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ORIGINAL PAPER

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The expression of several *Cbf* genes at the *Fr-A2* locus is linked to frost resistance in wheat

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Abstract The *C-repeat binding factor* (*Cbf*) gene family has been shown to have a critical role in the regulation of low-temperature stress response in Arabidopsis. In *Triticum monococcum*, a locus carrying a family of *Cbf*-like genes, orthologs of Arabidopsis *Cbf* genes, is tightly linked to the frost tolerance locus *Fr-A*^{m} *2*, representing candidates for the differences in frost tolerance mapped at this locus. In this work we show that several *Cbf* genes have dramatically different levels of induction after cold exposure in hexaploid wheat. The *Cbf*-transcription levels differ between substitution and single chromosome recombinant lines carrying different 5A chromosomes or chromosome segments of the chromosome 5A from frost-tolerant and frost-sensitive wheat varieties. When the expression of eight *Cbf* genes, previously mapped at the *Fr-A2* locus was investigated with gene specific primers using real-time RT-PCR, three *Cbf* sequences (*Cbf1A*, *Cbf1C*, *Cbf7*) showed a significantly higher relative transcription level (more than fourfold change) in lines differing for the *Fr-A2* region. Differences in *Cbf* expression were also associated with a variation in frost tolerance. These results suggest that the amount of some *Cbf* mRNAs might be a critical factor for determining the level of frost tolerance in wheat.

Keywords Wheat · Frost tolerance · *Cbf* · Transcription factor

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Introduction

The C-repeat Binding Factors (*Cbf*) are the most extensively studied among the stress-related transcription factors because of their critical role in the regulation of low-temperature stress response in Arabidopsis and other plant species (reviewed by Thomashow et al. 2001). *Cbf* transcriptional activators, namely *Cbf1*, *Cbf2* and *Cbf3* (*DREB1a*, *DREB1b*, *DREB1c*), bind to CRT/DRE elements found in the regulatory regions of many cold inducible genes and induce their transcription activating the plant response to low temperature.

The Arabidopsis *Cbf* genes are organized in a tandem arrangement localized on chromosome 4 and their amino acid sequences share a common AP2/EREBP-DNA binding domain (Stockinger et al. 1997; Gilmour et al. 1998; Medina et al. 1999). The expression of the *Cbf*-like transcripts is transiently upregulated by cold after 15 min of low temperature exposure (Medina et al. 1999; Gilmour et al. 1998). A sudden cold stress, transferring the plants directly from 20 to 4°C, leads to a fast accumulation of *Cbf* transcripts with a maximum after 3 h of stress. Then a drop of the mRNA steady-state level can be detected, and after 9–21 h of cold stress only a very low amount of *Cbf* mRNAs can be found. The same expression profile can be recorded after a gradual temperature decrease, suggesting that cold shock is not required to induce *Cbf* expression; rather, an absolute temperature is being sensed. The threshold temperature, promoting transcript accumulation, is approximately 14°C (Zarka et al. 2003).

The cold induction of *Cbf* genes is under the control of regulatory factors such as *ICE1* (inducer of *Cbf* expression 1; Chinnusamy et al. 2003), which promotes

Cbf transcription as well as under a negative feedback control based on the CBF proteins themselves or by the downstream gene products (Guo et al. 2002). *ICE1* is constitutively expressed and slightly upregulated by cold (Chinnusamy et al. 2003). In the absence of cold stress, Arabidopsis plants over-expressing *ICE1* do not show increased levels of any of the three *Cbf* transcripts, but at low temperatures both the *Cbf2* and *Cbf3* have higher induction levels. The *ICE1* mutation abolishes the *Cbf3* expression and slightly reduces the expression of *Cbf1* and *Cbf2* genes in the early phases of stress response (Chinnusamy et al. 2003). It has also been shown that the other regulators such as *ZAT12* have an influence on the expression of the Arabidopsis *Cbf* genes (Vogel et al. 2005).

Much less is known about the orthologs of Arabidopsis *Cbf* genes in cereals. Cold-induced transient accumulation of *Cbf*-like transcripts was reported by Jaglo et al. (2001) in wheat and rye, and by Shen et al. (2003) in wheat. The barley *Cbf3* gene was first mapped on barley chromosome 5H (Choi et al. 2002) and then, in the colinear region of chromosome $5A^m$ in einkorn wheat (*Triticum monococcum* L., $2n = 14$) linked to the frost tolerance locus *Fr-*A^m *2* (Vágújfalvi et al. 2003). This same chromosome region was then found to affect frost tolerance on chromosome 5B of common wheat (*Triticum aestivum* L., $2n = 42$, Tóth et al. 2003) and chromosome 5H of barley (Francia et al. 2004). The 5B locus was originally published as *Fr-B1* (Tóth et al. 2003) and later corrected to *Fr-B2* (McIntosh et al. 2004). The barley locus, which includes the *HvCbf3*, *HvCbf4* and *HvCBf8* genes (Francia et al. 2004) was designated as *Fr-H2* (McIntosh et al. 2004).

