Isolation and Molecular Characterization of a New CRT Binding Factor Gene from *Capsella bursa-pastoris*

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A new CRT binding factor (CBF) gene designated *Cbcbf25* was cloned from *Capsella bursa-pastoris*, a wild grass, by the rapid amplification of cDNA ends (RACE). The full-length cDNA of *Cbcbf25* was 898 bp with a 669 bp open reading frame (ORF) encoding a putative DRE/CRT (LTRE)-binding protein of 223 amino acids. The predicted CbCBF25 protein contained a potential nuclear localization signal (NLS) in its N-terminal region followed by an AP2 DNA-binding motif and a possible acidic activation domain in the C-terminal region. Bioinformatic analysis revealed that *Cbcbf25* has a high level of similarity with other CBF genes like *cbf1*, *cbf2*, and *cbf3* from *Arabidopsis thaliana*, and *Bncbf5*, *Bncbf7*, *Bncbf16*, and *Bncbf17* from *Brassica napus*. A cold acclimation assay showed that *Cbcbf25* was expressed immediately after cold triggering, but this expression was transient, suggesting that it concerns cold acclimation. Our study implies that *Cbcbf25* is an analogue of other CBF genes and may participate in cold-response, by for example, controlling the expression of cold-regulated genes or increasing the freezing tolerance of plants.

Keywords: AP2 domain, *Capsella bursa-pastoris*, *Cbcbf25*, CBF, RACE

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Introduction

Cold adversely affects the growth and productivity of plants (Thomashow, 1999), and many plants have the ability to respond and adapt to low temperatures. Previous studies have shown that cold can signal trigger a cascade of transcriptional regulation in plants, and that cold-regulated gene expression is a critical factor of chill tolerance (Gong *et al.*, 2002; Hsieh *et al.*, 2002) and in the cold acclimation of plants (Knight *et al.*, 1999; Thomashow, 1999; Tahtiharju and Palva, 2001). These cold-responsive genes encode a diverse array of proteins, such as, enzymes involved in respiration and in the metabolism of carbohydrates, lipids phenylpropanoids and antioxidants, molecular chaperones, antifreezing proteins, and others, which are presumed to impart tolerance to dehydration caused by freezing (Mohapatra *et al.*, 1989; Guy, 1990; Thomashow, 1999).

The CBF (CRT binding factor) cold-responsive pathway is an integral component of the cold acclimation response (Shinozaki and Yamaguchi-Shinozaki, 1996; Thomashow, 1999, 2001; Fowler and Thomashow, 2002). The *Arabidopsis* CBF family (*cbf1*, *cbf2*, and *cbf3*) (Gilmour *et al.*, 1992; Stockinger *et al.*, 1997; Liu *et al.*, 1998; Medina *et al.*, 1999), the winter *Brassica napus* CBF family (*Bncbf5*, *Bncbf7*, *Bncbf16*, and *Bncbf17*) (Gao *et al.*, 2002), and several other CBF genes from *Lycopersicon esculentum*, *Oryza sativa* and barley (Jagglo-Ottosen *et al.*, 2001; Choi *et al.*, 2002) have been identified and isolated, and CBF proteins have been found to share some common characteristic motifs such as NLSs (nuclear localization signals), an AP2 DNA-binding domain, and acidic C-terminal fragments. The CBF proteins were found to bind the CRT (C-repeat) elements (cis-regulator elements in the promoters of cold-responsive genes such as *Cor78* and *Cor15a*), and to activate the downstream genes (Thomashow, 1999). Further study revealed that the CRT

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element regulates gene expression in an ABA-independent pathway (Yamaguchi-Shinozaki and Shinozaki, 1994). The over expression of *cbf1* in *Arabidopsis* was found to increase the freezing tolerance of transformed plants in the absence of a prior cold treatment (Jagglo-Ottosen *et al.*, 1998; Medina *et al.*, 1999). In fact, all earlier studies have shown that CBF plays a positive role in the cold acclimation process, by controlling cold-responsive gene expression and by increasing freezing tolerance.

