DOI: 10.1007/s10535-014-0393-x BIOLOGIA PLANTARUM 58 (2): 247-255, 2014

Structural and expression analyses of three *PmCBF*s from *Prunus mume*

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Abstract

C-repeat binding factor (CBF), also called the dehydration-responsive element binding factor 1 (DREB1), can be induced by low-temperature (LT), and plays an important role in abiotic stress tolerance in higher plants. In present study, two new homologous genes of *CBF* from *Prunus mume* (*PmCBFb* and *PmCBFc*) have been identified and characterized. The complete coding sequences of *PmCBFb* and *PmCBFc* were 714 and 723 bp, respectively. They encoded putative proteins of 237 and 240 amino acids. Neither of them had introns. Genome PCR sequencing showed that *PmCBFb* was arranged in tandem with *PmCBFa* (another *CBF/DREB1* homolog in *P. mume*) within a region of nearly 4 kb. Promoter prediction analyses indicated that multiple types of *cis*-elements related to abiotic stress and irradiance existed in the putative promoter region of *PmCBFb*. LT treatment of seedlings showed that the expression of *PmCBF* genes were induced by 2 °C within 30 min, and their expression reached a peak after 8 - 12 h. In addition, *PmCBFa* and *PmCBFb* appeared more sensitive to LT than *PmCBFc*. However, the exact roles of *PmCBF* genes in plant cold tolerance need to be further investigated.

Additional key words: dehydration-responsive element binding factor, gene expression, low temperature, Mei flower, phylogenetic tree, tandem array.

Introduction

Low temperature (LT) is one of the important environmental stresses that severely affect plant growth and development, and limits their distribution. To cope with these adverse environmental conditions, plants have developed a number of defence mechanisms and processes to enhance their freezing tolerance. Among them, cold acclimation occurs after a period of exposure to low non-freezing temperature and results in a transient increase in freezing tolerance. This phenomenon is very complex, involving a variety of physiological and biochemical alterations regulated by global changes in gene expression (Hughes and Dunn 1996, Thomashow 1999).

The best characterized genetic control of the cold acclimation process is executed by the *CBF*/*DREB1* (Medina *et al.* 2011). Proteins encoded by *CBF/DREB1* belong to the APETALA2/ethylene-responsive (AP2/ERF) transcription factor superfamily and bind specifically to the C-repeat/dehydration-responsive (CRT/DRE) element which is present in the promoters of a large number of cold-regulated (COR) genes whose products are thought to contribute to a plant tolerance to abiotic stresses (Fowler and Thomashow 2002). The AP2 DNA binding domain of a CBF protein typically has 14th valine (V14) and 19th glutamic acid (E19), differing from 14th alanine (A14) and 19th aspartic acid (D19) in ERF proteins (Sakuma *et al.* 2002). In addition, two conserved amino acid sequences, PKK/RPAGRx KFxETRHP and DSAWR flanking the AP2 domains, are described as "CBF signature" motifs which distinguish CBF proteins from other members of the AP2 protein family (Jaglo *et al.* 2001).

Submitted 24 May 2013, *last revision* 6 October 2013, *accepted* 7 October 2013.

Abbreviations: ABA - abscisic acid; AP2/ERF - APETALA2/ethylene-responsive factor; CBF - C-repeat binding factor; COR - cold-regulated; DREB1 - dehydration-responsive element binding factor 1; EF1α - eukaryotic translational elongation factor 1α; GA - gibberellins; RT-PCR - real-time polymerase chain reaction.

Acknowledgements: This research was financially supported by the National Natural Science Foundation of China (grant No. 31070624) and the National 863 Project of China (No. 2011AA100207). C. Guo and J.Q. Zhang contributed equally to this paper.

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The *CBF* orthologs are sometimes organized in chromosomes as clusters of tandem duplicated genes. In *Arabidopsis*, *CBF1*, *CBF3*, and *CBF2* (also referred to as *DREB1b*, *DREB1a*, and *DREB1c*) are located on the bottom arm of chromosome IV in tandem array (Gilmour *et al.* 1998). Studies in *Arabidopsis* showed that they are all cold-induced mainly through an abscisic acid (ABA) independent pathway and act redundantly with some possibly specific functions (Medina *et al.* 1999, Novillo *et al.* 2004, 2007, Sharabi-Schwager *et al.* 2010). In addition to *Arabidopsis*, homologous genes of *CBF* have been reported in both freezing-tolerant and freezing-sensitive species from 58 genera, including 14 woody plants (Navarro *et al.* 2011). In cereals, the *CBF* gene family is much larger. There are about 25 *CBF* homologous genes in *Triticum aestivum* (Badawi *et al.* 2007) and at least 14 members belong to the lineage of *OsDREB1* in rice (Skinner *et al.* 2005). According to the research records, ectopic expression of *CBF* and its homologous genes results in an increased freezing resistance and an enhanced tolerance to drought and high salinity, in many cases accompanied by growth retardation (Achard *et al.* 2008, Huang *et al.* 2009, Yang *et al.* 2011, Gupta *et al.* 2012, Movahedi *et al.* 2012). The CBF pathway also contributes to plant dormancy, vernalization, and reproductive development (Seo *et al.* 2009, Knox *et al.* 2010). Taking *CBF* orthologs in *Prunus* as example, the ectopic expression of *P. avium DREB1/CBF* in *Arabidopsis* confers salt and freezing tolerances (Kitashiba *et al.* 2004). The ectopic expression of *PpCBF* (*CBF* homolog from *P. persica*) in *Malus* × *domestica* results in short-day induced dormancy (Wisniewski *et al.* 2011). In *P. dulcis*, two *PdCBF* genes were regulated by irradiance and they showed a temporal difference of transcript accumulation in field-grown trees (Barros *et al.* 2012).

