from 0= dead plants to 5= undamaged plants. The -8°C experiment included 22 mvp-1/mvp-1 homozygous mutants (black bars) and 35 Mvp-1/- plants (gray bars) carrying at least one functional VRN-1 copy. The -12°C experiment used 24 mvp-2/mvp-2 homozygous mutants and 65 Mvp-2/- plants. Plants were 35 days old before the cold acclimation treatment. P values correspond to ANOVAS comparing mvp/mvp (black bars) and Mvp/- (gray bars) plants. B) Average relative conductivity (ion leakage) of leaf segments from mvp-2/mvp-2 and Mvp-2/- plants. Error bars represent standard errors of the means (SE) based on 9 replications per genotype / temperature combination.
Methods used for Figure B.3:

A) Regrowth. Seeds were germinated in Petri dishes and seedlings were transplanted to wooden boxes (42 cm long x 30 cm wide x 14 cm high) with 9 cm of soil depth. Soil consisted of 4:1 soil to sand. Plants were grown for 35 days in a Conviron growth chamber using a light intensity of 260 µmol m\(^{-2}\) s\(^{-1}\), constant temperature of 20°C, and 75% relative humidity. Plants were genotyped with the marker described above in Fig. B.1. After 35 days temperature was decreased 4°C per day until the temperature reached 4°C, which was then held constant for 18 d. Both growth and cold hardening were carried out under long day conditions 16/8 hours (day/night). This was followed by acclimation at -2°C of 6h and 17h (separated by +2°C for 7 hours), and at -4°C for 22 h. Ice nucleation was induced by spraying the leaves with water. Temperature was then lowered 1°C/h to the target temperatures of -9°C, -12°C, and -13°C, which were held for 24 h.

After the freezing treatment temperature was increased 2°C per h to 17°C under a light intensity of 260 µmol m\(^{-2}\) s\(^{-1}\), a 16 h/8 h light/dark photoperiod cycle and 75% RH. After two days the leaves were cut several cm above the soil. Regrowth was scored after 14 days of recovery.