Capsella bursa-pastoris is a wild grass, which belongs to the Cruciferae like *Arabidopsis* and *B. napus*. It is considered to be a potential vegetable crop and can grow and set seeds normally at low temperatures, suggesting that it possesses a cold-acclimation pathway. Thus, we believed that *C. bursa-pastoris* was likely to contain CBF-like genes, and if so, the cloning of these genes could be useful for the study of the mechanism of cold tolerance in *C. bursa-pastoris*. To date no study has reported on the cloning of CBF genes from *C. bursa-pastoris*. In this paper, we describe the cloning and molecular characterization of a new CBF gene, named *Cbcbf25*, from *C. bursa-pastoris* by using RACE technology. Bioinformatic analysis and a cold acclimation assay revealed that the *C. bursa-pastoris* CBF gene (*Cbcbf25*) strongly resembles CBF genes from other species, and that this is relevant to cold acclimation.

Materials and Methods

Materials Young leaves of *C. bursa-pastoris*, collected from the Fudan University campus, Shanghai, China, were used for RNA isolation. Molecular cloning of *Cbcbf25* from *C. bursa-pastoris* was carried out using a RACE PCR Kit (GIBCO BRL, Gaithersburg, USA) and a SMARTTM RACE cDNA Amplification Kit (Clontech, Palo Alto, USA). Primers used in the study, shown in Table 1, were synthesized by the Shanghai Sangon Biotechnological Company, and DNA sequencing was performed by the Shanghai Genetech Biotechnological Company. The pGEM Teasy vector was purchased from Promega (Madison, USA). All other chemicals used were of analytical grade.

Methods

Isolation of total RNA Approximately 1.0 g of freshly harvested young leaves of *C. bursa-pastoris* were allowed to stand at 4°C for 12 h and then homogenized in liquid nitrogen. Total RNA was extracted using TRIZOL reagent according to the manufacturer's

instructions (GIBCO BRL, Gaithersburg, USA).

5' RACE of *Cbcbf25* According to the protocol of the SMARTTM RACE cDNA Amplification kit (Clontech), about 100 ng of total RNA was reverse transcribed with primer 5'-CDS primer coupled with (dC) tailing and SMART II A oligo, which annealed to the tail of the RNA and served as an extended template for PowerScript RT. The reverse primer used for the cloning of a partial coding sequence of *Cbcbf25* was designed and synthesized according to the conservative regions of the CBF gene sequences of *Arabidopsis* and *B. napus* in GenBank. First round PCR was performed with CBF5#GSP1 as the reverse primer and Universal Primer A Mix (UPM) as the forward primer. PCR was carried out in a total volume of 50 µl containing 2.5 µl cDNA, 10 pmol primer CBF5#GSP1, 5 µl UPM, 41.5 µl Master Mix (34.5 µl PCR-Grade Water, 5 µl 10× Advantage 2 PCR buffer, 10 mmol dNTP Mix, 1 µl 50× Advantage 2 Polymerase Mix) under the following condition: the template was firstly denatured at 94°C for 3 min and then subjected to 35 cycles of amplification (94°C for 1 min, 59°C for 1 min, 72°C for 1 min) followed by 10 min at 72°C. Subsequently, nested PCR was performed under normal PCR reaction conditions using CBF5#GSP2 as reverse primer and the Nested Universal Primer A (NUP, 5'-AAGCAGTGGTATCAACG CAGAGT-3') as forward primer. The nested-PCR product (CBCBF25a, about 550 bp) was purified and cloned into pGEM-T vector (Promega), and sequenced. A Blast Search revealed that the 5'RACE product CBCBF25a had high similarity with CBF genes from *Arabidopsis* and *B. napus*. Subsequently, the 3' RACE primer 3'jcCBF#GSP1 and the nested PCR primer 3'jcCBF#GSP2 were designed and synthesized according to the alignment results of CBCBF25a to clone the 3' end of *Cbcbf25*.

