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Authors
Knox, AK
Li, C
Vágújfalvi, A
et al.

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Identification of candidate CBF genes for the frost tolerance locus Fr-A’m2 in Triticum monococcum

Andrea K. Knox · Chengxia Li · Attila Vágújfalvi · Gabor Galiba · Eric J. Stockinger · Jorge Dubcovsky

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Abstract A cluster of eleven CBF genes was recently mapped to the Frost resistance-2 (Fr-A’m2) locus on chromosome 5 of diploid wheat (Triticum monococcum) using a cross between frost tolerant accession G3116 and frost sensitive DV92. The Fr-A’m2 locus was mapped at the peak of two overlapping quantitative trait loci (QTL), one for frost survival and the other for differential expression of the cold regulated gene COR14b. Seven lines with recombination events within the CBF cluster were used to identify CBF candidate genes for these QTL. The lines carrying the critical recombination events were tested for whole plant frost survival and for differential transcript levels of cold induced COR14b and DHN5 genes. The strongest effect for these traits was associated to the linked TmCBF12, TmCBF14 and TmCBF15 genes, with the G3116 allele conferring improved frost tolerance and higher levels of COR14b and DHN5 transcript at mild cold temperatures (12–15°C) than the DV92 allele. Comparison of CBF protein sequences revealed that the DV92 TmCBF12 protein contains a deletion of five amino acids in the AP2 DNA binding domain. Electrophoretic Mobility Shift Assays (EMSA) confirmed that the protein encoded by this allele cannot bind to the CRT/DRE (C-repeat/dehydration-responsive element) motif present in the promoters of several cold induced genes. A smaller effect on frost tolerance was mapped to the distal group of CBF genes including TmCBF16. Transcript levels of TmCBF16, as well as those of TmCBF12 and TmCBF15 were up-regulated at mild cold temperatures in G3116 but not in DV92. Higher threshold induction temperatures can result in earlier initiation of the cold acclimation process and better resistance to subsequent freezing temperatures. The non-functional TmCBF12 allele in DV92 can also contribute to its lower frost tolerance.

Keywords Frost tolerance · COR14b · DHN5 · WCS120 · CBF · Wheat · Triticum monococcum

Abbreviations
bp Base pair
CBF C-Repeat binding factor
COR Cold regulated
CRT/DRE C-Repeat/Dehydration response element
DHN Dehydrin
Fr Frost resistance
QTL Quantitative trait locus
SEM Standard errors of the means
SNP Single nucleotide polymorphism
SOM Supplementary online material
Introduction

Recent studies on frost tolerance in many plant species have focused on the CBF (C-repeat binding factor) gene family. CBF genes were originally characterized in Arabidopsis as transcription factors that activate COR (Cold-regulated) genes, whose proteins play a structural role in conferring cold tolerance to plants (Stockinger et al. 1997). Three AtCBF genes are clustered in tandem on chromosome four (Gilmour et al. 1998; Shinwari et al. 1998) and their encoded proteins are functionally redundant (Gilmour et al. 2004). Grass species have much larger CBF families with more complex transcriptional profiles (Jaglo et al. 2001; Dubouzet et al. 2003; Brautigam et al. 2005; Skinner et al. 2005; Vágújfálvai et al. 2005; Badawi et al. 2007; Stockinger et al. 2007). To differentiate the CBF genes from different species we will use a two-letter prefix in front of the CBF gene names throughout the text (At = Arabidopsis thaliana, Tm = Triticum monococcum, Ta = T. aestivum, Hv = Hordeum vulgare). Numbers assigned to wheat and barley CBF genes indicate similar genes (Skinner et al. 2005; Miller et al. 2006), but are not comparable with numbers assigned to CBF genes from Arabidopsis or other species.

We recently reported a large family of TmCBF genes in diploid wheat (T. monococcum) (Miller et al. 2006). Eleven CBF genes map to a 0.8 cM region on chromosome 5A at the Frost resistance-2 (Fr-A2) locus. This locus maps at the peak of two overlapping QTL, one for frost tolerance and the other for differential expression of the cold-regulated COR14b gene (Vágújfálvai et al. 2000, 2003). The Fr-2 locus also maps to colinear regions of homoeologous group 5 chromosomes in barley (Fr-H2) (Francia et al. 2004) and hexaploid wheat (Toth et al. 2003; Bága et al. 2007). In both wheat and barley a cluster of CBF genes maps to the Fr-2 locus (Vágújfálvai et al. 2003; Francia et al. 2004; Miller et al. 2006; Tondelli et al. 2006; Bága et al. 2007; Francia et al. 2007).

At least 10 rice (Dubouzet et al. 2003; Skinner et al. 2005), 20 barley (Francia et al. 2004; Skinner et al. 2005), and 18 diploid and polyploid wheat CBF genes (Skinner et al. 2005; Miller et al. 2006) have been described. Although it has been well established that the Fr-2 CBF cluster is linked to differences in frost tolerance in both wheat and barley (Vágújfálvai et al. 2003; Francia et al. 2004), it is currently not known which of the CBF gene(s) within this cluster is/are responsible for the observed differences in frost tolerance. One strategy to answer this question has focused on the transcription profiles of the CBF genes within the Fr-2 locus (Vágújfálvai et al. 2005; Stockinger et al. 2007).

In a previous study we characterized the transcription profiles of T. aestivum CBF genes using quantitative PCR and gene specific primers (Vágújfálvai et al. 2005). CBF genes TaCBF3, 10, 14, 15, 16, and 17 are all induced by cold treatment (2 h at 2°C), with TaCBF14 and 15 showing the highest transcript levels. Using ‘Cheyenne’ (CNN, frost tolerant) or T. spelta (TSP, highly frost sensitive) chromosomes 5A substitution lines in the ‘Chinese Spring’ background, and recombinant chromosome lines generated from the cross between them, we showed that the TaCBF14, 15, and 16 transcript levels are more than fourfold higher in the lines carrying the frost tolerant CNN Fr-A2 allele than in those carrying the frost sensitive TSP allele (Vágújfálvai et al. 2005). In a similar study in barley, we showed that higher transcript levels of HvCBF2 and HvCBF4 are present in recombinant lines harboring the frost tolerant ‘Nure’ Fr-H2 allele than in those harboring the frost susceptible ‘Tremois’ Fr-H2 allele (Stockinger et al. 2007).

An alternative strategy to dissect the complex Fr-2 locus is to generate high-density mapping populations, and identify recombination events among the CBF genes within this locus. We previously described seven recombination events within the Fr-A2 CBF cluster in a cross between T. monococcum genotypes DV92 (spring, frost sensitive) and G3116 (winter, frost tolerant) (Miller et al. 2006). In this study, we tested the lines carrying these critical recombination events for frost tolerance using whole plant frost survival tests, and for differential expression of COR14b using RNA blot analyses. The cold induced gene DHN5 (Van Zee et al. 1995) was also analyzed in the same blots to test if the COR14b results could be extended to other COR genes. Together, these data identified a region including TmCBF12, 14, and 15, which has the largest effect upon the differences in both traits. Sequence comparisons and transcription profiles of these CBF genes revealed key polymorphisms between the susceptible and tolerant parental lines that may explain the differences in frost tolerance mapped at the Fr-A2 locus.

