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

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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 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 transcript levels of COR14b and DHN5 at 12°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 (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 (Cold-regulated) genes, whose proteins play a structural role in conferring cold tolerance to plants1. We recently reported a large family of TmCBF genes in diploid wheat (T. monococcum). Eleven CBF genes map to a 0.8 cM region on chromosome 5A m at the Frost resistance-2 (Fr-A"m2) 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 gene3. 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, 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.

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 F2 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 (R²= 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).

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

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<thead>
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<th>Frost tolerance</th>
<th>COR14b expression</th>
<th>DHN5 expression</th>
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<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>% var</td>
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<tr>
<td>CBF12/15</td>
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<td>0.00</td>
<td>40.5</td>
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<tr>
<td>CBF12/14/15</td>
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<td>&lt;0.001</td>
<td>78.8</td>
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<tr>
<td>CBF16</td>
<td>22.7</td>
<td>&lt;0.001</td>
<td>67.3</td>
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The lines with recombination events within the Fr-A<sup>+</sup>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.

When similar quantities of full-length proteins were used in the EMSA, the G3116 TmCBF12 protein as well as the positive control AtCBF1 protein, were able to bind to the CRT/DRE sequences from both COR78-I and COR15a promoters; whereas, the DV92 TmCBF12 protein did not produce a mobility shift with either of CRT/DRE probes. Interaction between the G3116 TmCBF12 protein and the labelled CRT/DRE probe was eliminated by the addition of excess unlabeled CRT/DRE DNA in the binding reactions, suggesting binding selectivity (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. 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), CBF2 from G3116 (2) and CBF2 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 (HvDCBF12; AA01491) and hexaploid wheat (CBFIId-B12; ABK53566). The deletion is highlighted. Asterisks denote the AP2 DNA binding domain; dashes represent the flanking CBF signature sequence.

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.
DISCUSSION

The association of the Fr-A*2 locus with the central CBF cluster has important implications because it establishes that the Fr-A*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 TmCBF12/17 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*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*2 locus in this population.

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