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Isolation and expression of a cold-responsive gene *PtCBF* in *Poncirus trifoliata* and isolation of citrus *CBF* promoters

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Abstract

C-repeat/dehydration-responsive element binding factor (CBF) plays important roles in cold response network in plants. Here, one member of CBF coding gene family in trifoliate orange (*Poncirus trifoliata*), designated as *PtCBF*, was isolated. Semi-quantitative reverse transcription-polymerase chain reactions showed up-regulation of *PtCBF* not only under low temperature but also induced by abscisic acid. Additionally, the *CBF* genomic fragments in four citrus species including trifoliate orange, sweet orange (*Citrus sinensis*), pummel (*Citrus grandis*) and rough lemon (*Citrus jambhiri*) were isolated with complete open reading frames. According to the results of alignment analysis between full length cDNA and genomic DNA sequences in trifoliate orange, there were no introns in *PtCBF*. Moreover, the results of multiple sequence alignment analysis and phylogenetic analysis on putative protein sequences suggested that the AP2 DNA binding domains and CBF signature sequences were highly conserved in four citrus CBF proteins. Finally, the *CBF* promoters in above citrus species were isolated, which provides some information concerning promoter function.

Additional key words: abscisic acid, *Citrus grandis*, *Citrus jambhiri*, *Citrus sinensis*, low temperature, trifoliate orange.

Introduction

Citrus is one of the most important fruit crops, that is widely grown in tropical and subtropical regions of the world (Talon and Gmitter 2008). However, the productivity of citrus industry is usually limited by several environmental factors, such as low temperature (Zhang *et al.* 2005). In citrus species, trifoliate orange is the most cold-tolerant, it can survive at -20 °C if fully cold-acclimated and usually used as rootstocks in citrus cultivation (Champ *et al.* 2007). So, trifoliate orange is a very important germplasm source for citrus breeding to improve cold tolerance. However, cold tolerance is a quantitative trait and difficult to improve by multigene transformation. Fortunately, the findings on transcription factors could solve above problems to some extent and make the improvement much easier (Cai *et al.* 1995).

It has been proved that the C-repeat/dehydration-responsive element binding factor (CBF/DREB1) plays key roles by modulating expression of cold-related genes during cold acclimation, and *CBF/DREB1* coding genes have been identified as a small gene family from many plant species, such as *Arabidopsis* (Thomashow *et al.* 2001, Gilmour *et al.* 2004, Novillo *et al.* 2007), rape (Gao *et al.* 2002), tomato (Zhang *et al.* 2004), maize (Qin *et al.* 2004), wheat (Badawi *et al.* 2007), rice (Gutha and Reddy 2008), apple (Hellens *et al.* 2005), grape (Xiao *et al.* 2006), poplar (Benedict *at al.* 2006), *Eucalyptus* (El Kayal *et al.* 2006), *etc.*. Furthermore, over-expression of *CBF/DREB1* genes enhanced freezing tolerance successfully in *Arabidopsis* (Jaglo-Ottosen *et al.* 1998, Kasuga *et al.* 1999, Gilmour *et al.* 2000) and other species (Wang *et al.* 2008). Recently, *PtCBF1*, *CgCBF1* and *CpCBF1* were isolated from *Poncirus trifoliata*, *Citrus grandis* and *C. paradisi*, respectively, and their expression profiles during cold acclimation were reported

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Abbreviations: ABA - abscisic acid; CBF - C-repeat/dehydration-responsive element binding factor; RT-PCR - semi-quantitative reverse transcription-polymerase chain reaction.
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(Champ *et al.* 2007). Besides, another novel *CBF*-like gene named *Ptcbfb* (GenBank No. DQ790889.1) was cloned in *Poncirus trifoliata* and analyzed in expression under cold treatment (Wang *et al.* 2009).

In this study, a *CBF*-like gene, *PtCBF* (cloned independently in our lab) also designated as *PtCBF1* by Champ *et al.* (2007) was isolated from different vegetative organs and its expression was investigated under low temperature and abscisic acid treatments. Finally, four *CBF* promoters in citrus species were isolated.

Materials and methods

Young leaves of trifoliate orange [*Poncirus trifoliata* (L.) Raf.] and other related citrus species [*Citrus sinensis* (L.) Osbeck, *Citrus grandis* (L.) Osbeck and *Citrus jambhiri* Lush.] were collected from open orchard in National Indoor Conservation Center of Virus-free Germplasms of Fruit Crops, and then stored at -80 °C for future DNA extraction. Trifoliate orange seedlings for RNA analysis were grown in nutrient substrate for two months in controlled room at 16-h photoperiod, irradiance of 40 μmol m^{-2} s^{-1} and temperature of 26 °C, and then transferred to 4 °C for cold treatment of various duration (15 min, 1 h, 2 h, 8 h, 1 d, 2 d and 3 d), while untreated seedlings were used as control. For abscisic acid (ABA) treatment, the seedlings were placed in Hoagland's nutrient solution containing 100 μM ABA for the same time periods and control samples were put into nutrient solution without addition of ABA. After treatments, all samples were immediately ground to fine powder under liquid nitrogen.