In barley, one *Cbf*-like gene, *HvCbf1*, was shown to activate the expression of *COR* (COld Regulated) genes through the interaction with a (G/a)(C/t)CGAC responsive element (Xue 2002). However, not all the barley *Cbf* genes show a cold-induced ability; *HvCbf2* is constitutively expressed and several cold-induced conformational changes are thought to be responsible for the activation of HvCbf2 protein during cold exposure (Xue 2003). In bread wheat, two regions on the long arm of chromosome 5A, named *Rcg1* and *Rcg2*, are involved in the regulation of the cold-induced gene *Cor14b* (Vágújfalvi et al. 2000). The *Rcg1* was shown later to correspond to the *Fr-*A^m *2* locus mapped in einkorn wheat and will be referred as *Fr-A2* hereafter.

Taken together, these results suggest that the *Cbf* genes mapped at the *Fr-A2* locus are candidates for both the differential expression of *Cor14b* at certain temperatures and the differences in frost tolerance mapped at this locus. However, it is not yet clear which of the multiple *Cbf* genes present at the *Fr-A2* locus is responsible for these differences. Recently, we showed 11 of the 13 *Cbf*-like genes identified in the *T. monococcum* BAC library (Lijavetzky et al. 1999) mapped to a 0.8-cM region at the *Fr-*A^m *2* locus (Miller et al. 2005). In this work we show that several *Cbf* genes mapped at the *Fr-A2* locus have dramatically different levels of induction after cold exposure in hexaploid wheat. We also show that those transcription levels differ between substitution and single chromosome recombinant lines carrying different 5A chromosomes or 5A chromosome segments from frost-tolerant and -sensitive wheat varieties.

Materials and methods

Genetic materials, growth conditions and frost tolerance tests

The *Cbf* gene expression was tested in two wheat cultivars (Cheyenne—CNN—frost tolerant and Chinese Spring—CS—frost sensitive), chromosome substitution lines and single chromosome recombinant lines. A frost sensitive *T. spelta* (TSP) genotype was also included in the test. The following CS/CNN chromosome substitution lines were used: 1A, 2A, 3A, 4A, 5A, 6A, 7A, 2B, 5B, 5D, 7B and 7D. Eight single chromosome recombinant substitution lines (RSL 10-2, 19-4, 31-2, 35-2, 38-6, 46-1, 72-6, 74-7) originated from the cross between CS/TSP5A and CS/CNN5A were selected on the bases of the molecular marker map published by Galiba et al. (1995).

Seeds were germinated in Petri dishes and then grown in phytotron chambers in modified Hoagland solution (Nagy and Galiba 1995) for 2 weeks at 18°C, 16 h light (260 μmol/s m^2) and 15°C 8 h dark. The relative humidity was set at 75% in all experiments. The plants were cold treated at 2°C for 2–8 h in presence of light. The control plants were harvested at 18°C prior to the cold stress.

The frost tolerance test was carried out as described previously (Vágújfalvi et al. 2003). Seeds were potted in wooden boxes in a randomized block design arrangement. Seedlings were grown in a phytotron at 15/10°C (day/night), 75% relative humidity, and 260 μmol/s m^2 light intensity. The hardening started when the temperature was reduced to 10/5°C for 2 weeks, then to 5/0°C for another 2 weeks, and to +2/−2°C for 1 week. Then the plants were gradually subjected to 24 h freezing at −10°, −11°, −12° and −13°C. After freezing, the temperature was gradually increased to 17/16°C and the leaves were cut several centimeters above the soil. Frost tolerance was estimated as the assessment of the regrowth of the plants scored on a scale running from 0 (death) to 5 (undamaged).

Northern analysis

Total RNA was extracted from 100 mg of shoot tissue using Trizol® reagent (Invitrogen). Twenty micrograms of RNAs of each samples were loaded onto 1% formaldehyde-agarose gel, blotted onto Millipore Immobilon™-Ny+ membrane and UV cross-linked according to the manufacturer instructions. Pre-hybridization and hybridization was performed with ULTRAhyb®

Ultrasensitive Hybridization Buffer (Ambion). The *Hin*dIII–*Xba*I fragment (382 bp) cleaved from the barley *Cbf3* sequence (accession no. AF298231) encompassing the AP2-*Cbf* signature conserve domains (Jaglo et al. 2001) was labeled (Feinberg and Vogelstein 1983) and used as a probe. Filters were hybridized overnight at 42°C and washed at 65°C with 2× and 1× SSC-0.1% SDS. Images were developed using Typhoon 9210 (Molecular Dynamics) phosphoimager. To ensure the correct loading of RNA, the gels were stained with ethidium-bromide and photographed when the electrophoresis was completed, in addition the filters were normalized with the RLP12 probe coding for a constitutively expressed ribosomal protein gene (Baldi et al. 2001).

Reverse transcription-polymerase chain reaction

Three micrograms of total RNA of each sample was reverse transcribed using oligo(dT) primer with Improm-II™ Reverse Transcriptase (Promega) according to the manufacturer's standard reverse transcription protocol (without Ribonuclease Inhibitor). The reaction mixture was incubated at 25°C for 5 min then at 42°C for 60 min. The enzyme was heat inactivated at 75°C for 15 min. Subsequently, the cDNAs were quantified, diluted and used for PCR or real-time PCR amplifications with *Cbf* gene specific primers.