3' RACE of *Cbcbf25* According to the protocol of the 3' RACE System for Rapid the Amplification of cDNA ends (GIBCO BRL), an aliquot of isolated 100 ng RNA was reverse transcribed using the cDNA synthesis primer, AP (5'-GGCCACGCGTCGACTAGTAC (T)₁₇-3'), provided with the kit (GIBCO BRL). The gene-specific primer 3'jcCBF#GSP1 was used as the forward primer and Abridged Universal Amplification Primer (AUAP, 5'-GGCCACGC GTCGACTAGTAC-3') was used as the reverse primer to amplify the 3' end cDNA. PCR was performed in a total volume of 50 µl containing 2 µl cDNA, 10 pmol of each of the primers 3'jcCBF#GSP1 and AUAP, 10 mmol dNTPs, 1× cDNA reaction buffer and 8U *Taq* polymerase using 35 amplification cycles (94°C for 1 min, 60°C for 1 min, and 72°C for 1 min). Nested PCR was carried out in a total volume of 50 µl containing 1 µl of the product of the first round 3' RACE-PCR using the same PCR conditions. The nested-PCR product (CBCBF25b, about 400 bp) was purified, cloned into pGEM-T vector (Promega), and sequenced.

Generation of the full-length cDNA of *Cbcbf25* By comparing and aligning the sequences of the 5'RACE product CBCBF25a and the 3' RACE product CBCBF25b, the full-length cDNA sequence of *Cbcbf25* was deduced and subsequently amplified using the primer pair, FCBCBF and RCBCBF, using a One Step PCR Kit (Takara, Shiga, Japan). RT-PCR was carried out in a total volume of 50 µl containing 100 ng RNA, 10 pmol of each of FCBCBF and RCBCBF, 10 mmol dNTPs, 1× One Step RNA PCR buffer, 5 U

Table 1. Primers used for the cloning of *Cbcbf25*

Primers	Primer sequence (5' → 3')
CBF# 5'GSP1	5'-AGTATTCCATTGTACGGACGGAGG-3'
CBF# 5'GSP2	5'-ATCGCTCTGTTCGGCGTCAC-3'
3'jcCBF#GSP1	5'-AGATGGCTGCTCGTGCTCACGAC-3'
3'jcCBF#GSP2	5'-ACGACTACGGCTGCGACTACGAC-3'
FCBCBF	5'-ACGCGGGAACCTTATCCAAACAG-3'
RCBCBF	5'-TTTACATTCGTTTCTCACACTAGCCAC-3'

RNase Inhibitor, 10U AMV Reverse Transcriptase XL and 10 U AMV-Optimized *Taq* under the following conditions: 50°C for 30 min and 94°C for 2 min followed by 30 cycles of amplification (94°C for 30 sec, 60°C for 30 sec and 72°C for 5 min). The RT-PCR product (CBCBF25c, about 850 bp) was purified and cloned into pGEM-T vector and then sequenced.

Sequence analysis The full-length *Cbcbf25* and associated molecular information were analyzed using Vector NTI Suite 6.0, BioEdit, and Clustal W.

Secondary and three-dimensional structure analyses The secondary structure of CbCBF25 was analyzed by SOMPA (<http://npsa-pbil.ibcp.fr/cgi-bin/npsaautomat.pl?page=npsasopma.html>). Three-dimensional structure analysis and molecular modeling were performed using TOPITS (<http://www.embl-heidelberg.de/predictprotein/predictprotein.html>) (data submitted to database SWISS-MODEL, SDSC1, Loopp, Superfamily, DAS, JPRED, PHD, PROF, PSIPred, PSSP, SAM-T99 and SSpro separately), Frsvr (<http://www.mbi.ucla.edu/people/frsvr.html>) and 123D+ (<http://123d.ncicrf.gov/>).

Cold acclimation assay To determine whether the cloned *Cbcbf25* was cold-responsive, total RNA was extracted from young leaves of *C. bursa-pastoris* plants subjected to various treatments, and then subjected to semi-quantitative RT-PCR for *Cbcbf25* expression. These treatments included first growing the plant at

28°C for 4 d, subsequently treating at 4°C for 0.5, 1, 1.5, 2 or 4 h, and then at 28°C for 2 d or 4 d, respectively. RT-PCRs using a One Step PCR Kit (Takara, Japan) were performed at 50°C for 30 min followed by 30 cycles of amplification (94°C for 1 min, 62°C for 1 min and 72°C for 1 min), using gene-specific primers FCBCBF and RCBCBF. Two primers were also used to amplify ubiquitin gene (AY189972) from 1 nt to 250 nt as a positive control for semi-quantitative RT-PCR.

