Title

Identification of candidate CBF genes for the frost tolerance locus Fr-Am2 in Triticummonococcum

Journal

Plant Molecular Biology, 67(3)

ISSN

0167-4412

Authors

Knox, Andrea K
Li, Chengxia
Vágújfalvi, Attila
et al.

Publication Date

2008-06-01

DOI

10.1007/s11103-008-9316-6

Peer reviewed

Plant Mol Biol (2008) 67:257–270
DOI 10.1007/s11103-008-9316-6

Identification of candidate *CBF* genes for the frost tolerance locus *Fr-A^{m}2* in *Triticum monococcum*

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

Received: 27 August 2007 / Accepted: 15 February 2008 / Published online: 4 March 2008

Abstract A cluster of eleven *CBF* genes was recently mapped to the *Frost resistance-2* (*Fr-A^{m}2*) locus on chromosome 5 of diploid wheat (*Triticum monococcum*) using a cross between frost tolerant accession G3116 and frost sensitive DV92. The *Fr-A^{m}2* 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 *Tm*CBF12 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*

Andrea K. Knox, Chengxia Li and Attila Vágújfalvi contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11103-008-9316-6) contains supplementary material, which is available to authorized users.

A. K. Knox · E. J. Stockinger
Department of Horticulture and Crop Science, The Ohio State University/OARDC, Wooster, OH 44691, USA

C. Li · J. Dubcovsky (✉)
Department of Plant Sciences, University of California, Davis, CA 95616, USA
e-mail: jdubcovsky@ucdavis.edu

A. Vágújfalvi · G. Galiba
Agricultural Research Institute of the Hungarian Academy of Sciences, 2462 Martonvasar, Hungary

G. Galiba
Faculty of Information Technology, University of Pannonia, Veszprem, Hungary

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 *b*inding *f*actor) gene family. *CBF* genes were originally characterized in *Arabidopsis* as transcription factors that activate *COR* (*Co*ld-*r*egulated) 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; Vágújfalvi 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 $5A^m$ at the *F*rost *r*esistance-2 (*Fr-A*^{m}*2*) 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 (Vágújfalvi 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; Båga et al. 2007). In both wheat and barley a cluster of *CBF* genes maps to the *Fr-2* locus (Vágújfalvi et al. 2003; Francia et al. 2004; Miller et al. 2006; Tondelli et al. 2006; Båga 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 (Vágújfalvi 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 (Vágújfalvi 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 (Vágújfalvi 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 four-fold higher in the lines carrying the frost tolerant CNN *Fr-A2* allele than in those carrying the frost sensitive TSP allele (Vágújfalvi 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-A*^{m}*2 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-A*^{m}*2* locus.

Materials and methods

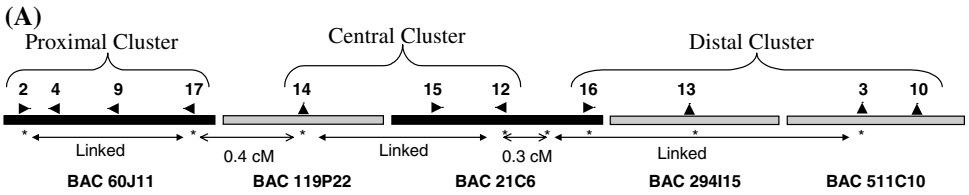
Plant materials

We screened 374 F_2 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-A*^{m}*2 CBF* cluster (lines 11, 268, 346, 394, 426, 495, and 532). Individual F_2 plants were self-pollinated and F_3 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-A^{m}2* locus (Miller et al. 2006). Genotypes of recombinant and control lines are shown in Fig. 1. Lines 16 (DV92 *Fr-A^{m}2* region) and 2 (G3116 *Fr-A^{m}2* region) were used as additional controls in the first frost tolerance experiment (Fig. 1). Homozygous recombinant lines used in all tests were in the F_4 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-A^{m}2* 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



(B)

