cDNA cloning and expression analysis of a *Poncirus trifoliata CBF* gene

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Abstract

The transcription factors CBF/DREB play an important role during low temperature, drought and high-salt stress in higher plants. A new *CBF* (CRT/DRE binding factor) gene was cloned from trifoliate orange [*Poncirus trifoliata* (L.) Raf.] by RT-PCR with degenerate primers and rapid amplification of cDNA ends (RACE) techniques. The full-length cDNA of *CBF* gene from trifoliate orange (designated as *Ptcbfb*) was 847 bp containing a 732 bp open reading frame (ORF), encoding a 243 amino acid protein. The predicted protein (designated as PtCBFb) had over 60 % identity to CBFs from some other plant species. Bioinformatical analysis showed that PtCBFb contained N-terminal bipartite nuclear targeting sequence, potential C-terminal acid domain and high conserved AP2 domain. Some other loci such as phosphorylation sites of several protein kinases, N-myristoylation site, tyrosine sulfation site and amidation site were also conserved in PtCBFb. Predicted three-dimentional structure of PtCBFb was similar to CBF from *Arabidopsis thaliana*. Expression pattern analysis revealed *Ptcbfb* expression in every tested organ, and *Ptcbfb* was cold induced.

Additional key words: full-length cDNA, PtCBFb, RACE, trifoliate orange.

Introduction

Many plants have inherent abilities to respond and adapt to low temperature (Thomashow 1990). Over the past two decades, a number of cold-regulated genes, such as *cor*, *rd*, *kin*, *lea* and *bn*, have been identified and characterized in several plants including *Arabidopsis*, rape, alfalfa, barley, wheat and tomato.

The transcription factors CBF/DREB1 (CRT/ DRE binding factor) play an important role during low temperature, drought and high-salt stress in higher plants. A number of CBF/DREB1 gene families, were isolated from *A. thaliana*, rape, soybean and tomato (Thomashow 2001, Choi *et al*. 2002, Francia *et al*. 2004, Gamboa and Rasmussen-Poblete 2007).

Trifoliate orange (*Poncirus trifoliata* (L.) Raf.) is the most resistant to cold among all citrus species. To elucidate its ability of cold acclimation, the full-length cDNA of CBF-like gene was cloned and the sequence and structure of deduced protein were analyzed.

Materials and methods

Three month old *Poncirus trifoliata* (L.) Raf. seedlings were exposed to 4 °C for 2 h to induce *CBF*-like genes. Young leaves were collected and frozen in liquid nitrogen, then kept under -70 °C.

All primers (Table 1) were synthesized by *Shanghai Sangon Biological Engineering & Technology and Service*, (Shanghai, China). All DNA sequences were measured by *BGI Life Tech* (Beijing, China).

Total RNA was extracted according to method of Liu *et al*. (1998a) with some modification and stored at -70 °C. The quality of RNA was identified by UV/Vis spectrometer (*UV2450*, *Shimadzu*, Japan) and agarose gel electrophoresis. About 0.1 μg of total RNA was reversely transcribed with M-MLV reverse transcriptase (*Toyobo*, Japan).

A pair of degenerate primers were designed for amplification of AP2 fragments of *CBF*-like genes according to conserved AP2 domain of CBFs from *A. thaliana*, *Capsicum annuum* and some other plants (Table 1). PCR reaction was as follows: 94 °C for 30 s,

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Abbreviations: CBF/DREB - CRT/ DRE binding factor; ORF - open reading frame; RACE - rapid amplification of cDNA ends; TMHs - transmembrane helix; NLS - nuclear localization signal; RT - reverse transcript.
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Table 1 Sequences of primers used in experiment (R = A/G, Y = C/T, V = A/C/G, N = A/G/C/T)