Results and Discussion

Isolation and characterization of the full-length cDNA of *Cbcbf25* Using the RT-PCR method and primers mentioned above, the full-length cDNA of *Cbcbf25* (Genbank accession No. AY491498) was obtained; this was subsequently confirmed by sequencing. The full-length cDNA was 898 bp long; a size similar to CBF genes from *A. thaliana* (*Cbf1*, *Cbf2*, *Cbf3*) and *B. napus* (*Bncbf5*, *Bncbf7*, *Bncbf16* and *Bncbf17*), and contained a 669 bp open reading frame encoding a putative DRE/CRT (LTRE)-binding protein of 223 amino acids with a calculated isoelectric point (pI) of 4.84 and a calculated molecular weight of ca. 25 kDa (Fig. 1).

Transmembrane helix prediction (TMHMMv2.0, <http://www.cbs.dtu.dk/services/TMHMM-2.0/>) showed that there were no transmembrane helices in the deduced CbCBF25

1	ACGCGG GAACCT TATCCA AACAGA GCAGAC TGAGTT AGTCCA AAGTCC ACCACA																			
1	M	N	S	S	S	F	S	A	F	S	E	M	F	G	S	D	Y	E	S	P
61	ATGAAC TCATCA TCATTC TCTGCT TTCTCT GAAATG TTTGGC TCCGAT TACGAG TCTCCG																			
21	G	S	S	G	G	G	D	Y	C	P	T	L	A	T	S	C	P	K	K	P
121	GGTTCT TCAGGC GGCGGA GATTAT TGTCCT ACGCTT GCCACG AGCTGT CCCAAG AAACCC																			
41	A	G	R	K	K	F	R	E	T	R	H	P	I	Y	R	G	V	R	Q	R
181	GCGGGT CGGAAG AAGTTT CGCGAG ACTCGT CACCCG ATTTAC AGAGGA GTTCGT CAAAGG																			
61	N	S	G	K	W	V	S	E	L	R	E	P	N	K	K	T	R	I	W	L
241	AACTCA GGTAAG TGGGTC TCTGAG CTAAGA GAGCCA AACAAA AAAACT AGGATT TGGCTC																			
81	G	T	F	Q	T	A	E	M	A	A	R	A	H	D	V	A	A	I	T	L
301	GGCACT TTCCAA ACCGCC GAGATG GCTGCT CGTGCT CACGAC GTCGCC GCCATT ACCCTC																			
101	R	G	R	S	A	C	L	N	F	A	D	S	A	W	R	L	R	I	P	E
361	CGTGGC AGGTGA GCCTGT CTCAAT TTCGCC GACTCG GCTTGC CGGCTA AGGATC CCGGAG																			
121	S	T	C	A	K	E	I	Q	K	A	A	A	E	A	A	L	A	F	Q	D
421	TCAACA TGCGCC AAGGAG ATCCAG AAGGCG GCGGCT GAAGCT GCCTTG GCTTTT CAAGAT																			
141	E	M	G	D	T	T	T	A	A	T	T	T	T	D	H	G	L	D	K	E
481	GAGATG GGTGAT ACGACT ACGGCT GCGACT ACGACG ACTGAT CATGGC CTTGAC AAGGAG																			
161	E	T	M	V	E	A	T	V	T	P	E	Q	S	D	S	L	Y	I	D	E
541	GAGACG ATGGTG GAAGCT ACTGTG ACGCCG GAACAG AGCGAT TCGTTA TATATA GACGAG																			
181	E	A	M	F	G	M	P	S	F	L	D	S	M	A	E	G	M	L	L	P
601	GAGGCA ATGTTC GGGATG CCGAGT TTTTTC GATAGT ATGGCC GAAGGG ATGCTT TTGCCT																			
201	P	P	S	V	Q	W	N	T	N	Y	D	V	D	G	D	D	D	L	S	L
661	CCTCCG TCCGTA CAATGG AATACT AACTAT GACGTC GACGGC GATGAT GACTTG TCGCTA																			
221	W	S	Y																	
721	TGGAGT TATTAA TATTTG ATTATT ATTATT ATATTG TTTTAT TTTTTC CATTTT TGGTAT																			
781	TATAGT TTTTTA TATGTT AGATCC TTTTTA TAGAAA CGGATC TTCTTA ATTTTT GTGGCT																			
841	AGTGTG AGAAAC GAATGT AAATGA CTCAGT AAAGGA GGTTGT CAAAAA AAAAAA AAAAAA																			
901	AAAA																			