	Proximal Cluster	Central Cluster	Distal Cluster	Frost Survival	*COR14b* transcript	*DHN5* transcript
346-14-1	S	T	T	3.24	0.25	0.59
346-6-5	S	S	S	1.40	0.12	0.15
346-9-11	S	S	S	0.78	0.07	0.06
346-12-2	S	S	S	1.88	0.01	0.12
268-17-2	S	T	T	2.91	0.13	0.44
532-1-4	T	S	S	1.40	0.15	0.13
532-4-1	T	S	S	1.00	0.18	0.14
532-8-1	T	S	S	0.96	0.11	0.41
532-19-6	T	T	T	2.26	1.00	0.80
532-23-5	T	T	T	2.68	0.42	0.47
426-5-5	T	S	S	1.64	0.02	0.09
426-4-2	T	T	T	2.50	0.07	0.08
426-12-2	T	T	T	2.61	0.27	0.27
394-12-8	T	T	S	0.96	0.00	0.08
394-12-2	T	T	T	1.68	0.22	0.38
394-12-9	T	T	T	1.63	n/a	n/a
495-4-1	S	S	T	1.46	0.18	0.08
495-8-3	S	S	T	1.79	0.23	0.35
495-11-2	S	S	T	1.32	0.23	0.02
495-12-1	S	S	T	0.95	0.02	0.13
495-9-1	T	T	T	1.56	0.69	0.20
495-15-1	T	T	T	2.03	0.22	1.00
495-23-3	T	T	T	2.12	0.22	0.42
495-24-4	T	T	T	1.98	0.64	0.45
11-1-9-1	T	T	S	1.77	0.12	n/a
11-1-9-7	T	T	S	n/a	0.15	n/a
11-1-9-10	T	T	S	2.19	0.12	n/a
11-1-4	S	S	S	1.61	n/a	n/a
11-1-9-15	S	S	S	0.88	0.04	n/a
DV92	S	S	S	0.60	0.11	n/a
16	S	S	S	1.91	0.14	n/a
G3116	T	T	T	2.36	0.28	n/a
2	T	T	T	2.71	0.23	n/a

Fig. 1 Characterization of recombinant lines. (**A**) Graphical representation of the *CBF* cluster at *Fr-A^{m}2*. 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 *DHN5* transcript data represents the average relative expression of *COR14b* and *DHN5* 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 cm^2 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).

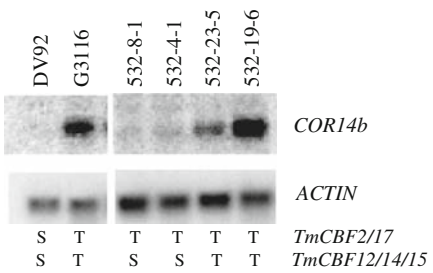


Fig. 2 RNA blot analyses of *COR14b* and *ACTIN*. Total RNA was extracted from crown tissue from plants transferred to 12°C. The parental lines and the recombinant and control lines of family 532 are shown as an example. Note the higher *COR14b* transcript accumulation in lines carrying the G3116 allele for *TmCBF12/14/15*. 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

reported in the text followed by the standard errors of the means (SEM).

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 $2^{-\Delta\Delta C}{}_{T}$ 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 *TmCBF12*, *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 *BamH*I