Type	Name	Sequences (5'→3')
Primers for reverse transcription	Oligo$(dT)_{18}$	TTTTTTTTTTTTTTTTTT
	3'-CDS	AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$VN
	5'-CDS	$(T)_{25}$VN
Degenerate primers for RT-PCR	DP1	CCNAARAARAGRGCNGGNAG
	DP2	TCNGCRAARTTYAARCA
5'-linker for 5'-RACE	BD-Oligo	AAGCAGTGGTATCAACGCAGAGTACGCGGG
Primers for RACE	UPL	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
	UPS	CTAATACGACTCACTATAGGGC
	NUP	AAGCAGTGGTATCAACGCAGAGT
	APB1	GTCATGTGCCCTTGCAGCCATT
	APB2	ATGCGTGTTTGCTTGTTGGGTTC
	SPB	TGAACCCAACAAGCAAACACG
Primers for ORF	SP1-1	GGGCTAACTTCAAACCAT
	AP1-1	TTCTCACAACTAAGCAATCT

53 °C for 45 s, 72 °C for 1 min. Primers DP1 and DP2 were used. Product of RT-PCR was separated using 1.5 % agarose gel electrophoresis. Bands matched the predicted fragments were extracted and purified using EZNA™ gel extraction kit (*Omega*, USA). Purified fragments were connected to PMD18-T vectors (*TaKaRa*, Japan). Then transferred into *E. coli* (DH5α). Positive ones were identified by PCR and two enzyme-cutting method (Pst I, ECOR I) and sequenced.

The 5'- and 3'-RACE was carried out according to *SMART*™ RACE (*Clontech*, USA) method with some modification. Two pairs of nested primers were used. First strand cDNA as PCR template for 5'-RACE were synthesized using powerscript reverse transcriptase (*Clontech*) with 5'-linker and 5'-CDS primer. Nested PCR was carried out using *Advantage 2* PCR kit (*Clontech*). Touch-down PCR was used as follow. Predicted fragments were extracted and purified. Subsequently, the nested PCR was performed with purified product of first PCR as template, APB2 and SPB as primers. Purified fragments were connected to PMD18-T vectors and then transferred into *E. coli* (DH5α). The recombinants were identified by two enzyme-cutting method (Pst I, ECOR), and sequenced.

First strand cDNA for 3'-RACE were synthesized through reverse transcription with 3'-CDS as primer. Primers for two PCR were APB2 and SPB respectively compared to 5'-RACE. Purified product of nested PCR was used as template for PCR with APB2 and SPB as primers to test whether purified product was positive or not. Detailed procedure was similar to 5'-RACE.

The 5'- and 3'-end with an overlap fragment of AP2 were linked together by *DNAMAN* software, formed the full-length cDNA. According to the sequence of full-length cDNA, a pair of primers located in 5'-UTR and 3'-UTR respectively were designed for RT-PCR using 5'-RACE ready cDNA as template to isolate the full ORF of CBF-like gene (Table 1). PCR reaction was as follows: 94 °C for 30 s, 58 °C for 30 s, 72 °C for 90 s. Primers SP1-1 and AP1-1 were used.

Semi-quantitative RT-PCR was used to investigate *Ptcbfb* expression profiling in different seedling organs of *P. trifoliata* with different exposing time to 4 °C. RNA was extracted separately from leaf, stem, cotyledon and root of two month old seedlings. These parts of the seedlings were treated at 4 °C for 0, 15, 30, 45, 60 or 120 min and first strand cDNA was prepared. Reverse transcription was done using a RT-PCR kit (*Toyobo*). Amplification for *Ptcbfb* was performed at 94 °C for 5 min, 94 °C for 30 s, 58 °C for 30 s, 72 °C for 90 s (35 cycles) and finally 72 °C for 10 min, using primers AP1-1 (5'-TTCTCACAACT AAGCAATCT-3'） and SP1-1 (5'- GGGCTAACTTCA AACCAT-3'), which amplified a fragment of 847 bp containing a 732 bp open reading frame (ORF). At the same time, actin was used in RT-PCR in parallel with the *Ptcbfb*, using primers Actin F (5'-ATTGTAAGCAACT GGGATGATA-3') and Actin R (5'-GCCTGGATGGCAA CGTACATGG-3'), which amplified a fragment of 191 bp, as the control in the same conditions. The amplified products were analyzed using agarose gel electrophoresis.

