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

H.L. WANG, J.J. TAO, L.G. HE, Y.J. ZHAO, M. XU, D.C. LIU and Z.H. SUN*

Key Laboratory of Horticultural Plant Biology, Ministry of Education, Citrus Research Institute, Huazhong Agricultural University, Wuhan 430070, P.R. China

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-dimensional 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 spectrophotometer (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,
Table 1 Sequences of primers used in experiment (R = A/G, Y = C/T, V = A/C/G, N = A/G/C/T)

<table>
<thead>
<tr>
<th>Type</th>
<th>Name</th>
<th>Sequences (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers for reverse transcription</td>
<td>Oligo(dT)18</td>
<td>TTTTTTTTTTTTTTTTTTTT</td>
</tr>
<tr>
<td></td>
<td>3’-CDS</td>
<td>AAGCAGTGGTATCAACGCAGAGTAC(T)30VN</td>
</tr>
<tr>
<td></td>
<td>5’-CDS</td>
<td>(T)30VN</td>
</tr>
<tr>
<td>Degenerate primers for RT-PCR</td>
<td>DP1</td>
<td>CCNAARARAGRRCNNGNAG</td>
</tr>
<tr>
<td></td>
<td>DP2</td>
<td>TCNGCRAARTTYAARCA</td>
</tr>
<tr>
<td>5’-linker for 5’-RACE</td>
<td>BD-Oligo</td>
<td>AAGCAGTGGTATCAACGCAGAGTACGCGGG</td>
</tr>
<tr>
<td>Primers for RACE</td>
<td>UPL</td>
<td>CTAATACGACTCATATAAGGCAAGCAGTATCAACGCAGATG</td>
</tr>
<tr>
<td></td>
<td>UPS</td>
<td>CTAATACGACTCATATAAGGCC</td>
</tr>
<tr>
<td></td>
<td>NUP</td>
<td>AAGCAGTGGTATCAACGCAGAGT</td>
</tr>
<tr>
<td></td>
<td>APB1</td>
<td>GTCATGTGCCCTTTGCCAGCCATT</td>
</tr>
<tr>
<td></td>
<td>APB2</td>
<td>ATGCCTGTTGTCTTGGGGTC</td>
</tr>
<tr>
<td></td>
<td>SPB</td>
<td>TGAACCCCAAACGAAACACAG</td>
</tr>
<tr>
<td>Primers for ORF</td>
<td>SP1-1</td>
<td>GGGCTAACTTCAACACAT</td>
</tr>
<tr>
<td></td>
<td>AP1-1</td>
<td>TTCTCACAACCTAAGCAATC</td>
</tr>
</tbody>
</table>

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 I) 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.

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 (Towell). 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’-TTCTCACAATCGAACTTCAACACAT-3’) and SP1-1 (5’- GGGCTAACTTCAACACAT-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’-ATTGTAAGCAACTTCCGT-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. 1A). 5'- and 3'-end of Ptcbfb were obtained by RACE and identified through sequencing (Fig. 1B,C). Then the full ORF was cloned by RT-PCR (Fig. 1D).

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 annum, Eucalyptus gunnii, and Lycopersicon esculentum, with the identity being 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 conservative.
Fig. 3. Multiple sequence 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. PKKrAGrKfERTRHP 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, Ptbfb 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. Ptbfb 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.

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 controlling its activity, thus a conclusion that PtCBFb might be the final receptor of Ca2+ and other second messengers induced by cold in transduction pathway. Analysis of three-dimensional structure showed that PtCBFb possessed an AP2 DNA binding domain which resembled to 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 constitutively 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.

References


