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ORIGINAL PAPER

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A cluster of 11 *CBF* transcription factors is located at the frost tolerance locus *Fr-A^{m}2* in *Triticum monococcum*

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Abstract Due to the adverse effects of cold temperatures on winter wheat, frost tolerance is an important trait for breeding programs in regions with severe winters. Frost tolerance locus *Fr-A^{m}2* was recently discovered in diploid wheat (*Triticum monococcum* L.). This locus was mapped as a QTL on chromosome 5A^m in the same region as a QTL for the level of transcription of the cold-regulated gene *COR14b* at 15°C. A *CBF* transcription factor was mapped in the center of these two overlapping QTLs. However, since the *CBF* gene family in wheat has numerous members, it was possible that multiple *CBF* genes were present at *Fr-A^{m}2*. To investigate this possibility we initiated a systematic characterization of the *CBF* family in *T. monococcum*. Here we report the molecular characterization of thirteen *TmCBF* genes. Nine of them were numbered according to the closest barley *HvCBF* gene, and the other four that have no clear barley orthologues were assigned numbers *TmCBF15* to *TmCBF18*. *TmCBF5* and *TmCBF18* were mapped on *T. monococcum* chromosomes 7A^m and 6A^m, respectively, and are thus not candidates for the *Fr-A^{m}2* gene. The remaining eleven *TmCBF* genes are clustered at the *Fr-A^{m}2* locus within five different Bacterial Artificial Chromosome (BAC) clones. These BACs were mapped using a high-density map and recombination events were found between most BACs. Lines carrying these recombination events will be useful to identify which of the *CBF* genes is responsible for the differences in frost tolerance between the *T. monococcum* parental lines at the *Fr-A^{m}2* locus.

Keywords Frost Tolerance · *COR14b* · *CBF* · *Triticum monococcum* · Wheat

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ACCESSION NUMBERS: Sequence data for the *T. monococcum CBF* genes is recorded in GenBank under accession numbers AY951944, AY951945, AY951946, AY951947, AY951948, AY951949, AY951950, and AY951951.

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Introduction

Frost tolerance is an important objective for wheat breeding programs in regions with severe winters, where low temperatures can result in severe yield reductions. The damage resulting from freezing temperatures to wheat plants can be reduced by exposing them to a period of cold acclimation at low non-freezing temperatures. During cold acclimation cold-responsive pathways are activated, preparing plants for more severe levels of cold (Sakai and Larcher 1985). Understanding the genetics of cold acclimation and frost tolerance can help breeders to better select frost tolerant wheat varieties.

Wheat chromosomes from homoeologous group five include critical genes for cold tolerance (Law et al. 1976; Sutka and Snape 1989; Roberts 1990; Sutka 1994; Dubcovsky et al. 1998; Vágújfalvi et al. 2003) in addition to several genes related to other abiotic stresses (Dubcovsky et al. 1995). Particularly relevant to cold tolerance are the vernalization (*VRN*) and frost tolerance (*Fr*) genes. *VRN* genes are responsible for the requirement of a long exposure to low temperatures (several weeks at 4°C) to initiate flowering, whereas the *Fr* genes affect the ability to survive freezing temperatures independently of flowering regulation.

VRN-1 and *VRN-2* are the main vernalization genes in wheat and barley. *VRN-1* is a meristem identity gene

up-regulated by vernalization (Yan et al. 2003) whereas *VRN-2* is a transcription factor down-regulated by vernalization and epistatic to *VRN-1* (Yan et al. 2004). *VRN-1* loci have been mapped in the central region of the long arm on chromosomes 5A, 5B, and 5D in wheat, as well as on chromosome 5H in barley, whereas *VRN-2* loci are present on chromosome 5A, 4B, and 4D in wheat and 4H in barley. The increased frost tolerance associated with the *VRN-1* and *VRN-2* alleles for winter growth habit (Doll et al. 1989; Hayes et al. 1997; Vágújfalvi et al. 2003; Francia et al. 2004) is likely associated with the protection of the sensitive floral meristem from low temperatures by preventing the transition from vegetative to reproductive apexes during the winter (Fowler et al. 2001).

We recently discovered a new locus for frost tolerance on chromosome $5A^m$ of diploid wheat (*Triticum monococcum* L.). This locus, named *Fr-A^m2*, was mapped 30 cM proximal to *VRN-1* (Vágújfalvi et al. 2003). This same chromosome region was found to affect frost tolerance on chromosome 5B of common wheat (Tóth et al. 2003) and chromosome 5H of barley (Francia et al. 2004). Although the 5B locus was originally published as *Fr-B1*, the authors later corrected the name to *Fr-B2* in the 2004 supplement of the Catalogue of Gene Symbols for Wheat (McIntosh et al. 2004). Finally, the QTL for frost tolerance on the long arm of chromosome 5D (Snape et al. 2001) was mapped on an intermediate location between *VRN-D1* and the 5D chromosome region orthologous to *Fr-2*. The authors indicated that this population might have a bimodal distribution for the frost response suggesting the possibility that the observed QTL may be a combination of the effects of two loci on chromosome 5D. An additional locus for frost tolerance, designated *Fr-1*, has been reported to be tightly but not completely linked to *VRN-A1* in hexaploid wheat (Galiba et al. 1995; Sutka et al. 1999).

The parental lines of *T. monococcum* used to map the *Fr-A^m2* locus also showed a differential regulation of the cold-regulated gene *COR14b* at 15°C. The *COR14b* gene is likely involved in the protection of the chloroplast membranes from freezing temperatures (Crosatti et al. 1999). At this temperature the tolerant parent showed high levels of *COR14b* transcripts whereas the susceptible parent showed almost undetectable levels. Characterization of *COR14b* transcription levels at 15°C in the mapping population revealed an expression QTL for *COR14b* that completely overlapped with the *Fr-A^m2* QTL for frost survival at −13°C. This result suggested that the frost tolerance at *Fr-A^m2* was mediated by differential regulation of the expression of the *COR* genes (Vágújfalvi et al. 2003). The same result was observed in the *Fr-H2* locus in barley, shown by a highly significant QTL for the accumulation of COR14b and other cold induced proteins that perfectly overlapped the QTL for frost tolerance (Francia et al. 2004).

