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

Knox A[1], Li C[2], Vágújfalvi A[3], Galiba G[3], Stockinger E[1], Dubcovsky J[2]
[1]Dept of Horticulture & Crop Science, Ohio State University/OARDC, Wooster, OH, 44691, USA
[2]Dept of Plant Sciences, University of California, Davis, CA, 95616, USA
[3]Agricultural Research Institute of the Hungarian Academy of Sciences, 2462, Martonvásár, Hungary

ABSTRACT

A cluster of eleven *CBF* genes was recently mapped to the *Frost resistance-2* (*Fr-A^{m}2*) locus on chromosome 5 of *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 transcript levels of *COR14b* and *DHN5* at 12°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 (12-15°C) 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.

INTRODUCTION

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[1]. We recently reported a large family of *TmCBF* genes in diploid wheat (*T. monococcum*)[2]. 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[3]. Although it has been well established that the largest *Fr-2 CBF* cluster is linked to differences in frost tolerance in both wheat and barley[3], it is currently not known which of the *CBF* gene(s) within this cluster is/are responsible for the observed differences in frost tolerance. In order to dissect the complex *Fr-2* locus, we generated a high-density mapping population and identified recombination events among the *CBF* genes within this locus. In this study, we scored 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.

RESULTS

Homozygous recombinant sister lines were isolated within the *TmCBF* cluster. Recombination events divided the locus into three clusters (Fig. 1). The proximal *CBF* genes (*TmCBF2, 4, 9* and *17*) are separated from the other seven *CBF* genes by four recombination events, thus four independent recombinant lines are used to characterize this cluster. The central cluster, containing *TmCBF14, 15,* and *12*, is separated from the distal cluster, containing *TmCBF16, 13, 3* and *10,* by three recombination events.

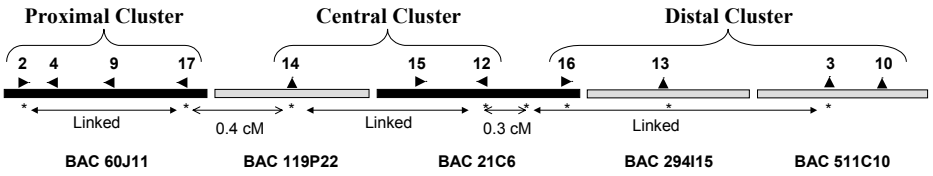


Fig 1: Graphical representation of the *CBF* cluster at *Fr-A^{m}2*. Arrowheads represent markers. Proximal, central and distal clusters are defined by recombination events.

Recombinant lines were tested for frost survival by freezing them at -11°C for 24 h. Survival was measured on a scale of 0 (death) to 5 (undamaged). Analyses of

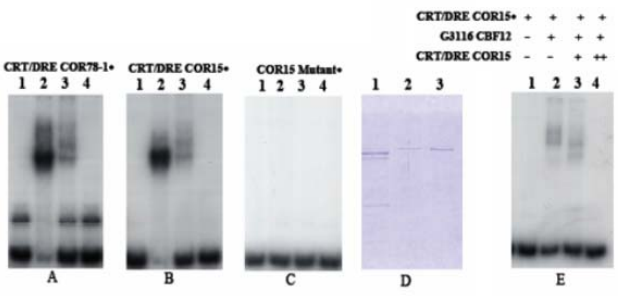
variance using the different *TmCBF* markers as classification values and the F_2 families as blocks showed that the largest *F* values (*F*= 48.3, *P*<0.0001) were detected with the marker for the central *TmCBF* genes *TmCBF12/14/15* (Table 1). Allelic differences at the central *TmCBF* locus also 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).

	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	0.0085	58.5
CBF16	22.7	<0.001	67.3	3.9	0.06	38.5	4.7	0.049	42.4

Table 1: Statistical analysis of frost tolerance and transcription levels of *COR14b* and *DHN5* in the recombinant plants.

The lines with recombination events within the *Fr-A^{m}2 TmCBF* cluster were also monitored for *COR14b* and *DHN5* (=WCS120) transcript levels at 12°C using RNA blot analyses. These analyses showed that the central *TmCBF* genes (*CBF12/14/15*) had higher *F* values (Table 1) and explained a larger proportion of the variation in *COR14b* and *DHN5* transcript levels than proximal and distal clusters. Average *COR14b* values for 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).

To determine if the sequences of 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.




```
DV92            PKRPAGRTKFKETRHPVFHGVRRR-----WVCEVRVPGKRGER
G3116           PKRPAGRTKFKETRHPVFHGVRRRGSNGRWVCEVRVPGKRGER
CBFIIId-B12     PKRPAGRTKFKETRHPVFHGVRRRGSNGRWVCEVRVPGKRGER
HvDtCBF12       PKRPAGRTKFKETRHPVFHGVRRRGSNGRWVCEVRVPGKRGER
                ----------------**************************

DV92            LWLGTHVTAEAAARAHDAAMLALYGRTPAARLNYPDSAW
G3116           LWLGTHVTAEAAARAHDAAMLALYGRTPAARLNYPDSAW
CBFIIId-B12     LWLGTHVTAEAAARAHDAAMLALYGRTPAARLNYPDSAW
HvDtCBF12       LWLGTHVTAEAAARAHDAGMLALYGRTPAARLNFPDSAW
                **********************************----   F
```

These comparisons revealed that the DV92 *TmCBF12* gene had a 15 base pair deletion beginning at nucleotide 165 (after the MET initiator codon) relative to the G3116 *TmCBF12* gene. 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 (EMSA) with the wild type (G3116) and mutant (DV92) recombinant proteins.