Analysis the full length sequence using *ProtParam* (http://us.expasy.org/tools/protparam.html). *Blastp* program in GenBank database (http://www.ncbi.nlm. nih.gov/) was used. Multi-align of amino acid sequences of PtCBFb and some other CBFs was performed using *ClustalW* (http://www.ebi.ac.uk/ clustalw/index.html) and *Jalview 2.08* (Fig. 3), phylogenetic tree was constructed. Analysis PtCBFb with via *PROSITE* database (http:// www.expasy. org/prosite/) and *InterProScan* (http:// www.ebi.ac.uk/ InterProScan/) and secondary structure modeling of PtCBFb were performed by *PredictProtein* (http://www. predictprotein.org/newwebsite/submit.html), garnier program of *EMBOSS* (http://emboss.nhri.org.tw/) and *Jnet* program of *Jalview 2.08*.

Results

Conserved AP2 domain of *CBF*-like gene from trifoliate orange was isolated by RT-PCR with degenerate primers (Fig. 1*A*). 5'- and 3'-end of *Ptcbfb* were obtained by RACE and identified through sequencing (Fig. 1*B*,*C*). Then the full ORF was cloned by RT-PCR (Fig. 1*D*).

Analysis using *ProtParam* revealed that the 847 bp full-length cDNA contained a 732 bp ORF encoding a 243 amino acid protein (Fig. 2) with a calculated isoelectric point (pl) of 5.56, instability index of 61.75 and molecular mass of about 27.21 kD. *Blastp* program in GenBank database was used to screen the amino acid sequence similarity. The result showed that PtCBFb had identity to CBFs from *Hevea brasiliensis*, *Populus tomentosa*, *Malus domestica, A. thaliana, Iris lactea var. chinensis, Capsicum annuum, Eucalyptus gunnii,* and *Lycopersicon esculentum*, with the identity being 62, 61, 60, 59, 58, 56, 55 and 52 %, respectively (Fig. 3). Multi-align of amino acid sequences of PtCBFb and some other CBFs was performed using *ClustalW* and *Jalview 2.08* (Fig. 3), and then phylogenetic tree was constructed (Fig. 4). As CBFs from other species, PtCBFb also possessed two CBF-specific signature sequences of PKKrAGRrvFkETRHP and DSvWR flanked on both termini of AP2 domain (Fig. 3). Both of them were highly

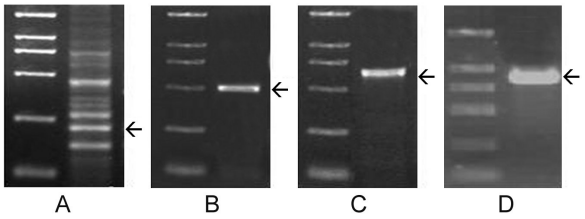

Fig. 1. *A* - Fragment of AP2, *B* - 5'-end of cDNA, *C* - 3'-end of cDNA, *D* - translated region. Left bands are DNA marker DL2,000 (*TaKaRa*) with 100, 250, 500, 750, 1000 and 2000 bp from the bottom up, *black arrows* point at objective bands.