Total RNA was isolated from trifoliate orange using 1 cm^3 of *Trizol* reagent added to 0.2 g ground powder, and then the samples were centrifuged for 5 min at 12 000 *g* at 4 °C. 0.2 cm^3 chloroform was added to supernatant, mixed for 5 min and incubated at room temperature for 15 min. Samples were centrifuged for 15 min at 12 000 *g* at 4 °C to separate the phases. The top layer was transferred to a new clean tube and 0.5 cm^3 isopropanol was added to precipitate the RNA. Samples were mixed gently, and incubated at room temperature for 10 min, centrifuged for 10 min at 12 000 *g* at 4 °C. Finally, pellet was washed by 75 % ethanol, air-dried and resuspended in 20 mm^3 RNase-free water. Isolated RNA was then treated with DNase I to remove genomic DNA contamination. First strand cDNA synthesis kit (*Toyobo*, Osaka, Japan) was introduced for cDNA preparation according to product manual. Final cDNAs were centrifuged shortly and stored at -20 °C for future use.

Genomic DNA was extracted with modified CTAB method (Cheng *et al.* 2003). 20 cm^3 CTAB extraction buffer preheated at 65 °C was added to ground leaf powder. The mixture was incubated at 65 °C for 1.5 h with occasional inversion. Equal volume of chloroform and isoamyl alcohol (24:1, v/v) was added, and mixed well. Samples were centrifuged at 5 000 *g*. Then the chloroform/isoamyl alcohol was added again to top aqueous phase and frozen isopropanol was used to precipitate the crude DNA. The pellet was washed with 76 % ethanol with 10 mM NH_4Ac and dissolved in 3 cm^3 TE buffer. After digestion with 20 mm^3 DNA-free RNase A, 3 cm^3 of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) was added. Samples were centrifuged at 5 000 *g* followed by addition of equal volume of chloroform/ isoamyl alcohol (24:1). 1 cm^3 5 M NaCl and 3 cm^3 water-saturated ether were added to get rid of pectin. Bottom layer was gathered and 5 cm^3 isopropanol was added for the second time. Pellet was washed with 70 % ethanol, and resuspended with 0.5 cm^3 TE buffer. Extracted genomic DNA was stored at -20 °C until use.

Semi-quantitative reverse transcription-polymerase chain reactions (RT-PCR) were carried out for expression analysis. 0.5 mm^3 of 20 mm^3 reverse transcription reaction products was used for RT-PCR in a final volume of 20 mm^3 with gene-specific primers. All RT-PCR were performed for 25 cycles under suitable cycle parameters. Constitutively expressed *β-actin* was used as an internal control (Liu *et al.* 2007). The gene-specific primers are listed as follows: *PtCBF*: 5'-CTTATCATCTCCGT GTTCGC-3' and 5'-AGAGCCCGCTTGATTACTG-3'; *β-actin*: 5'-CCAAGCAGCATGAAGATCAA-3' and 5'-ATCTGCTGGAAGGTGCTGAG-3'. RT-PCR reactions were repeated three times for each gene. The electrophoresis photographs from RT-PCR were analyzed and quantified with *Band Leader* software (version *3.00*). The *PtCBF* expression was calculated as a percentage after normalization with *β-actin* values.

Trifoliate orange and other citrus *CBF* genomic DNA fragments were amplified by normal PCR using gene specific primers designed according to trifoliate orange *CBF*-like full-length cDNA sequence (GenBank No. DQ790888.1). PCR was performed under following conditions: denaturation for 45 s at 94 °C, annealing at 50 °C for 45 s, elongation 72 °C for 60 s, 35 cycles in total. 50 mm^3 PCR reaction mixture contains 200 ng genomic DNA templates, 2.5 U *ExTaq* DNA polymerase (*Takara*, Dalian, China), 5 mm^3 10× PCR buffer, 200 μM each dNTP, 200 nM each gene specific primer. PCR product was excided from 0.8 - 1.0 % agarose gel, then purified by *E.Z.N.A*™ gel extraction kit (*Omega*, USA). At the last step, 40 mm^3 of elution buffer was added and concentration was measured by spectrophotometer (*U-0080D*, *Hitachi*, Tokyo, Japan). Ligation was according to manufacturer's manual of *pMD18-T* vector kit (*Takara*). 10 mm^3 ligation system consisted of 25 ng *pMD18-T* vector, 200 ng purified DNA sample, 5 mm^3

solution I, ddH_2O was added as well. Ligation was conducted at 16 °C for overnight. DH5α *Escherichia coli* genetic transformation was operated with the method of $CaCl_2$-mediated freeze-thaw. Single colony elected by blue-white colour was examined by double digestion with two restriction enzymes *Pst*I and *Eco*RI after plasmid DNA isolation by alkaline lysis. Then the positive colony was finally sent to sequencing at least three repeats for definite confirmation. Phylogenetic tree for species was constructed using Neighbor-Joining method in *MEGA* (*v. 4.0.2*) software.

