Pak. J. Bot., 44(3): 961-968, 2012.

MOLECULAR CLONING AND EXPRESSION ANALYSIS OF TWO CALMODULIN GENES ENCODING AN IDENTICAL PROTEIN FROM *CAMELLIA OLEIFERA*

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Abstract

Calmodulins, members of the EF-hand family of Ca^{2+}-binding proteins, play significant regulatory roles in the processes of plant cell proliferation, growth, development, photosynthesis and stress resistance. Here, the two full-length complementary DNA (cDNA) clones were isolated from the constructed *Camellia oleifera* cDNA library. They are 953 base pair (bp) and 1024 bp in the length, respectively, and designated as *CoCaM1 and CoCaM2* (GenBank access numbers EU856536 and FJ649316). They contain the complete protein-coding region of 447 bp encoded an identical polypeptide of 149 amino acids and various lengths of untranslated segments. The result supported the hypothesis "Multigenes possess an identical amino acid sequence". The structure analyzed results indicated the putative CoCaM protein possessed four EF-hand domains, hydrophobic residues and crucial residues, in which the amphipathic helices might possess lipid affinity via binding and altering the associated plasma or organelle membranes. In addition, the protein had one to seven substitutions, and displayed more than 89% identity at the amino acid level in comparison to other species CaMs. Finally, the expression of the 2 cDNA genes in matured seeds of 6 *C. oleifera* varieties was analyzed by real-time fluorescence quantitative PCR, and the result showed that there were different expression levels. In conclusion, the structure properties together with the observed expression levels indicate that they may play different roles in the processes of development, lipid biosynthesis and stress responses of *C. oleifera* seeds.

Introduction

Camellia oleifera is one of the most important ligneous edible oil trees in China, and widely planted due to its strong adaptability. Its oil possesses higher edible and medicinal value for enriching unsaturated acid of oleic acid and linoleic acid, and thus is beneficial to human health (Zhuang, 2008). Its lower oil-yield, however, is a main bottleneck of *C. oleifera* production for a long time. Numerous efforts have been carried out to explore the mechanism of lipid biosynthesis. One of our representative researches is that the cDNA library and EST library of nearly matured seeds have been constructed, in which 2 clones of calmodulin (CaM) abundantly expressed draw our attention, suggesting that CaM might be correlated with lipid biosynthesis (Hu *et al*., 2004; Tan *et al*., 2006; Tan *et al.*, 2010).

Calmodulin (CaM) is a small acidic protein, and possesses eight helices, four EF-hand "motifs" (helix-loop-helix) and a central helix region. And the hallmarkers of CaM are apparent ubiquitous distribution among eukaryotic organisms and high degree of amino acid sequence conservation (Nakayama *et al.*, 1992; Perera & Zielinski, 1992). Although CaM has no enzymatic activity of its own, the Ca^{2+}/CaM complex is able to regulate a variety of processes by modulating the activities of numerous target proteins, and thus processes regulatory roles of phytohormone signaling, protein folding, protein phosphorylation and dephosphorylation, phospholipid metabolism, transcription, environmental biotic and abiotic stimuli (Hepler & Wayne, 1985; Rao *et al*., 1998; Kim *et al*., 2009).

Researches have been focused on divergent CaM genes in a singe species encoding an identical protein. In animals, there are at least three CaMs (Chien & Dawid, 1984; Fischer *et al*., 1988), each of which encodes an identical CaM polypeptide; and in plants, there also present the same cases, *e.g. AtCaM-2* and *AtCaM-3* of *Arabidopsis thaliana* , and *SCaM-1* and *SCaM-3* of soybean encode identical isoforms, respectively (Perera & Zielinski, 1992; Lee *et al.*, 1995). These CaM genes are under selective pressure to encode an identical protein while maintaining their divergent nucleotide sequences (Chien & Dawid, 1984; Fischer *et al*., 1988; Perera & Zielinski, 1992). Accumulating evidences also suggest that each of the different CaM genes may have distinct and significant functions. Until now, there is no any information on CaM gene and its function in *C. oleifera*. As an initial step toward understanding the biological role(s) in *C. oleifera*, this study describes the cloning and expression of the two CaM cDNA genes encoding for an identical protein, and aims to ascertain their roles in the processes of seed development, lipid biosynthesis and stress resistance.

Materials and Methods

Materials: The constructed cDNA library and EST library of nearly matured *C. oleifera* seeds were used (Hu *et al.*, 2004; Tan *et al*., 2006). Six *C. oleifera* matured seeds, 'Huashuo', 'Huajin', 'Huaxin', 'Hengdong 31', 'Hengdong 65' and 'Hengdong 17' were collected and stored in -80°C for RNA preparation.