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1    ACGCGGGCTAACTTCAAACCATGGACATATTATTCGGCCAAGTTTCTGATCCTGGTTTG
1                         M  D  I  L  F  G  Q  V  S  D  P  G  L
60   ACTGACAATAAGCCGGAGTCATCAACACAGTCAGATGCCAGCAGCACGGCGCCACCAAGG
14    T  D  N  K  P  E  S  S  T  Q  S  D  A  S  S  T  A  P  P  R
120  CGAGGTGCCCACTCAGACGAGGAAGTAGTACTGCTAGCAACGAGCAGGCCGAAGAAGCGG
34    R  G  A  H  S  D  E  E  V  V  L  L  A  T  S  R  P  K  K  R
180  GCGGGGAGGAGAGTGTTTAAGGAGACACGTCATCCGATTTTCAGAGGAGTCAGAAGGAGA
54    A  G  R  R  V  F  K  E  T  R  H  P  I  F  R  G  V  R  R  R
240  AACAATAATAAATGGGTATGTGAGCTACGTGAACCCAACAAGCAAACACGCATTTGGCTA
74    N  N  N  K  W  V  C  E  L  R  E  P  N  K  Q  T  R  I  W  L
300  GGTACCTATCCTTCTCCAGAAATGGCTGCAAGGGCACATGACGTGGCAGCGCTGGCACTG
94    G  T  Y  P  S  P  E  M  A  A  R  A  H  D  V  A  A  L  A  L
360  AGAGGAAAATCCGCTTGCCTGAATTTCGCTGACTCAGTGTGGAGGTTACCGGTGCCGGCT
114   R  G  K  S  A  C  L  N  F  A  D  S  V  W  R  L  P  V  P  A
420  TCCACGGATGCTAAGGATATAAGGAAAGCTGCAGCTGAGGCAGCAGAAGCATTTAGACCT
134   S  T  D  A  K  D  I  R  K  A  A  A  E  A  A  E  A  F  R  P
480  CGTGATGATGAACTTGAAGAAAGCAATATTCACCATGTGAAGCTTGAGGAAGTACAGAAA
154   R  D  D  E  L  E  E  S  N  I  H  H  V  K  L  E  E  V  Q  K
540  ACGATGCAAGAAAACACATTGCTGCCAGAAAATGTGTTATATATGGATGAACAGGCGGTG
174   T  M  Q  E  N  T  L  L  P  E  N  V  L  Y  M  D  E  Q  A  V
600  TTTGATATGCCGGGATTGCTTGCGGACATGGCGGAGGGCTTGCTTCTTTCACCACCGCCT
194   F  D  M  P  G  L  L  A  D  M  A  E  G  L  L  L  S  P  P  P
660  TTAATTTTTGGAGATGATGATGTTATGAAGTGGGATTATCATAATAACGGGGAAAGTGAT
214   L  I  F  G  D  D  D  V  M  K  W  D  Y  H  N  N  G  E  S  D
720  GGCTGTGTGTCGTTGTGGAGTCATTCAATTTGACAAGTAATTTCTACCGCAGTTTTTTTT
234   G  C  V  S  L  W  S  H  S  I  *
780  TTTCACTTTAATCTTTTCACAGTGTATTTACTGTGCATGTAGATTAATTTAAATTTATCT

840  AGGGCAGTTCTGCAGATAAATAAAATTCAGATTGCTTAGTTGTGAGAATCTGAGAATAAC

900  AAGGGAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 2. The nucleotide and deduced amino acids sequence of Ptcbfb. Analysis using *ProtParam* revealed that the 847 bp full-length cDNA contained a 732 bp ORF encoding a 243 amino-acid protein with a calculated isoelectric point (pl) of 5.56, instability index of 61.75 and molecular mass of about 27.21 kD.

