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Identification of a cold-inducible gene encoding calmodulin-binding protein from *Eucalyptus dunnii* through suppression subtractive hybridization

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Abstract

Low temperature is the main limiting factor for cultivation expansion, fast growth, and high yield of *Eucalyptus* species. To investigate the mechanism of their cold tolerance, a cDNA subtraction library representing the cold-induced genes of *Eucalyptus dunnii* was constructed using suppression subtractive hybridization (SSH) technique. A gene encoding a calmodulin-binding protein (*CaMBP*) was identified from the SSH library, and the expression pattern of *CaMBP* under cold stress was further evaluated through reverse transcription - quantitative polymerase chain reaction (RT-qPCR). The expressions of *EguCBF1a* and *EdCaMBP* increased already after 4 h of the cold stress, supporting the idea that the *CaMBP* gene may have a function in the survival of *Eucalyptus* during winter. A full-length mRNA sequence of 1 808 bp was obtained *via* a rapid amplification of cDNA ends method, and the sequence was subsequently deposited to *GenBank* (accession No. JX401571). The *CaMBP* cDNA of *E. dunnii* contained a single open reading frame of 1 362 bp, a 5′ untranslated region of 175 bp, and a 3′ untranslated region of 268 bp. Multiple sequence alignment and phylogenetic analysis indicated that *CaMBP* of *E. dunnii* shared 56 to 84 % identities with the *CaMBP*s of other plants and was similar to that of *Ricinus communis*, *Medicago truncatula*, and *Gossypium hirsutum*.

Additional key words: cold acclimation, eucalypt, phylogenetic tree, RT-qPCR, stress tolerance.

Introduction

Eucalyptus genus originates from Australia and comprises more than 700 species. They are important in both commercial plantation and ecological restoration of degraded lands (Eldridge *et al*. 1993, Yu *et al*. 1994, Yu and Wang 1995, Brooker 2000, Zhou *et al*. 2002). Low temperature is one of the main limiting factors for cultivation expansion, fast growth, and high yield of *Eucalyptus* species. A considerable effort has been exerted toward understanding how *Eucalyptus* species respond and adapt to low temperature, but the majority of studies have been mainly concerned on extensively planted species, such as *E. globulus*, and species that are resistant to cold temperatures, such as *E. gunnii* (Travert *et al*. 1997, Fernández *et al*. 2006, Moraga *et al*. 2006, Rasmussen-Poblete *et al*. 2008, Costa *et al*. 2009). *Eucalyptus dunnii* has a limited natural distribution compared with other widely planted species, but may have a potentially significant function in *Eucalyptus* plantations. Trials in Australia and in limited-scale commercial plantations in South America have proven that *E. dunnii* is capable of superior growth and survival under cold (as low as -5 °C), and drought stresses (Jovanovic *et al*. 2000). In a study that we conducted in China, we used different *Eucalyptus* species to prove that *E. dunnii* has wide temperature tolerance from low to high temperature. However, few studies have been performed on the mechanism of cold tolerance in *E. dunnii*.

Molecular studies have demonstrated that CRT/DRE-binding factor (CBF) transcription factors have central functions in the control of cold tolerance in plants (Fowler and Thomashow 2002, Cook *et al*. 2004, Gilmour *et al*. 2004). Characterization and functional studies on *Eucalyptus CBF* genes have also been reported (El *et al*. 2006, Navarro *et al*. 2009, 2011). Previous studies have

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Abbreviations: CaMBP - calmodulin-binding protein, CBF - CRT/DRE-binding factor, ORF - open reading frame, RACE - rapid amplification of cDNA ends, RT-qPCR - reverse transcription - quantitative polymerase chain reaction; SSH - suppression subtractive hybridization.

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suggested that the expressions of *CBF* genes in response to cold may be the key to winter survival in *Eucalyptus*. Despite numerous studies about *CBF* genes, their expressions, regulation, and roles in freezing tolerance and cold acclimation remain undetermined. Large-scale expressed sequence tags (EST) have revealed other cold-related genes in some cold-inducible *Eucalyptus* trees (Rasmussen-Poblete *et al*. 2008, Keller *et al*. 2009). Whether isolated ESTs are closely related to cold tolerance of *Eucalyptus* species remains undetermined, and no other *CBF* genes have been further isolated and characterized using the aforementioned basis.