COR genes in *Arabidopsis* are regulated by *CBF* transcriptional activators (reviewed in Thomashow 2001). *CBF* genes have also been identified in monocot species including rye (Jaglo et al. 2001), rice (Dubouzet et al. 2003), barley (Choi et al. 2002; Xue 2002; Francia et al. 2004; Skinner et al. 2005), and wheat (Jaglo et al. 2001; Kobayashi et al. 2005). Therefore, it was not surprising to find that wheat and barley *CBF* orthologues were mapped at the peak of the *Fr-A^m2* and *Fr-H2* QTLs (Vágújfalvi et al. 2003; Francia et al. 2004). This association suggests that *CBF* transcriptional activators may play a major role in the determination of frost tolerance in cereals. However, testing this hypothesis is complicated by the presence of multiple *CBF* copies in wheat as revealed by multiple RFLP bands co-segregating at the *Fr-A^m2* locus and additional bands from unlinked loci (Vágújfalvi et al. 2003).

Therefore, a more detailed characterization of the cereal *CBF* genes is necessary to determine which of the multiple *CBF* genes is (are) responsible for the observed differences in frost tolerance at the *Fr-A^m2* locus. The goals of this study were to: (1) sequence most of the *CBF* genes present in *T. monococcum* and compare their sequences with previously characterized *CBF* genes from other plant species, (2) map these genes to determine which ones are located at the *Fr-A^m2* locus and determine their physical organization, and finally (3) screen a large mapping population from the same cross used to map *Fr-A^m2* to identify lines with recombination events between the different *CBF* genes present at this locus.

Materials and methods

Bacterial artificial chromosome clone selection and contig construction

High density filters for the BAC library from *T. monococcum* L. accession DV92 (Lijavetzky et al. 1999) were screened with two probes from barley, *BCBF1* (Accession No. BF631103) and *BCBF3*, kindly provided by Patrick Hayes (Oregon State University). *BCBF3* was obtained from barley variety Dicktoo, and is allelic to *BCBF3* from Morex (Accession No. AF298231; Choi et al. 2002). The *BCBF3* probe was previously used in the RFLP mapping of several bands to the *Fr-A^m2* locus (Vágújfalvi et al. 2003). The translated sequences of the *BCBF1* and *BCBF3* probes are 77 and 98% identical to the HvCBF4A and HvCBF3 proteins used for the phylogenetic studies (Skinner et al. 2005).

Triticum monococcum genes with approximately 80% similarity to the barley probes were detected under the hybridization conditions used in the BAC library screening. Radioactively labeled probes were prepared by the random-primer method (Feinberg and Vogelstein 1983) and hybridization was carried out overnight at 65°C. BAC membranes were washed with a 0.5 × SSC-0.1% SDS buffer for 30 min at 65°C and exposed to Kodak Scientific film. Positive BAC clones were fingerprinted with *Hin*dIII, and the contigs were confirmed by hybridization of the fingerprint's Southern blots with the two *BCBF* probes.

Cloning

*Hin*dIII fingerprinting bands from BAC clones 119P22 (5.2 kb), 284I15 (1.8 and 6.0 kb), 289H4 (3.0 kb), 511C10 (2.0 and 4.5 kb) and 584E14 (2.2 kb) that hybridized to the *BCBF* probes were cloned. BAC DNA for cloning was prepared using the QIAGEN (Valencia, CA, USA) Large-Construct Kit. Excised bands indicated above were purified using the QIAGEN QIAquick gel extraction kit. Fifty nanograms of each fragment were ligated with fifty nanograms of *Hin*dIII digested pBluescript II (Strategene, La Jolla, CA, USA) and 3 units of T4 ligase (New England Biolabs, Beverly, MA, USA) overnight at 4°C. Two microliters of the ligation reaction were chemically transformed into DH10B competent *E. coli* cells (Invitrogen, Carlsbad, CA, USA). Positive clones were selected with *lacZ* blue-white color selection and verified by *Hin*dIII digestion, Southern blot, and hybridization with *BCBF3*.

Subclone and BAC sequencing

Subclones were sequenced at the University of California DNA Sequencing Facility in the Department of Biological Sciences (Davis, CA, USA). Sequencing was done with ABI BigDye Terminator Version 3.1 Cycle Sequencing chemistry on an ABI 3730 Capillary Electrophoresis Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Initial sequencing used pBluescript cloning site primers M13(−21) and M13-R. Based on sequencing results, primers were developed using the WEB program Primer3 (Rozen and Skaletsky 2000) and sequencing was repeated until entire inserts were sequenced.

BAC clones 21C6 and 60J11 were sequenced at Purdue University in the laboratory of Philip SanMiguel as previously described (Dubcovsky et al. 2001). Base calling and quality assessment were done using PHRED (Ewing and Green 1998), assembled by PHRAP, and edited with CONSED (Gordon et al. 1998). Gaps were filled by primer walking. Difficult regions were sequenced using proprietary methods by MTR Scientific (Ijamsville, MD, USA).

Restriction maps of 21C6 and 60J11 were constructed to experimentally validate the computer sequence assembly. This experimental confirmation is important in the *Triticeae* species because of the abundance of similar retroelements within the same BAC. BACs were individually digested with 8 bp specificity restriction enzymes *Asc*I, *Not*I, *Pac*I, *Pme*I, and *Swa*I. All possible single and double digestions were analyzed. Restriction fragments were separated by pulse field electrophoresis in 0.8% (w/v) agarose gels (14°C, 16 h, 6 V/s, pulse 5–15 s).