B) Electrolyte leakage assay: Electrolyte leakage assays from plants treated at -9, -12 and -13 °C (Fig. B.3B) utilized the middle one-third section of the oldest leaf. After removal from the plant, the weight and length of this leaf section was determined. Leaf sections were rinsed with DI-H\(_2\)O, and then incubated overnight in 10 ml of DI-H\(_2\)O in tubes, during which time they were gently shaken.
Maximum ion leakage was determined by boiling three independent mvp/mvp homozygous mutant class leaf samples and three independent Mvp/- leaf samples for 20 minutes. Conductance was measured using a MultiSample Conductometer (Mikro KKn., Hungary) in an average of 9 plants per genotype temperature combination. Relative conductance was calculated using the equation, Relative conductance = (Measured conductance – Average conductance of di-H$_2$O)/ (Average maximum conductance - Average conductance of di-H$_2$O)* 100.
Figure B.4. Quantitative RT-PCR analysis of transcript levels of the CBF genes present at the FR-2 locus. Values are expressed relative to the TEF1 endogenous control (COR14b transcript levels are included as reference). Leaf samples were collected from 8-weeks old plants 8 h after transferring plants from 20°C to 4°C. Values in the Y axes were normalized and calibrated using the 2^{ΔΔCT} method (Livak and Schmittgen 2001). The same calibrator was used for all genes to ensure scales are comparable across genes. Homozygous mvp-1/mvp-1 plants are indicated in black and those of Mvp-1/- plants carrying 1 or 2 functional VRN-1 copies in gray. Values are averages of 10 biological replications ± SE of the means. The inset shows the CBF genes with relatively lower transcript levels using a different scale. P values were calculated using ANOVA of log(n+1)transformed values *: P<0.05 and **: P<0.01.
Appendix C: Pedigree analysis of the spring barley ‘Tremois’ reveals a historical origin of its $CBF2$, $CBF4$, and $CBF13$ alleles and of spring growth habit.
Table C.1. Barley genotypes used in this study.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Accession number</th>
<th>Hort. Class.</th>
<th>Row-type</th>
<th>G. H.</th>
<th>Pedigree</th>
<th>Location</th>
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<tbody>
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<td>Abacus-1</td>
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<td>LR</td>
<td>6</td>
<td>S</td>
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<tr>
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<td>CV</td>
<td>2</td>
<td>S</td>
<td>Vada / Zephyr</td>
<td>England, UK</td>
</tr>
<tr>
<td>Agio</td>
<td>CIho 9560</td>
<td>CV</td>
<td>2</td>
<td>S</td>
<td>Kenia / Schweigers Georgine</td>
<td>South Holland, Netherlands</td>
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<td>N.A.</td>
<td>California, USA</td>
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<td>6</td>
<td>S</td>
<td>N.A.</td>
<td>USA and Algeria</td>
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<tr>
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<td>Germany</td>
</tr>
<tr>
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<td>Algeria</td>
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<td>S</td>
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<tr>
<td>Archer-3</td>
<td>PI 467655</td>
<td>CV</td>
<td>N.A.</td>
<td>S</td>
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<tr>
<td>Armelle</td>
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<td>CV</td>
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<td>S</td>
<td>Ceres / Clermont</td>
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<td>A. Bavaria</td>
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<td>CV</td>
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<tr>
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<td>S</td>
<td>Bavaria / Danubia</td>
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<tr>
<td>A. Isaria-2</td>
<td>PI 328618</td>
<td>CV</td>
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<td>Bavaria, Germany</td>
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<td>U</td>
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<td>Balder</td>
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<td>PI 467696</td>
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<tr>
<td>Berac</td>
<td>PI 355136</td>
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<td>Balder / Erica</td>
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<tr>
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<td>PI 186416</td>
<td>CV</td>
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<td>W</td>
<td>Lignee 66 / Lignee 125</td>
<td>Namur, Belgium</td>
</tr>
<tr>
<td>Bruens Wisa</td>
<td>PI 269155</td>
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<td>S</td>
<td>WMI / Ackermans Isaria</td>
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</tr>
<tr>
<td>Carlsberg</td>
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<td>CV</td>
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<td>S</td>
<td>Prentice / Maja</td>
<td>Denmark</td>
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<tr>
<td>Carlsberg II</td>
<td>CIho 10114</td>
<td>CV</td>
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<td>S</td>
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<th>Genotype</th>
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<th>Pedigree</th>
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<td>S</td>
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<td>Term / RMGH 61229</td>
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<td>S</td>
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<td>CV</td>
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<td>U</td>
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<td>Gelderland, Netherlands</td>
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Continued
Table C.1 continued

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<th>Genotype</th>
<th>Accession number</th>
<th>Hort. Class.</th>
<th>Row-type</th>
<th>G. H.</th>
<th>Pedigree</th>
<th>Location</th>
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<tbody>
<tr>
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<td>CV</td>
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<td>S</td>
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<td>WMI / Morgenrot</td>
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<td>S</td>
<td>WMI / Morgenrot</td>
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</tr>
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<td>CV</td>
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<tr>
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<td>CV</td>
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<tr>
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<td>CV</td>
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<td>S</td>
<td>Single plant selection from stock of Archer</td>
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<tr>
<td>Proctor</td>
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<td>CV</td>
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<td>S</td>
<td>Kenia / Plumage Archer</td>
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<td>S</td>
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<td>Malmo, Sweden</td>
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<td>Tern</td>
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<td>S</td>
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<td>S</td>
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<td>S</td>
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**Known variants**

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<td>Selection No. 2 from an unknown cross made prior to 1917 at Dickinson, ND</td>
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Continued
Table C.1 continued

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a All genotypes in the ‘Tremois’ pedigree and relevant information were obtained from the National Plant Germplasm System (NPGS; [http://www.ars-grin.gov/npgs/index.html](http://www.ars-grin.gov/npgs/index.html)). Shortened accession names include: A. Bavaria = ‘Ackermanns Bavaria’; A. Fruhe = Australische Fruhe; A. Isaria = ‘Ackermanns Isaria’; H. Franken = ‘Heils Franken’; P. Archer = ‘Plumage Archer’; Pflugs = ‘Pflugs Intensiv’; WMI = ‘Weihenstephan Mehltauresistente I’. Accessions unavailable from the NPGS are only depicted in Fig. 3.1 and include Binder, C403 (Crewner 403), Ecken. Winter. (Eckendorfer Wintergerste), Granat, Haisa I, Heine 2149, Heines Haisa I, H204 (subsp. spontaneum), HP 5466, Kalk. Winter. (Kalkreuther Wintergerste), Lignee 66, Pyrthyjarven, RMGH 61229, Scania barley, S. Georgine (Schweigers Georgine), and PI 57753.