Fig. 1. The nucleotide acid sequence of *Cbcbf25* full-length cDNA and its deduced amino acid sequence.

protein, which is consistent to the hydrophobicity analysis result obtained using BioEdit software. According to the result obtained from the PROSITE database (<http://www.ExPASy.org/prosite/>) and InterProScan (<http://www.ebi.ac.uk/interpro/scan.html>), CbCBF25 shows high similarity to all other CBF proteins by possessing a bipartite nuclear localization sequence (NLS) domain from R₄₃ to R₆₀ and an AP2 domain from I/V₅₃ to S₁₁₃, both of which are suspected to function as

DNA-binding domains (Ohme-Takagi and Shinshi, 1995). In order to explore the function of the AP2 domain in CBF proteins, we carried out further bioinformatic analysis of the AP2 domain using PROSITE, InterProScan, NCBI (<http://www.ncbi.nlm.nih.gov/blast>), WU-Blast2 (<http://www.dove.embl-heidelberg.de/Blast/>) and FASTA (<http://www2.ebi.ac.uk/fasta3/>). The results obtained showed that the AP2 domain contained some potential ATP/GTP-binding sites and two

Fig. 2. Multi-alignment of CbCBF25 with other known CBF proteins from *A. thaliana* (CBF1, CBF2, CBF3) and *B. napus* (BnCBF5, BnCBF7, BnCBF16, BnCBF17). Identical amino acid residues in the aligned sequences are indicated in yellow. Nuclear localization signals (NLS), the ALA-RICH domain, and the AP2 domain are also indicated. The conserved AP2 domain elements YRG and RAYD are marked by a dotted line and a broken line, and the amino acid residues in the RAYD conserved element predicted to form an amphipathic α -helix and promote DNA binding are boxed. The potential ATP/GTP-binding sites are underlined by an unbroken line. Underlined amino acids indicate potential recognition sites for protein kinase C (■) and casein kinase II (●).

strikingly conserved elements YRG and RAYD. The YRG element, a sequence of 22 amino acids, is highly basic and contained the conserved YRG amino acid motif. It was noticeable that the RAYD element, a sequence of 36 amino acids, contained a highly conserved core region consisting of 21 amino acids, which was predicted to form an amphipathic α -helix that might play an important role in recognizing the cis-regulator sequence and in promoting DNA binding (Okamuro *et al.*, 1997) (Fig. 2). An ALA-Rich domain (Alanine existed comparatively mostly) from amino acid A₈₆ to K₁₂₉ was also identified (Fig. 2). According to previous studies, the ALA-Rich domain is presumed to maintain the helical structure, which is of importance in DNA binding (Bird *et al.*, 2002). Furthermore, based on homology analysis using NCBI, WU-Blast2 and FASTA, we found that CbCBF25 contains an acidic C-terminal fragments that might act as a transcriptional activation domain and participate in protein-protein interactions, which is an essential aspect of transcriptional regulation (Hahn, 1993). Some potential recognition sites for protein kinase C and casein kinase II (Kennelly and Krebs, 1991) were also noticed, particularly a kinase C recognition site, Ser-62, located in the AP2 DNA-binding domain (Fig. 2). Most of these sites were evolutionarily conserved among the six CBF proteins and might function in transcriptional activation via phosphorylation and dephosphorylation mechanisms (Gao *et al.*, 2002).

Through above analyses, it can be concluded that CbCBF25 shares characteristic motifs, i.e., NLS, the AP2 domain, and acidic C-terminal fragments, with other CBF proteins; moreover, these motifs are suspected to play a critical role in DNA binding and in the activation of cold-responsive genes (Choi *et al.*, 2002). This strongly implies that CbCBF25 protein may function as a trans-acting factor in cold-responsiveness in *C. bursa-pastoris*, by controlling the expressions of cold-regulated genes and by increasing the plants freezing tolerance.