Suppression subtractive hybridization (SSH) technique (Diatchenko *et al*. 1996) has been used for the identification of novel genes related to plant tolerance to various stresses (Zhu *et al*. 2008, Liu *et al*. 2010, Long *et al*. 2012, Kumar *et al*. 2013). To investigate the mechanism of cold tolerance in *Eucalyptus*, a cDNA subtraction library representing the cold-induced genes of *E. dunnii* has been constructed using SSH technique. In the present study, we screened the SSH library using sequencing and the reverse transcription - quantitative polymerase chain reaction (RT-qPCR). The candidate genes sensitive to cold temperatures like *CBF-like* genes were further selected and analyzed by comparing the expressional pattern in *E. dunnii.*

Materials and methods

Plants cold treatments: Forty *Eucalyptus dunnii* Maiden seeds were germinated on sterilized filter paper. Seven-day-old seedlings were transferred to a hydroponic facility using a Murashige and Skoog (1962, MS) medium. Upon reaching the five-leaf stage (about 30 d after transplanting), a low-temperature treatment was initiated. A half of the seedlings were removed from a normal culture temperature of 25 ºC and transferred into gradually decreasing temperatures of 22 ºC for 1 d, 19 ºC for 1 d, 16 ºC for 1 d, 13 ºC for 1 d, 10 ºC for 1 d, 7 ºC for 1 d, 4 ºC for 1 d, 1 ºC for 1 d, and 0 ºC for 1 d. The other seedlings remained at 25 ºC were used as control. The hydroponic culture was conducted in a greenhouse with a 16-h photoperiod, an irradiance of 180 μmol(photon)·m^{-2} s^{-1}, and a relative humidity of 60 - 70 %. After 9 d, leaves of 39-d-old plants were harvested from two treatments separately, quickly frozen in liquid nitrogen, and stored at -80 °C until further use.

Suppression subtractive hybridization: Total RNA extraction from *E. dunnii* leaves cultured in both the treatments (control and the cold stress) was performed using a *Trizol* reagent (*Gibco*, Eggenstein, Germany) according to the manufacturer's instructions. Total RNA was then reverse transcribed and amplified with a *SUPER SMART* cDNA synthesis kit (*BD Biosciences*, Clontech, USA). The amplified cDNA samples were then used for the subtraction library construction using a PCR-select cDNA subtraction kit (*BD Biosciences*) according to the manufacturer's instructions and Li *et al*. (2014). The final secondary PCR products were cloned into *pMD19-T* vector (*Takara*, Dalian, China) and transformed into ultracompetent JM109 *Escherichia coli* cells. Positive clones were randomly picked and sequenced by an automated DNA sequencer *ABI 377* (*Applied Biosystems*, Foster City, CA, USA.

RT-qPCR analysis: The RT-qPCR technique was used to evaluate and compare gene expressions in *E. dunnii* cultured in the normal temperature of 25 ºC and the low temperature of 4 ºC. The culture conditions and cold treatments were the same as those described. After 1 d of the low temperature treatment, 31-d-old leaves were harvested separately, quickly frozen in liquid nitrogen, and stored at -80 °C until further use. The cDNA was synthesized as described above using *E. dunnii* leaves under the two treatments. The specific primer sets for RT-qPCR were designed with the *Oligo 7.57* software (*MBI*, NY, USA) based on the sequenced cDNA from the SSH cDNA library (Table 1). The cDNA was diluted and amplified using a *7500 Fast* real-time PCR system (*Applied Biosystems*) and *SYBR Premix Ex Taq*™ kit (*Takara*) according to the manufacturer's instructions. Standard curves for individual unigenes were calculated using a dilution series of pMD19-T vector containing the target gene. The relative expressions of the genes in the low temperature-treated seedlings compared with the untreated ones were determined based on comparisons with the reference gene *18S* rRNA. The expression of *EuCBF1a* was also used for comparison according to El *et al*. (2006). All RT-qPCR experiments were performed in triplicate with RNA isolated from at least three different seedlings. For the data analysis, three methods were used to compare results: the conventional $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen 2001) based on *StepOne Software V2.2* result data, the miner method (Zhao and Fernald 2005) from raw data, and the FPK-PCR method (Lievens *et al*. 2012) from raw data.