Prunus mume is a deciduous tree that blooms beautifully in late winter. Compared with other *Prunus* species, it is more freezing-sensitive. The early coming, long-standing winter and the cold-dry wind mostly restrict its growth and development. To find and investigate the cold-related genes in *P. mume*, we have previously reported the isolation and identification of a *CBF* ortholog from *P. mume* named *PmCBFa* (Genbank acc. No. HM099909; Guo *et al.* 2012). Sequence analyses showed that *PmCBFa* encodes a typical CBF transcription factor most closely related to the DREB1 protein in *P. persica*. Based on these results, the aim of our recent study was to isolate more homologous genes of *PmCBFa*, and to investigate the character of each *PmCBF* under a LT stress. In this article, we described cloning two new *CBF* orthologs, *i.e.*, *PmCBFb* and *PmCBFc*. The expression patterns of three *PmCBF* genes under a 2 °C treatment have been analyzed by quantitative RT-PCR. Besides, we have discovered the head-to-tail linked pair of *PmCBFa* and *PmCBFb* in the genome of *P. mume*.

Materials and methods

Seeds of *Prunus mume* Xue Mei) were obtained from trees grown at the Huazhong Agricultural University, Wuhan, China (30° 27' N). They were germinated and grown in the field for 4 months. After that time, the seedlings were approximately 60 cm high with more than 15 branches. Before the low temperature treatment, four seedlings were planted in the pot of a 22 cm diameter in a mixture of peat, sand, and *Vermiculite* (6:2:1, v/v/v). Then the plants were moved to a phytotron with a 12-h photoperiod, irradiance of 120 µmol m^{-2} s^{-1}, temperature of 24 °C, and relative humidity of 75 % for one week. Then three of them used for the LT treatment were transferred into another chamber of the same type but with temperature of 2 °C, and the remaining one was used as negative control. After 0, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, and 168 h, leaves were harvested. All samples were frozen in liquid nitrogen and stored at -80 °C until RNA extraction.

Cloning *PmCBF* gene was performed using a 3'/5'-rapid amplification of cDNA ends (RACE) with the *SMART*$^{\text{TM}}$ cDNA library construction kit (*Clontech*, Mountain View, USA). For cDNA synthesis, total RNA was isolated from flower buds of a *P. mume* adult tree. Double-stranded cDNA was synthesized according to the procedure recommended by *Clontech*. Genome DNA (gDNA) was extracted from young leaves by the modified cetyltrimethylammonium bromide (CTAB) method. A pair of degenerate primers (PmCBFF1 and PmCBFR2) was designed to amplify AP2 fragments of *CBF*-like genes according to the conserved AP2/ERF domain and its flanking motifs as described before (Guo *et al.* 2012). The PCR conditions were 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 59 °C for 30 s, 72 °C for 30 s, and a final extension of 72 °C for 5 min. PCR products were separated by 1.2 % (m/v) agarose gel electrophoresis. Bands matched the expected size were cut from the gel, purified using the *AxyPrep*$^{\text{TM}}$ DNA gel extraction kit (*Axygen*, Hangzhou, China), and cloned into the *pMD18-T* vector (*Takara*, Dalian, China). Sequencing using universal M13 primer pairs was performed in the *BGI* (*Huada Gene*) company in Wuhan.