The *pMD18-T* vector used as adapter source was digested with restriction enzymes *Pst*I and *Bam*HI for 3 - 4 h at 37 °C, vector fragments were then purified for ligation by 0.8 - 1.0 % agarose gel electrophoresis. Trifoliate orange genomic DNA was single-digested with *Bam*HI at 37 °C for overnight, then ligated with vector fragments at 16 °C for another overnight after reaction termination. Using ligation products as templates, adapter-mediated PCR reaction was performed under cycle parameters referred to modified TAIL-PCR procedures (Terauchi *et al.* 2000). Four gene-specific primers were used in primary and secondary PCR reactions: pMD18_F (5'-GAAACAGCTATGACC ATGATTACG-3'), IASP9 (5'-CAAACCCACTTGCC CGAATC-3'), IASP14 (5'-GGCGATGACCAGCT TGATAG-3') and IASP8 (5'-GCGAACACGGAG ATGATAAG-3') (Fig. 1). PCR reaction system was the same as described above. PCR products from primers pMD18_F and IASP14 with longer known overlap were purified by *E.Z.N.A*™ gel extraction kit, and then sequenced for rough results. Two gene-specific primers were designed according to sequencing results and known genomic DNA sequences, SP1 (5'-GTGATAGCT TTTGTCCGTACTC-3') and AP3 (5'-TGAATATCCTTG GGGTCAGT-3'). PCR reactions were conducted in 50 mm^3 reaction system under cycle parameters: 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 2 min, 35 cycles in total. PCR products were purified from 0.8 - 1.0 % agarose gel by electrophoresis, and sequenced finally.

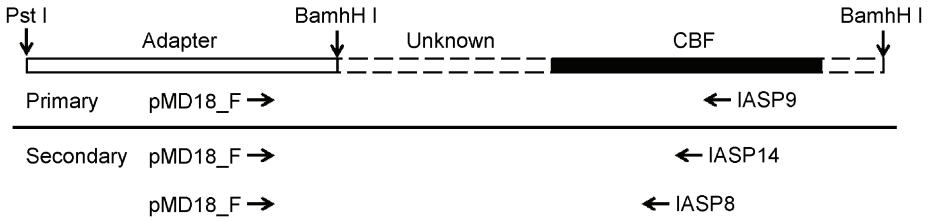

Fig. 1. Localization of nested gene-specific primers in citrus genomic DNA. Nested gene-specific primers were used in adapter-mediated PCR for citrus *CBF* promoter isolation: pMD18_F and IASP9 for primary PCR, IASP14 or IASP8 with the same forward primer pMD18_F for secondary PCR.

Results and discussion

To learn more about roles of *PtCBF* during cold acclimation, the expression of *PtCBF* from different organs under low temperature was analyzed using semi-quantitative RT-PCR. As reported in previous work, the expression increased transiently in leaves after cold treatment, although *PtCBF* expressed obviously in control treatment (Fig. 2*A*). Under cold treatment, the expression in leaves increased to maximum value within 1 h, and then decreased gradually to less than control level within 3 d of low temperature. In stems, the expression decreased at first, then increased within 8 h, then decreased again 8 h later, and finally increased to maximum value after 3 d. Differently, the expression in roots remained decreased after 2 d of treatment.

For abscisic acid treatment, *PtCBF* in roots was more sensitive than in leaves and stems, and showed similar expression trend as in leaves under cold treatment (Fig. 2*B*). In roots, the expression increased rapidly to maximum value within 15 min, and then decreased until the end of treatment. The expression in stems fluctuated from 76 to 103 % of control through the whole treatment. However, the expression in leaves decreased sharply after treatment, and then increased after 2 h. After 8 h, the expression decreased again, then increased slightly and finally decreased until the end.

The genomic DNA fragments of *PtCBF* and other CBF coding genes in related citrus species (designated as *CsCBF* in *Citrus sinensis*, *CgCBF* in *Citrus grandis*, and *CjCBF* in *Citrus jambhiri*) were isolated and sequenced. Finally, lengths of isolated *CBF* homologs were 874 bp for *PtCBF*, 1 029 bp for *CsCBF*, 1 030 bp for *CgCBF*, and 1 021 bp for *CjCBF*, respectively. All *CBF* gene sequences contained complete ORFs encoding 214 amino acids with no introns, according to the results of alignments between *PtCBF* cDNA and DNA nucleotide sequences or *ORF Finder* (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) on *NCBI* website (http://www.ncbi.nlm.nih.gov/).