Materials and methods

Plant materials

We screened 374 F2 lines (748 chromosomes) from the cross between T. monococcum genotypes DV92 (spring, frost sensitive) and G3116 (winter, frost tolerant) and identified seven recombination events within the Fr-A2 CBF cluster (lines 11, 268, 346, 394, 426, 495, and 532). Individual F2 plants were self-pollinated and F3 plants homozygous for both the chromosome carrying the critical recombination event and for the non-recombinant chromosome (sister control lines) were recovered. Plants within each family were also selected for homozygosity at the
VRN2 locus to avoid segregation for vernalization requirement. All lines from family 11 were homozygous for the Vrn2 allele for winter growth habit, whereas all other families were homozygous for the vrn2 allele for spring growth habit. These seven recombination events were previously used to generate a genetic and physical map of the Fr-Am2 locus (Miller et al. 2006). Genotypes of recombinant and control lines are shown in Fig. 1. Lines 16 (DV92 Fr-Am2 region) and 2 (G3116 Fr-Am2 region) were used as additional controls in the first frost tolerance experiment (Fig. 1). Homozygous recombinant lines used in all tests were in the F4 generation.

Frost tolerance tests

Frost tolerance tests were carried out as described previously (Vágújfalvi et al. 2003), but the photoperiod was changed from long days (16 h light and 8 h dark) to short days (8 h light and 16 h dark) to eliminate the effect of the differences in vernalization requirement on frost tolerance. Short day conditions preclude the induction of VRN1 and the transition between the reproductive and vegetative stages in T. monococcum (Dubcovsky et al. 2006). Plants were grown in wooden boxes with 9 cm of soil. Two frost tolerance tests were performed (2006 and 2007) both in the same chamber and at the same time of the year. The first experiment included families 268 (1 line), 346 (4 lines), 394 (2 lines), 426 (3 lines), 495 (8 lines), and 532 (5 lines); and the second experiment included only families 11 (4 lines), 394 (3 lines), and 495 (8 lines). All lines were homozygous for the Fr-Am2 locus. Both experiments included control parental lines DV92 and G3116, but only the first one included the additional control lines 2 and 16. Each line was represented by five plants (subsamples). Lines within each box were randomized independently. Five boxes (replications) were used in the first experiment and ten in the second experiment (6 lines had seeds for only 8 replications). Seedlings were grown in a Conviron growth chamber (Martonvásár, Hungary) at 15°C during the day and 10°C during the night for 2 weeks and then

![Diagram](image)

**Fig. 1** Characterization of recombinant lines. (A) Graphical representation of the CBF cluster at Fr-Am2. Arrowheads represent CBF genes; asterisks represent markers. Proximal, central and distal clusters are defined by recombination events as described. (B) Recombinant lines are listed on the left. Bolded lines represent recombinants; italicized lines are the non-recombinant sister lines. Letters represent genotypes based on mapping data of the markers shown within each cluster in (A). Letter S represents the susceptible genotype DV92. Letter T represents the tolerant genotype G3116. Frost survival data is the average of all survival scores for each line on a scale of 0 (death) to 5 (undamaged). COR14b and DHNS transcript data represents the average relative expression of COR14b and DHNS on Northern blot. n/a = not analyzed
cold hardened for two weeks at 10°C day/5°C night, two weeks at 5°C day/0°C night, and finally for one week at +2°C day/−2°C night. For the frost tolerance test, the temperature was slowly lowered to −11°C. During this last period the humid soil cools down uniformly and freezes. After 24 h at −11°C, the temperature was gradually increased to 17°C day/16°C night. Survival and re-growth was manually scored on a scale from 0 (death) to 5 (undamaged).

**COR14b and DHN5 expression analyses**

Two independent expression analyses experiments were carried out. The first one included families 346 (4 lines), 394 (2 lines), 426 (3 lines), 495 (8 lines), and 532 (5 lines); and the second only families 11 (4 lines), 394 (2 lines), and 495 (8 lines). Plants were sown in 11.4 cm² pots with 4 or 5 plants of the same recombinant line per pot. Plants were grown at 20°C for 4 weeks in short day conditions in Conviron (Pembina, ND) growth chambers. After 4 weeks, the chamber temperature was decreased by 2°C every 48 h until the final temperature of 12°C was reached. After 48 h, tissue was collected in liquid nitrogen. This temperature was selected because preliminary experiments showed that 12°C was optimal for maximizing differences in COR14b transcript levels between G3116 and DV92. The same preliminary study revealed larger differences in crown samples than in leaf samples. Crown tissues, consisting of a 1–1.5 cm segment of the white, non-photosynthetic tissue between the upper photosynthetic shoot and the primary root, were pooled from each pot.

Crowns were ground using liquid nitrogen and total RNA was extracted using the Qiagen (Valencia, CA) RNeasy extraction kit. Formaldehyde gels containing 7 μg of total RNA were blotted onto Hybond-N membranes using 10X SSC and were fixed by UV cross-linking with a Stratalinker (Stratagene, La Jolla, CA). The radioactively labeled probe for COR14b was prepared by random-priming (Feinberg and Vogelstein 1983), using the clone for barley EST BF625933. Overnight hybridizations were at 42°C in 50% formamide, 5X SSC, 20 mM Na-phosphate buffer pH6.8, 1X Denhardt’s solution, 0.1% SDS, 10% dextran sulfate and containing 100 μg/ml herring sperm DNA. Final washes were in 0.5X SSC-0.1% SDS. Membranes were exposed for two days and autoradiographs were scanned using ImageQuant software (GE Healthcare, Piscataway, NJ). Relative expression was determined by normalizing COR14b (Fig. 1B) transcript levels to those from ACTIN (Fig. 2). To test if the COR14b results could be expanded to other COR genes the blots from the first expression experiment were re-hybridized with a probe for the DHN5 gene, generated from barley EST BF625933 (Fig. 1B).

**Fig. 2** RNA blot analyses of COR14b and ACTIN. Total RNA was extracted from crown tissue from plants transferred to 12°C. The parental lines and the recombinant and control lines of family 532 are shown as an example. Note the higher COR14b transcript accumulation in lines carrying the G3116 allele for TmCBF2/17. The genotype of each line is indicated below the figure: the letter S represents the allele from the susceptible parent (DV92) and the letter T the allele from the tolerant parent (G3116).