To obtain the 3' and 5' terminal regions of *PmCBFb*, 3' and 5' RACE reactions were performed using primers PmCBFbrF1 and PmCBFbrR1, respectively. Universal 3' and 5' PCR primers (3RaceR and 5RaceF) were supplied by the *SMART*™ cDNA library construction kit Fusion primer and nested integrated (FPNI)-PCR, a new high-efficiency PCR version based on TAIL-PCR, was used to clone the 3' and 5' flanking sequences of the *PmCBFc* gene. Published fusion arbitrary degenerate primers (FAD1-9; Wang *et al.* 2011) were synthesized. Six modified specific primers, PmCBFcTF1-3 and PmCBFcTR1-3, were designed. The FPNI-PCR conditions and reactions were as reported previously (Wang *et al.* 2011). Gene-specific primers (PmCBFbZF and PmCBFbZR, and PmCBFcZF and PmCBFcZR) were used to amplify the complete cDNA and gDNA sequences of *PmCBFb* and *PmCBFc*. Gene-specific primer pairs (PmCBFaF1 and PmCBFaR1, PmCBFbF1 and PmCBFbR1, and PmCBFcF1 and PmCBFcR1) were designed to amplify the potential tandem arrays of the *PmCBF* genes. The PCR conditions were as follows: 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 59 °C for 30 s, 72 °C for 90 s, and a final extension of 72 °C for 10 min. Primers except universal ones were all designed using the *Primer Premier 5* software. All primers are described in Table S1.

Fragments obtained were firstly analyzed through the nucleotide *BLAST* program in *National Center for Biotechnology Information* (*NCBI*) service (http://blast.ncbi.nlm.nih.gov). Protein sequences were deduced using the vector *NTI Advance 9* suit and the *Primer Premier 5*. To verify the protein homology of PmCBFs, the protein *BLAST* program in NCBI service was used. The multiple protein sequence alignments were done using the *ClustalX 1.83* (Thompson *et al.* 1997) and refined using *Genedoc* (Nicholas and Nicholas 1997). Phylogenetic analyses on alignments were conducted using the *MEGA 4* software (Tamura *et al.* 2007). Phylogenetic trees were generated with *MEGA 4* neighbor-joining and minimum evolution default methodologies on 1 000 bootstrap replications. The promoter region of *PmCBFb* was identified by the prediction of plant promoters (TSSP) analysis in softberry database (http:// linux1.softberry.com/berry.phtml). The *cis*-element analysis was performed by signal scan searching in the *PLACE* database (Prestridge 1991, Higo *et al.* 1999).

Total RNA for quantitative real time (RT)-PCR analysis was extracted from leaves of one-year-old *P. mume* seedlings as described previously (Li *et al.* 2008). Digestion by *RQ1* RNase-free DNase (*Promega*, Madison, WI, USA) was carried out to avoid all non-RT-dependent background. The integrity of the purified RNA samples was ascertained by agarose gel electrophoresis. DNase pre-treated total RNA (2 µg) was reverse transcribed with 0.5 µg oligo(dT)$_{15}$, 0.75 mM dNTPs, a 20 U RNase inhibitor (*Takara*) and 200 U *M-MLV* reverse transcriptase (*Promega*, Madison, USA) in a total volume of 20 mm^3. Primers (Table S1) for quantitative RT-PCR were designed within the non-conservative regions of each *PmCBF* genes and tested to ensure the amplification of single discrete bands with no primer-dimers. The *P. mume eukaryotic translational elongation factor 1 alpha* (*EF1α*) gene was used as reference gene.

Reactions were performed with the *SYBR Premix Ex Taq* (*Takara*, Shiga, Japan) and analyzed in the *ABI 7500* real-time system (*Applied Biosystems*, Foster City, CA, USA). Quantitative RT-PCR products were amplified with the 1 mm^3 template of the RT reaction mixture, 10 mm^3 of the 2× *SYBR Green Master* mix, 1 mm^3 of forward and reverse primers (100 µM stock solutions), and water to a final volume of 20 mm^3. The gene expression was calculated by the *ABI Prism 7500* sequence detection system software (*Applied Biosystems*) on the basis of Ct values of the gene in different samples using the mathematical term $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen 2001) and normalized with *PmEF1α*. For better comparisons among time points of each gene and among genes in each time point, the relative mRNA level was calculated when the expression level of *PmCBFa* at time 2 h was used as calibrator. Quantitative RT-PCR was performed in four replicates for each of three samples. The relative gene expression analysis was carried out by the *Microsoft Office Excel 2003* software.

Results and discussion

CBF/DREB1 homologous genes and their corresponding proteins have been isolated from a wide range of plants. Sequences of early reported *CBF/DREB1* homologs from the genus *Prunus* and the genus *Arabidopsis* were aligned prior to the designation of a pair of degenerated primers to amply the AP2 DNA-binding domain and its flanking sequences. Two cDNA fragments of different lengths closely related to *PmCBFa* (Guo *et al.* 2012) were isolated and designated as *PmCBFb* and *PmCBFc* from the cDNA library of flower buds. The 5' and 3' untranslated regions of *PmCBFb* and *PmCBFc* were obtained by RACE and FPNI-PCR separately. The full-length of *PmCBFb* had a 714 bp open reading frame (ORF) that encoded a deduced protein of 237 amino acids with a molecular mass of 26.38 kDa and a theoretical isoelectric point of 6.25, whereas *PmCBFc* contained a 723 bp ORF of 240 amino acids and encoded a putative protein with a predicted molecular mass of 26.84 kDa and a theoretical isoelectric point of 7.01. PCR detection indicated that they were intron-less genes (Fig. 1). The sequences of *PmCBFb* and *PmCBFc* have been submitted to the *NCBI* GenBank, and the accession numbers are HM099910 and HM099911, respectively.