Table 1 Primers used in this study

Name	Forward (5′ → 3′)	Reverse (5′ → 3′)	Function
QCBF12	GTCCCACTCCCACTCACAG	ACATGTCGTGGCACAATGC	QPCR (95%)[a]
QCBF14	CATGGAGTCGCCGGACACCAGACC	GCCCTCCCCAAAAATAGACAGCGGAG	QPCR (84%)
QCBF15	CATGTTCGAGCTGGATATGTCCGGGG	GGGAACAGCTTCGGTTTGTTCATGC	QPCR (100%)
QCBF16	GCGGCATGCCTCCAACAGCGCAG	ACGTGCCCAGGTCCATCTCCCCG	QPCR (93%)
ACTIN	AATGGCTGACGGTGAGGA	GCTGACACCATCACCAGA	QPCR (98%)
CBF12-GST	AAGGATCCATGGACACGGGCCCGGAG	CCGAATTCTCAGTGGTTCCATAGCGCC	EMSA
CBF2	GATTGAGCCGGCATATCATT	CAAACATCAAACGGTCATGG	Mapping
CBF3	ATCCCACACTCTCGCTCAAG	GCTGGGAATTATCGGCACTA	Mapping
CBF12a	ATTAGCAAGTTGGCCGTCAC	CTCGGTGTCATCAGTGGTTC	Sequencing
CBF12b	GCCGTGGTGGACTTCCTG	TTATGGTAACCGCACGTGAA	Sequencing
CBF14a	GCCGATATAAGCGAGCTGTC	CACCGAGCTCAAAGTCTTCC	Sequencing
CBF14b	TCTCTCAACGCAGCAGCTAA	TCAAGTGATTCAACTCAACAACAA	Sequencing
CBF15a	TAATTGGTCACGCGAATGAA	TTTGCAGCGTTGAAATGAAG	Sequencing
CBF15b	AAGGTGCCTTCATTTCAACG	ATGCTGACAGGGAACAGCTT	Sequencing
CBF16a	ACACCCAGTTCCGTCTCAAC	CCATCCCTTTGATTTTCACG	Sequencing
CBF16b	CGTGAAAATCAAAGGGATGG	GCTCCAGCTGGTTCAGTAGC	Sequencing

[a] The efficiency of the primer pairs used for quantitative PCR is indicated between brackets

and *Eco*RI were incorporated into the oligos to facilitate cloning. PCR products were digested with *BamH*I and *Eco*RI, 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 *Eco*RI and *Sal*I, radiolabeled with α-^{32}P-dATP and α-^{32}P-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 $MgCl_2$, 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 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 at room temperature.

Results

Update of the *Fr-A^{m}2 CBF* cluster genetic and physical map

The eleven *CBF* genes identified at the *Fr-A^{m}2* 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 F_2 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*s than the sister lines carrying the DV92 allele (60–140% increases, SOM). These differences were highly significant for families 346 ($P < 0.0001$) and 532 ($P < 0.0001$). 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 *TmCBF*s. Family 11 and 495 segregate for the central *TmCBF*s, whereas family 394 segregates for the distal *TmCBF*s (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*.

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

	Frost tolerance			*COR14b* expression			*DHN5* expression		
	F	*P*	% var	F	*P*	% var	F	*P*	% var
CBF2/17	5.1	0.03	40.5	3.9	0.06	35.4	4.6	0.047	31.7
CBF14/15/12	48.3	<0.001	78.8	9.5	0.005	54.4	9.6	0.008	58.5
CBF16	22.7	<0.001	67.3	3.9	0.06	38.5	4.7	0.049	42.4

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

Mapping of the *COR14b* and *DHN5* differential induction phenotype

The lines with recombination events within the *Fr-A*^{m}*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

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 *Tm*CBF12 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 *Tm*CBF14 and *Tm*CBF15 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

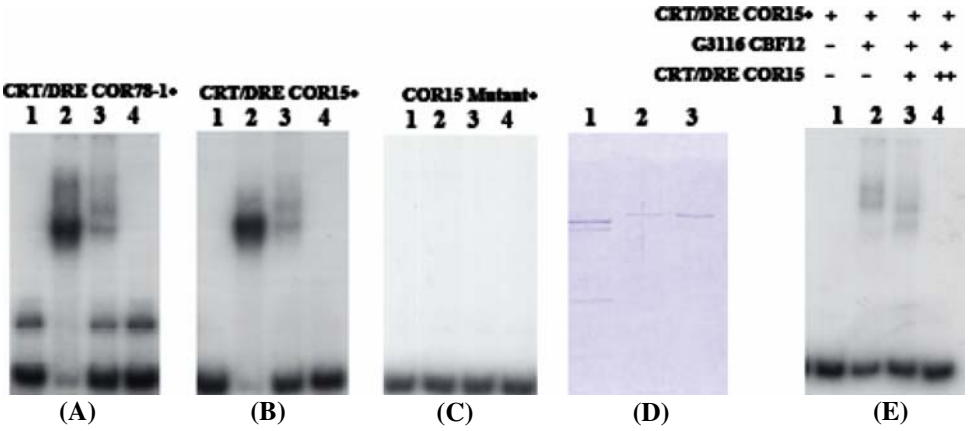
The 15 bp deletion in the DV92 *TmCBF12* allele occurs within the AP2 DNA binding domain and therefore has the potential to affect this protein's ability to bind to the CRT/DRE (C-repeat/dehydration-responsive element) motif present in the *COR* gene promoters. To test this hypothesis we carried out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