Blastp (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis with four putative proteins showed great similarity to CBFs or DREB1s from other plant species including herbaceous and woody plant, such as strawberry, poplar,

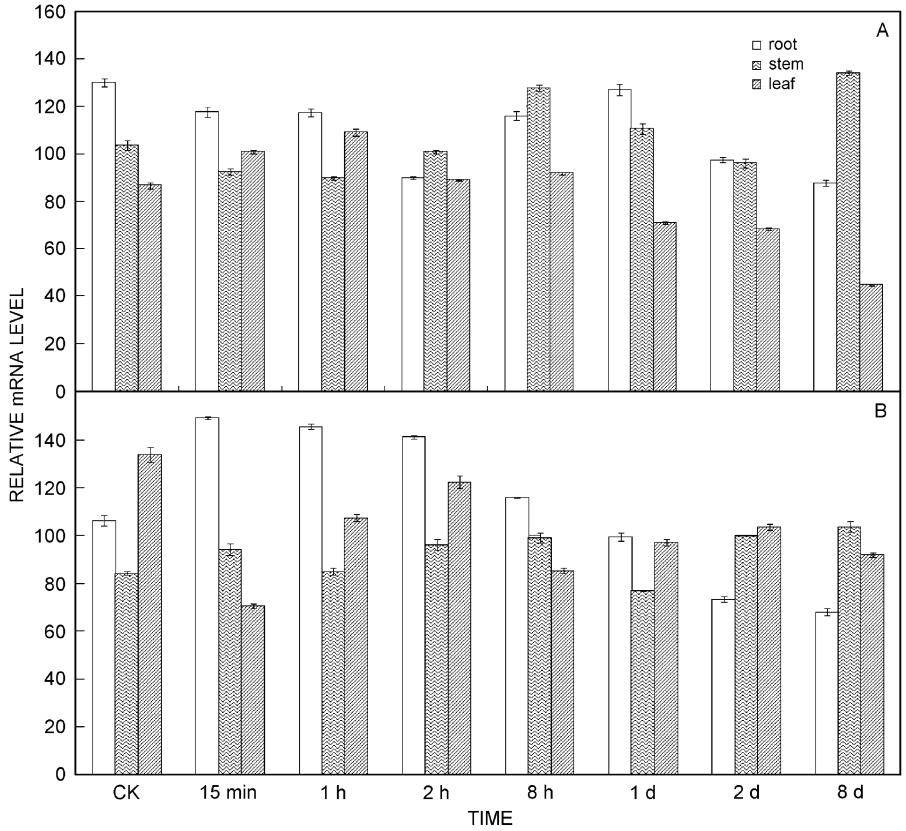

Fig. 2. Expression analysis of *PtCBF* in trifoliate orange in response to low temperature and abscisic acid (ABA). *A* - two-month-old trifoliate orange seedlings grown in nutrient substrate were transferred to 4 °C for cold treatment, while untreated seedlings were used as control. *B* - two-month-old trifoliate orange seedlings were transferred to Hoagland's nutrient solution containing 100 μM ABA, while seedlings in Hoagland's solution with no addition of ABA were used as control. CK indicates the controls. The *PtCBF* relative mRNA levels were normalized as a percentage with internal control *β-actin* values.

apple, and others. Multiple sequence alignment analysis using *ClustalW2* online (http://www.ebi.ac.uk/Tools/clustalw2/index.html) indicated that CBF signature sequences and AP2/ERF DNA binding domain were highly conserved in four citrus CBF proteins (Fig. 3). PtCBF shared more than 95 % of residues with other three CBFs from related citrus species, and showed 60 % identity to *PtCBF1* (GenBank No.: ABH08746.1, different from *PtCBF1* mentioned by Champ *et al.* (2007) from trifoliate orange itself, 80 % identity to strawberry, and only 24 % to rice. For CBF family members in model plant *Arabidopsis*, PtCBF was more similar to AtCBF1 and AtCBF3, which may contribute to functional characterization of PtCBF in trifoliate orange. Phylogenetic analysis revealed the genetic relationship in evolution among all the CBFs/DREB1s studied based on the result of full polypeptide alignment (Fig. 4). Above data indicated that PtCBF was one member of CBF transcription factor family in trifoliate orange. Like CBF in other plant species, CBFs form a small family with at least two members, PtCBF and PtCBF1 in trifoliate orange. The conclusion provided the evidence for multiple CBF-like proteins in *Citrus spp.* and complexity of CBF cold regulatory network.