b Hort. Class. = Horticultural Classification; CV = Cultivar; LR = Landrace; U = Uncertain.

c N.A. = Information not available.

d G.H. = Growth Habit; S = Spring; F = Facultative; W = Winter. Note: growth habits are NPGS annotations.

e Pedigrees are given in Purdy format. N.A. = Information not available.

f The location indicated is the place of development for genotypes whose pedigree information is provided and the place of collection or origin for the genotypes whose pedigree information is not available.

g These barley cultivars served as known variants in all genotyping and phenotyping assays.

h PMH = P.M. Hayes, Oregon State University, Corvallis, OR; NP = Nichola Pecchioni, Instituto Sperimentale per la Cerealicoltura Section of Fiorenzula, Italy.
Table C.2. Primers and genotyping markers used for ‘Tremois’ pedigree analysis.

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<td>CBF13</td>
<td>CCAATCCATCACTCAATCA</td>
<td>TGGCCTCAGATGGATTTGC</td>
<td>SalI &amp; BglII CAPS</td>
</tr>
<tr>
<td>VRN-HI promoter</td>
<td>GCTGCAATAGTTTCAGACGA</td>
<td>GTGACCAGCTTCTCGGTA</td>
<td>InDel</td>
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<td></td>
<td>GCGTCAAAAGTCCAGCT</td>
<td>GTAGGAGGCTGTTCTCTTG</td>
<td>BglII CAPS</td>
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<tr>
<td>VRN-HI intron 1</td>
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<td>CGTTTATCACTGACGAC</td>
<td>InDel</td>
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<tr>
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<td>CCATAGAGCAATTGATGTTGG</td>
<td>GATCGTTCGTTGACTATAGT</td>
<td>Dominant</td>
</tr>
<tr>
<td>ZCCT-Hc</td>
<td>AGAGAGGCGAAGCAGATGAT</td>
<td>GGTGACAGCTTTCCGGAT</td>
<td>Dominant</td>
</tr>
<tr>
<td>PPD-H1</td>
<td>ATGCGAATGGTTGATCGGC</td>
<td>TATAGCTAGGTGAGTCGG</td>
<td>BstUI CAPS</td>
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Appendix D: *HvCBF2A* overexpression accelerates the early stage of cold acclimation in the spring habit barley ‘Golden Promise’
Figure D.1. CBF2, COR14B, DHN5, and DHN8 transcript levels in the early transformant generations as compared to non-transformed ‘Golden Promise’ (GP) and ‘Dicktoo’ (Dt). Lines 2, 6, and 10 were T2, and line 13 was T3 generation, respectively. Transcript levels were measured using qRT-PCR. Barley plants were grown under short day photoperiod (8 h light/16 h dark) and harvested after 0, 8, and 96 hours at 3°C ± 1°C. A minimum of four independent plants per transgenic line were used for qRT-PCR. Error bars represent standard error. Note different scales are used for each gene.
Appendix E: CBF gene copy number variation and the role it plays in regulating expression of FR-2 CBF genes
Table E.1. Barley cultivars used for the analysis of CBF2–CBF4B copy numbers.

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<td>Gamma ray mutant of Maythorpe.</td>
<td>S</td>
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<td>UK</td>
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<tr>
<td>Dicktoo</td>
<td>PMH</td>
<td>Selection No. 2 from an unknown cross made prior to 1917 at Dickinson, ND</td>
<td>F</td>
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<tr>
<td>Nure</td>
<td>NP</td>
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<tr>
<td>Beardless</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>Carstens</td>
<td>NPGS</td>
<td>Introduction from Europe</td>
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<tr>
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<td>NPGS</td>
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Table E.2. Primer sequences used in the ‘Admire’ study.