Comparison of CBF proteins from different species

CBF genes within subclones were annotated using NCBI BLASTN and TBLASTX (Altschul et al. 1997). BACs were annotated using a combination of BLASTN and TBLASTX through NCBI and the TREP (Triticeae Repeats) database. *T. monococcum* CBF proteins more than 77% identical to previously published barley CBF proteins were assigned the same identification number as Skinner et al. (2005), whereas those with no clear barley homologues were assigned consecutive numbers to those used in the barley study.

For the cluster analyses, the *T. monococcum* CBF proteins were compared with previously published CBF proteins from barley, wheat and rice (Skinner et al. 2005). Preliminary studies including all sequences revealed some pairs of closely related proteins. To simplify the analysis only one member of these clusters was retained in the analysis used for Fig. 2. In the final analysis we included barley proteins *Hv*CBF1, 2A, 3, 4A, 5, 6, 7, 9, 10A, 11, 12, 13, and 14, but excluded closely related proteins *Hv*CBF2B, 4B, 4D, and 10B, and pseudogenes *Hv*CBF8A, B, and C (Skinner et al. 2005). We also included eight rice proteins designated *Os*DREB1A to H (Skinner et al. 2005) and excluded OsDREB1I and OsDREB1J, which were closely related to OsDREB1A and OsDREB1D (Skinner et al. 2005). Finally two *T. aestivum* (*Ta*CBF6 and *Ta*CBF11) and one *T. monococcum* accessions (*Tm*CBF7) that had no close homologues among our *T. monococcum* sequences were also included in the comparison. *T. aestivum* proteins *TaCBF*2, 5, 9, and 14 (Skinner et al. 2005) were 83–99% similar to the *T. monococcum* CBF proteins with the same numbers and were excluded in the final cluster analysis. GenBank accession numbers for the *T. monococcum* CBF genes can be found in Table 2, whereas all others are available in Skinner et al. (2005). Previously published wheat (AF376136) and rye (AF370730) CBF protein sequences (Jaglo et al. 2001) were very similar to *TmCBF14* and *TmCBF9* sequences from this study, respectively, and were excluded from the final cluster analysis. Protein alignments were made using CLUSTALW (Thompson et al. 1994). Cluster analysis of the CBF proteins was conducted using MEGA Version 2.1 (Kumar et al. 2001) using the Unweighted Pair Group Method using Arithmetic Averages (UPGMA) and the pairwise deletion option. A bootstrap consensus tree was made using 1,000 replications.

Mapping

Markers for each of the seven BACs including *CBF* genes were mapped in the 74 F_2 plants from the cross DV92 (spring, frost susceptible) × G3116 (winter, frost tolerant) previously used to construct the *T. monococcum* RFLP map (Dubcovsky et al. 1996), and in the SSD lines derived from that population that were previously used to map the *Fr-A*^{m}*2* locus (Vágújfalvi et al. 2003). Primers for the CAPS markers used to map the seven BAC clones including *CBF* genes are listed in Table 1. Two markers were developed for 21C6 in order to orient the BAC within the genetic map. A larger population of

Table 1 Primers for the CAPS markers used to map the seven BAC clones including different *CBF* genes

BAC	Forward (5′ → 3′)	Reverse (5′ → 3′)	CAPS enzyme
21C6a	GATGGCTGGATGGGTCTCTA	CAGAAATAGGTGCCGTTGGT	*Msl*I
21C6b	GATGCACGGTTTCCTCTGAT	TTCAACGGTTGCATCCAATA	*Mbo*II
60J11	AATCCAAGCTGAGCCTAGCA	TACCGTCAGGAGAACCCAAC	*Hph*I
289H4	GGCGGCTCAGGAAGTCAC	ACGCTTAAAAGCGCAAACAC	*Eco*RV
511C10	GACTGCTGCCTTCTCTTTGC	TGATGCTGGAGGTTCAAGTG	*Mnl*I
284I15	GCCACGCATATTGCCTTATT	AAGAAGTGGTCAGGCCAGTG	*Ava*II
584E14	CGATGCAAAGTGTGCAATTC	GGCTTGTGATCGAGGTTTGT	*Hpy*99I
119P22	CAGCCAGCACTTACACCAAA	AAAATGCACCCCAAAACAAG	*Sau*96I

300 F_2 plants from the same cross was screened with RFLP probes ESI14 and WG530, flanking the *Fr-A*^{m}*2* locus (Vágújfalvi et al. 2003). Plants with recombination events between these two markers were further characterized with the CAPS markers for the different *CBF* genes previously mapped on chromosome 5A^m.

Results

Selection of *CBF*-containing BAC clones

Screening of the *T. monococcum* BAC library with barley *BCBF1* (BF631103) and *BCBF3* (AF298231) probes resulted in twenty positive clones. These 20 clones were organized into seven contigs based on their *Hin*dIII fingerprints and on the hybridization of the Southern blots of these fingerprints with the *CBF* probes. The representative BAC clones from each contig used for subcloning and sequencing are indicated in Fig. 1. Hybridization intensities of specific bands varied between the hybridizations with *BCBF1* and *BCBF3* probes, notably a more intense hybridization signal was observed when the 119P22 BAC fingerprint was hybridized with the *BCBF1* probe (data not shown). However, no band was unique to one probe. Based on the fingerprinting results, seventeen fragments that hybridized with the CBF probes were selected for sequencing.

Bacterial artificial chromosome sequencing

Triticum monococcum BAC clones 21C6 and 60J11 showed four and six *Hin*dIII fragments hybridizing with the CBF probes, respectively, (Fig. 1), and were completely sequenced. Annotation of the BAC sequences revealed three *CBF* genes and one *CBF* pseudogene in *Tm*BAC 21C6 (AY951944, 190 kb, 72% annotated as repetitive elements) and four *CBF* genes and two *CBF* pseudogenes in *Tm*BAC 60J11 (AY951945, 117.5 kb, 53% annotated as repetitive elements). The *Tm*BAC 21C6 pseudogene is an incomplete *CBF* gene with a degenerated nuclear localization sequence (NLS) and an AP2 domain truncated by a frame shift mutation. The first *Tm*BAC 60J11 pseudogene shows similarity to the last 360 bp of other *CBF* genes after the AP2 domain, but the predicted protein is interrupted by several stop codons. The second *CBF* pseudogene from *Tm*BAC 60J11 shows similarity to the end of the AP2 domain specific to *CBF*, but again is disrupted by several stop codons and has many frame-shift mutations.