PCR was performed with 100 ng of cDNA as template using the following conditions: 94°C for 4 min then 32 or 35 cycles (94°C for 30 s, 65°C for 30 s, 72°C for 60 s) with a final extension of 7 min at 72°C. PCR products were analyzed on 1.5% agarose gel.

Real-time PCR was performed with SYBR Green fluorescence detection in a real-time PCR thermal cycler (GeneAmp® 5700, Perkin-Elmer). PCR mix was prepared with 100 ng of cDNA, 15.75 µl of SYBR Green RT-PCR Master mix (Applera), forward and reverse primers (final concentration 0.3 µM) in a total volume of 50 µl. The cycling conditions were: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s/65°C for 90 s. Melting curve analysis was performed after PCR to evaluate the presence of non-specific PCR products and primer dimers. Normalization was carried out with the *β-actin* constitutively expressed gene.

Gene specific primer pairs (Table 1) were designed based on the sequences of 11 *T. monococcum Cbf* genes mapped on chromosome $5A^m$ linked to the *Fr-*A^m *2* locus (Miller et al. 2005). The PCR fragments amplified in bread wheat using the *T. monococcum* primers were sequenced on both strands using the "BigDye" terminator cycle sequencing kit (Applera) to confirm their identity.

The real-time PCR data were plotted as the ΔR_n fluorescence signal versus the cycle number. The PE Biosystems 5700 Detection System software calculates the ΔR_n using the equation $\Delta R_n = (R_n^+) - (R_n^-)$, where R_n^+ is the fluorescence signal of the product at any given time and R_n^- is the fluorescence signal of the baseline emission during cycles 6–13. An arbitrary threshold was set at the midpoint of the log ΔR_n versus cycle number at which the ΔR_n crosses the threshold. The efficiency *(E)* of the target amplification was evaluated for each primer pairs and the corresponding value was used to calculate the fold changes (FC) with the following formula: $FC = 2^{-\Delta\Delta C_t}(1+E)^{-\Delta\Delta C_t})$, where $\Delta\Delta C_t = (C_{t\text{target}} - C_{t\beta-\text{act}})_{\text{resistant}} - (C_{t\text{target}} - C_{t\beta-\text{act}})_{\text{susceptible}}$.

The mean concentration of the *β-actin* gene was used as a control for input RNA. The data are expressed as average of four independent biological experiments with standard deviation.

Results

Wheat chromosome 5A plays a key role in the genetic control of *Cbf* gene expression

Northern analysis was performed to test the expression level of the *Cbf* genes at low temperature in Cheyenne (CNN—frost tolerant), Chinese Spring (CS—frost sensitive) and in 12 CS/CNN chromosome substitution lines, each carrying one of the CNN chromosomes from the A genome as well as other chromosomes known to be involved in the regulation of frost tolerance, namely 2B, 5B, 5D, 7B, 7D (Sutka 1994 Veisz and Sutka 1989). To get an overall view of *Cbf* expression, Northern blots were hybridized with a DNA insert corresponding to the conserved AP2 domain plus the *Cbf* signatures (Jaglo et al. 2001).

Under control conditions (18/15°C day/night temperature) the recipient CS and the substitution lines did not expressed *Cbf* genes, while the frost resistant CNN showed a faint level of expression (Fig. 1, left panel). When plants were exposed to 2°C for 2 h all genotypes accumulated *Cbf* transcripts. A sharp difference in the *Cbf* mRNAs steady-state level was observed between the frost-tolerant variety CNN and the frost-sensitive CS (Fig. 1, right panel), suggesting that a fast and/or higher *Cbf* cold induction might be associated with the differences in frost resistance between these lines. Among all the substitution lines only one carrying the 5A chromosome of CNN showed the same expression level of CNN (Fig. 1, right panel). This result proved the central role of this chromosome in the regulation of *Cbf* genes.

The higher Cbf expression is associated with the Fr-A2 region on chromosome 5A

A further experiment was carried on to map more precisely the chromosome 5A region controlling the higher *Cbf* expression in CNN compared to CS. Eight RSLs originated from the cross between CS/CNN5A and CS/TSP5A were selected based on the presence of recombination events between the *Fr-A2* locus and the *Rcg2–Vrn1* locus, which also affects the cold-induced expression of *cor14b* (Fig. 2b and Vágújfalvi et al. 2000).