**Statistical analyses**

To test the effect of each of the three CBF clusters on frost tolerance and transcript levels of COR14b and DHN5 we performed three independent ANOVAs using the genotype at each CBF cluster as a classification factor with two levels (Table 2). All the recombinant families and their respective controls were included in these analyses but the parental lines were excluded. The ANOVA model included genotype, family, and the genotype*family interactions. We included families as blocks to control for the variability between families. This is particularly important in comparisons across families because this population is derived from a cross between cultivated (DV92) and wild (G3116) T. monococcum accession and therefore shows large phenotypic differences among families. For families 394 and 495, for which we have two years of data, each year was treated as a separate block. Lines within a family (recombinant and control) were used as replications. In the overall frost tolerance test each line was represented by the average of all boxes used in each experiment.

The overall statistical analysis for frost tolerance using all lines was complemented by individual statistical tests for each family (Supplementary Online Materials, SOM hereafter), using boxes as blocks in a completely randomized block design (RCBD). The frost scores from the five plants from the same line within each box were averaged (subsamples). Individual statistical analyses per family were not possible for the COR14b and DHN5 transcription data because only a single value was available per line. In the analysis of the COR14b and DHN5 expression results, lines were used as replications and families as blocks as in the overall frost tolerance test. All statistical analyses were performed using SAS version 9.1 (SAS 2003). For all the unbalanced designs we used Type III Sums of Squares and least significant means. Means are
Quantitative RT-PCR

Transcript levels of CBF12, 14, 15 and 16 were determined using the primers described in Table 1. For the leaf tissue experiment DV92 and G3116 plants were initially grown under long day conditions (16 h light/8 h dark) at 20–25°C for three weeks and were then transferred to the three targeted temperatures: 15°C, 10°C, and 4°C. At the time of the transfer to the target temperature the apices of both DV92 and G3116 plants were still at the vegetative stage. This was confirmed by the presence of low levels of VRN1 transcript in both G3116 (6.8 ± 2.6) and DV92 (5.0 ± 1.6) relative to older DV92 control plants already induced to flower (2,518.9 ± 493.5), as determined by quantitative PCR. A last experiment was performed to measure CBF12, 14, 15 and 16 transcript levels in the same tissue (crowns), and under the same temperature (12°C), and photoperiod (short day) conditions as the COR14b and DHN5 experiments.

Samples were collected at the time of transfer to the targeted temperature and then 2 h and 4 h later (only 4 h for the crowns). All experiments were initiated at approximately the same time in the day/night cycle. RNA samples were extracted from leaves using the TRIZOL method (Invitrogen, Carlsbad, CA). Transcript levels of CBF12, 14, 15, and 16 at the different collection times were compared by quantitative PCR using SYBR GREEN systems. All quantitative PCR experiments were performed in an ABI7000. The 2−ΔΔCT method (Livak and Schmittgen 2001) was used to normalize and calibrate transcript values relative to the endogenous ACTIN control. Six to eight independent plants were measured in all quantitative PCR experiments to calculate average transcript level.

Sequence comparisons

Sequences for the TmCBF genes from the frost susceptible parent DV92 were published before (Miller et al. 2006). To determine the sequences from the frost tolerant parent G3116 we designed gene specific primers for CBF12, 14, 15, and 16 (Table 1), amplified the fragments from G3116 genomic DNA, and directly sequenced the PCR products. The G3116 allele sequences were deposited in GenBank under accession numbers: EU076381 (CBF12), EU076382 (CBF14), EU076383 (CBF15), and EU076384 (CBF16). Protein alignments were made using CLUSTALW (Thompson et al. 1994).

Electrophoretic mobility shift assays (EMSA)

Two primers, CBF12-GST forward and reverse, were used to PCR amplify the full-length TmCBF12 coding sequence from G3116 and DV92 (Table 1). Restriction sites BamHI

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**Table 1** Primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward (5' → 3')</th>
<th>Reverse (5' → 3')</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>QCBF12</td>
<td>GTCCCACTCCCCACTCACAG</td>
<td>ACATGTCGTGGACACATGC</td>
<td>QPCR (95%)a</td>
</tr>
<tr>
<td>QCBF14</td>
<td>CATGGAGTCGCCGGACACCGGACC</td>
<td>GCCCTCCCCAAAAATAGACAGGAG</td>
<td>QPCR (84%)</td>
</tr>
<tr>
<td>QCBF15</td>
<td>CATGTTCAGCTGTATATGTCCGGGG</td>
<td>GGGAACAGCTTCGTGGTTCTCATGC</td>
<td>QPCR (100%)</td>
</tr>
<tr>
<td>QCBF16</td>
<td>GCGGATGTGCTCTCCAAACACGCGAG</td>
<td>ACCTGCCGAGTCTCCCTCCCG</td>
<td>QPCR (93%)</td>
</tr>
<tr>
<td>ACTIN</td>
<td>AATGGCTGACGGGCTAGGAG</td>
<td>GCTGACACCATCAGGACCA</td>
<td>QPCR (98%)</td>
</tr>
<tr>
<td>CBF12-GST</td>
<td>AAGGATCATGGACACGGGGAGGCCCAGGAG</td>
<td>CGGAATTCTCAGTGGTTCCATAGGAGGC</td>
<td>EMSA</td>
</tr>
<tr>
<td>CBF2</td>
<td>GATTGAGCCGGATGATGTTCTTCTTCCG</td>
<td>TTATGTTGATACCGGCTGGAGA</td>
<td>Sequencing</td>
</tr>
<tr>
<td>CBF3</td>
<td>ATCCCACTCTCGTCTCAAG</td>
<td>GCTGGAGATTACCGGCACTA</td>
<td>Mapping</td>
</tr>
<tr>
<td>CBF12a</td>
<td>ATTAGACAGTGGGCGGTCAC</td>
<td>CTCGGTGTCATCAGTGGTTC</td>
<td>Sequencing</td>
</tr>
<tr>
<td>CBF12b</td>
<td>GCCGTTCGGATGAGTTCCCTTCTTG</td>
<td>TTATGTTGATACCGGCTGGAGA</td>
<td>Sequencing</td>
</tr>
<tr>
<td>CBF14a</td>
<td>GCCGTTAGAAGGCTGAGCTG</td>
<td>CACCCGACCTAAAGCTTCC</td>
<td>Sequencing</td>
</tr>
<tr>
<td>CBF14b</td>
<td>TCTCTCAACAGCGAGAGCTA</td>
<td>TCAAAGTGATCTACAGGACAA</td>
<td>Sequencing</td>
</tr>
<tr>
<td>CBF15a</td>
<td>TATAATGGTACAGGATGAAA</td>
<td>TTGAGTGGTACTGAAATGAG</td>
<td>Sequencing</td>
</tr>
<tr>
<td>CBF15b</td>
<td>AAGGATGCTCCTTTACCTACAG</td>
<td>ATGCGAGAGGAGACAGCTT</td>
<td>Sequencing</td>
</tr>
<tr>
<td>CBF16a</td>
<td>ACACCCGAGTCCCTGCTCAAC</td>
<td>CCATCCCTTTGTATACGTC</td>
<td>Sequencing</td>
</tr>
<tr>
<td>CBF16b</td>
<td>CGTGAAAATCAAGGGGATGG</td>
<td>GCTCGACCTGTTACGATGC</td>
<td>Sequencing</td>
</tr>
</tbody>
</table>