Homology analysis According to the alignments of *Cbf1*, *Cbf2*, *Cbf3*, *Bncbf5*, *Bncbf7*, *Bncbf16* and *Bncbf17* by Clustal W/X, the regulatory sequences of CBF genes diverge more than the coding regions, but still retain a high level of similarity, which might explain the identical expression patterns shown by these genes (Medina *et al.*, 1999; Gao *et al.*, 2002). Amino acid sequence comparisons between CbCBF25 and other CBF proteins were performed using Clustal W/X and NCBI Blast. The results showed that CbCBF25 shares high homology with the CBF proteins of *Arabidopsis* and *B. napus* (Fig. 2), with identities of 83.0, 83.0, 79.6, 74.7, 73.7, 71.1, and 62.5% to CBF1, CBF2, CBF3, BnCBF5, BnCBF7, BnCBF16, and BnCBF17, respectively (using bio-software Vector NTI suite 7.0). CbCBF25 was more like CBF1 and CBF2 than the other CBF proteins. The phylogenetic tree analysis of the six CBF proteins was consistent with the evolutionary relationship between these species (Fig. 3) (Fowler and Thomashow,

2002), suggesting that CbCBF25 and other CBF family members possess similar functions. Homologous analysis also demonstrated that the CBF gene family might have been conserved during evolution, again suggesting that they have the same functions, probably associated with survival in a harsh cold environment. The amino acid sequences of the AP2 domains of CbCBF25 showed high identity with its counterparts in other species, indicating that the AP2 domain is extremely well conserved in CBF proteins between different species (Fig. 2).

All our homology analyses showed that *Cbcbf25* strikingly resembles all of the other known CBF genes in *Arabidopsis* and *B. napus*, and that the AP2 domain and NLS have been evolutionarily conserved, in *Arabidopsis*, *B. napus* and *C. bursa-pastoris*, which implies that they have a critical role in controlling gene expression in *C. bursa-pastoris* (Jagglo-Ottosen *et al.*, 2001).

Secondary and three-dimensional structural analyses of CbCBF25 protein Secondary structure analysis revealed that CbCBF25 consisted of 42 α -helices, 19 β -turns jointed by 41 extended strands and 161 random coils, like other CBF proteins such as BnCBF5, BnCBF7, and BnCBF16 (Gao *et al.*, 2002). Homology modeling analysis was carried out by SWISS-MODEL, SDSC1, Frsvr and 123D+ separately, while threading modeling was performed using Loopp and Superfamily. Structure analysis and molecular modeling revealed that CbCBF25 strongly resembles the other CBF proteins (Fig. 2). The presumed three-dimensional structure of CBF1, which was most similar to CbCBF25 in amino acid sequence, closely resembled that of CbCBF25. Although BnCBF17 was least similar to CbCBF25 in amino acid sequence and had much fewer H-bonds, helices, strands and turns, its three-dimensional structure also resembled CbCBF25 and CBF1 (Fig. 4).

All of CBFs have a common amino sequence element known as the AP2 domain, a 62-amino acid sequence encoded by the AP2 motif in the CBF genes, and which is evolutionarily conserved in plants (Stockinger *et al.*, 1997). Molecular modeling of the AP2-domain of CbCBF25 by SWISS-MODEL showed that embedded within the AP2 domain is a large helix, four strands, and six turns, a typical feature of the AP2 domain of CBF proteins (Stockinger *et al.*, 1997), and which might act as a DNA-binding domain (Fig. 4). The α -helix might fit into the major groove with several of its side chains touching DNA base pairs where it recognizes and binds to the cis-regulator sequence (Ohme-Takagi and Shinshi, 1995). The structure of the protein might form a hydrophobic surface that promotes its binding with another protein of identical or similar structure to form a dimer, which would be expected to play an important role in regulating gene expression (Gong *et al.*, 2002; Chinnusamy *et al.*, 2003). As a result of dimerization, two recognition helices, one from each subunit of the dimer, are symmetrically extended into two adjacent major grooves of the target DNA (Mushegian and

Fig. 3. Phylogenetic tree analysis of the deduced amino acid sequences of CbCBF25 and other CBF proteins from *A. thaliana* (CBF1, CBF2, CBF3) and *B. napus* (BnCBF5, BnCBF7, BnCBF16, BnCBF17).