A comparison of the sequences of the *CBF* ORFs in *Prunus* showes that *PmCBFb* showed 97 % identity with homologs from *P. mira* and *P. davidiana* (acc. Nos. JX464664 and JX464666), whereas 96 % identity existed between *PmCBFc* and orthologous genes from *P. avium* (*PaCIG-B*) and *P. canescens* × *P. cerasus* (Nos. AB121674 and EU854320). The identity between

PmCBFb and *PmCBFc* genes was 82 %, and between their corresponding proteins 93 %. Protein alignments (Fig. 2) suggests that a CBF-type AP2 domain with "CBF signatures" (which is also called CMIII-3) were highly conserved in three PmCBF proteins which shared high identities with CBF/DREB1s from other *Prunus* species and CBF1 - CBF3 in *Arabidopsis* (Jaglo *et al.* 2001, Sakuma *et al.* 2002). Furthermore, all the deduced PmCBF proteins were highly conserved in their COOH-terminus with four CMIII motifs (the CMIII-3 motif was "CBF signature" sequences). In the study by Nakano *et al.* (2006), the CMIII-1 - CMIII-4 are highly conserved in the CBF/DREB1 subgroup in *Arabidopsis* and rice, and the CMIII-2 motif is absent in DREB1E and DREB1F proteins which are also called dwarf and delayed-flowering (DDF2 and DDF1), respectively (Sakuma *et al.* 2002, Magome *et al.* 2004). The sequences from CMIII-2 motif to CMIII-4 motif of the deduced PmCBF proteins also fitted the intermittent pattern of HC2-HC5 hydrophobic cluster motifs that were involved in trans-activation as described by Wang *et al.* (2005).

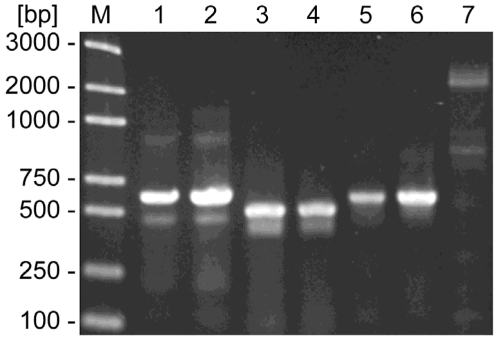

Fig. 1. Comparison of the cDNA and gDNA structures of *PmCBF* genes and PCR detection of the tandem array of *PmCBFab*. PCR amplifications of *lanes* 1 and 2 were performed using *PmCBFaZF* and *PmCBFaZR*, of *lanes* 3 and 4 using *PmCBFbZF* and *PmCBFbZR,* of *lanes* 5 and 6 using *PmCBFcZF* and *PmCBFcZR*, and of lane 7 using *PmCBFaF1* and *PmCBFbR1*. *Lane* M - a 3 000 bp DNA ladder. *Lanes* 1, 3, and 5 - PCR products generated from cDNA templates. *Lanes* 2, 4, 6, and 7 - PCR products generated from gDNA templates.

To characterize the relation between the putative proteins encoded by the *PmCBF* genes and 32 other full-length DREB sequences obtained from GenBank, a phylogenic tree was constructed (Fig. 3). The tree revealed a clear separation between monocotyledons and dicotyledons in the CBF/DREB1 group, and PmCBFs were classified into a dicotyledon subclade. Interestingly, the CBF/DREB1s within the dicotyledon clade were distributed clearly due to the genus category, in contrast within the monocotyledon clade. Similar results were obtained by Skinner *et al.* (2005) and Barros *et al.* (2012).

The evolutionary relationship among all the CBFs in *Prunus* was coincident with the protein alignment (Fig. S1). In the phylogenic analysis based on the result of full polypeptide alignment (Fig. S1*A*), most CBF orthologs from *Prunus* were explicitly clustered in three major subgroups within a clade, which were named PmCBFa, PmCBFb, and PmCBFc, respectively. Fig. S1*B* illustrated that the PmCBFb subgroup was distinct for an AP2 domain including 19th Q and a DSGWR flanking sequence that was different from traditional DSAWR, and the PmCBFc subgroup showed a substantial variability in its COOH-terminus. However, the greatest variability among the three subgroups came from the amino acid deletion in the NH3-terminus upstream of "the CBF signature" (the PmCBFb subgroup) and the region between CMIII-1 and CMIII-2 (the PmCBFa subgroup). Nevertheless, this kind of division and characterization needs to be approved by more CBF homologous proteins, and further studies need to be performed on whether this phylogenetic distribution is related to gene function divergence.