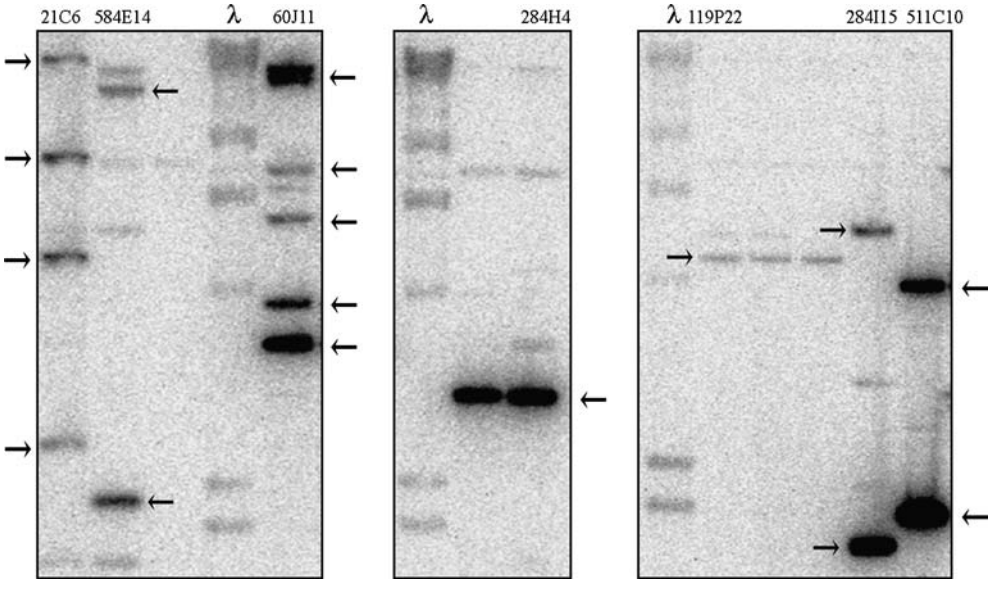

Fig. 1 Southern blot of *Hin*dIII fingerprint of seven contigs of *T. monococcum* BAC clones with *BCBF3* probe. Marker is Lambda digested with *Hin*dIII. *Arrows* indicate fragments containing putative *CBF* genes. The largest fragment in *Tm*BAC 60J11 represents two bands. *Unlabeled lanes* represent overlapping BAC clones to those selected in this study

In addition to the *CBF* genes and pseudogenes, *Tm*BAC 60J11 contains three additional genes. The translated protein from the first gene, located proximal to *Tm*CBF17, showed 81% similarity (72% identity) to rice protein BAD46702.1, annotated as a putative single-strand DNA endonuclease-1e. This gene includes an XPG domain that is known in humans to be involved in nucleotide excision repair. The translated protein from the second gene, adjacent to the previous one, showed 78% similarity (67% identity) to rice protein BAD44792.1, which has an SPX domain involved in vacuolar polyphosphate accumulation and an AraJ Arabinose efflux permease domain generally involved in carbohydrate transport and metabolism (Wang et al. 2004). The third gene, located between *Tm*CBF2 and *Tm*CBF4 is a short protein, 95% similar to a putative wheat powder tolerance protein (AAP94873.1) but with no clear orthologues in the complete rice genome.

The rice genes BAD46702.1 and BAD44792.1 are located within the same fosmid clone OSJNOa273B05 (AP006859) on rice chromosome 9 and are adjacent to three *CBF* genes *DREB1H* (OSJNOa273B05.9, BAD46703), *DREB1A* (OSJNOa273B05.11, AF300970) and *DREB1B* incorrectly annotated as a pseudogene (OSJNOa273B05.10). The *XPG*, *SPX* and *CBF* genes are located in the same order in wheat and rice indicating that these two regions are orthologous. This agrees with the general colinearity of this region of wheat chromosome 5 with a large segment of rice chromosome 9 (Linkiewicz et al. 2004).

CBF sequences and comparisons

Bacterial artificial chromosomes 511C10 and 284I15 have two *Hin*dIII fragments that showed a strong hybridization with the *BCBF3* probe. The two bands from BAC 511C10 corresponded to two *CBF* genes whereas the two bands from BAC 284I15 corresponded to a single *CBF* gene with an internal *Hin*dIII restriction site. BAC clones 289H4, 584E14, and 119P22 each contain one *CBF* gene. Thus, together with the seven genes from the shotgun sequenced BAC clones, a total of 13 *CBF* genes were found in *T. monococcum*.

Triticum monococcum CBF genes were named according to the most similar barley *CBF* gene (identity $>77\%$). Consecutive numbers were assigned to the other *T. monococcum* CBF proteins (*Tm*CBF15 to *Tm*CBF18) following the last numbers assigned to *HvCBF*14 by Skinner et al. (2005). These *TmCBF* genes were originally given different names based on their location in different BAC clones (Miller 2005 and Vágújfalvi et al. 2005). However, to facilitate future comparative studies we adjusted the wheat CBF nomenclature to match the most similar barley *Hv*CBF proteins (Skinner et al. 2005). The correspondence between the old *TmCBF* nomenclature and the one used in this study is presented in Table 2.

All the *Tm*CBF proteins sequenced in this study contain a putative Nuclear Localization Sequence (NLS) followed by an AP2 DNA binding domain flanked by the CBF characteristic K(K/R)PAG-RxKFxETRHP and DSA(W/A)(R/L) conserved amino acid sequences (Jaglo et al. 2001). Conservation in regions outside of the NLS and AP2 domain is low, even among genes located within the same BAC clone. Some areas of the C-terminal domain are conserved, notably several clusters of hydrophobic residues and the characteristic LWSY motif found at the end of most CBF proteins (Dubouzet et al. 2003; Wang et al. 2005).