a The efficiency of the primer pairs used for quantitative PCR is indicated between brackets.
and EcoRI were incorporated into the oligos to facilitate cloning. PCR products were digested with BamHI and EcoRI, and ligated into the corresponding sites of pGEX-6p-1 (GE Healthcare, Piscataway, NJ) generating CBF12 (G3116)-GST and CBF12 (DV92)-GST fusion constructs. Constructs were transformed into *E. coli* BL21 for protein expression. Protein induction was done at 30°C for 2–4 h with addition of 0.5 mM IPTG. GST SpinTrap purification columns (GE Healthcare, Piscataway, NJ) were used to purify GST-tagged proteins. Purified proteins CBF12-G3116 and CBF12-DV92 were cleaved from the GST tag using PreScission protease (GE Healthcare, Piscataway, NJ) and then re-purified.

Wild type CRT/DRE (C-repeat/dehydration-responsive element) motifs present in the promoters of *COR78-1* and *COR15a*, and a mutated version of the *COR15* CRT/DRE cloned in pUC118 (Stockinger et al. 1997) were used for EMSA. DNA fragments were excised with EcoRI and *Sal*I, radiolabeled with α-32P-dATP and α-32P-dTTP (3000 Ci/mole, Perkin Elmer) using the Klenow subunit of DNA polymerase, and purified with *MicroSpin*™ G-25 columns (GE Healthcare, Piscataway, NJ). Binding reactions were conducted at room temperature for 20 min in 25 μl containing 20 mM Tris-HCl (pH 8.5), 50 mM KCl, 5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 200 ng/μl BSA, 10% glycerol, 1 μg poly(dI-dC), 1 ng of labeled probe and 0.1–1 μg of protein. For competition experiments, a 20- to 50-fold molar excess of unlabeled competitor DNA was added 10 min prior to addition of the labeled probe. 5% non-denaturing polyacrylamide gels were used to resolve protein:DNA complexes from the labeled probe. 5% non-denaturing polyacrylamide competitor DNA was added 10 min prior to addition of the labeled probe and 0.1–1 μg of protein. For competition experiments, a 20- to 50-fold molar excess of unlabeled competitor DNA was added 10 min prior to addition of the labeled probe. 5% non-denaturing polyacrylamide gels were used to resolve protein:DNA complexes from free DNA probes in a Tris–glycine electrophoresis buffer (25 mM Tris–Cl, 190 mM Glycine, 1 mM EDTA, pH 8.3). Gels were dried and exposed to Kodak X-AR films (25 mM Tris–Cl, 190 mM Glycine, 1 mM EDTA, pH 8.3). Gels were used to resolve protein:DNA complexes from the labeled probe. 5% non-denaturing polyacrylamide competitor DNA was added 10 min prior to addition of the labeled probe. 5% non-denaturing polyacrylamide gels were used to resolve protein:DNA complexes from free DNA probes in a Tris–glycine electrophoresis buffer (25 mM Tris–Cl, 190 mM Glycine, 1 mM EDTA, pH 8.3). Gels were dried and exposed to Kodak X-AR films.

### Results

**Update of the *Fr-A*m2 CBF cluster genetic and physical map**

The eleven CBF genes identified at the *Fr-A*m2 locus are distributed along five non-overlapping BAC clones, which we physically ordered using genetic analyses (Miller et al. 2006). To obtain a greater resolution of the CBF genes within this cluster we developed additional markers and mapped them in the homozygous recombinant lines summarized in Fig. 1. Lines beginning with the same number (i.e. 426-5-5 and 426-4-2) are sister lines within the same F2 family. Lines showing the name in bold carry the recombinant chromosomes whereas names in italics identify the non-recombinant sister controls (Fig. 1B).

**Proximal CBF genes (TmCBF2, 4, 9, 17)**

A Cleavage Amplified Polymorphic Sequence (CAPS) marker was developed 1 kb distal to CBF2 using primers CBF2F and CBF2R (Table 1) and the restriction enzyme *Mnl*I. TmCBF2 is located on *T. monococcum* BAC clone 60J11 (AY951945), 68 kb away from TmCBF17 (Miller et al. 2006). Genetic analyses of the TmCBF2 and TmCBF17 markers showed that these two genes are completely linked in all our recombinant lines (Fig. 1). For simplicity, the linked TmCBF2, 4, 9, and 17 will be referred hereafter as the “proximal CBF cluster” (Fig. 1). As no recombination events were detected within BAC clone 60J11, we used the order of these four genes relative to CBF14 in barley (Francia et al. 2007) as a best estimate of the orientation of this BAC in diploid wheat (Fig. 1). The proximal CBF genes are separated from the other seven CBF genes by four recombination events detected in families 268, 346, 426, and 532 (Fig. 1).

**Central CBF genes (TmCBF14, 15, 12)**

Even though TmCBF14, 15, and 12 are completely linked to each other in the genetic map their relative order can be established using the physical map and the sequence of BAC 21C6 (AY951944). This BAC includes TmCBF15, TmCBF12, and TmCBF16 (Fig. 1). TmCBF16 is separated from TmCBF15 and TmCBF12 by three recombination events (lines 11, 394, and 495). Since TmCBF14 is located in a separate BAC, it is possible to establish that the gene order from proximal to distal is TmCBF14, TmCBF15, and TmCBF12 (Fig. 1).

Recombinant lines 394 and 495 harbored a recombination event between the TmCBF12 marker 21C6a, and marker 21C6b, located 15 kb proximal to TmCBF16 (Miller et al. 2006). Recombinant line 11 showed a more distal recombination event that was mapped between 21C6b and two new SNP (single nucleotide polymorphism) markers located 305 bp upstream of the TmCBF16 start codon and within the TmCBF16 coding region.