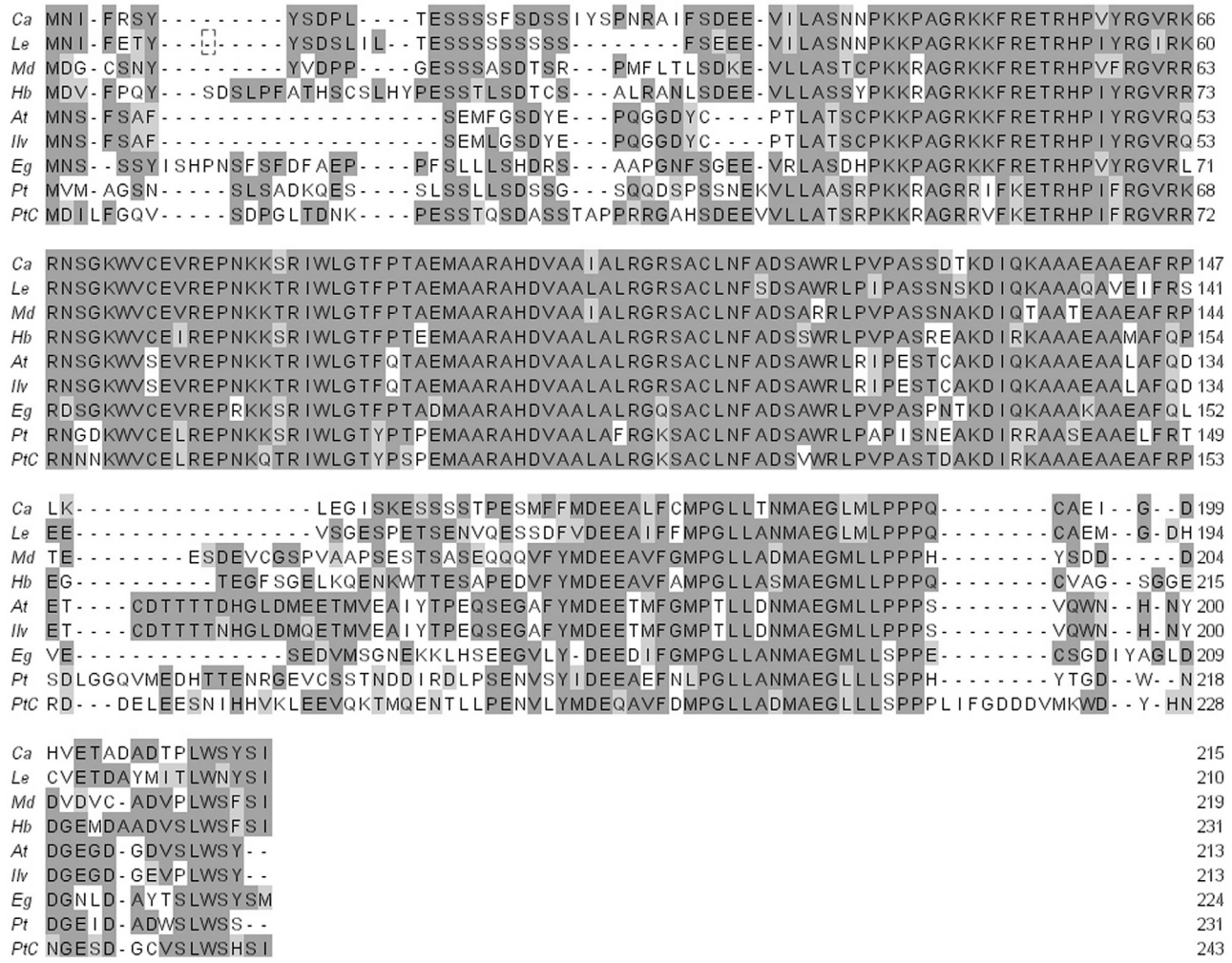

Fig. 3. Mutiple sequences alignment of CBFs. Conserved sequence are *in shadow*.

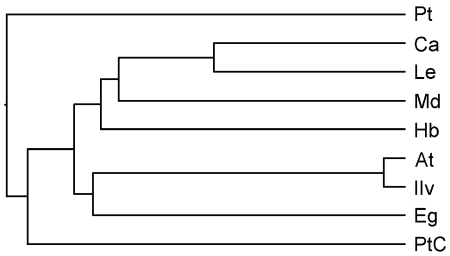

Fig. 4. Phylogenetic tree of CBFs. Multi-align of amino acid sequences of PtCBFb and some other CBFs was performed using *ClustalW* and *Jalview 2.08*.

conserved in different species, implying their important roles. PKKrAGRrvFkETRHP might be involved in transportation of proteins for its resemblance to sequence of nuclear localization signal (NLS).

To gain insight into the expression pattern of this gene, RT-PCR were done using different seedling organs subjected to different 4 °C cold treatment. Total RNA was isolated separately from leaf, stem, cotyledon and root of *P. trifoliata* seedlings. Some of the seedlings were exposed to 4 °C for 15, 30, 45, 60, and 120 min, and the others not. The result showed that, *Ptcbfb* expressed in every organ but at different levels (Fig. 5). High expression was found in cotyledon and root treated with low temperature, while low expression was found in root

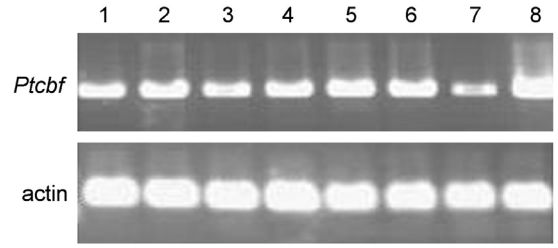

Fig. 5. *Ptcbf* expression in various organs. Total RNA was isolated separately from leaf, stem, cotyledon and root of *P. trifoliata* and subjected to semi-quantitative RT-PCR analysis. *Lanes* 1, 3, 5, 7 were leaf, stem, cotyledon and root of control and 2, 4, 6, 8 were leaf, stem, cotyledon and root of cold-treated for 2 h.