Using adapter-mediated PCR protocol, CBF upstream regulatory sequences were isolated successfully in trifoliate orange and related citrus species. After sequencing confirmation, 1 043 bp of *PtCBF* promoter, 1 126 bp of *CsCBF* promoter, 1 083 bp of *CgCBF* promoter, 934 bp of *CjCBFs* short promoter and 1 126 bp of *CjCBFl* long promoter were determined in four citrus species. *NNPP* (http://www.fruitfly.org/seq_tools/promoter.html) was introduced to search for the transcription start site. The potential transcription start sites were predicted successfully and located at 185 bp away from translation start codon in citrus *CBF* promoters. *PLACE* (http://www.dna.affrc.go.jp/PLACE/signalscan.htmL) and *PlantCARE* (http://bioinformatics.psb.ugent.be/webtools/plantcare/htmL/) were both used for *cis*-acting element analysis.

Results showed that several stress-related elements were detected in upstream region of citrus *CBF*-like genes, such as G-Box, MYB, MYC and ABRE, and so on (Fig. 5*A*; Table 1). Furthermore, a LTRE element was present only in *PtCBF* promoter, while the 5 bp core motif CCGAC was changed to CCGTC because of A/T mutation in other *CBF* promoters. However, the CBFHV element was predicted in promoters of *CsCBF*,

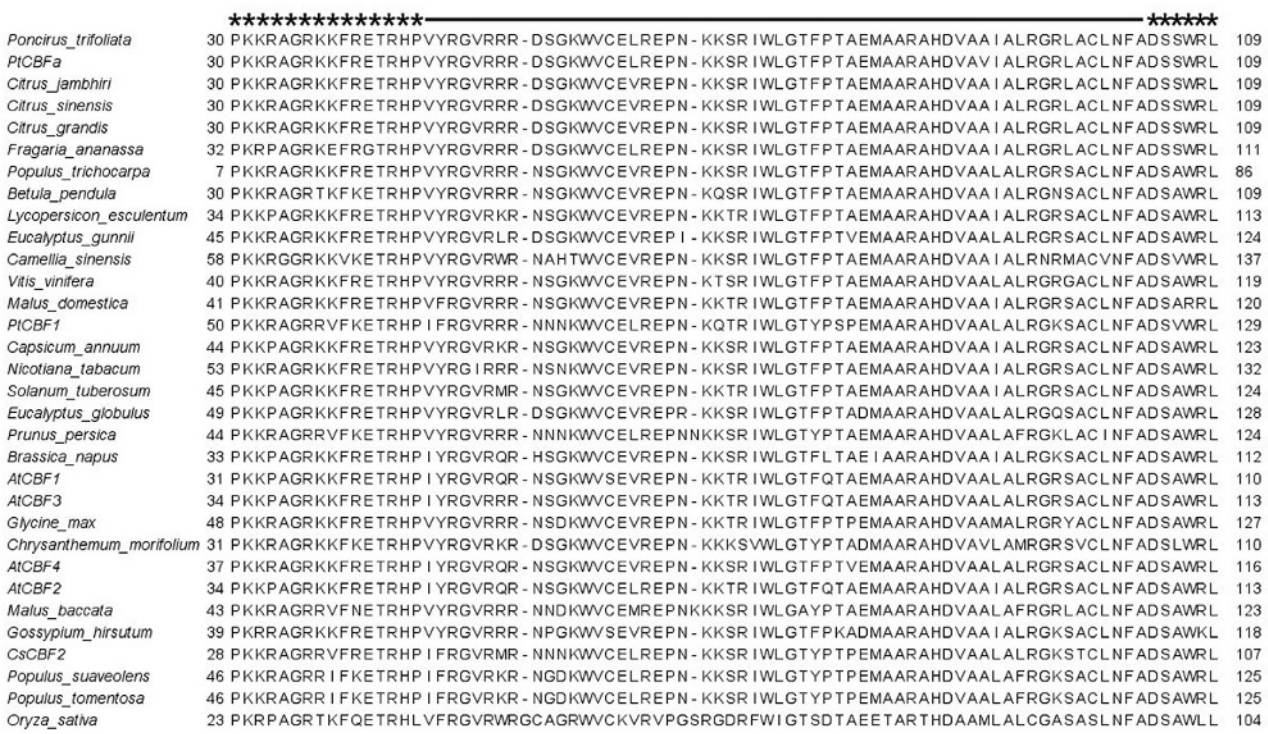

Fig. 3. Multiple sequence alignment analysis of CBF/DREB1 in citrus and other plant species. The *asterisks* indicated CBF-like signature sequence, *black bold line* represents AP2 DNA binding domain, which is the common and highly conserved feature in AP2 transcription factor family.