The cluster analysis of 13 barley, eight rice, and 16 wheat CBF proteins presented in Fig. 2 suggests the existence of three major groups (accession numbers in Table 2 and in Skinner et al. 2005). The cluster indicated by an "A", which corresponds to the *Hv*CBF3-subgroup (Skinner et al. 2005), includes *Os*DREB1A and H, five pairs of related wheat and barley CBF proteins (CBF3, 10, 12, and 13) and four *T. monococcum* proteins (*Tm*CBF15, 16, 17, and 18) that do not have current orthologues in barley. The "B" cluster, corresponding to the *Hv*CBF4-subgroup (Skinner et al. 2005), includes only one rice CBF protein (*Os*DREB1B) and four pairs of related wheat and barley CBF proteins (CBF2, 4, 9, and 14). The "C" cluster was supported by lower bootstrap values (90%) than the previous two groups. This cluster corresponds to the *Hv*CBF1-subgroup (Skinner et al. 2005) and includes the rice CBF proteins (*Os*DREB1C, E, and G), two pairs of related wheat-barley CBF proteins (5 and 11) and the *Hv*CBF1 protein, for which no close homologues have yet been identified in wheat.

Groups A and B also can be differentiated by the sequence of the region directly after the AP2 domain. Group A has the conserved sequence A(W/E)LL(A/S)VPX(A/S) whereas Group B has the conserved sequence AWRMXP(V/L)(L/H)A. Group C containing *Tm*CBF5 and *Ta*CBF11, does not have a conserved motif among all of its members in this region (data not shown). These two groups appeared as separated clusters in Neighbor Joining trees generated from the same multiple sequence alignment (data not shown) and in the Minimum Evolution tree published by Skinner et al. (2005)

Mapping of *CBF* genes

CAPS markers for each of the seven BAC clones were designed and mapped in the DV92 (spring) × G3116 (winter) *T. monococcum* F_2 mapping population (74 F_2 plants), and in the SSD population derived from the 74 F_2 lines that was used to map the *Fr-A*^{m}*2* locus. *TmCBF5* was mapped to chromosome 7A^{m} between loci *Xmwg530* and *XksuD91* (Dubcovsky et al. 1996), and *TmCBF18* was mapped to chromosome 6A^{m} between loci *Xgwm617* and *Xabg652* (Dubcovsky et al. 1996),

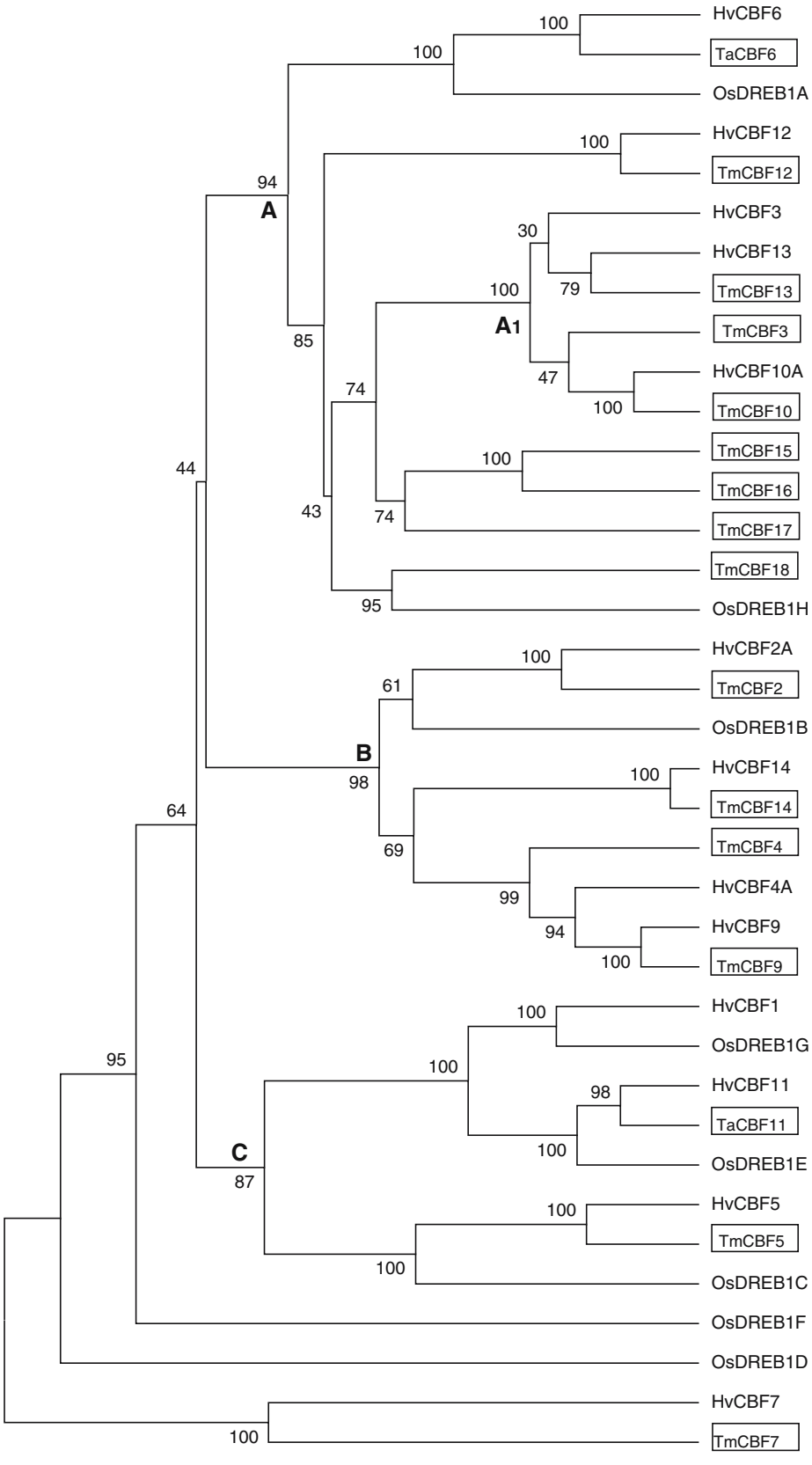

Fig. 2 Bootstrap consensus UPGMA tree of CBF proteins from wheat, barley, rice, and *Arabidopsis*. Values on top of the branches are the result of 1,000 bootstrap replications. *Letters A*, *B*, and *C* indicate the most divergent groups of *Tm*CBF proteins. Subgroup A1 includes closely related CBF proteins within group A