The head-to-tail tandem cluster of multiple *CBF* homologous genes is a feature that arises in diverse species (Pennycooke *et al.* 2008, Tondelli *et al.* 2011). Here, we designed an experiment to isolate the potential arrays of *PmCBF*s. A 2 897 bp DNA fragment, named *PmCBFab*, was obtained from gDNA of *P. mume* using primers PmCBFaF1 and PmCBFbR1 (Fig. 1). The fragment contained the partial 3'-terminal region of *PmCBFa*, the partial 5'-terminal region of *PmCBFb*, and their inner sequence which suggests that *PmCBFa* and *PmCBFb* were indeed linked closely in tandem in one chromosome. A fragment of 1 914 bp between *PmCBFa* and *PmCBFb* was assigned as the promoter of *PmCBFb*, named as *PmCBFbp*. However, fragments of other presumed tandem arrays of *PmCBF* genes had not yet been obtained. Promoter analyses show that the transcription start site (TSS) was located about 62 bp upstream of its translation start codon. Promoter scan searching in the *PLACE* database revealed that *cis*-elements involved in responses to radiation, ABA, gibberellin, heat shock, and in tissue-specific expression, especially in pollen and guard cell, were found in *PmCBFbp*. Furthermore, motifs as binding sites for DOF, MYB, MYC, WRKY, and RAV transcription factors have also been found in *PmCBFbp* (Table S2). These elements and binding sites are hot topics in recent CBF pathway researches (Medina *et al.* 2011). Previous research has described two ICE elements, the induction of *CBF* expression regions 1 and 2 (ICEr1 and ICEr2), and seven conserved DNA motifs (CAM1-7) presented in the promoters of *CBF2* and other CBF/DREB1 homologous genes (Zarka *et al.* 2003, Doherty *et al.* 2009). But in this research, only CM1 and CM3 boxes were found in *PmCBFbp*, and the location of them were quite different from that in *Arabidopsis*. The absence of the other motifs was probably due to the genetic variation in the *CBF* promoter region. However, the exact functions of the *PmCBF* promoters need to be analyzed and verified in further studies.

In order to study the roles of the *PmCBF* genes in cold-acclimation, we investigated the expression pattern of three *PmCBF* genes in response to LT. The expression patterns of the genes were detected by quantitative RT-PCR (Fig. 4). The three *PmCBF* genes were cold (2 °C) induced within 30 min and the transcript accumulation of the three genes peaked at 8 -12 h after the exposure to cold. This was consistent with the findings in *Arabidopsis* and other species, which have revealed that *CBF* orthologs are transiently induced by LT with mRNA abundance peaking at 4 - 12 h after cold exposure (Medina *et al.* 2011, Tondelli *et al.* 2011). In the whole process, the maximum mRNA content of all the three *PmCBF* genes was about 10^3 ~ 10^5 times as much as their minimum (or before treatment). Undulate *PmCBF* expression profiling in the control line was used to reflect and eliminate the disturbance of potential circadian rhythm of *PmCBF*s (Harmer *et al.* 2000, Fowler *et al.* 2005). It was evident that when *CBF* transcripts of the cold-treated lines accumulated to their peak at 8 - 12 h, the mRNA content of the control line was at a low tide. After 168 h of the LT exposure, the transcription of the three *PmCBF* genes was still higher than at 0 h. Of the three *CBF* genes, the increase in the *PmCBFa* transcription was 10 - 100 times higher