**Distal CBF genes (TmCBF16, 13, 3, 10)**

We also created a new and more robust PCR-based marker for TmCBF3 (Table 1). Re-examination of the homozygous recombinant lines with this new marker and with TmCBF13 showed that all three distal crossovers were located between TmCBF12 and TmCBF16. Therefore, TmCBF16, 13, 3 and 10 are all completely linked in this genetic map (corrected relative to Miller et al. 2006). As a best estimate of the physical order of linked genes TmCBF13, 3 and 10 (Fig. 1), we used the relative order of the orthologous barley genes (Francia et al. 2007).
Mapping frost survival

Mean plant survival scores are presented in Fig. 1, together with the genotypic data for each of the lines used in the experiments. Values for families 394 and 495 represent the average of two experiments. Families tested in two different experiments were included as two separate blocks in the statistical analyses. Analyses of variance using the different TmCBF markers as classification variable and families as blocks showed that the largest F values ($F = 48.3, P < 0.0001$) were detected with the marker for the central TmCBF genes TmCBF12/14/15 (Table 2). Allelic differences at the central TmCBF locus also explained the largest proportion of the variation in frost tolerance among these lines ($R^2 = 0.78$). Average survival scores for the lines carrying the G3116 allele at the TmCBF12/14/15 locus (2.02 ± 0.13 SEM) were 52% higher than those found in lines carrying the DV92 allele (1.33 ± 0.09).

Smaller F values were obtained when the lines were classified using the genotypic data for the proximal and distal TmCBF markers (Table 2). However, these values were still significant due to their linkage with the central TmCBF genes. To further test the effect of the different groups of TmCBF loci we performed separate ANOVAs for the individual families (SOM). Family 268 was excluded from this analysis because only one homozygous recombinant line was recovered from the progeny test (Fig. 1).

Families 346, 426, and 532 have recombination events between the proximal and central TmCBF genes and segregate for the central TmCBF genes but are invariant for the proximal TmCBF genes. All three families had higher frost tolerance values in lines carrying the G3116 allele for the central TmCBF genes than the sister lines carrying the DV92 allele (60–140% increases, SOM). These differences were still significant due to their linkage with the central TmCBF genes but are invariant for the proximal TmCBF genes (Table 2). Interestingly, family 394 which is invariant for the central TmCBF genes and segregates for the distal group of TmCBF genes also showed significant differences ($P = 0.006$) between lines carrying the G3116 and DV92 alleles at the distal cluster of TmCBF genes, suggesting the possibility of either an additional effect of the distal cluster of genes on frost tolerance or of other genes distal to TmCBF12.

Mapping of the COR14b and DHN5 differential induction phenotype

The lines with recombination events within the Fr-A"2 TmCBF cluster were also monitored for COR14b and DHN5 transcript levels at 12°C in the crowns using RNA blot analyses. Quantification of the hybridization signals relative to ACTIN, showed that the transcript levels from both genes were significantly correlated ($R = 0.55; P = 0.002$). The hybridization signals from all lines (Fig. 1) were analyzed using families as blocks, using methods similar to those used for the frost tolerance scores (Table 2). Families tested in two different experiments were included as two separate blocks. This analysis showed that the central TmCBF genes (CBF12/14/15) had higher F values and explained a larger proportion of the variation in COR14b and DHN5 transcript levels than the proximal and distal clusters (Table 2).

Average COR14b values for the lines carrying the G3116 allele at the CBF12/14/15 locus (0.31 ± 0.06 SEM) were 180% higher than those found in lines carrying the DV92 allele (0.11 ± 0.02) (Fig. 2). Average DHN5 values for the lines carrying the G3116 allele (0.43 ± 0.08) were also 182% higher than those found in lines carrying the DV92 allele (0.15 ± 0.04) (Fig. 1B). The ANOVA using the CBF12/14/15 alleles confirmed that these differences were highly significant ($P = 0.005$, Table 2). In contrast, analysis of the COR14b and DHN5 values using the

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Table 2 Statistical analysis of frost tolerance and transcription levels of COR14b and DHN5 in the recombinant plants

<table>
<thead>
<tr>
<th>Frost tolerance</th>
<th>COR14b expression</th>
<th>DHN5 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
</tr>
<tr>
<td>CBF2/17</td>
<td>5.1</td>
<td>0.03</td>
</tr>
<tr>
<td>CBF14/15/12</td>
<td>48.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CBF16</td>
<td>22.7</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Each row is an independent ANOVA using the alleles from the CBF cluster indicated in the first column as a classification variable. Family-year combinations were used as blocks and lines as replications. See SOM for complete ANOVA analyses. Higher F values in the CBF14/15/12 region are equivalent to a peak in a QTL analysis ($P < 0.0001$). Family 426 was just below the level of significance ($P = 0.06$). These data parallel the results of the complete analysis (Table 2) and indicate that the locus affecting frost tolerance is distal to the TmCBF2/4/6/17 group of linked genes.

Families 11, 394, and 495 all have recombination events between the central and distal TmCBFs. Family 11 and 495 segregate for the central TmCBFs, whereas family 394 segregates for the distal TmCBFs (Fig. 1). Highly significant effects of the central TmCBF genes were detected for family 495 ($P < 0.0001$, SOM) and 11 ($P = 0.007$, SOM). These two families showed higher frost tolerance values in the lines carrying the G3116 allele at the central TmCBF cluster than in the sister lines carrying the DV92 allele (SOM). These results suggest that the TmCBF12/14/15 group of genes has a major effect in frost tolerance. Interestingly, family 394 which is invariant for the central TmCBF genes and segregates for the distal group of TmCBF genes also showed significant differences ($P = 0.006$) between lines carrying the G3116 and DV92 alleles at the distal cluster of TmCBF genes, suggesting the possibility of either an additional effect of the distal cluster of genes on frost tolerance or of other genes distal to TmCBF12.
flanking CBF markers TmCBF2/17 and TmCBF16/13 as classification variables yielded small F values that were either not significant or borderline significant (P = 0.05–0.06, Table 2). These results indicate that the central TmCBF genes play a significant role in the differential regulation of COR14b and DHN5 transcript levels at 12°C. Separate analyses by family were not possible for these datasets because only a single estimate is available per line.

The results for COR14b were consistent between years as demonstrated by non-significant year by genotype interactions (P > 0.90) for any of the TmCBF clusters tested both years (separate factorial ANOVA including families 394 and 495, data not shown). DHN5 was analyzed only for the first year samples.

Sequence analysis

To determine if the central TmCBF12, 14, 15, and 16 genes differ between the parental lines of our mapping population we sequenced G3116 coding and promoter regions of these genes and compared them with previously published DV92 sequences (Miller et al. 2006). These comparisons revealed that the DV92 TmCBF12 gene (AY951944) had a 15 base pair deletion beginning at nucleotide 165 (after the MET initiator codon) relative to the G3116 TmCBF12 gene (EU076381, Fig. S3, SOM). This deletion resulted in the loss of five amino acid residues in the AP2 DNA binding domain (Fig. 3). Alignment of the TmCBF12 proteins to those of barley and hexaploid wheat indicated that the five amino acids present in G3116 were common to other CBF12 proteins (Fig. 3). This suggests that the difference arose as a result of a deletion event in the DV92 allele rather than an insertion event in the G3116 allele. Other minor differences distinguished the G3116 and DV92 TmCBF12 genes including one SNP in the promoter, and two SNPs in the coding region, both of which result in conservative amino acid changes: an Ala (DV92) to Val (G3116) at residue 166 (based on G3116), and an Arg (G3116) to His (DV92) substitution at residue 238 (SOM).