Purified recombinant *Tm*CBF12 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 *At*CBF1 protein used as positive control (Fig. 3A–C; lane 2). Interaction between the G3116 *Tm*CBF12 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 *Tm*CBF12 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 Vágújfalvi 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 *CBF*s 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




```
DV92          PKRPAGRTKFKETRHPVFHGVRRR-----WVCEVRVPGKRGERLWLGTHVTAEAAARAHD
G3116         PKRPAGRTKFKETRHPVFHGVRRRGSNGRWVCEVRVPGKRGERLWLGTHVTAEAAARAHD
CBFIIId-B12   PKRPAGRTKFKETRHPVFHGVRRRGSNGRWVCEVRVPGKRGERLWLGTHVTAEAAARAHD
HvDtCBF12     PKRPAGRTKFKETRHPVFHGVRRRGSNGRWVCEVRVPGKRGERLWLGTHVTAEAAARAHD
              ----------------**********************************************

DV92          AAMLALYGRTPAARLNYPDSAW
G3116         AAMLALYGRTPAARLNYPDSAW
CBFIIId-B12   AAMLALYGRTPAARLNYPDSAW
HvDtCBF12     AGMLALYGRTPAARLNFPDSAW
              ********************----
```

(F)

Fig. 3 Recombinant CBF12 protein from G3116 binds to the CRT/DRE, whereas CBF12 from DV92 does not. Radiolabeled probes are marked with an asterisk. (**A**, **B**, and **C**) Radiolabeled wild type and mutant CRT DNA sequences were used for *in vitro* binding reactions with probe alone (lane 1), recombinant *Arabidopsis* CBF1 protein (lane 2) and recombinant CBF12 proteins from winter wheat G3116 (lane 3) and spring wheat DV92 (lane 4). (**D**) Coomassie brilliant blue stained gel with 5 µl each of the purified proteins: Arabidopsis CBF1 (lane 1), CBF12 from G3116 (lane 2) and CBF12 from DV92 (lane 3). (**E**) Unlabeled wild type CRT/DRE DNA was used as a competitor in the competition binding reactions: no competitor (lane 1 and 2), 20-fold molar excess of competitor (lane 3) and 50-fold molar excess of competitor (lane 4). (**F**) Alignment of the CBF12 proteins from DV92 and G3116 to those from barley (HvDtCBF12; ABA01491) and hexaploid wheat (CBFIIId-B12; ABK55366). The deletion is highlighted in yellow. Asterisks denote the AP2 DNA binding domain; dashes represent the flanking *CBF* signature sequence