Table 1 Sequences of the primers used in the RT and real-time RT-PCR experiments

Gene	Forward primer	Reverse primer	Size	Efficiency (%)
Cbf1A	CATGTTCGAGCTGGATATGTCCGGGG	GGGAACAGCTTCGGTTTGTTCCATGC	213	100
Cbf1B	GGCGAAATGGACGCGGGCACGTAC	TGGGTCCACACCGTCCACGTACG	ND	ND
Cbf1C	GCGGCATGCCTCCAACAGCGCAG	ACGTGCCCAGGTCCATCTCCCCG	200	93
Cbf2A	ACTTCGAGTTCGACGTGTCCTGGGTG	ACTACTGACTCCAGAGCGCGGCGTC	303	88
Cbf2B	AGATCATTCTTCCAGTCGCGTGCCTG	CGCTTCCCAAATCGAGAAGAAGGCG	278	91
Cbf2C_1	GAGCCTGGCGAGAGGACAGCGAG	ATAAGCAATCTAGCCCTTTCTTC	ND	ND
Cbf2C_2	GGAGACGCAGACGCCGTTGTGGAG	TCTATTTGATTTGGATTCATGAC	ND	ND
Cbf2D	TCGCTGTGGAGCTACTGGTTGGAC	TTGGTCCATGTCCATGGAGCGAATG	ND	ND
Cbf4A	GCGGTGGACACCGATATGTTCAG	GCGAGGGGAATTATCGACTGTAC	204	87
Cbf4B	TGTTCAGTAGGCTTGACTTGTTCCCG	GCAGAATCGGCTACAAGCTCCAG	180	86
Cbf5	TGGACATCGACATGTTCAGGCTTG	CAGAGCAGAATCAGATGGGGAATC	214	91
Cbf7	CATGGAGTCGCCGGACACCAGACC	GCCCTCCCCAAAAATAGACAGCGGAG	208	84
β-Actin	ATGTGGCCATCCAGGCAGTGCTTT	TGGTCTCATGGATTCCAGCAGCTTCC	ND	95

Gene specific primer pairs were designed based on the sequences of 11 *T. monococcum Cbf* genes mapped on chromosome $5A^m$ linked to the *Fr-*A^m *2* locus (Miller et al. 2005). Primers for *Cbf2C* (no amplification), *Cbf1B* and *Cbf2D* (more than one *Cbf* mRNA amplified) were not used for analysis of *Cbf* expression. Fragment length was calculated after DNA sequencing*ND* not determined

The analysis of *Cbf* expression after 2 h at 2°C detected a different amount of *Cbf* corresponding mRNAs between the two parental genotypes (CS/CNN5A and CS/TSP5A) as well as a segregation of the trait among the recombinant lines (Fig. 2b). Five RSLs (38-6, 35-2, 72-6, 74-7, 31-2) showed a low level of *Cbf* expression similar to CS/TSP5A, while three lines (10-2, 19-4 and 46-1) showed a high level of *Cbf* expression level similar to CS/CNN5A. The genotypes of the recombinant lines based on the RFLP markers for the critical region of chromosome arm 5AL is presented in Fig. 2b. All the genotypes showing a low level of expression carried *T. spelta* (T) alleles at the *Xpsr911* and *Xpsr637* linked loci. On the other hand, all the lines with high *Cbf* transcript levels share the Cheyenne (C) allele at these loci. The *Xpsr911* locus was previously mapped linked to the *Fr-2* locus in wheat (Vágújfalvi et al. 2000, 2003) and barley (Francia et al. 2004) (Fig. 2a). This result indicates that the higher level of *Cbf* transcripts is linked to the *Fr-A2* locus responsible for the differential regulation of the *Cor14b* locus at 15°C between the frost tolerant and frost susceptible parents (Vágújfalvi et al. 2000).

No recombination events were observed between the *Rcg2, Fr-A1*, and *Vrn-A1* loci in the recombinant substitution lines used in this study, and therefore the effects of these putatively different loci cannot be separated here. In this group of lines these three loci are linked to molecular markers *Xcdo504*, *Xwg644* and *Xpsr426*.

Three Cbf sequences are differentially expressed in resistant and susceptible genotypes

Since the results described above are based on Northern hybridizations made with a probe corresponding to the conserved region of a known gene family, these experiments provide only a general overview of *Cbf* expression. The recent availability of the genomic sequence of the *Cbf* genes of *T. monococcum* (Miller et al. 2005) has provided the required information for a more detailed analysis of each member of the *Cbf* gene family.

By comparing the sequences of the 13 *Cbf* genes detected so far in *T. monococcum* (Miller et al. 2005) we designed gene specific primers for the 11 *Cbf* genes mapped on the *Fr-*A^m *2* locus on chromosome $5A^m$ (Table 1). Comparison of the *T. monococcum* sequences with the wheat EST database showed that only four *T. monococcum Cbf* sequences (*Cbf1A*, *Cbf2B*, *Cbf4A* and *Cbf7*) showed high similarity (higher than 94% at

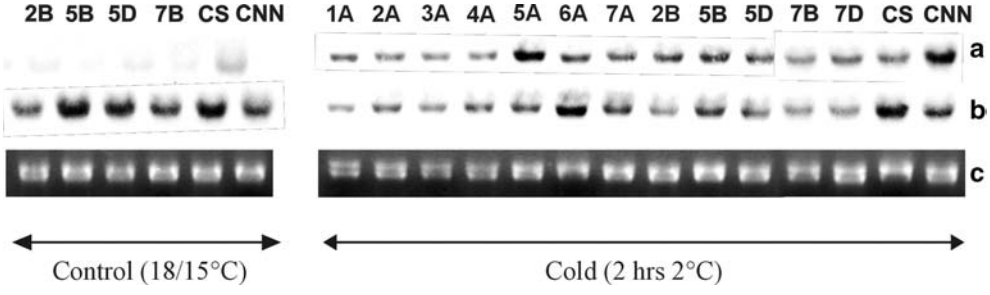

Fig. 1 Northern analysis of selected Chinese spring/Cheyenne single chromosomal substitution lines. Control samples were grown at 18/15°C, while cold-stressed plants were subjected to 2°C for 2 h. **a** The blots probed with a fragment corresponding to the conserved *Cbf* domain (AP2 and *Cbf* signatures). The equal loading of all samples was assessed probing the blots with RPL12 ribosomal probe (**b**) as well as staining the total RNA separated on the gel before blotting (**c**). *CS* Chinese Spring, *CNN* Cheyenne; all CS/CNN chromosomal substitution lines are indicated with the name of the substituted chromosome