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<td>ACGTTTAACGCTTGGGTGTC</td>
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<td>CBF4</td>
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<td>TTCTAGAGCTAGCTAGTGTAGTGA</td>
<td>5’ DBP</td>
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<td>CBF12</td>
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<td>ChIP-qPCR</td>
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<td>CTCTCTTCGTGGCGTCTTTCT</td>
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<tr>
<td>Pc</td>
<td>GCACCTTGGCCTAGTTTCTA</td>
<td>TGGCTGCTTTGCTACGTACTG</td>
<td>ChIP-qPCR</td>
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</table>

^a DBP: DNA blot probe.
Figure E.1. Time course analyses of CBF2 in ‘Golden Promise’ (GP) and ‘Admire’. Plants were grown under short day (SD; 8 h light/16 h dark) for three weeks at 18°C. Warm samples were collected on day 21 at 18°C (A) and on day 22 following a temperature decrease to 6°C (B) at daybreak. Harvesting was carried out at two-hour intervals starting at daybreak until the 10 h time point in the subjective day. Each sample comprised of crown tissues of five seedlings. Seven μg of total RNA was loaded per lane. RNA blot was hybridized with CBF2 and actin CDS probes.
Figure E.2. DNA blot analyses of two-row parents of MO B lines alongside ‘MEB’.

BamHI- or BglII-digested DNAs were hybridized to CBF2 3’ probe (A and C) or CBF4B 5’ probe (B). BglII digest did not discriminate between the two CBF2 paralogs. Relative copy numbers of CBF2A and CBF4B as estimated from CBF2A/CFB2B ratios (A) and CBF4B/CFB2 ratios (C), respectively, are provided below the individual DNA lanes. MEB = ‘Missouri Early Beardless’. All MO B accessions are identified as ‘Tschermak’ selections (Table D.1).
Appendix F: Variation in CBF14 copy numbers in A, B, and D genomes of wheat at all ploidy levels
Table F.1. List of wheat genotypes used to study CBF14 copy number variations (CNVs).

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<th>G.H.&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Pedigree&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Site of collection or development&lt;sup&gt;f&lt;/sup&gt;</th>
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<td>Punjab, Pakistan</td>
</tr>
<tr>
<td>PI 70711</td>
<td>CItr 9054</td>
<td>NPGS</td>
<td>S</td>
<td>n.a.</td>
<td>Iraq</td>
</tr>
<tr>
<td>PI 83402</td>
<td>CItr 10911</td>
<td>NPGS</td>
<td>W-F</td>
<td>n.a.</td>
<td>Shanxi, China</td>
</tr>
</tbody>
</table>

\(^a\) Abbreviations: CS = ‘Chinese Spring’, CNN = ‘Cheyenne’, WI = ‘Wichita’, NE = Nebraska, KS = Kansas, WY = Wyoming.

\(^b\) n.a. = not applicable.

\(^c\) NPGS = National Plant Germplasm System (http://www.ars-grin.gov/npgs/index.html); WGGRC = Wheat Genetic and Genomics Resources Center (http://www.k-state.edu/wgrc/Resources/resources_index.html); DFG = David F. Garvin, University of Minnesota; PSB = P.S. Baenziger, University of Nebraska-Lincoln; CAG = Carl A. Griffey, Virginia Polytechnic Institute and State University. Durum wheats obtained from CAG were in F\(_7\)-F\(_8\) generation.

\(^d\) Growth habit is listed as winter (W), facultative (F), or spring (S). *T. urartu* accession, PI 427328 is listed at the NPGS to be a mixture of winter and spring types. When grown in the greenhouse, this accession flowered in about 3 months (sowing 6/21/2006 – harvesting 8/28/2006), suggesting the seed used in this study was of spring growth habit. Growth habit of ‘Wichita’ is reported as winter in research articles and as facultative by the NPGS. In the case of hexaploid wheat germplasm, growth habit is replaced by market class is used. Abbreviations for market classes include SWS = Soft white spring; SWW = Soft white winter; HRS = Hard red spring; SRW = Soft red winter; HRW = Hard red winter. U refers to unknown or unclear growth habit or market class.

\(^e\) Pedigree information is as listed by NPGS. n.a. = information not available.

\(^f\) The location listed is the site of development of the genotype, if it’s pedigree information is available and the site of collection, if pedigree information is not available (except for the genetic stocks).
Table F.2. *CBF14/Pinb* copy number estimates\(^a\) for the A, B, and D genomes of hexaploid wheats based on their *CBF14/Pinb* hybridization ratios.