3′-and 5'- rapid amplification of cDNA ends: A *SMART RACE* cDNA amplification kit (*BD Biosciences*) was used to perform both 5′ and 3′ RACE. First-strand cDNA was synthesized from 5 μg of total RNA by using the adapter primer provided by the manufacturer. The 3′ and 5′ target cDNAs were initially amplified using the provided universal primer and gene-specific primers (C4C10F for 3' RACE and C4C10R for 5' RACE, Table 1) based on the sequence of the original positive clone C4C10. PCR consisted of 30 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. The PCR products were then diluted five times and used as templates for following nested PCR using a second set of nested, gene-specific primers (C4C10fr for 3' RACE, C4C10rf for 5' RACE, Table 1).

Twenty five rounds of PCR were carried out according to the parameters described above. The 5′- and 3′- RACE products were excised from 1.5 % agarose gels and purified using a *BioSpin* gel extraction kit (*Bioer*, Hangzhou, China). The bands were then ligated into pMD-19T vector (*Takara*). The inserted DNA fragments were sequenced by the *ABI 377* automated DNA sequencer. The nucleotide sequences of the cloned PCR products were determined using at least three samples isolated from different colonies.

Sequence analysis: The 5′-and 3'-RACE products were edited and assembled using the *ContigExpress* software (*Vector NTI*, *Invitrogen*, Carlsbad, CA, USA). The conceptual translation of the nucleotide sequence was made using the *Open Reading Frame Finder* program (ORF, www.ncbi.nlm.nih. gov/gorf/gorf.html). Percent identities were obtained by blasting two sequences with the *BLASTP* server by using the *BLOSUM 62* matrix in the National Center for Biotechnology Information's web server. Sequences were aligned with *ClustalX v. 1.81* (Thompson *et al*. 1997) and the multiple sequence alignment was assembled by using *BOXSHADE SERVER 3.21* (http://www.ch.embnet.org/ software/BOX_form. html). A phylogenetic tree was constructed using *MEGA v. 5* based on the neighbor- joining (NJ) method (Tamura *et al*. 2011). All the unambiguous characters and character transformations were weighed equally. A bootstrap test was performed using 1 000 replicates. Gaps were considered by selecting the pairwise deletion option in *MEGA*. The full-length nucleotide sequences for *E. dunnii CaMBP* were deposited in the *GenBank* database and assigned the accession No. JX401571.

Table 1. Main primers used in this study.