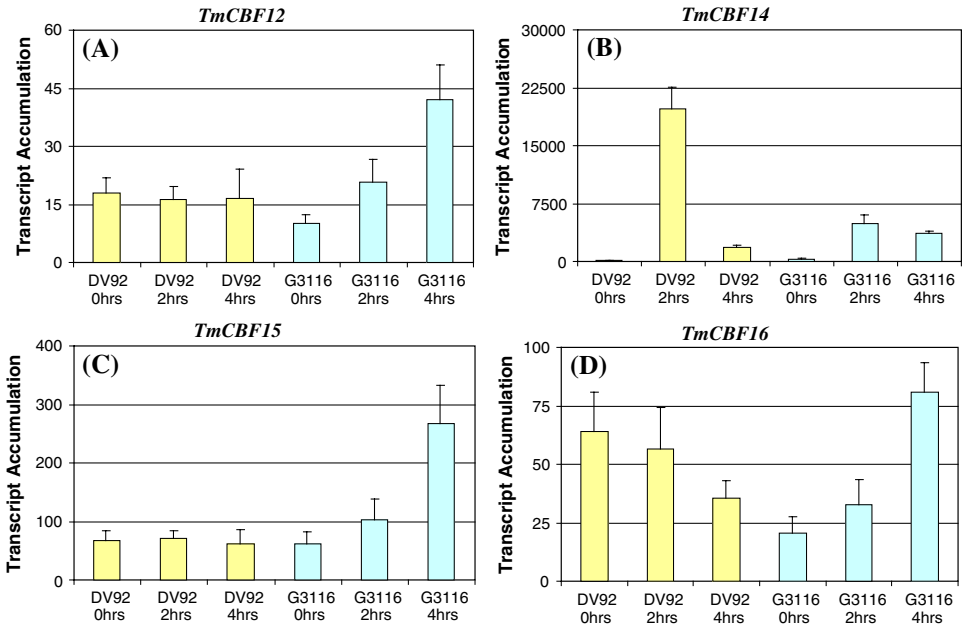


Fig. 4 Quantitative real time PCR analyses of *TmCBF12*, *14*, *15*, and *16*. *CBF* transcript levels measured at room temperature and after 2 h and 4 h at 15°C in DV92 (yellow) and G3116 (blue) were normalized to *ACTIN*. (**A**) *CBF12*; (**B**) *CBF14*; (**C**) *CBF15*; (**D**) *CBF16*. Error bars represent standard error. Units are values linearized with the $2^{(-\Delta\Delta CT)}$ method, where CT is the threshold cycle. All analyses were performed using the same corrector so scales for the four graphs and for those in the SOM are comparable

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*, *15*, and *16* 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*, *15*, and *16*, 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*, *15*, and *16* 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*, *14*, *15*, and *16* 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 *Triticeae* has been a subject of intense research in recent years. The cluster of *CBF* genes located at the peak of the *Fr-A^{m}2* 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^{m}2* 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^{m}2 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^{m}2* locus with the central *CBF* cluster has important implications because it establishes that the *Fr-A^{m}2* 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 60J11 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-A^{m}2* 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. spelta* (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 *Tm*CBF 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 *Tm*CBF12, 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 (β1-3) antiparallel β-sheet, and an α-helix packed almost parallel to the β-sheet (Allen et al. 1998). The deletion in the *Tm*CBF12 DV92 allele is located between β-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^{m}2* 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^{m}2* 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^{m}2* 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).

Acknowledgements This project was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant number 2004-01783, the Hungarian Wheat Spike Consortia (NKFP 406404) and the Hungarian National Research Fund No. T046573. Work in the Stockinger lab is supported in part by grants from the NSF Plant Genome Program (DBI 0110124) and the Ohio Plant Biotechnology Consortium (Proposal Number 2007-020). The Hungarian authors thank Alexandra Soltész for her technical assistance.

References

Allen MD, Yamasaki K, Ohme-Takagi M, Tateno M, Suzuki M (1998) A novel mode of DNA recognition by a beta-sheet revealed by the solution structure of the GCC-box binding domain in complex with DNA. EMBO J 17:5484–5496

Badawi M, Danyluk J, Boucho B, Houde M, Sarhan F (2007) The *CBF* gene family in hexaploid wheat and its relationship to the phylogenetic complexity of cereal *CBF*s. Mol Genet Genomics 277:533–554

Båga M, Chodaparambil SV, Limin AE, Pecar M, Fowler DB, Chibbar RN (2007) Identification of quantitative trait loci and associated candidate genes for low-temperature tolerance in cold-hardy winter wheat. Funct Integr Genomics 7:53–68

Baker SS, Wilhelm KS, Thomashow MF (1994) The 5′-region of *Arabidopsis thaliana Cor15a* has cis-acting elements that confer cold-, drought- and ABA-regulated gene expression. Plant Mol Biol 24:701–713

Brautigam M, Lindlof A, Zakhrabekova S, Gharti-Chhetri G, Olsson B, Olsson O (2005) Generation and analysis of 9792 EST sequences from cold acclimated oat, *Avena sativa*. BMC Plant Biol 5:18