Table 1. Cis-acting elements predicted by *PLACE* and *PlantCARE* tools in citrus *CBF* promoters

Motif Name	Sequence	Sites(+/-) Pt	Cs	Cg	Cl_s	Cl_l	Function
ABRE	ACGTG	2/4	2/3	¾	2/3	2/3	abscisic acid responsiveness
ASF	TGACG	2/2	1/3	½	1/2	1/3	auxin and/or salicylic acid, abiotic and biotic stress
CARGCW8GAT	CWWWWWWWWG	1/1	0/0	0/0	0/0	0/0	a variant form of CArG box
CBFHV	RYCGAC	0/0	0/1	0/1	0/1	0/1	binding site of barley CBF1 and CBF2
Circadian	CAANNNNATC	1/0	3/1	1/1	1/1	3/1	circadian clock control
G-Box	SACGTB	5/2	4/2	6/2	4/2	4/2	light responsiveness
GATA BOX	GATA	4/2	2/3	3/2	2/2	2/3	light regulated, and tissue specific expression
GT1	GRWAAW	10/2	9/2	9/2	9/2	9/2	pathogen- and salt-induced gene expression
LTRE	CCGAC	1/0	0/0	0/0	0/0	0/0	low temperature responsiveness
MYB	CNGTTR	3/4	3/5	2/5	3/4	3/5	water stress and dehydration responsiveness
MYC	CANNTG	6/6	5/5	7/7	5/5	5/5	stress responsiveness
SP1	GGGCGG	1/0	1/1	1/1	1/1	1/1	light responsiveness
TATC Box	TATCCCA	0/0	1/0	0/0	0/0	1/0	gibberellin responsiveness
TC-rich repeats	ATTCTCTAAC	0/0	1/0	0/0	0/0	1/0	defense and stress responsiveness
W-Box	TGAC	7/6	6/7	7/7	6/6	6/7	salicylic acid, wound and pathogen responsiveness

CgCBF and *CjCBF*, which was binding site of barley CBF1 and CBF2 (Xue 2002, Svensson *et al.* 2006). Meanwhile, more than one circadian clock-responsive element was found in all citrus *CBF* promoters, which suggested that CBFs in citrus might be gated by circadian clock as in *Arabidopsis* (Fowler *et al.* 2005). Additionally, the variant form of CArG motif was detected only in *PtCBF* promoter, while missing in other citrus *CBF* promoters, which implied that the expression of *PtCBF* may be regulated by SOC1-like transcription factor in *Poncirus trifoliata* as *AtCBF*s in *Arabidopsis* (Seo *et al.* 2009).

Alignment analysis with all citrus *CBF* promoters suggested two conserved regions and one intensively variable area. The conserved regions were located in upstream of 735 and downstream of 156, and full of