Clone	Primer	Sequence	Clone	Primer	Sequence
C3F05	C3F05F	TTTCTGCAATAACCTTCCCTGAA	C2G10	C2G10F	AACGGGAAGCTGGTTTACCA
	C3F05R	CCGGCAACATCGTTGAAAG		C2G10R	CCTTGTTGCTTGAAGCTTTGAA
C3B10	C3B10F	CTTCATCAATCTCTGTGGTCATCAT	C3G11	C3G11F	TCCCGTATTTGACCGCTCTCT
	C3B10R	TGTGTGGGATGCTGGTGAAC		C3G11R	CGCCACCAATCGAAGATCTT
C4A10	c4a10f1	GCATGTGCAAGAGATTCATTCC	7B7E9	7b7e9f1	TCCTCCCATTCTTCGTTGATTT
	c4a10r1	AGTTCCATGGGTTTTGTTGAAGA		7b7e9r1	TGGCGACCGAGACGAAAC
C4C10	C4C10F	TCCCGTTTCATACATGATTGCT	C3E11	C3E11F	AACGACCTTTCCCCACTACAAG
	C4C10R	CATCGAGTTGGTGAGCTTTGG		C3E11R	GGCTTAAACCATCACCATGACTT
C4D09	C4D09F	TCTGCACCTCCAATGTTGCA	7B7H5	7b7h5f1	TTACAGTGGAGAGAACACTTTTGGTT
	C4D09R	TACTAAAGCCAGGAGGACCATGA		7b7h5r1	GTCACCAAGTCGAAAGAGGAAGA
C3G02	C3G02F	AATACTTTGTGGTTTCCCATTCTTG	7B5F6	7B5F6F	CGATACTCGGAGGGTTCACTAGTT
	C3G02R	GAAACCCGCTGGAAAGTTTG		7B5F6R	CAGCACCCCAACCAGTTCTC
C3D03	C3D03F	TGCAGGATCCGGAGATTCA	7B5G10	7B5G10F	GCTTTTTGCCGAGATTAACCTAAG
	C3D03R	CGCTGACCAGCTTCTGGATAT		7B5G10R	CAAAAATTCTCTTTTCTCCCTATGAAC
C1D11	C1D11F	CCAAGGTGGGTTGCATTTG	C2G07	C2G07F	TTACCTGTACTTTAGCAGCGTCTTG
	C1D11R	TTCACAAGATTTGCAGCAATTTG		C2G07R	ACCCCAACCATTAGCCATCA
7B5G12	7B5G12F	AAGTTTCCCAGGCCCATTGT	C1E05	c1e05f1	GCTTGGATAGGAAAAATTCGATGA
	7B5G12R	GGCCAACAGACTTGTCAATGTG		c1e05r1	CAGCCGACACCATTCAAATTG
7B5G6	7B5G6F	AACATGATGATAGCAAATGGAAGGT	C4E12	C4E12F	TCCATACATTCGCACCATTGA
	7B5G6R	CTCCATAGCCAGCATTCATGTC		C4E12R	CAATACCTATGGAGGCACTTTGC
C1E06	C1E06F	TTCTGGGCTCGTCCGTCTT	C1H05	C1H05F	CTTGTTCTCGTTCCTGGTCTGA
	C1E06R	CAGGAGACTGGCCAAAGTAGTTTC		C1H05R	ACTTGGAGCTCTGCAACTAGGAAT
C4E11	C4E11F	GGTCCCACCTTCTCAGGACAT	C3D10	C3D10F	GAAGGACAATGCCGGTAACG
	C4E11R	TTGCCATTGCAGAACCCAAT		C3D10R	GCTGATGGGCAAACCAGAAG
C1E12	C1E12F	TCCCCTTTGAATAATCCGATCA	C3E02	C3E02F	TTCAGAGCTAGCACTTCCGAGAT
	C1E12R	TGTCACAATTAAGGGTGCTTATGAG		C3E02R	TAGACACCCATCCGTCATCATC
7B7F2	7b7f2f1	AACTGCCTTTGATGATTATTTTGCT	C2G01	C2G01F	AGAGGCTACTTGCCGATGATTT
	7b7f2r1	GCCCCGGACCTTGAATTT		C2G01R	TCTCTTCGCCGCTCCAACT
7B7G7	7b7g7f1	GCCATTACTTCTGCCCAACAA	C2G12	c2g12f1	AATCGCCTCCAATTCAATCG
	7b7g7r1	CCTTGCCGTCTTCAGTTACGA		c2g12r1	GGGCAGAAGACGAGCGTAAG
C4B07	c4b07f1	AGTGCAGGACTCTGGGAAGCT	C2D06	c2d06fi	TGGCAACTTACATTCGGGTTAA
	c4b07r1	GGCGCAGGCCAAGGAT		c2d06r1	GCCTGATTATGCTTGAGAAGATATTTC
C1C08	c1c08f1	GCACGTGCTCTTCAGCTTGA	C3F10	c3f10f1	ATGCTGCGACTGGCTGCTAT
	c1c08r1	ATAGCCTTCGTGCGCCAAT		c3f10r1	CTAGAGCCAATGCTCCTGCTAAA
18s	Eg18Sf1	CGCGCTACACTGATGTATTC	EguCBF1a	Egc1af1	GGTACGAAGCCATTTTTTTGGT
	Eg18Sr1	GTACAAAGGGCAGGGACGTA		Egc1ar1	GCCCAACATCATCATCGATATG
EuCaMBP	C4C10fr	AGCAATCATGTATGAAACGGG			
	C4C10rf	CCAAAGCTCACCAACTCGATG			

Results

The RT-qPCR technique was used to evaluate the expressional differences of genes found in the cold-treated samples and controls. A total of 34 positive clones from the SSH library were randomly selected for the RT-qPCR analysis. The conventional RT-qPCR analysis based on the $2^{-\Delta\Delta C_T}$ method requires a standard curve for the estimation of amplification efficiency (E) and deduces the Cq (quantification cycle, formerly known as Ct) value subjectively. However, the $2^{-\Delta\Delta C_T}$ method cannot satisfy the need to screen plenty of genes (Livak and Schmittgen 2001). Therefore, we compared the conventional $2^{-\Delta\Delta C_T}$ method with two newly developed methods (the miner method and FPK-PCR method) that individually calculate E and Cq from the amplification data. We found that the result based on the miner method was closer to that of the $2^{-\Delta\Delta C_T}$ method and comparatively more stable during analysis, whereas the FPK-PCR method required more reaction cycles and a strict amplification curve to obtain reasonable results (Table 1 Suppl.). Moreover, approximately one-third of the genes did not show a higher expression in the cold-treated plants compared with the controls. In addition, most genes were expressed obviously in both the cold-stressed and control plants, but the genes expression was higher under the cold-stress. Our results indicate that conventional SSH had a low positive rate, and mainly detected genes with a higher expression under the cold-stress instead of specific genes. Five genes (*C3F05*, *C3B10*, *C4A10*, C4C10, and *C4D09*) with the highest expression ratio between the cold-stressed and control plants were chosen as candidates for further study using the $2^{-\Delta\Delta C_T}$ and miner methods.