thus these genes are not linked to the frost tolerance locus *Fr-A^{m}2*. The remaining *CBF* genes were all mapped at the *Fr-A^{m}2* locus on chromosome 5A^m (Fig. 3).

To define the relative order of the five BACs mapped at the *Fr-A^{m}2* locus, we first screened an additional 300 F_2 plants with RFLP probes ESI14 and WG530, which

Table 2 Nomenclature of *T. monococcum CBF* genes and GenBank accession numbers

T. monococcum BAC	Original *TmCBF* nomenclature	New *TmCBF* nomenclature	GenBank accession No.
21C6	1A	15	AY951944
	1B	12	AY951944
	1C	16	AY951944
60J11	2A	17	AY951945
	2B	9	AY951945
	2C	4	AY951945
	2D	2	AY951945
289H4	3	18	AY951946
511C10	4A	3	AY951949
	4B	10	AY951950
284I15	5	13	AY951951
584E14	6	5	AY951947
119P22	7	14	AY951948

The original nomenclature used by Miller (2005) and Vágújfalvi et al. (2005) reflected the BAC location of the different *TmCBF* genes. The new proposed nomenclature uses the numbering of the closest barley homologue according to Skinner et al. (2005)

flank the *Fr-A^{m}2* locus (Vágújfalvi et al. 2003). The 72 plants with recombination events within this region plus those identified previously in the original F_2 and SSD populations were analyzed with the CAPS markers for the BACs previously mapped on chromosome 5A^m. Recombination was found between all BACs except between 119P22 and 21C6 that were completely linked in this mapping population. A second PCR marker was developed near *TmCBF16* that was linked to BAC 511C10, thus a recombination event was detected within the 21C6 BAC. This allowed us to determine the order of the BAC clones and to orient BAC 21C6 within the genetic map. BAC 60J11 was oriented to maximize the proximity of *TmCBF*2, 4, 9, and 14 that were related based on their protein sequences (Fig. 2).

The lines showing recombination events within this region were self-pollinated and the F_3 seed was screened with the PCR markers to select homozygous recombinant plants. Seeds from these lines will be increased in the future to test their frost tolerance and their *COR14b* transcription levels at 15°C.

Discussion

A cluster of *CBF* genes was mapped to the frost tolerance locus *Fr-A^{m}2* suggesting that the differences in frost tolerance and in the regulation of *COR14b* between the parental lines might be due to differences in one or more of these *CBF* genes. The detailed characterization of the *TmCBF* family presented here provides an insight into the complexity of the *CBF* gene family in wheat.

Organization of *CBF* genes in *T. monococcum*

The 13 *TmCBF* genes found in *T. monococcum* are not randomly distributed. Eleven of these genes are clustered at the *Fr-A^{m}2* frost tolerance locus. The presence of clusters of *CBF* genes has been also observed in other species. The three *CBF* genes in *Arabidopsis* are organized as a tandem array on chromosome four, suggesting that local duplication events resulted in this *CBF* cluster (Shinwari et al. 1998; Gilmour et al. 1998). Likewise, in rice, three *CBF* genes are located within the same fosmid (AP006859). In barley, at least *HvCBF3* and *HvCBF4* were mapped at the *Fr-H2* locus (Choi et al. 2002; Francia et al. 2004). *HvCBF8* was mapped approximately 20 cM distal to the *Fr-H2* locus by Francia et al. (2004), but was later identified as a pseudogene by Skinner et al. (2005).

Based on the cluster analyses, we have identified three subgroups of closely related CBF proteins (Fig. 2) that correspond with similar subgroups previously identified in barley (Skinner et al. 2005). Subgroup A (equivalent to the *Hv*CBF3 subgroup) includes more than half of the wheat CBF proteins. Within subgroup A, proteins *Tm*CBF3, 10, and 13 form a tight group supported by high bootstrap values (100) with high levels of sequence identity (70–79%) (Fig. 2). These three genes are in tandem at the distal end of the *CBF* cluster (Fig. 3). Since *Tm*CBF3 is more closely related to *Tm*CBF13 (Fig. 2), we have oriented BAC 511C10 to parallel this relationship. *Tm*CBF18, another member of subgroup A was mapped on chromosome 6A^m, suggesting a relatively old duplication from a CBF member from Group A. Group B, including proteins *Tm*CBF2, 4, 9, and 14 is clearly separated from the other CBF proteins, and its members are physically clustered together. We have oriented BAC 60J11 to reflect this relationship. The *TmCBF5* gene, from the C subgroup, was mapped on chromosome 7A^m. The chromosome location of the other wheat gene from this group (*TaCBF11*) is currently not known.