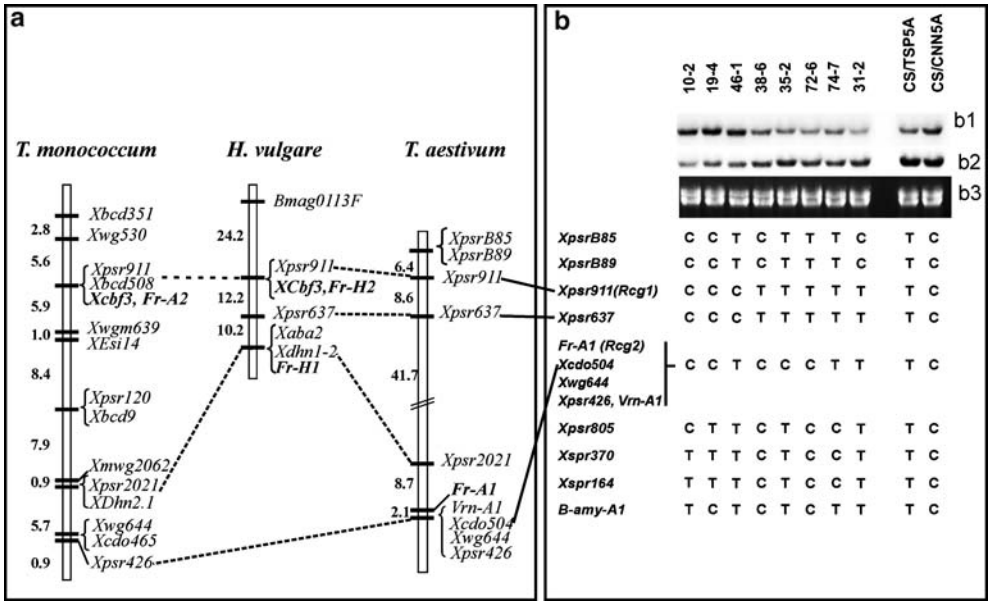

Fig. 2 **a** Chromosome map alignment among the long arm of the *T. monococcum* 5A^{m} (Vágújfalvi et al. 2003), *H. vulgare* 5H (Francia et al. 2004) and *T. aestivum* 5A (Vágújfalvi et al. 2000). The common markers are connected with *broken lines*, the *Cbf* and *Fr* loci are indicated in *bold*. Distances are given in centiMorgans. **b** Northern analysis of CS/TSP5A, CS/CNN5A and some selected single chromosome recombinant lines in comparison with their marker composition. The RFLP probes representing TSP or CNN alleles are indicated by T and C, respectively. The plants were cold treated for 2 h at 2°C and the blot was probed with a fragment corresponding to the conserved *Cbf* domain (AP2 and *Cbf* signatures, **b1**). The equal loading of all samples was assessed probing the blot with RPL12 ribosomal probe as well as staining the total RNA separated on the gel before blotting **b2, b3**, respectively)

nucleotide level) with wheat ESTs (CK211510, CK217052, CK209009 and CK211824, respectively). This level of similarity suggests that these EST sequences might be from orthologous genes. The search in the wheat database with the *T. monococcum* sequences *Cbf1B*, *Cbf2C*, *Cbf4B* and *Cbf5* yielded sequences with 85–90% identity suggesting that they correspond to paralogous loci, while *Cbf1C*, *Cbf2A* and *Cbf2D* did not identify any wheat EST sequences with significant similarity. Despite the absence of clear homologous wheat EST sequences for some of the *T. monococcum Cbf* genes, all primer pairs, except the primers for *Cbf2C*, amplified a single product of the expected size with the same efficiency from CNN, CS, CS/CNN5A and CS/TSP5A genomic DNAs, confirming that these genes were also present in hexaploid wheat. None of the two primer pairs designed based on the *T. monococcum Cbf2C* sequence amplified any products of the expected size. Sequencing analysis of the PCR products from the other *Cbf* genes showed that eight out of ten fragments amplified from bread wheat using the *T. monococcum* primer pairs correspond to single mRNA species sharing more than 98% of identity with the corresponding *T. monococcum Cbf* sequences. This level of similarity is usually find between orthologous genes in the A and A^{m} genomes. The amplicons of *Cbf1B* and *Cbf2D* genes included a mixture of different mRNAs, and therefore, were discarded from further analyses.