In contrast to TmCBF12, the TmCBF14 (EU076382), TmCBF15 (EU076383), TmCBF16 (EU076384) alleles of G3116 were very similar to the corresponding DV92 alleles. A single SNP in the coding region distinguished the two TmCBF16 genes, resulting in a Thr-to-Met transition at amino acid 218. The TmCBF14 and TmCBF15 proteins of G3116 and DV92 were invariant from one another, while the promoter regions of the genes harbored one (TmCBF14), two (TmCBF15), and several (TmCBF16) SNPs.

Electrophoretic mobility shift assays

The 15 bp deletion in the DV92 TmCBF12 allele occurs within the AP2 DNA binding domain and therefore has the potential to affect this protein’s ability to bind to the CRT/DRE (C-repeat/dehydration-responsive element) motif present in the COR gene promoters. To test this hypothesis we carried out electrophoretic mobility shift assays with the wild type (G3116) and mutant (DV92) recombinant proteins.

Purified recombinant TmCBF12 protein from G3116 produced a mobility shift with both the COR78-1 and COR15a wild type CRT/DRE sequences (Fig. 3A, B; lane 3) but not with a mutated COR15a CRT/DRE sequence (Fig. 3C; lane 3), as did the recombinant AtCBF1 protein used as positive control (Fig. 3A–C; lane 2). Interaction between the G3116 TmCBF12 and the labeled CRT/DRE probe was eliminated by the addition of excess unlabeled CRT/DRE DNA in the binding reactions (Fig. 3E; lane 3, 4). In contrast, the DV92 TmCBF12 protein did not produce a mobility shift with either CRT/DRE probe (Fig. 3A, B; lane 4). Examination of the G3116 and DV92 recombinant proteins by gel electrophoresis confirmed that similar quantities of full-length proteins were used in the EMSA (Fig. 3D).

Quantitative PCR

We also carried out quantitative PCR experiments to determine whether the central cluster TmCBF genes were differentially expressed in G3116 and DV92 plants in the vegetative stage. Gene specific primer pairs were developed for the three genes located within the central TmCBF region (TmCBF12, 14 and 15). We also included TmCBF16 in the expression analyses because the orthologous gene in hexaploid wheat is expressed to higher levels in frost resistant genotypes than in frost sensitive ones (Vágújfalvi et al. 2005). TmCBF transcript levels were quantified in the leaves at 15°C (Fig. 4), the temperature at which Vágújfalfalvi et al. (2003) observed differences in COR14b transcript levels between DV92 and G3116 leaves. TmCBF transcript levels were also quantified in the crowns at 12°C, using the same conditions as the COR14b and DHN5 experiments (SOM, S2). For comparative purposes, TmCBF transcript levels at 10°C and 4°C are also included in the SOM (S3). These lower temperatures did not show clear differences between DV92 and G3116 induction profiles.

The experiments using leaf tissue indicated that TmCBF16 and the three central cluster CBFs were transcribed in both parents, although their cold temperature responsiveness at the different time points and temperatures was complex (Fig. 4 and SOM, S2-3). TmCBF14 showed the largest increase and the highest transcript levels at each of the different temperatures (Fig. 4 and SOM, S2-3). For all three temperatures, the TmCBF14 transcript levels were two orders of magnitude higher than those

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observed for the three other TmCBF genes (Fig. 4 and SOM, S2 and 3). At its maximum values, TmCBF14 transcript levels were four-fold higher than ACTIN. After 4 h at 15°C the transcript levels of TmCBF14 were significantly higher in G3116 than in DV92 (P < 0.0001). In G3116, transcript levels of TmCBF14 in plants transferred
to 4°C for 4 h were 16-fold higher than in those transferred to 15°C and four-fold higher than in those transferred to 10°C (P < 0.01), indicating a stronger response at lower temperatures. 

*TmCBF12, TmCBF15 and TmCBF16* showed lower transcript levels than *TmCBF14*, but similar differential profiles between DV92 and G3116 when transferred to mild cold temperatures (12°C and 15°C). Transcript levels of *TmCBF12, TmCBF15* and *TmCBF16* in the leaves showed four-fold increases (P < 0.01) within 4 h of transferring the G3116 plants to 15°C (Fig. 4A, C, and D). However, in DV92 the transfer to 15°C had no effect upon the transcript levels of these same genes (Fig. 4A, C, and D). After 4 h at 15°C transcript levels of *TmCBF12, TmCBF15*, and *TmCBF16* were significantly higher (P < 0.03) in G3116 than in DV92. Slightly higher transcript levels in G3116 relative to DV92 were also visible at 10°C (SOM, S3) for *TmCBF12, TmCBF15*, and *TmCBF16*, but the differences were significant only for *TmCBF16*.

The transcription profiles observed in the crowns at 12°C (SOM, S2) were very similar to those found in the leaves at 15°C (Fig. 4). These two independent experiments support the existence of differences in *TmCBF12, TmCBF15*, and *TmCBF16* threshold induction temperatures between DV92 and G3116 at mild cold temperatures. In G3116, the four *CBF* genes showed higher transcript levels after 4 h at 12°C relative to the room temperature values (significant for *TmCBF12* and *TmCBF14*), whereas in DV92 that pattern was only observed for *TmCBF14*. *TmCBF14* showed the highest transcript levels in both leaves and crowns (Fig. 4 and SOM S2).

In G3116, *TmCBF12, TmCBF14, TmCBF15*, and *TmCBF16* showed increased transcript levels in the leaves when the plants were moved from room temperature (20–25°C) to any of the lower temperatures (Fig. 4 and SOM, S3). However, only *TmCBF12* and *TmCBF14* showed significantly higher transcript levels at 4°C compared to 10 or 15°C (P < 0.01). All of these genes showed a decrease in transcript levels from 4 h to 8 h at 10°C (SOM, S3). This decrease was less evident at 4°C, and was only significant for *TmCBF12* (SOM, S3).