Crosatti C, Polverino de Laureto P, Bassi R, Cattivelli L (1999) The interaction between cold and light controls the expression of the cold-regulated barley gene *Cor14b* and the accumulation of the corresponding protein. Plant Physiol 119:671–680

Danyluk J, Kane NA, Breton G, Limin AE, Fowler DB, Sarhan F (2003) TaVRT-1, a putative transcription factor associated with vegetative to reproductive transition in cereals. Plant Phys 132:1849–1860

Dubcovsky J, Dvorak J (2007) Genome plasticity a key factor in the success of polyploid wheat under domestication. Science 316:1862–1866

Dubcovsky J, Loukoianov A, Fu D, Valarik M, Sanchez A, Yan L (2006) Effect of photoperiod on the regulation of wheat vernalization genes *VRN1* and *VRN2*. Plant Mol Biol 60: 469–480

Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. Plant J 33:751–763

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

Fowler DB (2008) Cold acclimation threshold induction temperatures in cereals. Crop Sci (in press)

Francia E, Rizza F, Cattivelli L, Stanca AM, Galiba G, Toth B, Hayes PM, Skinner JS, Pecchioni N (2004) Two loci on chromosome 5H determine low-temperature tolerance in a 'Nure' (winter) × 'Tremois' (spring) barley map. Theor Appl Genet 108:670–680

Francia E, Barabaschi D, Tondelli A, Laidò G, Rizza F, Stanca AM, Busconi M, Fogher C, Stockinger EJ, Pecchioni N (2007). Fine mapping of a Hv*CBF* gene cluster at the frost resistance locus *Fr-H2* in barley. Theor Appl Genet 115:1083–1091

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998) Low temperature regulation of the *Arabidopsis CBF* family of AP2 transcriptional activators as an early step in cold-induced *COR* gene expression. Plant J 16: 433–442

Gilmour SJ, Fowler SG, Thomashow MF (2004) *Arabidopsis* transcriptional activators *CBF1*, *CBF2*, and *CBF3* have matching functional activities. Plant Mol Biol 54:767–781

Hao D, Yamasaki K, Sarai A,Ohme-Takagi M (2002) Determinants in the sequence specific binding of two plant transcription factors, *CBF1* and *NtERF2*, to the DRE and GCC motifs. Biochemistry 41:4202–4208

Houde M, Danyluk J, Laliberte J-F, Rassart E, Dhindsa RS, Sarhan F (1992) Cloning, characterization, and expression of a cDNA encoding a 50-kilodalton protein specifically induced by cold acclimation in wheat. Plant Physiol 99:1381–1387

Jaglo KR, Kleff S, Amundsen KL, Zhang X, Haake V, Zhang JZ, Deits T, Thomashow MF (2001) Components of the *Arabidopsis* C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. Plant Physiol 127:910–917

Kobayashi F, Takumi S, Kume S, Ishibashi M, Ohno R, Murai K, Nakamura C (2005) Regulation by *Vrn1*/*Fr-1* chromosomal intervals of CBF-mediated *Cor*/*Lea* gene expression and freezing tolerance in common wheat. J Exp Bot 56:887–895

Kume S, Kobayashi F, Ishibashi M, Ohno R, Nakamura C, Takumi S (2005) Differential and coordinated expression of *Cbf* and *Cor/Lea* genes during long-term cold acclimation in two wheat cultivars showing distinct levels of freezing tolerance. Genes Genet Syst 80:185–197

Limin AE, Fowler DB (2006) Low-temperature tolerance and genetic potential in wheat (*Triticum aestivum* L.): response to photoperiod, vernalization, and plant development. Planta 224:360–366

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 10:1391–1406

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\Delta\Delta C(T))}$ Method. Methods 25:402–408

Miller AK, Galiba G, Dubcovsky J (2006) A cluster of 11 *CBF* transcription factors is located at the frost tolerance locus *Fr-A^{m}2* in *Triticum monococcum*. Mol Genet Genomics 275:193–203