The eight pairs of *Cbf* gene-specific primers that amplified single PCR products from bread wheat were used to assess *Cbf* expression in CNN, CS, CS/CNN5A and CS/TSP5A. RT-PCR analyses showed that all the tested *Cbf*s were expressed in CS or CNN, but at different levels. Transcript levels of *Cbf2B* were similar in CS and CNN and were not modified by temperature, while all other *Cbf* genes showed some cold induction and/or some differences in the expression levels between CNN and CS already detectable after RT-PCR (Fig. 3).

A real-time RT-PCR analysis was then carried out to compare the expression of the eight *Cbf* genes in CS/CNN5A, CS/TSP5A and in RSL 46-1 and 38-6 previously characterized by the different levels of *Cbf* expression in Northern blots. RSL 46-1 carried the CNN allele at the *Xpsr911* and *Xpsr637* markers associated to the *Fr-A2* locus and showed a high overall level of *Cbf* transcription (Fig. 2), whereas RSL 38-6 has the *T. spelta Fr-A2* allele and exhibits low overall levels of *Cbf* transcription (Fig. 2). Figure 4a shows the amount of the different *Cbf* mRNAs in CS/CNN5A after 2 h at 2°C relative to *Cbf2B*. The *Cbf2B* was selected as a standard (transcript level = 1 in Fig. 4a) because it is highly expressed, not cold-induced, and shows similar transcript level in CNN and *T. spelta* chromosome substitution lines. The *Cbf7* and *Cbf1A* showed the highest transcript levels, while *Cbf2A* had the lowest transcript level among this group of *Cbf* genes.

Figure 4b shows the amount of the different *Cbf* mRNAs in the CS/CNN5A chromosome substitution line and in the TSP5A/CNN5A RSLs 46-1 and 38-6 relative to the transcript levels in CS/TSP5A chromo-

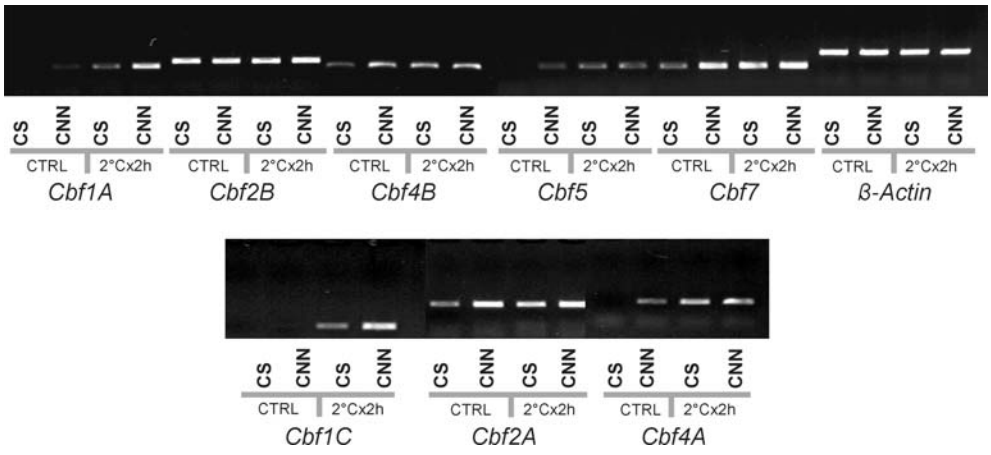

Fig. 3 RT-PCR analysis of eight *Cbf* sequences in CS and CNN. Plants were grown at 18/15°C (ctrl) and then exposed at 2°C for 2 h, total mRNA has been amplified with the gene specific primers listed in Table 1

some substitution line (transcript level = 1, last accession in Fig. 4b). After 2 h at 2°C three *Cbf* genes (*Cbf1A*, *Cbf1C* and *Cbf7*) showed significantly higher (more than fourfold changes) relative transcription levels in CS/CNN5A and RSL 46-1 than in the RSL 38-6. Based on the RSLs results, it was possible to map the differential regulation of these three *Cbf* genes to the *Fr-A2* region. Of note, *Cbf1A* and *Cbf7* are, among the differentially expressed genes between resistant and susceptible genotypes, those with the highest expression level.

A higher Cbf expression is associated with a higher frost resistance

The RSLs used in this work were previously tested for frost tolerance by Galiba et al. (1995). The survival data,