The expression patterns of the five candidate genes were compared with that of *EguCBF1a* which is the most sensitive gene among *Eucalyptus* cold-inducible genes according to the literature (Navarro *et al*. 2011). Two typical states (4 h and 24 h) of *EguCBF1a* after the cold treatment were chosen in addition to the room temperature control according to a previous report (El *et al*. 2006). Four genes, namely *C3F05*, *C3B10*, *C4C10*, and *C4D09*, all showed a higher expression after the 24-h cold treatment unlike *C4A10* that was not amplified because of a low template concentration. Among these genes, only *C4C10* was expressed as early as 4 h after the treatment indicating that *C4C10* responded quickly to the low temperature and was most sensitive to cold induction among all four candidate genes (Fig. 1). Furthermore, the expression pattern of *C4C10* was similar to that of *EguCBF1a*, which suggests that *C4C10* may have a function in the cold response of *Eucalyptus*.

To obtain full-length cDNA of the SSH clone *C4C10,* its original partial cDNA sequence was used to design gene-specific primers for 5′and 3′ RACE. The resulting *C4C10* PCR products for 5′ and 3′ RACE were 1 267 and 93 bp long. Both sequences overlapped with the initial *C4C10* and yielded a total product size of 1 808 bp with an 1 362 bp open read frame (ORF), as well as 175 bp 5′ and 268 bp 3′ untranslated regions (*GenBank* acc. No. JX401571). The *BLAST* analysis revealed that *C4C10* was a calmodulin (CaM)-binding homologous gene unlike in previous studies where analysis yielded ‘no hits’. Therefore, most gene fragments isolated from SSH occupied the 3′ region of genes which are not

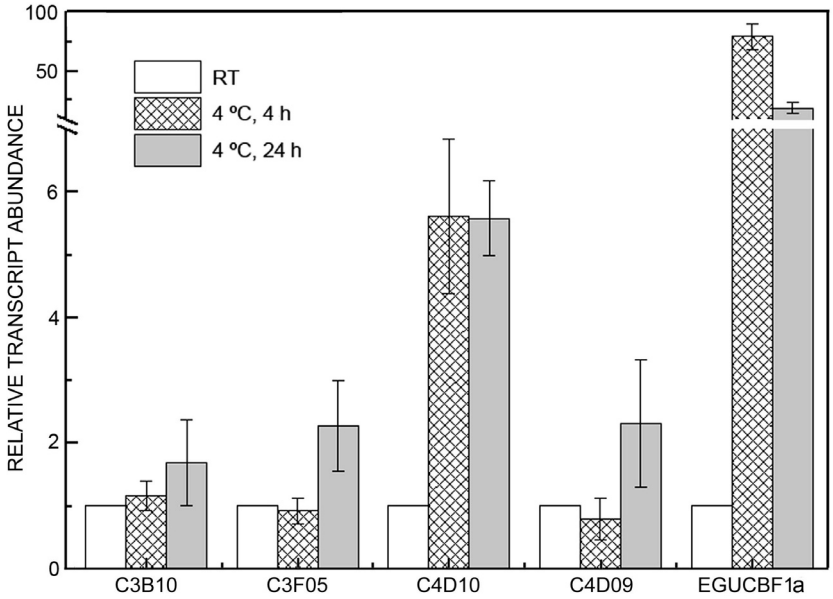

Fig. 1. Comparison of gene expressions after the cold treatment among the selected genes and *EguCBF1a* in *E. dunnii*. The expressions of genes are normalized by the *18S* endogenous control. The transcript abundance of each treatment is normalized by the expression value at room temperature (RT). Four seleted genes *C3B10*, C3F05, *C4C10*, and *C4D09* corresponded to the four clones listed in Table 1 Suppl.