<table>
<thead>
<tr>
<th>Market Class(^b)</th>
<th>Genotype</th>
<th><em>CBF14</em>-A</th>
<th><em>CBF14</em>-B(^c)</th>
<th><em>CBF14</em>-D</th>
<th>Total</th>
</tr>
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<td></td>
<td></td>
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<td>Baart</td>
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<td>0.1</td>
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Continued
Table F.2 continued

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Table F.3. Estimates of *CBF14* copy numbers relative to *Pinb* in diploid wheats.

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<th>Genotypes</th>
<th>Growth Habit</th>
<th>CBF14/Pinb</th>
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<tbody>
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<td></td>
</tr>
<tr>
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<tr>
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<td>W-S</td>
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</tr>
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</tr>
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<td>PI 487272</td>
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</tr>
<tr>
<td>PI 428316</td>
<td>W</td>
<td>7.4</td>
</tr>
<tr>
<td><em>T. monococcum</em> (A&lt;sup&gt;m&lt;/sup&gt;A&lt;sup&gt;m&lt;/sup&gt;)</td>
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<td></td>
</tr>
<tr>
<td>G3116</td>
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</tr>
<tr>
<td>PI 277121</td>
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<tr>
<td>PI 538722</td>
<td>S</td>
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</tr>
<tr>
<td>PI 355549</td>
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<td>PI 427927</td>
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<td><em>Ae. speltoides</em> (BB)</td>
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Table F.4. *CBF14*-A/*CBF14*-B ratios of tetraploid wheats (*T. turgidum* subsp. *durum*).

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<tr>
<td>VA05WD-12</td>
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<td>VA05WD-31</td>
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<sup>a</sup> *CBF14*-A/*CBF14*-B ratios that could not be estimated due to absence of hybridization to the B genome are listed as n.a.
Figure F.1. CBF14 and Pinb hybridization patterns in different market classes of hexaploid wheat (*T. aestivum* subsp. *aestivum*). *Sac*1-digested DNAs were hybridized with CBF14 5' (A) and Pinb CDS (B) probes. The A, B, and D genomes of CBF14 are labeled. A second weakly cross-hybridizing fragment of the B genome is indicated with an arrowhead. Copy numbers of CBF14 in each homoeolog relative to Pinb are provided in Table F.2. Differences in signal intensity of Pinb cross-hybridizing bands across the lanes are attributed to differences in the quantity of DNA loaded on the gel despite the normalizations using PicoGreen. WS = White spring; WW = White winter; HRS = Hard red spring; SRW = Soft red winter; HRW = Hard red winter.
Figure F.2. *CBF14* and *Pinb* hybridizations in 16 unclassified hexaploid wheats (*T. aestivum*). Digests, hybridizations, and labeling of the cross-hybridizing bands is the same as described in Fig. F.1. This collection of hexaploid wheats includes genotypes whose growth habit and market class are ambiguous, or are annotated as facultative by the NPGS (Table F.1). Also included are those that exhibit a different *CBF14* banding pattern.
Figure F.3. *CBF14* and *Pinb* hybridizations of diploid wheats. *SacI*-digested DNAs were hybridized with *CBF14* 5' (A) and *Pinb* CDS (B) probes.

<table>
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<th>T. urartu</th>
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<th>Ae. speltoides</th>
<th>Ae. tauschii</th>
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<tr>
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<td>W</td>
<td>F</td>
<td>S</td>
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<td>Pi 4.79227</td>
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![Image of hybridization results](image-url)
Figure F.4. *CBF14* hybridization patterns in tetraploid wheats (*T. turgidum* subsp. *durum*). *Sac*I-digested DNAs were hybridized with *CBF14* 5′ probe. A and B genomes of *CBF14* are labeled based on their similarity in MW with hexaploid wheat accessions (Fig. F.1 and F.2). A second weakly cross-hybridizing fragment of the BB genome is indicated using an arrowhead.
Figure F.5. *CBF14* hybridizations to *BglII*-digested DNAs of wheat chromosome 5 substitution lines. CNN = ‘Cheyenne’, WI = ‘Wichita’, and CS = ‘Chinese Spring’. The CS-CNN substitution lines and their parents are presented as evidence to support that the four fragments of the A homoeolog in CNN, WI, and their substitution lines reflect the *BglII* restriction pattern and are not the result from a partial digest.