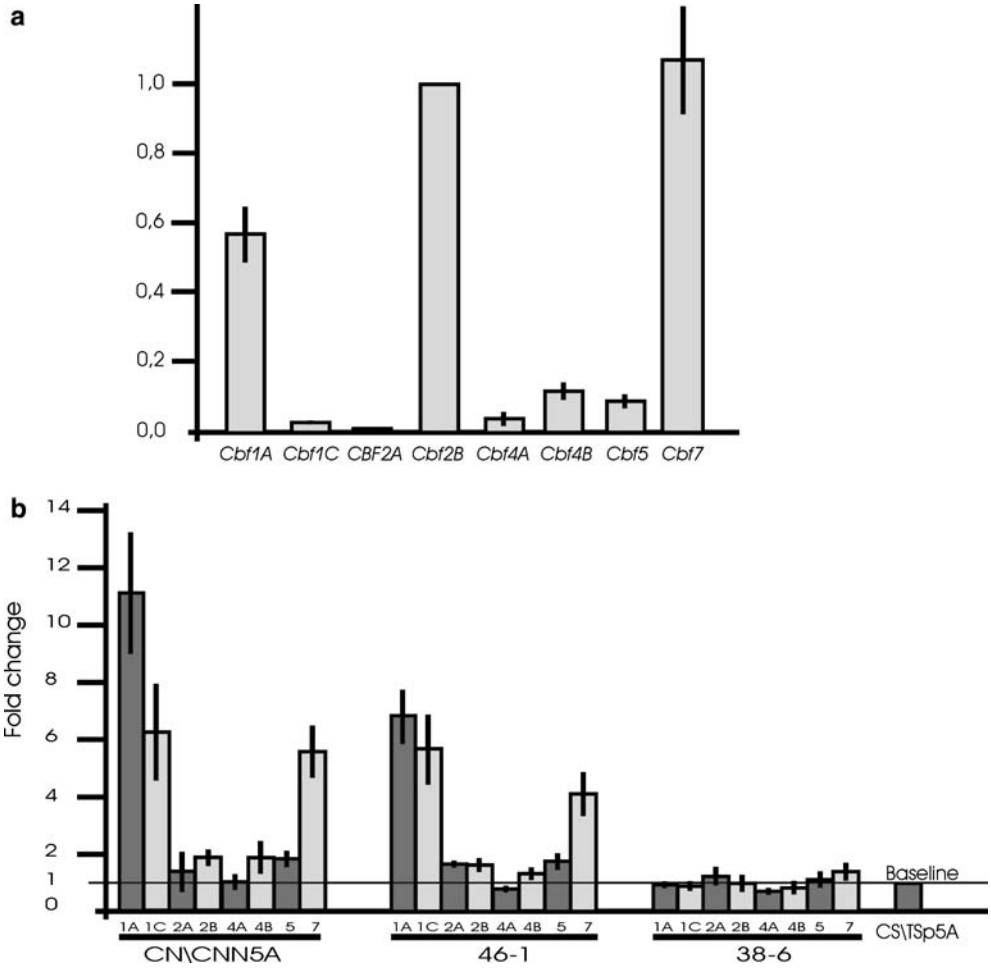

Fig. 4 Real-time RT-PCR analysis of eight *Cbf* sequences in the CS/CNN5A, CS/TSP5A and in two recombinant lines (46-1 and 38-6) characterized by different levels of *Cbf* expression. Plants were exposed at 2°C for 2 h, total mRNA has been amplified with the gene specific primers listed in Table 1. **a** relative amount of the different *Cbf* mRNAs in CS/CNN5A after 2 h at 2°C. The expression level of each gene is expressed in comparison with the expression level of *Cbf2B*, a *Cbf* sequence highly expressed and not cold induced. *Bars* represent the standard deviation. **b** variations in the expression the *Cbf* genes in resistant (CS/CNN5A and 46-1) and susceptible (CS/TSP5A and 38-6) genotypes exposed at 2°C for 2 h. The results are expressed as fold changes in the RNA steady state level of each *Cbf* in CS/CNN5A, 46-1 and 38-6 compared with CS/TSP5A (baseline sample). *Bars* represent the standard deviation.

recorded after freezing test, was interpreted by considering the presence of a single frost tolerance locus (*Fr-A1*) associated with the molecular markers *Xcdo504*, *Xwg644* and *Xpsr426*. The lines designated 10-2, 19-4, 35-2 and 72-6 were classified as frost tolerant, while RSLs 74-7, 31-2 and 46-1 were classified as frost sensitive. The recent finding in *T. monococcum* (Vágújfalvi et al. 2003) and in barley (Francia et al. 2004) of a QTL for frost tolerance associated with the *Cbf* locus and with the marker *Xpsr911* prompted us to repeat the frost assay of the key genotypes differing for the *Xpsr911-Rcg1* region. The RSL 46-1, characterized by high *Cbf* expression and by *T. spelta* alleles at all markers associated with *Fr-A1* locus (*Xcdo504*, *Xwg644* and *Xpsr426*) was subjected to freezing test together with the CS/CNN5A and CS/TSP5A parental lines using four freezing temperatures (from −10 to −13°C). At −10°C, the scores for frost tolerance of RSL 46-1 was significantly higher than the value of CS/TSP5A (1.30 and 0.30 respectively, $LSD_{(0.05)} = 0.7$, details in Table 2) demonstrating that RSL 46-1 carries some factor(s) affecting positively the ability to withstand frost. Freezing at lower temperatures (−12°C and −13°C) resulted in increased damage, the RSL 46-1 behaved as the sensitive parental line due to the absence of *Fr-A1*, the major determinant of frost tolerance (Table 2). The new freezing data suggest that the difference in *Cbf* expression is linked with the difference in frost tolerance, suggesting the presence of an additional QTL in wheat chromosome arm 5AL corresponding to the *Fr-2* found in *T. monococcum* (Vágújfalvi et al. 2003) and barley (Francia et al. 2004).

Discussion

Studies in Arabidopsis indicate that the *Cbf* family of transcriptional activators is one of the key components of the cold acclimation process (Shinozaki et al. 2003). Constitutive expression of the *Cbf* genes results in the constitutive expression of the *Cbf* regulon and increased freezing tolerance without a low-temperature stimulus (Fowler and Thomashow 2002; Gilmour et al. 2004). Arabidopsis metabolome is also extensively reconfigured in response to low temperature. In this species 325 metabolites showed increased levels in response to low temperature, out of these 256 were also increased in non-acclimated plants in response to overexpression of *Cbf3* (Cook et al. 2004).