Riechmann JL, Meyerowitz EM (1998) The AP2/EREBP family of plant transcription factors. Biol Chem 379:633–646

Sarhan F, Ouellet F, Vazquez-Tello A (1997) The wheat *Wcs120* gene family: a useful model to understand the molecular genetics of freezing tolerance in cereals. Physiol Plant 101:439–445

SAS version 9.1. (2003) SAS Institute Inc., Cary, NC, USA

Shinwari Z, Nakashima K, Miura S, Kasuga M, Seki M, Yamaguchi-Shinozaki K, Shinozaki K (1998) An *Arabidopsis* gene family encoding DRE/CRT binding proteins involved in low-temperature-responsive gene expression. Biochem Biophys Res Commun 250:161–170

Skinner JS, von Zitzewitz J, Szucs P, Marquez-Cedillo L, Filichkin T, Amundsen K, Stockinger EJ, Thomashow MF, Chen TH, Hayes PM (2005) Structural, functional, and phylogenetic characterization of a large *CBF* gene family in barley. Plant Mol Biol 59:533–551

Skinner JS, Szucs P, von Zitzewitz J, Marquez-Cedillo L, Filichkin T, Stockinger EJ, Thomashow MF, Chen TH, Hayes PM (2006) Mapping of barley homologs to genes that regulate low temperature tolerance in *Arabidopsis*. Theor Appl Genet 112:832–842

Stockinger EJ, Gilmour SJ, Thomashow MF (1997) *Arabidopsis thaliana CBF1* encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc Natl Acad Sci USA 94:1035–1040

Stockinger EJ, Skinner JS, Gardner KG, Francia E, Pecchioni N (2007) Expression levels of barley *Cbf* genes at the *Frost resistance-H2* locus are dependent upon alleles at *Fr-H1* and *Fr-H2*. Plant J 51:308–321

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol 50:571–599

Thompson JD, Higgins DG, Gibson TJ (1994) Improved sensitivity of profile searches through the use of sequence weights and gap excision. Comput Appl Biosci 10:19–29

Tondelli A, Francia E, Barabaschi D, Aprile A, Skinner JS, Stockinger EJ, Stanca AM, Pecchioni N (2006) Mapping regulatory genes as candidates for cold and drought stress tolerance in barley. Theor Appl Genet 112:445–454

Toth B, Galiba G, Feher E, Sutka J, Snape JW (2003) Mapping genes affecting flowering time and frost resistance on chromosome 5B of wheat. Theor Appl Genet 107:509–514

Vágújfalvi A, Crosatti C, Galiba G, Dubcovsky J, Cattivelli L (2000) Two loci on wheat chromosome 5A regulate the differential cold-dependent expression of the *Cor14b* gene in frost-tolerant and frost-sensitive genotypes. Mol Gen Genet 263:194–200

Vágújfalvi A, Galiba G, Cattivelli L, Dubcovsky J (2003) The cold-regulated transcriptional activator *Cbf3* is linked to the frost-tolerance locus *Fr-A2* on wheat chromosome 5A. Mol Gen Genomics 269:60–67

Vágújfalvi A, Aprile A, Miller A, Dubcovsky J, Delugu G, Galiba G, Cattivelli L (2005) The expression of several *Cbf* genes at the *Fr-A2* locus is linked to frost resistance in wheat. Mol Genet Genomics 274:506–514

Van Zee K, Chen FQ, Hayes PM, Close TJ, Chen THH (1995) Cold-specific induction of a *Dehydrin* gene family member in barley. Plant Physiol 108:1233–1239

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel cis-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell 6:251–264

Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T, Dubcovsky J (2003) Positional cloning of wheat vernalization gene *Vrn1*. Proc Natl Acad Sci USA 100:6263–6268

Yan L, Loukoianov A, Blechl A, Tranquilli G, Ramakrishna W, SanMiguel PJ, Bennetzen JL, Echenique V, Dubcovsky J (2004) The wheat *Vrn2* gene is a flowering repressor down-regulated by vernalization. Science 303:1640–1644