Koonin, 1996). Other segments in the protein, through hydrophobic interaction with the α -helix, might create a stable structural domain that locks the α -helix into its DNA interface. In addition, amino acid sequences outside the DNA-binding domain might affect the DNA-binding activity of AP2 domain (Mushegian and Koonin, 1996). It was also revealed that the AP2 portion of CbCBF25 comprises almost 30% of

its protein mass. Perhaps the activation domain of AP2 domain is capable of interacting with RNA polymerase and of activating transcription through protein-protein interactions with a subunit of RNA polymerase, or interacting with other intermediary proteins (Ohme-Takagi and Shinshi, 1995).

Cold acclimation assay Semi-quantitative RT-PCR was carried out to investigate the expression of *Cbcbf25* in *C. bursa-pastoris* under cold stress. The expression pattern of *Cbcbf25* was found to be similar to those of other CBFs in *Arabidopsis* and *B. napus* (Gilmour *et al.*, 1992; Stockinger *et al.*, 1997; Liu *et al.*, 1998; Medina *et al.*, 1999; Gao *et al.*, 2002) (Fig. 5). The *Cbcbf25* transcripts increased within 30 min of exposing plants to low temperature 4°C and reached a maximal level after 2 h. The low-temperature regulation of *Cbcbf25* appeared to be temporary, since the transcript levels decreased thereafter and no expression could be detected after the plant was returned to a normal temperature (28) for 4 d. This result indicated that *Cbcbf25* is activated by cold triggering and indeed is involved in the cold acclimate process.

Fig. 4. Molecular modeling of CBF proteins using SWISS-MODEL (<http://www.expasy.org/swissmod/SWISS-MODEL.html>). (A) Molecular modeling of CBF1 (213 amino acids) from *A. thaliana*, the most similar to CbCBF25 in terms of amino acid sequence, showed that it contained 312 H-bonds, 3 helices, 16 strands and 17 turns. (B) Molecular modeling of CbCBF25 (213 amino acids) from *C. bursa-pastoris* showed that it contained 312 H-bonds, 3 helices, 15 strands and 17 turns. (C) Molecular modeling of BnCBF17 (251 amino acids) from *B. napus*, the least similar to CBCBF in terms of amino acid sequence, revealed that it contained 269 H-bonds, 2 helices, 11 strands, and 10 turns. (D) Molecular modeling of the AP2-domain.

Fig. 5. Expression of *Cbcbf25* during cold acclimation. Total RNA was extracted from young leaves of *C. bursa-pastoris* plants that had been subjected to various treatments and examined by semi-quantitative RT-PCR analysis for *Cbcbf25* expression (upper panel). The treatments include growing plant at 28°C for 4 d (Lane 1), transferring and growing the plants at 4°C over 0.5 h (Lane 2), 1 h (Lane 3), 1.5 h (Lane 4), 2 h (Lane 5), and 4 h (Lane 6), and then returning the plants to 28°C for 2 d (Lane 7) or 4 d (Lane 8). M: Molecular size marker. The ubiquitin gene (AY189972) was amplified by RT-PCR as positive control (lower panel).

Since CbCBF25 transcripts began accumulating within 30 min of exposing the plant to cold, it was supposed that a transcription factor recognizes the *Cbcbf25*s promoter and induces *Cbcbf25* expression. Recently, a study in *A. thaliana* showed that *ice1* (the inducer of CBF expression) acts as an upstream factor that positively regulates the expression of *cbf* (Chinnusamy *et al.*, 2003). It was found that *Ice1* is expressed constitutively and that the overexpression of *Ice1* in wild-type plants could enhance the expression of *cbf*. Another upstream gene *hos1* was found to encode a protein that contains a RING-finger motif, which might function in the degradation of CBF and ICE proteins (Lee *et al.*, 2001). A study of the interaction between these genes would provide us with a better understanding of the pathway through which plants respond to cold temperature and also provide us with a useful tool for enhancing or inducing cold tolerance in plants (Thomashow, 2001).

Based on the analysis above, we suggest a potential role for CbCBF25 in the cold acclimation and freezing tolerance of *C. bursa-pastoris*. The cloning of *Cbcbf25* enables us to test whether *Cbcbf25* can increase the freezing tolerance of transgenic plants by transferring it into non-cold-tolerant plants such as tobacco in the future.

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