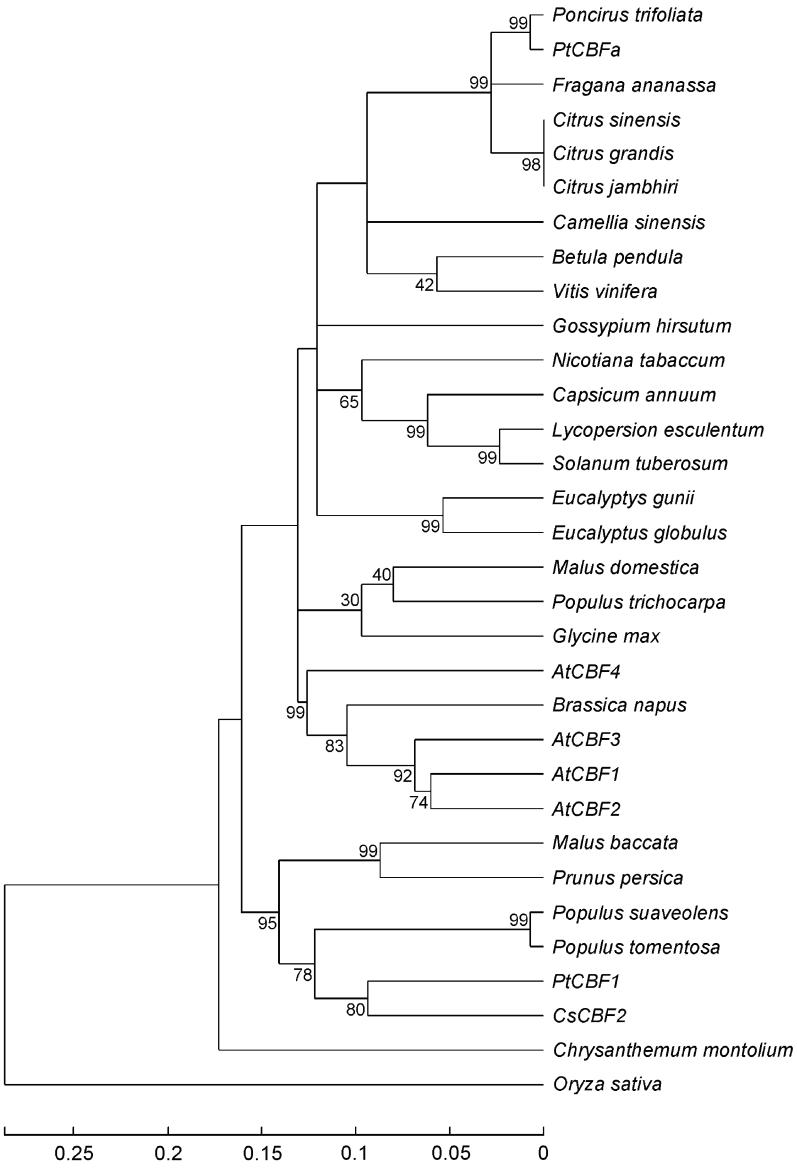

Fig. 4. Phylogenetic tree of CBF/DREB1 proteins was computed using Neighbor-Joining method in *MEGA* software (*v. 4.0.2*, http:/www.megasoftware.net/). The numbers showed the bootstrap values, and labels were the same as Fig. 3.

common *cis*-acting elements. The variable area was constituted by three subregions, named as VR1, VR2 and VR3 (Fig. 5*A*). Many special motifs were detected in variable regions, which could be involved in differential responses of CBF expression in citrus (Fig. 5*A*,*B*; Table 2). In addition, ICE1 was known to be another MYC-like transcription regulator of CBF expression *via* two regions ICEr1 and ICEr2 in CBF2 promoter in *Arabidopsis*, here ICEr1 and ICEr2 were predicted as well from -118 to -42 in conserved region of citrus *CBF* promoters (Fig 5*C*).

In a recent study, *PtCBF* was isolated and reported to have important roles during cold acclimation in trifoliate orange. Results showed that *PtCBF* transcripts became detectable within 2 h of cold treatment, and peak at 12 h (Champ *et al.* 2007). However, our data showed that *PtCBF* was expressed over the whole course of treatment, even in control. The difference may result from different daytime, irradiance and nutrition, which might affect the expression of *PtCBF* after treatments (Kim *et al.* 2002, Fowler *et al.* 2005). With regard to differential expression in various vegetative organs, we could infer that *PtCBF* expression is organ-specific. In this case, effects of direct contact with respective treatment should be considered.

ABA plays important roles in mediating stress tolerance and induces expression of many stress-related genes. Application of ABA could induce the *CBF* transcription and subsequent expression of cold-regulated genes *via* CRT/DRE promoter element in *Arabidopsis* (Knight *et al.* 2004). According to the data of ABA treatment in current study, a similar conclusion was obtained

Table 2. Some special motifs present in citrus *CBF* promoters.

No.	Sequence	Length	Notes
IR1	TACAGA	6	inverted complementary
DR1	TTGTTTTTT	9	direct repeat
IR2	TAGCTTACAGA	11	inverted complementary repeat
DR2	TACAGATTCTGTAA	14	direct repeat
DR3	AACAAACATATCCCATCATCCCACCATCCG	30	tandem direct, with two differential bases
VR3	AGCTAAACGGATAGTATAAAATTGAGACTGAAGTTTGACGCTG ACTCTGACAAGTACGGGTTCATTCCCTTAA	73	direct repeat, with four differential bases
VR2	ATGATCTCATAATTAACAGCTAAACGGATAGTAAAAAATTGAGA CTGAAGTTTGACTCTGACTCTGCCAGGTGCGGGTTCATTCCCTT AATAAAATAGAGTTAAAAAAATCAGGGGAAAGAAACTTTATTT AACACGTGTCAAACA	146	tandem direct repeat, with nine differential bases