The presence of close rice and *T. monococcum* orthologues within each of the three major CBF protein groups suggests that the duplication that originated these major CBF groups occurred before the divergence between wheat and rice, more than 50 million years ago. This hypothesis was also supported by the mapping of several wheat *CBF* genes in regions orthologous with rice. Rice fosmid clone AP006859 is located in a region of rice chromosome 9 colinear with the *Fr-A^{m}2* region in wheat chromosome 5 that also contains *CBF* genes *OsDREB1A* and *OsDREB1H* (Fig. 2), both belonging to group A. In addition, this fosmid contains *OsDREB1B* (annotated erroneously as a pseudogene) the only rice member from group B. Therefore, members of the A and B groups were likely adjacent before the wheat and rice divergence. Rice fosmid clone AP006859 also includes genes *XPG* and *SPX* in the same order as is found on *Tm*BAC 60J11 confirming that this region in rice is orthologous to *Fr-A^{m}2* in diploid wheat. Unfortunately, this region of the rice genome has a gap adjacent to AP006859 and we cannot currently tell if other rice *CBF* genes are located in this region. Genes from group C also map in colinear regions between rice chromosome 6

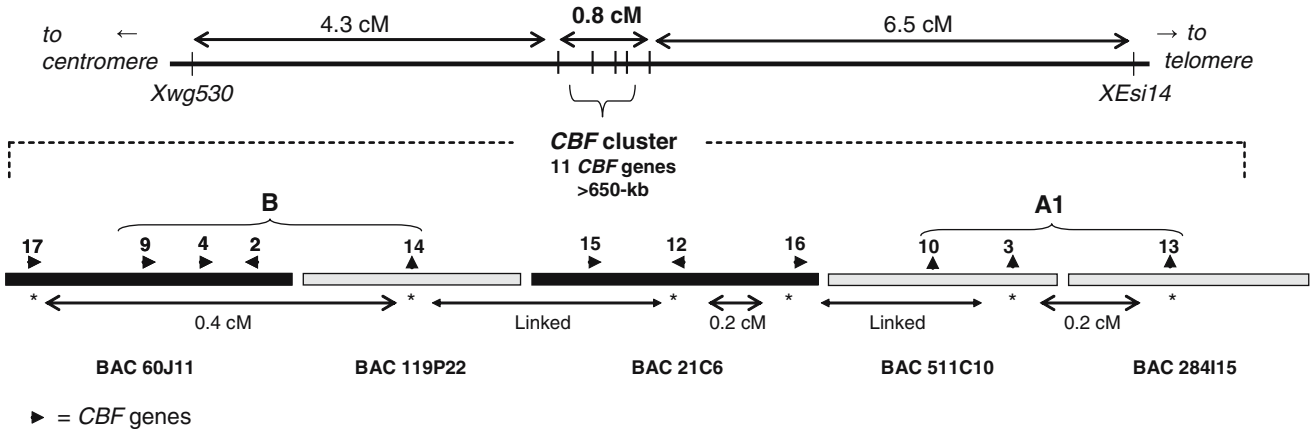

Fig. 3 Hypothesized organization of *CBF* genes at the *Fr-A*^{m}*2* locus. *Black rectangles* indicate BAC clones that were completely sequenced. *Asterisks* indicate location of CAPS markers. *Upward facing triangles* indicate the orientation of *CBF* genes in unknown. The locations of *CBF* genes within each BAC are also unknown except in the sequenced BAC clones. *Tm*BAC 21C6 is ordered based on markers at *TmCBF12* and *TmCBF16*. Capital A1 and B letters refer to groups of related sequences based on Fig. 2. *Tm*BAC60J11 was oriented to maximize the proximity of the *CBF* genes from group B

(*OsDREB1C*) and wheat chromosome 7A^{m} (*TmCBF5*) (Hossain et al. 2004).

Despite growing data about *CBF* genes in many plant species, it is still not possible to determine from sequence comparison alone which genes are responsible for frost tolerance. In fact, it has been shown that some *CBF* genes with high sequence similarity to cold-responsive *CBF* genes do not respond to cold stress, and some do not respond to any stresses at all. For example, *TmCBF5* is most similar to *HvCBF5* and *OsDREB1C* (Dubouzet et al. 2003) as shown in Fig. 2. *OsDREB1C* is not cold-responsive, rather is constitutively expressed (Dubouzet et al. 2003). This is in contrast to the expression data for *HvCBF1* from the same Subgroup C, which is strongly up-regulated due to cold and unresponsive to other stresses (Xue 2003).

The CBF1, 2, and 3 transcriptional activators in Arabidopsis have redundant functional activities (Gilmour et al. 2004). However, the sequence divergence among the three Arabidopsis *CBF* genes is minimal compared with the *CBF* sequence divergence in the temperate cereals. Therefore, it is also possible that the wheat and barely *CBF* genes may show more diverse functional activities. The recombinant lines developed in this study will be a valuable tool to explore these differences.

Regulation of *CBF* genes

Some progress has been made toward the characterization of *CBF* genes and the *CBF* pathway in *Arabidopsis*. The *AtCBF* genes transcriptionally activate *COR* and other cold-responsive genes by binding to the CRT/DRE present in their promoters (Stockinger et al. 1997; Liu et al. 1998; Gilmour et al. 1998; Shinwari et al. 1998). *AtCBF3* is partially regulated by another transcription factor, Inducer of *CBF* Expression 1, (*ICE1*), which binds to MYC recognition sites in *AtCBF* promoters (Chinnusamy et al. 2003). *ICE1* also has a minimal effect on the expression of *AtCBF1* and *2*. It is thought that *ICE1* binds more specifically to *AtCBF3* due to the presence of five MYC recognition sequences in the promoter, as compared with one MYC site in *AtCBF1* and *2* (Shinwari et al. 1998). Analysis of the available promoter sequences indicates that the *TmCBF* genes also contain MYC recognition sequences in their promoters, and some appear to have more than others (e.g. *TmCBF10* has nine MYC sites in the 800 bp upstream region, while *TmCBF17* has only one MYC site in the same region). However, without complete upstream gene sequence for all the *TmCBF* genes, it would be premature to speculate as to the preference of the ICE1 protein to any of the *TmCBF* genes. Other factors have also been shown to affect *CBF* expression including ZAT12 (Vogel et al. 2005) and VRN (Danyluk et al. 2003), so there is the possibility that a varying and complex mode of *CBF* activation exists.