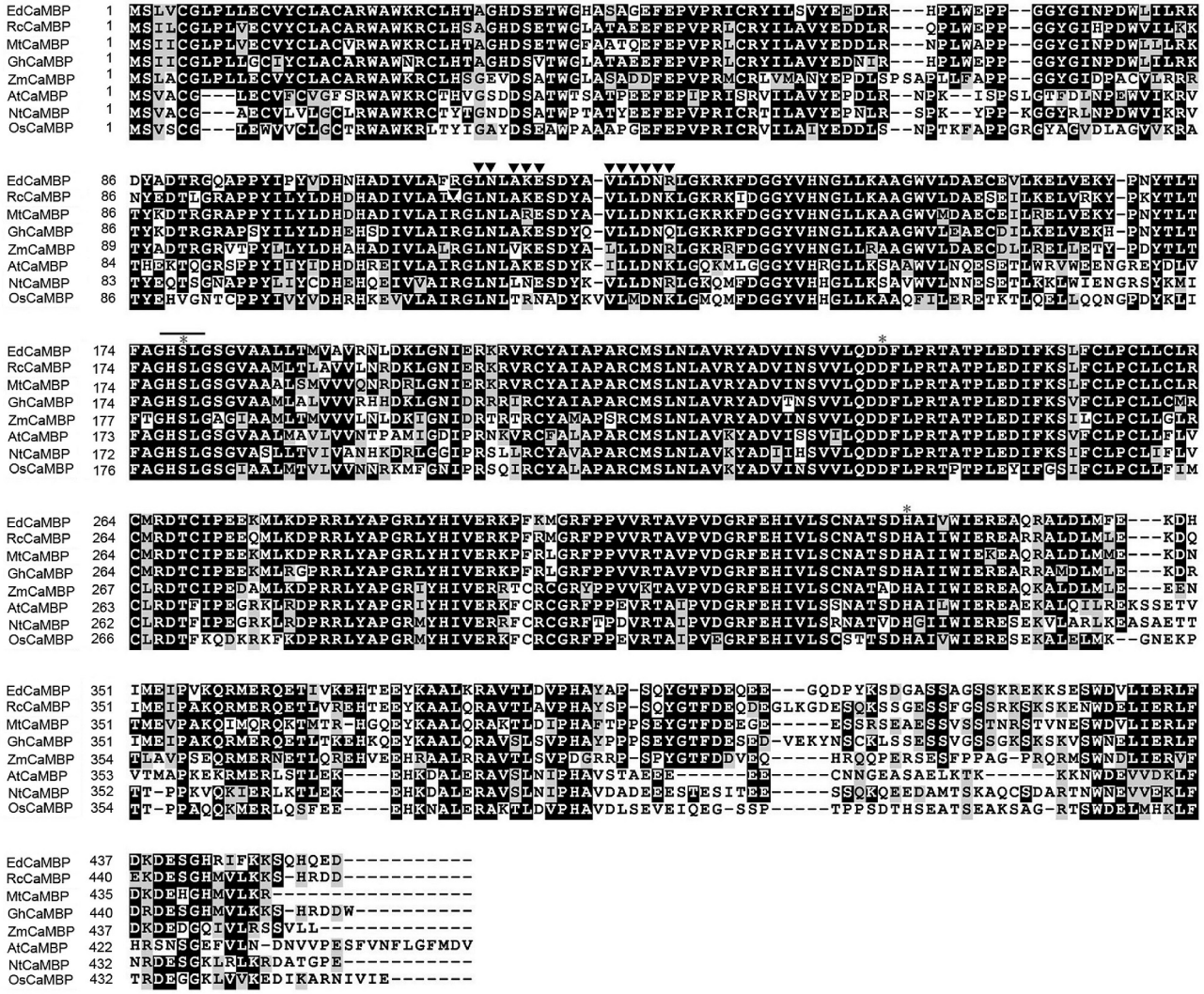

Fig. 2. The multiple sequence alignment of the deduced *E. dunnii* CaMBP with other plant CaMBPs retrieved from *GenBank*. Amino acids identical to *E. dunnii* are *shaded*. The *solid bars* denote the nucleophilic elbow (GHSLG). The residues of the catalytic triad (Ser_Asp_His) are denoted by the *asterisks*. The residues of active site flap/lid are denoted with the *triangles*. Calmodulin-binding proteins (CaMBPs): EdCaMBP - *Eucalyptus dunnii*, JX401571; AtCaMBP - *Arabidopsis thaliana*, BAB08316.1; GhCaMBP - *Gossypium hirsutum*, AAV73918.1; MtCaMBP - *Medicago truncatula*, XP_003592266.1; NtCaMBP - *Nicotiana tabacum*, AAB34987.1; OsCaMBP - *Oryza sativa*, BAD46303.1; RcCaMBP - *Ricinus communis*, XP_002513688.1; ZmCaMBP - *Zea mays*, NP_001149987.1.