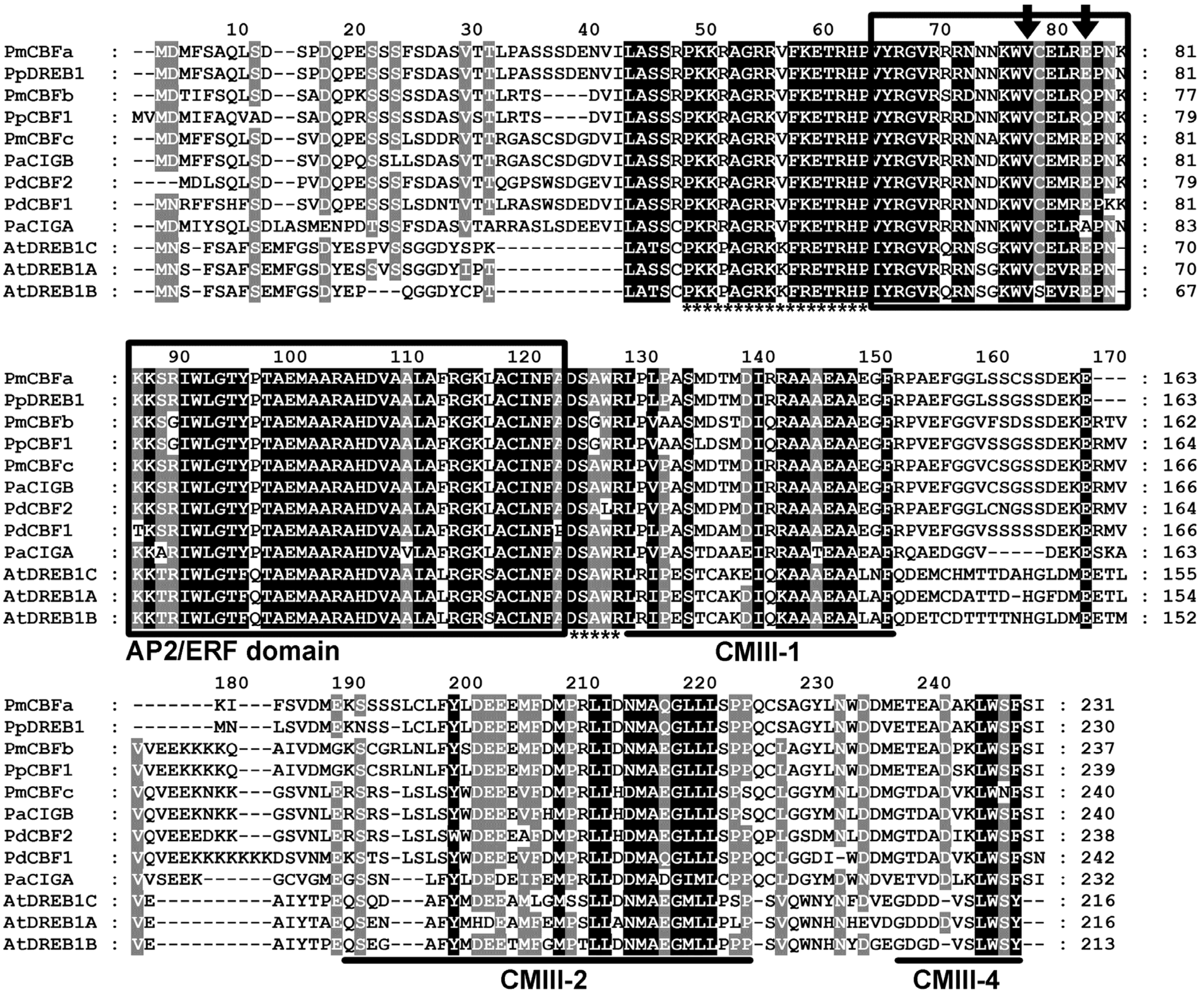

Fig. 2. The amino acid sequence alignment of PmCBF proteins from *Prunus* and *Arabidopsis*. *Marker line* and *dashes* indicate gaps introduced to optimize the alignment. Identical amino acids and conserved substitutions are *shaded black* and *gray*, respectively. The total number of amino acids for each deduced protein is indicated at the end of each sequence. The AP2/ERF-DNA binding domain is *boxed*. The CMIII-1,2,4 conserved motifs are *underlined by solid lines*. The "CBF signature" sequences (also called CMIII-3) are *underlined by asterisks*. The *arrows* indicate the conserved valine and glutamic acid residues. The CBF sequences and their GenBank accession numbers are as follows: PmCBFa (ADF43033.1), PmCBFb (ADF43034.1), and PmCBFc (ADF43035.1) from *Prunus mume*; PpDREB1 (ABR19831.1) and PpCBF1 (ADU03762.1) from *P. persica*; PaCIGA (BAC20183.1) and PaCIGB (BAC20184.1) from *P. avium*; PdCBF1 (AFL48190.1) and PdCBF2 (AFL48191.1) from *P. dulcis*; AtDREB1A (NP_567720.1), AtDREB1B (NP_567721.1), and AtDREB1C (NP_567719.1) from *Arabidopsis thaliana*.