**Discussion**

The *CBF* gene family in the *Triticaceae* has been a subject of intense research in recent years. The cluster of *CBF* genes located at the peak of the *Fr-A^m2* QTL for frost tolerance (Vágújfalvi et al. 2003) was later found to be important in the regulation of frost tolerance in barley (Francia et al. 2004) and polyploid wheat (Toth et al. 2003; Båga et al. 2007). Since *CBF* transcription factors are known to bind to the promoters of *COR* genes, it was not surprising to find a QTL for *COR14b* transcript levels associated with the *Fr-2* locus in *T. monococcum* (Vágújfalvi et al. 2003) and for *COR14b* protein accumulation in barley (Francia et al. 2004). The *COR14b* gene product is likely involved in the protection of the chloroplast membranes from freezing temperatures (Crosatti et al. 1999) and its transcript accumulation is a good marker for assessing cold acclimation capacity and frost tolerance. In this study we show that differences in transcript levels of *DHN5* at 12°C are also associated with the *Fr-A^m2* locus. *DHN5* is a cold induced dehydrin from barley (Van Zee et al. 1995) with homology to the wheat *WCS 120* gene, which is known to accumulate to higher levels in freezing-tolerant genotypes than in less tolerant genotypes (Houde et al. 1992; Sarhan et al. 1997).

The analysis of the *Fr-2* locus has been complicated by the presence of multiple *CBF* genes in both wheat (Miller et al. 2006) and barley (Francia et al. 2004; Skinner et al. 2006). Therefore, the goal of our study was to further dissect this complex locus in *T. monococcum* using a multidisciplinary approach including genetics, expression profiles, and biochemical characterization of critical *CBF* genes.

Genetic dissection of the *Fr-Am2 TmCBF* gene cluster

The seven recombinant lines discovered within the *Fr-A^m2* *CBF* cluster (Miller et al. 2006) provided the tools to dissect the relative effect of the multiple *CBF* genes present within this locus. These recombination events divided the large *TmCBF* cluster into three smaller gene clusters, each separated from the other by at least three recombination events. These multiple recombination events provided replicated measures for the differences in frost tolerance and transcript levels of the cold induced genes in different genetic backgrounds. The different statistical analyses of the frost tolerance data (among and within families) indicated that the major effect was located between the two groups of crossovers and completely linked to the central *CBF* cluster including *TmCBF12, TmCBF14* and *TmCBF15*. The differences in *COR14b* and *DHN5* transcript levels at the threshold temperature were also mapped to the central *CBF* cluster (Table 2). It is important to note that the frost tolerance tests and the expression experiments of the cold induced genes were performed in separate laboratories under each individual lab’s optimized growth conditions. The fact that two independent data sets returned the same results provides additional validation to our statistical analyses.

The association of the *Fr-A^m2* locus with the central *CBF* cluster has important implications because it establishes that the *Fr-A^m2* locus is within the *CBF* cluster. All previous QTL studies (Vágújfalvi et al. 2003; Toth et al. 2003; Francia et al. 2004; Båga et al. 2007) have
established that the CBF genes were located at the peak of the QTL, but because of the resolution of the QTL analyses it was not possible to rule out the presence of a different regulatory gene closely linked to the CBF cluster. Our results indicate that the differences in frost tolerance and COR14b/DHN5 transcript levels are distal to the TmCBF2/17 gene cluster and proximal to the TmCBF16/13 gene cluster (Fig. 1), and therefore at the central region of the CBF cluster. However, since BAC 60111 includes genes that do not belong to the CBF group (XPG and SPX, AY951945) we cannot completely rule out the presence of a yet unknown gene within the gap in the physical map between TmCBF14 and TmCBF15 (Fig. 1).

Even though all families segregating for the central gene cluster were consistent in pointing at TmCBF12, 14, and 15 as the source of the major differences in frost tolerance and COR14b/DHN5 transcript levels (Fig. 1 and SOM), it is still possible that an additional factor with a smaller effect on frost tolerance might be still segregating distal to the central CBF cluster. Family 394, which is the only family segregating for the distal cluster, also showed significant differences in frost tolerance between the DV92 and G3116, with the latter showing the higher tolerance values (Fig. 1). Since this result is based on a single family, it should be considered with caution.

To provide independent evidence on the relative importance of the TmCBF genes from the central cluster on frost tolerance and regulation of cold induced genes we characterized the transcription profile and the sequence variation of the TmCBF12, TmCBF14, and TmCBF15 genes. We also included the closely linked TmCBF16 (Fig. 1) because the sequence of this gene is closely related evolutionarily to the candidate genes TmCBF12 and TmCBF15 (Miller et al. 2006; Badawi et al. 2007). Moreover, TmCBF16 showed higher transcript levels in frost tolerant than in frost susceptible T. aestivum lines (Vágújfalvi et al. 2005), and therefore is a candidate for the putative distal effect suggested by family 394.

Transcription profiles differ among TmCBF genes at the Fr-Am2 locus

All TmCBF genes tested were up-regulated when plants were transferred from room temperature to 4°C or 10°C. However, the frost susceptible parental line DV92 showed no significant induction of TmCBF12, TmCBF15 and TmCBF16 when the plants were transferred to 15°C (Fig. 4), whereas the frost tolerant parental line G3116 showed a significant four-fold increase in transcript levels under the same conditions. Similar results were observed in the crowns at 12°C (SOM, S2). Since CBF proteins bind to the COR gene promoters and regulate their transcription (Stockinger et al. 1997; Liu et al. 1998), the differential transcription profiles of these CBF genes provide a possible explanation for the difference in COR14b and DHN5 induction at 12°C observed between DV92 and G3116. We hypothesize that the up-regulation of these CBF genes (and of the downstream COR genes) at these mild cold temperatures could result in an earlier initiation of the cold acclimation process in nature and consequently, in a better response to later exposures to freezing temperatures.

TmCBF12, TmCBF15 and TmCBF16 have a close evolutionary relationship (Miller et al. 2006; Badawi et al. 2007) and show similar up-regulation at 15°C in G3116. However, it is puzzling that all three CBF genes lost their ability to be induced at 15°C in DV92. One possible explanation is the occurrence of three independent mutations in the regulatory regions of these three CBF genes precluding their up-regulation at mild cold temperatures. However, an alternative and more parsimonious hypothesis would be a mutation in a single regulatory factor responsible for the up-regulation of these three CBF genes at mild cold temperatures. Additional transcriptional studies of these genes using segregating lines would be necessary to distinguish between these two hypotheses.

Some of the differentially expressed CBF genes identified in this study have been found previously to show differential transcription profiles between frost tolerant and susceptible accessions in other Triticeae species. For example, when T. aestivum plants were transferred to 2°C, transcript levels of TaCBF14, TaCBF15, and TaCBF16 (TaCBF12 was not included in the study) were higher in lines carrying the Fr-A2 allele from the frost tolerant cultivar Cheyenne than in those carrying the allele from susceptible T. aestivum (Vágújfalvi et al. 2005). The differential expression of TaCBF15 and TaCBF16 in frost tolerant and susceptible cultivars parallels our results for the orthologous genes in T. monococcum.