usually conserved. The multiple sequence alignment of the deduced *E. dunnii* CaMBP protein, labeled as EdCaMBP, along with other plant *CaMBP*s are shown in Fig. 2. The results indicate that the EdCaMBP homologous protein included one conserved lipase3_N region spanning residues 9 to 74 (N-terminus) and one conserved lipase_3 region spanning residues 109 to 244. *EdCaMBP* ORF shared 56 to 84 % identities with *CaMBP*s from *Ricinus communis* (84 %), *Medicago truncatula* (82 %), *Gossypium hirsutum* (81 %), *Zea mays* (70 %), *Nicotiana tabacum* (57 %), *Oryza sativa* (57 %), and *Arabidopsis thaliana* (56 %) indicating that this gene is highly conserved because *EdCaMBP* shares 70 % identity with its counterpart in monocot plant *Z. mays*. The comparison of *E. dunnii* CaMBP with the other CaMBPs revealed the existence of a serine motif (nucleophilic elbow, GHSLG) common to serine hydrolases, a catalytic triad (Ser_Asp_His), and three active site flap/lid (LN, AKE, and VLLDNR). The catalytic motif has been found at the active sites of enzyme families with different functions, such as serine proteases, lipases, and feruloyl esterases suggesting that the Ser_Asp_His triad is an independent catalytic motif that has been coupled to different binding sites, in nature to perform different functions (Sotirios *et al*. 2006). The presence of a catalytic triad in *Eucalyptus* CaMBP suggests that the protein has a similar catalytic activity involved in the cold response pathway.

Further, a phylogenetic tree resulting from the analysis of full-length CaMBP amino acid sequences based on the consensus neighbor-joining (NJ) method presents that

E. dunnii CaMBP was grouped with CaMBP from *R. communis*, *G. hirsutum*, and *M. truncatula*, supported by a 100 % bootstrap value that corresponds to the high identity of amino acid sequences of CaMBP in the three plants (Fig. 3).

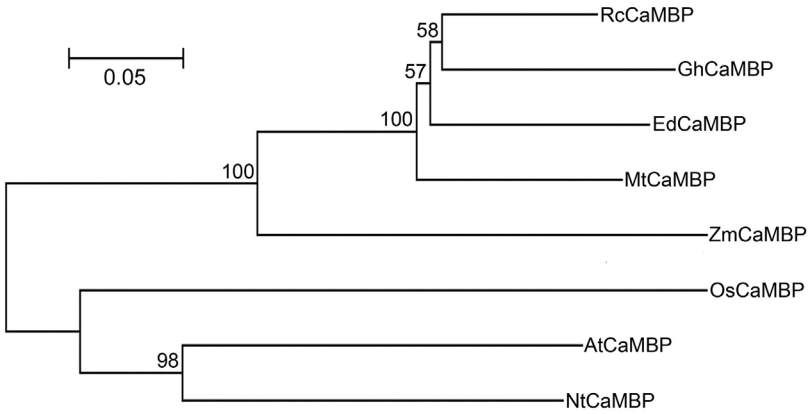

Fig. 3. A neighbor-joining phylogenetic tree generated for the deduced full length *Eucalyptus dunnii* CaMBP sequence and similar CaMBP sequences from other plants. Amino acids: the Kimura two-parameter distance model, partial deletion, bootstrap = 1 000, the numbers in each node represent bootstrap support values (those lower than 50 are not shown). Calmodulin-binding proteins (CaMBPs): EdCaMBP - *Eucalyptus dunnii*, JX401571; AtCaMBP - *Arabidopsis thaliana*, BAB08316.1; GhCaMBP - *Gossypium hirsutum*, AAV73918.1; MtCaMBP - *Medicago truncatula*, XP_003592266.1; NtCaMBP - *Nicotiana tabacum*, AAB34987.1; OsCaMBP - *Oryza sativa*, BAD46303.1; RcCaMBP - *Ricinus communis*, XP_002513688.1; ZmCaMBP - *Zea mays*, NP_001149987.1.