We used the sequence information from the *T. monococcum CBF* genes to design specific primers for eight *T. aestivum CBF* genes (TaCBF3, 9, 10, 13, 14, 15, 16, and 17) located at the *Fr-2* locus (Vágújfalvi et al. 2005). Real-time RT-PCR experiments showed that all these genes except *TaCBF9* were induced by cold. Transcript levels of *TaCBF14* and *TaCBF15* were the highest whereas transcript levels of *TaCBF17* were the lowest during exposure to cold. Interestingly, transcript levels of TaCBF14, 15, and 16 were more than fourfold higher in lines carrying the *Fr-A2* allele from a frost tolerant variety than in those carrying the allele from a frost susceptible line. These results suggest that the amount of some *CBF* mRNAs might be a critical factor for determining the level of frost tolerance in wheat (Vágújfalvi et al. 2005). Future studies using the re-

combinant lines created in this study will help to further examine the action of the different *TmCBF* genes located at the *Fr-A^{m}2* locus.

Other factors affecting frost tolerance in wheat

Initial studies on the control of *COR14b* expression detected the presence of two loci, Regulator of *COR* genes 1 and 2 (*Rcg*1 and *Rcg*2) on chromosome 5A of hexaploid wheat (Vágújfalvi et al. 2000). *Rcg*1 was linked to RFLP locus *Xpsr911*, 35 cM proximal to the vernalization gene *VRN-A1* (Yan et al. 2003). *Fr-A^{m}2* was also mapped linked to *Xspr911*, and thus *Rcg*1 was re-designated *Fr-A^{m}2* (Vágújfalvi et al. 2003). *Rcg*2 was mapped closely linked to the *VRN-A1* and *Fr-A1* (Galiba et al. 1995) loci, but no *CBF* genes were found in this region, suggesting that *Rcg*2 regulates *COR14b* gene expression in these hexaploid recombinant lines by a different mechanism than *Fr-A2*. We currently do not know if the differential regulation of *COR14b* at the *Rcg*2 locus is the result of *VRN-A1*, *Fr-A1*, or a different gene located in this region.

The vernalization requirement observed in the winter varieties prevents the transition from vegetative to reproductive apices during the winter, protecting the sensitive floral meristem from the harmful effects of cold (Fowler et al. 2001). Lines carrying alleles for winter growth habit in barley and diploid wheat mapping populations segregating for the *VRN-1* and *VRN-2* vernalization genes, respectively, showed increased tolerance to freezing temperatures relative to the lines carrying the alleles for spring growth habit (Vágújfalvi et al. 2003; Francia et al. 2004). In the *T. monococcum* study, allelic variation at both the *Fr-A^{m}2* and the *VRN-2* loci was associated with frost tolerance, but only the *Fr-A^{m}2* locus was associated with the differential *COR14b* transcript accumulation at 15°C (Vágújfalvi et al. 2003). Similarly, in the barley study, QTLs for frost tolerance were detected at the *VRN-H1* and the *Fr-H2* loci, but only the QTL for *Fr-H2* overlapped a QTL for differential accumulation of COR14b protein in leaf samples collected from the field at the beginning of the winter (Francia et al. 2004). These results suggest that the mechanisms conferring frost tolerance at the *Fr-2* and the *VRN* gene regions might involve different regulatory mechanisms, namely differential activation of the cold machinery regulated by the *Fr-2* locus and protection of the floral meristem regulated by the *VRN* loci.

However, later during the vernalization process there seems to be a link between these two mechanisms. When winter hexaploid lines were grown at constant vernalization temperatures (4°C), a significant decrease in the cold induced wheat genes *Wcs19* and *Wcs120* was observed by the time the vernalization requirement was satisfied and the *VRN-1* gene started to be transcribed (35–42 days). In the isogenic spring lines where the *VRN-1* gene was constitutively expressed, *Wcs19* and *Wcs120* showed lower and more uniform transcription profiles (Danyluk et al. 2003). Kobayashi et al. (2005) also reported that wheat NILs with *Vrn-1* alleles have a lower *WCOR15* expression and that the *Vrn-1* NILs have lower up-regulation of *WCBF2* (an orthologue of *TmCBF2*). However, these authors only tested *WCBF2* and their results may differ for other *CBF* genes. Currently it is not possible to determine if the effects described above are the result of variation at the *VRN-A1* locus or at the closely linked *Fr-A1* locus.

Galiba et al. (1995) reported the separation of the *Fr-A1* and the *VRN-A1* loci based on one recombinant plant showing winter growth habit and frost susceptibility. Using RFLP data they mapped *Fr-A1* 2 cM distal to *VRN-A1*. In a later study, however, the same authors concluded that *Fr-A1* is proximal to *VRN-A1* based on the analysis of deletion lines (Sutka et al. 1999). Although the critical deletion lines did show a significant difference (13%) in frost survival, it is not possible to rule out the possibility that the larger deletions present in the susceptible lines also included important genes that reduced the general adaptability of the plants and made them more susceptible to frost. Therefore, additional studies will be necessary to confirm the existence of *Fr-1* as a separate locus from *VRN-1*.

Concluding remarks

The determination of the sequence and chromosome location of the majority of the members of the *CBF* gene family in diploid wheat provides the foundation to study the contribution of the individual *CBF* genes to the observed differential frost tolerance phenotypes. Based on these sequences it is now possible to design *CBF* specific primers to determine the differential expression of each of these under different environmental conditions and study the allelic variation in these transcription profiles in frost tolerant and frost susceptible lines (Vágújfalvi et al. 2005). These gene specific primers can be used to characterize the allelic variation at each of these genes to study their association to varying levels of cold stress in different regions of the world.

The recombinant lines we have isolated in this study will be useful tools to determine which of the *CBF* genes present at the *Fr-A^{m}2* locus is responsible for the differences in frost tolerance observed in diploid wheat (Vágújfalvi et al. 2003). A detailed characterization of the frost tolerance response of these recombinant lines will be necessary to identify the critical *CBF* genes within the *Fr-A^{m}2* locus. These recombinant lines are also segregating for vernalization requirement, so they will also be a useful tool to characterize the relationship between frost tolerance and vernalization.

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