without low temperature treatment. Stem and leaf have the medium expression. Without cold induced, expression of *Ptcbfb* was found in various organs. After cold induction under 4 °C for 2 h, a little increased expression was found in leaf and stem, and distinctly increased expression was found in root, but there was no expression change in cotyledon. When treated with cold temperature for different duration, *P. trifoliata* leaves have different expression (Fig. 6). Low expression was found in seedlings with no cold treatment, while high expression was found in seedlings exposed to 4 °C for 30 min. When exposed to 4 °C for 15 or 45 min, the seedlings have the medium expression. When exposed to 4 °C for 60 min, *Ptcbfb* had the lowest expression in all cold treated seedlings than but still higher than in the seedlings without cold treatment.

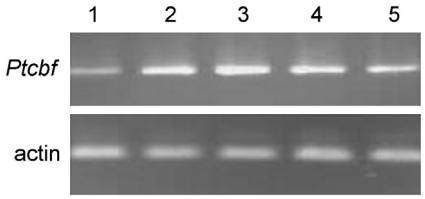

Fig. 6. *Ptcbf* expression during cold treatments. Total RNA was isolated from *P. trifoliata* leaves cold-treated for 0, 15, 30, 45 and 60 min (*lanes* 1 - 5) and subjected to semi-quantitative RT-PCR analysis.

Discussion

Analysis of predicted amino acid sequence of PtCBFb showed that it possessed a high conserved AP2 DNA binding domain, a nuclear targeting sequence and a potential C-terminal acid domain, all of which were typical motifs in many transcriptional factors. It predicted that PtCBFb was an important factor in controlling expression of target *COR* genes in trifoliate orange. Many phosphorylation sites including Protein kinase C phosphorylation site and Casein kinase II phosphorylation site in PtCBFb might function in controling its activity, thus a conclusion that PtCBFb might be the final receptor of Ca^{2+} and other second messengers induced by cold in transduction pathway. Analysis of three-dimentional structure showed that PtCBFb possessed an AP2 DNA binding domain which resembled to *CBF* from *A. thaliana*. The AP2 DNA binding domain contained one α-helix and three β-sheets participating in interaction with DNA and other transcriptional factors (Allen *et al*. 1998).

In this study, the full-length cDNA of a novel *CBF*-like gene was first cloned from trifoliate orange, which was the most cold-resistant species of citrus, declared that CBF-like factors also existed in trifoliate orange. PtCBFb and some other potential CBFs from citrus might activate the expression of many target *COR* genes, consequently enhance the cold-resistance of plants.

Cold induces the expression of most reported *CBF* genes, and *Ptcbfb* is no exception. The expression profiling indicated that *Ptcbfb* was cold induced and have somewhat constitutive transcripts. This is different from the previous results. In previous study, *CBF* was not constitutivly expressed (Jaglo *et al*. 2001, Liu *et al*. 1998a, Shinwari *et al*. 1998). When exposed to 4 °C, *Ptcbfb* have higher expression than without cold treatment, and the expression peak was found after 30 min. This is similar to results previously described in *Eucalyptus globulus* (Gamboa and Rasmussen-Poblete 2007), but different from those in *Arabidopsis* (Liu *et al*. 1998b, Shinwari *et al*. 1998). Surprisingly, Champ *et al*. (2007) isolated in citrus one *CBF* gene named *Ptcbf1*, which had a completely different expression pattern in comparison with *Ptcbfb*. The expression peak of *Ptcbf1* was found when exposed to 4 °C for 12 h. However, we cannot exclude the possibility that the expression level of *Ptcbfb* may increase again after 2 h of cold exposure as it occurred with *CBF* genes from *B. napus* and *T. aestivum* (Jaglo *et al*. 2001).

Further study on *Ptcbfb* promoter, upstream elements, target genes and corresponding cis-elements will be necessary to illuminate the whole pathway of cold signal transduction and the mechanism of citrus cold acclimation on molecular level.

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