Discussion

The isolation of differentially expressed genes is extremely important in molecular studies of trees because the genetic background is usually absent for trees, leading to an inconvenient performance of large-scale functional studies. When using the SSH method, positive clones are usually screened in the library using the hybridization method which probably neglects many valuable genes that could exhibit changes in their expression to some extent during stress. With the reduction of sequencing cost and development of the RT-qPCR method, screening the positive clones from the SSH cDNA library using the high-throughput qPCR method became more convenient. The qPCR screening experiments indicate that the majority of genes from SSH cDNA clones showed a higher expression under the cold treatment than in the controls. However, only a few genes showed a significant difference in terms of expression. The *BLAST* search on EST sequences also revealed plenty of homologous genes related to the cold stress. Therefore, it is a valuable strategy for differential screening the SSH library using EST sequencing combined with RT-qPCR.

In this study, RT-qPCR was used in the gene selection and expression analysis. RT-qPCR is a sensitive and accurate method for gene detection. However, the reliability of qPCR depends on the proper analysis method used for plenty of raw data. The $2^{-\Delta\Delta C_T}$ method is usually used for data analysis in qPCR which has an ideal amplification efficiency of 100 % for all samples. The $2^{-\Delta\Delta C_T}$ method strictly requires the calculation of PCR efficiency based on the standard curves made using gradient dilution, which is a time-consuming process that cannot fulfill the demand for filtering plenty of genes. FPK-PCR and miner based on individual amplifications are two new methods that were used in our study to estimate the expression level of cold-inducible genes. FPK-PCR claims to provide more reliable results. However, the FPK-PCR method is unacceptable in some analyses, possibly because the method requires more than 40 cycles (usually 60 cycles) (Lievens *et al*. 2012). The miner method is relatively more flexible and acceptable for expressional screening plenty of genes in most treatments. We found that the result of approximate estimation using the $2^{-\Delta\Delta C_T}$ method without the standard curve is often similar to the results obtained using the miner method, suggesting that the $2^{-\Delta\Delta C_T}$ method may also be acceptable in large-scale pre-screening candidate genes.

CaMBP homologous genes encode transduction proteins, such as protein kinases and transcription factors and effector proteins, such as ion transporters and enzymes (Bouché *et al*. 2005). The *CaMBP* homologous genes are closely related to calcium regulation in the cell, which is essential for cell responses to a variety of environmental stimuli including low temperature. Plant *CaMBP* genes have important functions in development, metabolism, cell skeleton formation, phytohormone response, and stress tolerance by interaction with CaM, which is the most important Ca sensor protein in the cell (Bouché *et al*. 2005). Evidence on the links between calcium signaling and the CBF pathway during cold induction was found upon discovering that CaM binding transcription activator (CAMTA) factors bind to a regulatory element in the

CBF2 gene promoter (Eckardt 2009). However, researchers have obtained limited information on the relationship of the *CaMBP* homologous genes with cold induction in model plants like *Arabidopsis* (Reddy *et al*. 2011). The *EuCBF1a* and *EdCaMBP* expressions increased during the early stage and then slowly declined. The expressions of *EuCBF1a* and *EdCaMBP* were obviously different from the expressions of other cold-inducible genes isolated in this study and also from seven C2H2-type zinc finger genes (*EgrZFP1-7*) from *E. grandis* isolated by Wang *et al*. (2014), suggesting *EdCaMBP* may have an important function in the cold tolerance of *E. dunnii*. However, further studies should be performed to decide which kind of protein EdCaMBP belongs to. Future research should include cloning more *Eucalyptus CaMBP* paralogous genes, more detailed phylogenetic analysis, and functional verification.

Cold acclimation involves changes in gene expression and cell metabolism. In the process of cold acclimation, the transcriptional activation and repression of genes by low temperature are important (Thomashow 1999). The phenotype of cold acclimation in *Eucalyptus* may vary among species. Most *Eucalyptus* species are neither the most resistant nor the most sensitive to cold; therefore, *E. dunnii* has been used in our studies on cold acclimation because of its ability to grow under low as well as high temperature and also under drought. The data collected in this study can serve as important comparison materials for further studies on the cold acclimation of *Eucalyptus* species.

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