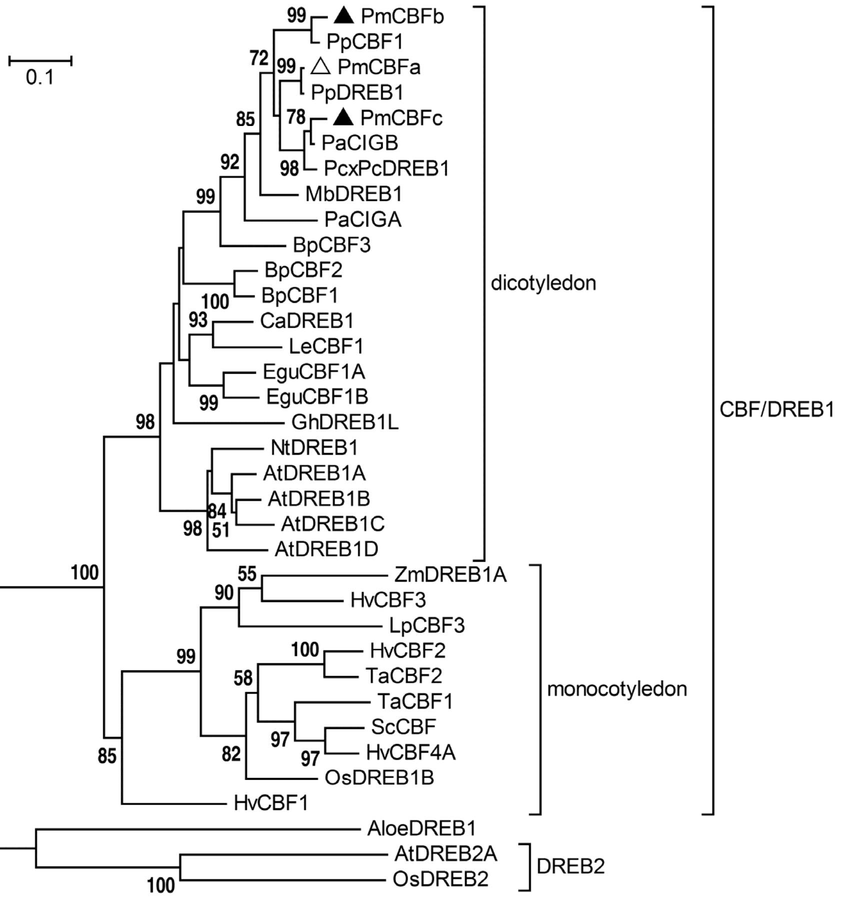

Fig. 3. A phylogenetic tree caculated using the neighbor-joining method. The *open triangle* indicates PmCBFa which was previously reported (Guo *et al.* 2012). The *closed triangles* indicate PmCBF homologs obtained in present work. Groups are indicated on the right. The bootstrap confidence values [%] from 1 000 replicates are indicated on the branches. The scale indicates the average substitutions per site. The sequences used for this analysis are as follows: PmCBFa (ADF43033.1), PmCBFb (ADF43034.1), and PmCBFc (ADF43035.1) from *Prunus mume*; PpDREB1 (ABR19831.1) and PpCBF1 (ADU03762.1) from *P. persica*; PaCIGA (BAC20183.1) and PaCIGB (BAC20184.1) from *P. avium*; Pc×PcDREB1 (ACF94686.1) from *P. canescens* × *P. cerasus*; MbDREB1 (ABQ59086.1) from *Malus baccata*; BpCBF1 (ABP98987.1), BpCBF2 (ABP98988.1), and BpCBF3 (ABP98989.1) from *Betula pendula*; CaDREB1 (AAR88363.1) from *Capsicum annuum*; LeCBF1 (NP_001234123.1) from *Solanum lycopersicum*; EguCBF1A (ABB51637.1) and EguCBF1B (ABB51638.1) from *Eucalyptus gunnii*; GhDREB1L (ABD65473.1) from *Gossypium hirsutum*; NtDREB1 (ACE73693.1) from *Nicotiana tabacum*; AtDREB1A (NP_567720.1), AtDREB1B (NP_567721.1), AtDREB1C (NP_567719.1), AtDREB1D (NP_200012.1), and AtDREB2A (NP_001031837.1) from *Arabidopsis thaliana*; HvCBF1 (AAL84170.1), HvCBF2 (AAM13419.1), HvCBF3 (ABE02630.1), and HvCBF4A (AAK01088.1) from *Hordeum vulgare*; ZmDREB1A (NP_001105651.1) from *Zea mays*; LpCBF3 (AAX57275.1) from *Lolium perenne*; TaCBF1 (AAL37944.1) and TaCBF2 (AAX28961.1) from *Triticum aestivum*; ScCBF (AAL35761.1) from *Secale cereale*; OsDREB1B (NP_001063710.1) and OsDREB2 (NP_001042107.1) from *Oryza sativa*; AloeDREB1 (ABJ09421.1) from *Aloe vera*.

compared with other *PmCBF* genes. Furthermore, the mRNA content of *PmCBFc* was always relatively lower than of *PmCBFa* and *PmCBFb*, especially after 168 h (Fig. 4). It would be reasonable to speculate that there were few *PmCBFc* transcripts in flower buds of *P. mume* harvested in late winter, which partly explained the loss of *PmCBFc* in gene cloning through RACE strategy.