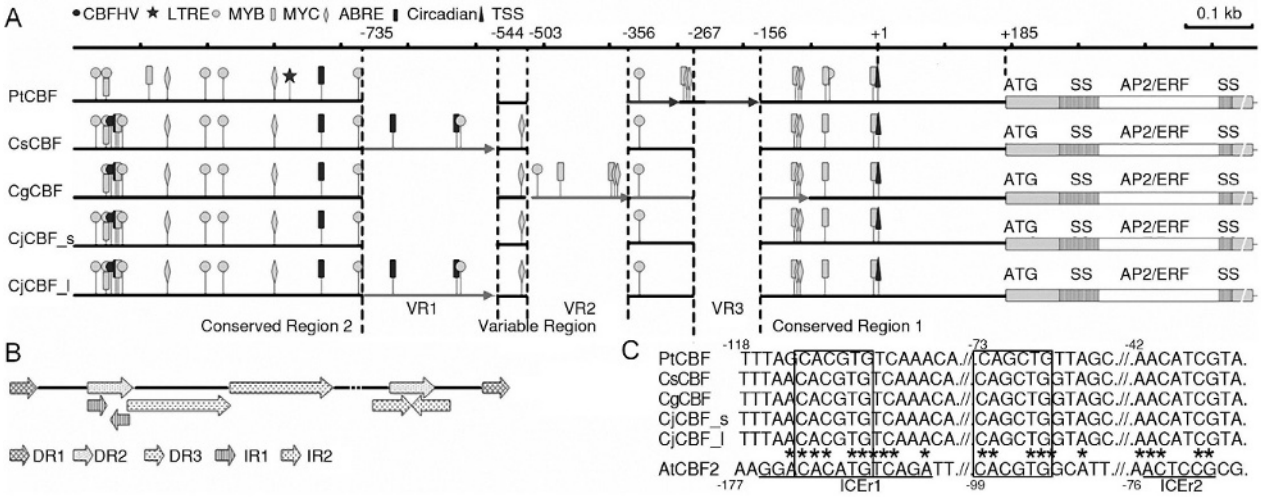

Fig. 5. Structural model of *CBF* promoters and partial coding sequences in trifoliate orange and related citrus species. *A* - main *cis*-acting elements and transcription start sites (TSS) predicted in citrus *CBF* promoters (SS - CBF signature sequence, AP2/ERF - APETALA2/ethylene-responsive factor, the *arrows* indicates the variable regions in citrus *CBF* promoters. *B* - direct repeats (DR) and inverted complementary repeats (IR) present in VR1. *C* - alignment of ICEr1 and ICEr2 regions in citrus *CBF* and *AtCBF2* promoters, *black boxes* represents MYC recognition sites between ICEr1 and ICEr2; *underlines* point to ICEr1 and ICEr2 regions, respectively.

in trifoliate orange, which implied that some uncertain interaction between ABA and CBF existed in citrus, although CBF cold response pathway was proved to be ABA-independent in many plants. Moreover, deletion of potential ABRE element in *CBF2* promoter still responded to ABA in *Arabidopsis* (Knight *et al.* 2004). In order to clarify the regulation mechanism, more work need to be done on functional characterization of *PtCBF* promoter elements in citrus.

The cytoskeletal protein β-actin is involved in several basic developmental processes including the establishment of cell polarity, cell division plane determination, cell wall deposition, and cell elongation (Gilliland *et al.* 2003). In our experiment, the expression of β-actin varied with times in the same vegetative organs, which demonstrated that some basic developmental processes may be influenced by low temperature and ABA in trifoliate orange. Similar results were reported in previous paper (Meng *et al.* 2008). To our knowledge, environmental stresses such as cold and drought could trigger the production of endogenous ABA, which lead to a number of physiological adaptations, including growth regulation. Therefore, changes of expression levels were more obvious especially in roots during ABA than cold treatment.

Interestingly, two upstream fragments were isolated from rough lemon, which meant that two promoters shared the same coding sequence with each other. Comparison analysis between these two promoters indicated that the long promoter contained a 200 bp insertion designated as VR1 between 735 and 536 away from transcription start site. After alignment with other citrus *CBF* promoters, the long promoter showed 99 % identity to *CBF* promoter from sweet orange with only three different bases. So, the long promoter in rough lemon was active possibly, but the activity of the short

promoter here and the function of VR1 in long promoter were unknown.

LTRE is known as an important *cis*-acting element in response to low temperature (Baker *et al.* 1994). The core sequence CCGAC also was a portion of *cis*-acting element CRT/DRE present in the promoter of cold-regulated (COR) genes, the target of CBF protein in *Arabidopsis*. In recent paper, PtCBF was confirmed to bind specifically to the CRT/DRE core CCGAC in CORc115 promoter *via* yeast-one-hybrid analysis (Champ *et al.* 2007). Thus, PtCBF could perform similar functions as CBF family in *Arabidopsis*, the existence of 5 bp CRT/DRE core motif CCGAC in *PtCBF* promoter created the potential for self-regulation, which could lead to more freezing tolerance than other citrus species.

In conclusion, plants have developed many mechanisms to accommodate adverse environmental conditions. The response network and signal pathway become more and more complicated and staggered in evolution. The ABA-independent CBF network plays important roles in response to cold, drought and other stresses. In current work, a *CBF*-like gene in trifoliate orange, *PtCBF* was isolated and characterized that *PtCBF* expression was up-regulated in response to cold and ABA; no introns were in *PtCBF*; AP2 DNA binding domain and CBF signature sequences were highly conserved in citrus *CBF* proteins. To understand the context of CBF regulatory pathway in citrus, the preliminary analysis of citrus *CBF* promoter could provide some information on functional characterization of *cis*-acting elements present in *CBF* promoters.

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