In cereals some molecular and genetic data suggest a role for *Cbf* genes in determining low-temperature tolerance. *Cbf* homologous sequences are present in barley and wheat genomes and expressed in response to cold treatment (Choi et al. 2002; Xue 2003). In addition, a barley *Cbf*-like sequence activates the expression of *COR* genes through the interaction with a GCCGAC motif (Xue 2002). Finally, clusters of *Cbf* genes were mapped at the center of the QTLs for frost tolerance and *Cor14b* regulation in wheat and barley suggesting that they are strong candidate genes for the *Fr-A2* and *Fr-H2* frost tolerance loci (Vágújfalvi et al. 2003; Francia et al. 2004). However, the presence of a multigene family with 11 *Cbf* members clustered together in the same region (Miller et al. 2005), makes difficult to pinpoint which member of the *Cbf* locus is the best candidate to explain the phenotypic variation in frost tolerance.

In this work we have demonstrated that a higher *Cbf* transcript level is associated with the frost tolerant allele at the *Fr-A2* locus. We further analyzed the transcript levels of eight *T. aestivum* orthologous members of the *Cbf* family located at the *Fr-A2* locus and showed that the transcript levels of three of them (*Cbf1A*, *Cbf1C* and *Cbf7*) were significantly higher in the resistant than in the susceptible genotypes. Since the expression analysis was carried out using single chromosome recombinant lines, the variation observed in *Cbf* expression can be ascribed only to allelic differences at the 5A chromosome. *Cbf* homeologous loci present on 5B and 5D were also active as confirmed by RT-PCR experiments with 5A nulli-tetrasomic lines (data not shown), nevertheless 5B and 5D chromosomes were not responsible for the different mRNA expression level detected between CNN and CS observed here (Fig. 1).

The well-known role of *Cbf*s in cold acclimation together with the genetic association between *Cbf* expression level and frost tolerance loci, suggests that the amount of the *Cbf* mRNAs and proteins more than a specific *Cbf* sequence might be the critical factor in determining the level of frost tolerance. The high level of homology among the CBF protein sequences, and the evidence in Arabidopsis, that the overexpression of three different *Cbf*s lead to very similar phenotypes (Gilmour et al. 2000, 2004), suggest that *Cbf* genes have redundant functional activities. Examples are known where different levels of gene expression were found associated to significant phenotypic variations. In barley, for example, a functional association approach was employed to identify genes involved in the variation of the complex trait "malting quality". Gene expression was monitored during grain germination in a set of barley genotypes that had been characterized for quality-associated trait components, and seven genes were identified whose expression was significantly correlated to malting quality. When the candidate genes were mapped, five of them displayed

Table 2 Frost scores of CS/CNN5A, CS/TSP5A and of the RSL 46-1 at different temperatures

	−10°C	−11°C	−12°C	−13°C
CS/CNN5A	2.0	2.4	2.0	1.0
46-1	1.3	0.8	0.4	0.2
CS/TSP5A	0.3	0.2	0.0	0.0
$LSD_{(0.05)}$	0.7	0.6	0.6	0.5

Frost tolerance was estimated as the assessment of the re-growth of the plants scored on a scale ranging from 0 (death) to 5 (undamaged)

linkage to known QTLs for malting quality traits (Potokina et al. 2004).

On the bases of their sequences, the *Cbf* genes in the temperate cereals are significantly more diverse than the *Arabidopsis Cbf* genes (Miller et al. 2005). The three *Arabidopsis Cbf* genes are more related to the *T. monococcum Cbf6* gene, a sequence located on the chromosome $7A^m$, than to the 11 *Cbf* genes located at the *Fr-*A^m *2* locus (Miller et al. 2005). Therefore, specific studies of the functions and redundancy of the *Cbf* genes present in the temperate cereals will be necessary to clarify the roles of the wider variety of *Cbf* genes. The comparative analyses of *Cbf* expression in a number of wheat, barley and einkorn genotypes characterized by different level of frost resistance might provide a new insight on the role of *Cbf* genes in the regulation of frost resistance. If CBF proteins have redundant functional activities, frost resistance might be found associated to the over-expression of different members of the *Cbf* gene family. On the contrary, if frost resistance is strictly associated to the over-expression of *Cbf1A*, *Cbf1C* and *Cbf7*, these genes might be more important than other *Cbfs* in the achievement of frost resistance.

The mapping results presented here suggest that the differences in *Cbf* transcript levels are directly regulated at the *Fr-2* locus. The co-localization of the *Cbf* genomic sequences and of the *Fr-A2* locus (Miller et al. 2005) imply the presence of polymorphism in regulatory regions of *Cbf1A*, *Cbf1C* and *Cbf7* genes, and, or the presence of sequences involved in the regulation of some cold-induced *Cbf*s in this region. More extensive genomic sequences than the ones currently available (Miller et al. 2005) would be required to compare the promoters from different alleles.

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