Just when this article was being written, the genome of *P. mume* grown in nature (Tongmai, Tibet, China) was published. Genome sequencing indicates that there are about 13 possible *CBF* homologous genes and 7 downstream regulons predicted in *P. mume* (Zhang *et al.* 2012). We have aligned their published *CBF* homologous genes with ours, and the predicted proteins as well (data not shown), which show that there is great variation between them. This may be due to the genetic polymorphism of the *CBF/DREB1* family and may help to interpret the cold tolerance variation among populations within *P. mume* (Zhen and Ungerer 2008, Mboup *et al.* 2012). Furthermore, it is rare in literature that a *CBF/DREB1* family containing more than ten members exists in a woody perennial plant, so the small size of CBF regulon (7 dehydrin orthologs) is speculated to be partially responsible for the cold sensitiveness of *P. mume*. For instance, cold-sensitive tomato has a much smaller regulon in its CBF cold response pathway than freezing-tolerant *Arabidopsis* (Zhang *et al.* 2004).

Anyway, the recent work illustrates that PmCBFb and PmCBFc are orthologs of AtCBF and they are highly conserved with other CBF homologs in *Prunus*. *PmCBFa PmCBFb*, and *PmCBFc* have fluctuant constitutive expressions under normal conditions and they are distinctly up-regulated by LT. It is worth to investigate deeper the regulatory pathway of *PmCBF*s, especially that involved in cold tolerance. Since genome sequencing of *P. mume* has been finished, the main aspects of our work in the near future, *i.e.*, isolation of more *PmCBF* genes and their promoters, definition of their upstream transactivation factors, expression and functional analyses of *PmCBF* genes and their regulon in conditions simulating wintertime in nature, and transgenic programs, are in a position to uncover the genetic mechanism in cold tolerance of *P. mume* and add new contents in CBF pathway research.

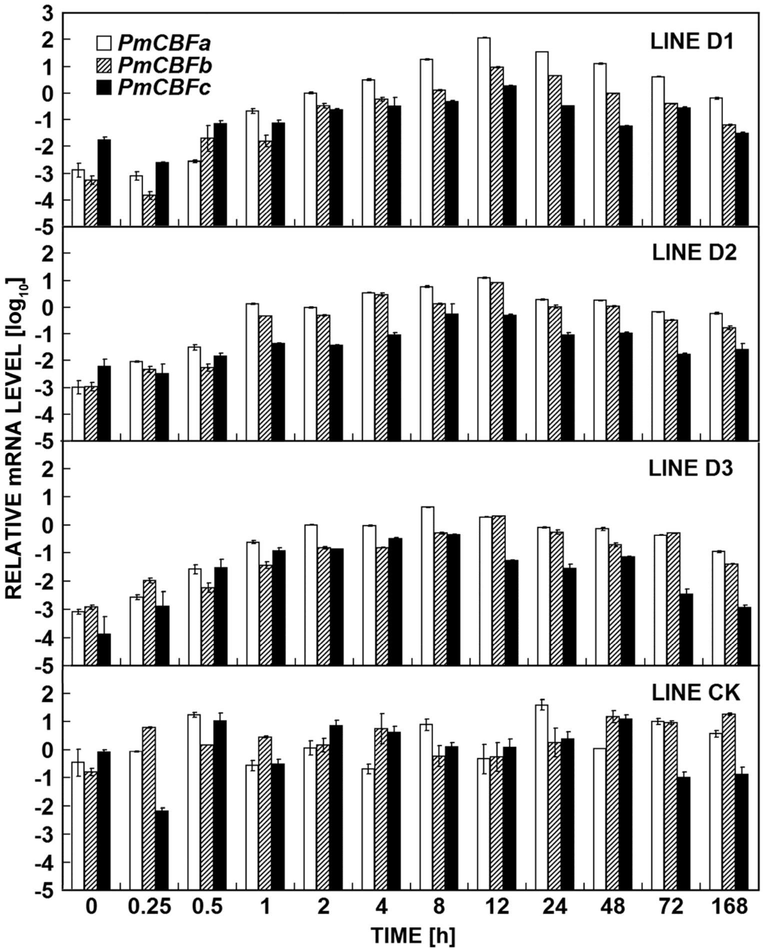

Fig. 4. The quantitative RT-PCR expression analysis of *PmCBF* genes in response to low temperature (2 °C). The relative mRNA content is transformed into the logarithm scale. Means ± SE, $n = 4$. The mRNA cntent of *PmCBFa* at time 2 h was used as calibrator to determine the fold change of mRNA abundance during treatments.

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