A similar study performed in barley plants showed that HvCBF2, 6, 12, and 14 showed higher transcript levels in the frost tolerant parent ‘Nure’ relative to the frost susceptible cultivar ‘Tremois’ (Stockinger et al. 2007). Within this set, HvCBF12 overlaps with the differentially transcribed CBF genes identified in our study. In a different study in barley, HvCBF2 and 9 showed higher transcript levels in the frost tolerant parent ‘Dicktoo’, whereas HvCBF4 showed higher transcript levels in the frost susceptible cultivar ‘Morex’ (Skinner et al. 2005). The previous differences can be explained by the use of different induction temperatures, but also by the presence of different polymorphic CBF genes segregating in the different mapping populations. Since the deletion in the DV92 CBF12 allele is not conserved in barley or other wheat genomes (Fig. 3F), it is likely that those mapping populations segregated for other CBF polymorphisms.
An additional factor confounding the interpretation of differentially transcribed CBF genes is the simultaneous segregation of vernalization genes VRN1 (Yan et al. 2003) and VRN2 (Yan et al. 2004) in some of these populations. Lines homozygous for dominant Vrn1 alleles or recessive vrn2 alleles have a spring growth habit and do not require vernalization to initiate the transition of the apices to the reproductive stage. Once this transition is initiated, the ability of wheat to acclimate to cold temperatures stops and plants became more susceptible to frost (Limin and Fowler 2006). In wheat and barley populations segregating for both Fr-2 and vernalization genes, CBF genes tend to show higher transcript levels in the lines with a winter growth habit than in those with a spring growth habit (Danyluk et al. 2003; Kobayashi et al. 2005; Kume et al. 2005; Badawi et al. 2007; Stockinger et al. 2007). We avoided the effect of the VRN2 gene on frost tolerance by growing and acclimating the plants under short day, which precludes the transition of the vegetative apices to the reproductive stage in both DV92 and G3116 (Dubcovsky et al. 2006). This was not necessary in the CBF quantitative PCR experiments because these experiments were performed with very young plants, before the differentiation of the vegetative apices and induction of VRN1.

Biochemical differences in TmCBF proteins from frost tolerant and susceptible alleles

In addition to the differences in transcription levels described above, the candidate TmCBF genes may differ in their coding sequences and/or translated proteins. To test this possibility we compared the sequences of the predicted TmCBF12, 14, 15, and 16 proteins between the frost tolerant and susceptible parents.

The most compelling difference in the candidate gene sequences is a deletion in the DV92 CBF12 AP2 domain. The AP2 DNA binding domain is common to many transcription factors (Riechmann and Meyerowitz 1998), but in the CBF proteins is flanked by unique CBF signature sequences (Jaglo et al. 2001). The AP2 domain is critical for the binding of the CBF proteins to the CRT/DRE (C-repeat/dehydration-responsive element) CCGAC motifs present in the promoters of many cold induced genes (Baker et al. 1994; Yamaguchi-Shinozaki and Shinozaki 1994; Stockinger et al. 1997; Gilmour et al. 1998; Liu et al. 1998; Thomashow 1999). The structure of the AP2 domain in CBF proteins consists of a three-stranded (b1-3) antiparallel b-sheet, and an a-helix packed almost parallel to the b-sheet (Allen et al. 1998). The deletion in the TmCBF12 DV92 allele is located between b-sheets 1 and 2, in a region known to bind to the CCGAC motif in the COR gene promoters (Allen et al. 1998; Hao et al. 2002).

Our gel shift data confirmed that the five missing amino acids in the AP2 domain of the DV92 CBF12 eliminate the ability of this protein to bind to the COR promoter (Fig. 3). Therefore, it is very likely that this protein can no longer regulate its target COR genes, which may alter the plant’s ability to cold acclimate and to tolerate freezing temperatures.

The fact that one of the three candidate genes predicted by the genetic analysis (TmCBF12, TmCBF14, and/or TmCBF15) has a mutation that disrupts its DNA binding ability provides independent support to this prediction. The biochemical results suggest that TmCBF12 is a putative candidate for the differences in frost tolerance associated with the Fr-A m2 locus in this population. However, we can not rule out the hypothesis that the linkage between the differential regulation of COR14b/DHN5 at mild cold temperatures and the central CBF cluster at the Fr-A m2 locus is generated by the differential threshold induction temperature of TmCBF15, or by more complex interactions among the TmCBF genes located within this cluster.

The TmCBF12 deletion found in T. monococcum accession DV92 has not been observed in any of the accessions of barley or polyploid wheat sequenced so far, and is absent from the majority of the T. monococcum accessions (SOM, Table S1). This suggests that different mutations in CBF12 coding or regulatory sequences or mutations in other CBF genes might be the source of natural allelic variation in frost tolerance in other Triticeae species. This possibility is supported by the different results obtained in the expression analyses of CBF genes in barley and hexaploid wheat. The presence of a large number of related CBF alleles at the Fr-2 locus provides multiple opportunities to alter the effect of this locus by modifications of different CBF genes.

Conclusions

In summary, our study indicates that the Fr-A m2 locus maps within the large TmCBF cluster on the long arm of homoeologous group 5, suggesting that polymorphisms in the TmCBF genes are the most likely explanation for the differences in frost tolerance and COR14b/DHN5 transcript levels mapped to the Fr-2 locus.

The early induction of TmCBF12, TmCBF15, and TmCBF16 at mild cold temperatures in G3116 and the lack of induction in DV92 provide a simple explanation for the differential response of COR14b and DHN5 between the same lines at mild cold temperatures. It is also possible that the mutation in the TmCBF12 AP2 domain that blocks its ability to bind the COR gene promoters may block the transmission of the mild cold temperature signal to COR14b, DHN5, and other COR genes (or other CBF
genes). COR genes from lines carrying the TmCBF12 deletion or TmCBF12/15/16 alleles that are not responsive to mild cold temperatures, will be induced only when temperatures reach lower levels than in the lines carrying the frost tolerant allele, delaying the initiation of the cold acclimation response and resulting in a lower tolerance to subsequent freezing temperatures. The importance of the differences in cold acclimation threshold induction temperatures on frost tolerance is also supported by the good correlation between these two parameters within and among Triticeae species. Wheat, barley and rye accessions with higher cold acclimation threshold induction temperatures are usually more frost tolerant than genotypes with lower threshold induction temperatures, likely due to longer acclimation times and a better preparation for later freezing temperatures (Fowler 2008).

It is important to keep in mind that each different segregating population may have different polymorphisms leading to the diversity of cold tolerance levels seen in different wheat and barley cultivars. Detailed genetic analyses will be necessary to identify the best alleles at the different CBF genes and to then breed for optimum CBF allele combinations at the Fr-2 loci. The presence of a large number of CBF genes, each with slightly different functions, or temperature induction thresholds may have provided the flexibility required for the successful adaptation of the temperate grasses to most of the cold regions of the world. It is also possible that the redundancy of the CBF gene family provides some protection to wheat and barley from the negative effects of the fast rate of deletions occurring in the large Triticeae genomes (Dubcovsky and Dvorak 2007).

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