When similar quantities of full-length proteins were used in the EMSA, the G3116 *Tm*CBF12 protein as well as the positive control *At*CBF1 protein, were able to bind to the CRT/DRE sequences from both *COR78-1* and *COR15a* promoters; whereas, the DV92 *Tm*CBF12 protein did not produce a mobility shift with either of CRT/DRE probes. Interaction between the G3116 *Tm*CBF12 protein and the labelled CRT/DRE probe was eliminated by the addition of excess unlabeled CRT/DRE DNA in the binding reactions, suggesting binding specificity (Fig. 2).

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 (Fig. 3). *TmCBF* transcript levels were quantified in the leaves at 15°C, the temperature at which Vágújfalvi et al.[3] observed differences in *COR14b* transcript levels between DV92 and G3116 leaves. In G3116 plants transferred to 4°C for 4h, transcript levels of *TmCBF14* were 16-fold higher than those observed at 15°C and four-fold higher than transcript levels at 10°C (*P*<0.01) indicating a stronger response at lower temperatures. Transcript levels of *TmCBF12, TmCBF15* and *TmCBF16* in the leaves showed four-fold increases (*P*<0.01) within four hours of transferring the G3116 plants to 15°C. However, in DV92 the transfer to 15°C had no effect upon transcript levels of these same genes.

Fig. 2: 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 (1), recombinant *Arabidopsis* CBF1 protein (2) and recombinant CBF12 proteins from winter wheat G3116 (3) and spring wheat DV92 (4). **D**: Coomassie brilliant blue stained gel with 5 µl each of the purified proteins: Arabidopsis CBF1 (1), CBF12 from G3116 (2) and CBF12 from DV92 (3). **E**: Unlabeled wild type CRT/DRE DNA was used as a competitor in the competition binding reactions: no competitor (1 & 2), 20-fold molar excess of competitor (3) and 50-fold molar excess of competitor (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. Asterisks denote the AP2 DNA binding domain; dashes represent the flanking *CBF* signature sequence.

DISCUSSION

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 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.

The transcription profiles differ among the *TmCBF* genes at *Fr-A^{m}2*. All *TmCBF* genes tested were up-regulated when plants were transferred from room temperature to 4°C or 10°C. The frost susceptible parental line DV92 showed no significant induction of *TmCBF12*, *TmCBF15* and *TmCBF16* when the plants were transferred to 15°C (Fig. 3), whereas the frost tolerant parental line G3116 showed a significant four-fold increase in transcript levels under the same conditions. *TmCBF12*, *TmCBF15* and *TmCBF16* have a close evolutionary relationship 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.

In addition to the differences in transcription levels described above, the candidate *TmCBF* genes may differ in their coding sequences and/or translated proteins. 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. 2). 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 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.

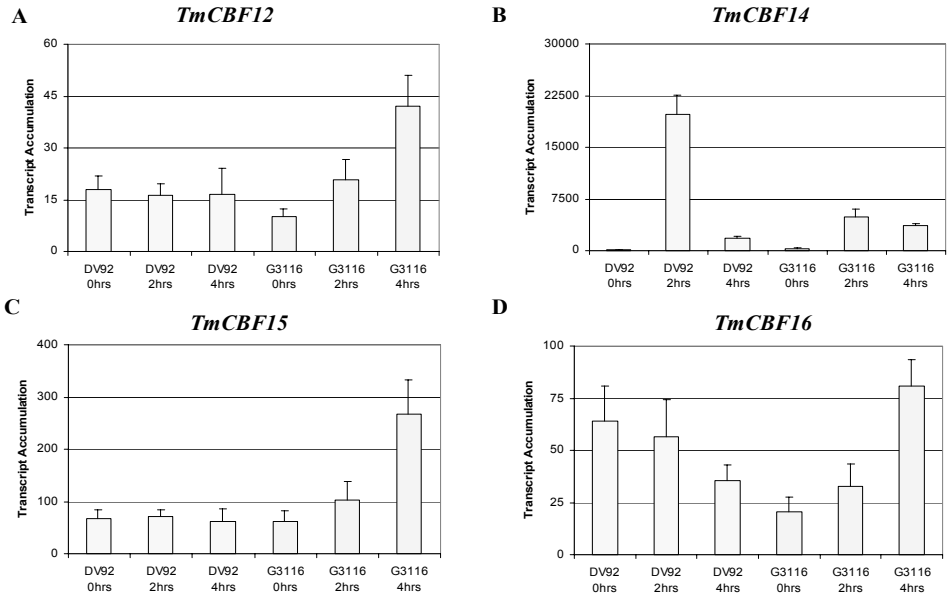


Fig. 3: Quantitative real time PCR analyses of *TmCBF12*, *14*, *15*, and *16*. *CBF* transcript levels measured at room temperature and after 2 and 4 h at 15°C in DV92 and G3116 were normalized to *ACTIN*. **A**: *CBF12*; **B**: *CBF14*; **C**: *CBF15*; **D**: *CBF16*. Error bars represent standard error. Units are values linearized with the $2^{(-\Delta\Delta C_T)}$ method, where C_T is the threshold cycle. All analyses were performed using the same calibrator so scales for the four graphs are comparable.

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 "Plant Resource" OMFB – 000515/2007 (2006-2009). Work in Stockinger's 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).

REFERENCES

1 Stockinger EJ, Gilmour SJ, Thomashow MF (1997) Proc Natl Acad Sci USA 94:1035-1040

2 Miller AK, Galiba G, Dubcovsky J (2006) Mol Genet Genomics 275:193-203

3 Vágújfalvi A, Galiba G, Cattivelli L, Dubcovsky J (2003) Mol Gen Genomics 269:60-67