Cloning and identification of cDNAs: The two Mono Clones (rzots0_000451.y1.scf and rzots0_002160.y1.scf) from the *C. oleifera* cDNA library were chosen (Hu *et al*., 2004), and their incubation and plasmid DNA preparation

(Minipreparation) were carried out as described by Sambrook & Russell (1999). The polymerase chain reactions (PCR) were performed with vector-based primers T3: 5'-ATTAACCCTCACTACCCGGGA-3' and T7: 5'-GCGTAATACGA- CTCACTATA-3' in a 20-µl reaction containing 1×PCR buffer, 0.5 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 mM of each primer, 1U *Taq* polymerase and 10 ng DNA template. Cycling conditions consisted of precycling at 94°C for 5 min, and then 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s and elongation at 72°C for 1 min 30 s, and an elongation phase at 72°C for 7 min. The PCR products were analyzed by 1.5% (w/v) agarose gel and then sequenced (data not shown).

Analysis of the deduced amino acid sequence and homology: Database was retrieved using the NCBI server (http: //www.ncbi.nlm.nih.gov), and the analysis properties of the protein were performed by ProtParam (http://www.expasy.ch/tools/protparam.html), TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), SignalP 3.0 sever (http://www.cbs.dtu.dk/services/SignalP/). The secondary structure, central helix region, EF-hand region and Ca binding region were predicted by SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page= /NPSA/npsa_sopma.html), ScanProsite (http://kr.expasy.org/ tools/scanprosite/) and DNAStar Protean. The amphipathic helix structure was analyzed by Antherprot V4.3. And hydrophilicity and flexibility analyses were carried out by DNAStar Protean. The homologue relationship between deduced amino acid sequence of *C. oleifera* CaM and those of other species CaMs from GenBank data was analyzed by Vector NTI10.0, and displayed by Gendoc.

RNA extraction and cDNA synthesis: Total RNAs were isolated from the frozen seeds of six varieties *C. oleifera*, lysised by the 600µl of 3×CTAB with 1% 2-mercaptoethanol, followed by plant RNA kit reagent (OMEGA) according to the manufacture's instructions. The RNase-free DNase (OMEGA) was used to remove any remaining contaminating DNA from the total RNA extractions. An aliquot of each sample was run on an agarose gel. The concentration and purity (i.e. the A260/A280 ratio) of each RNA sample was checked with Genequant pro (Amersharm Biosciences). Reverse transcription was carried out with 2.2-µg total RNA as template to synthesize the cDNA first strand in a 20-µl reaction volume using RevertAid ^{TM} M-MuLV reverse transcriptase (Fermentas) and an oligo $(dT)_{18}$ primer.

cDNA standards and real-time fluorescence quantitative PCR (qPCR): *CoCaM1* foward primer 5'-CTTGGAACAGTGATGCGGTC-3', and *CoCaM1* reverse primer 5'-CAGCCTCACGAATCATCTCA-3'; *CoCaM2* forward primer 5'-CTTGGAACGGTGATGCGGTC-3', and *CoCaM2* reverse primer 5'-CAGCCTCACGAATCATC-TCA-3', designed by Primer Primer 5, were used to amplify 292-base pair (bp) cDNA standards *CoCaM1* and *CoCaM2* in 20-µl reaction volumes. The reaction volumes contain 1×PCR buffer, 0.5 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 mM of each primer, 1U *Taq* polymerase, and the 2.0-µl synthesized cDNA first strand of 'Hengdong 65' as template. Conditions for the PCR reaction were as follows: 1 cycle at 94 °C for 5 min, 36 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min. The program was terminated by a 7-min incubation step at 72 °C. These amplicons were purified using the PCR purification kit (Ambio) and cDNA fragment concentrations were measured with the Biophotometer (Eppendorf). The corresponding copy number was calculated using the following equation: copy number = $9.1 \times 10^{11} \times$ (mg of cDNA standard)/size of cDNA in kb). Serial dilutions from the cDNA standard were used to generate a standard curve in the range of 10^1-10^{10} copy number.

The total volumes of the qPCR reaction were 10 µl, and consisted of 5 µl of Maxima ^{TM} SYBR Green qPCR Master Mix (2×)(Fermentas), 3.4 µl RNase free water, 0.3 µl of each primer (10µM), and 2 µl of 20-fold cDNA dilutions as PCR templates. The primers were described as those of the standard generation. The cyclings consisted of 94°C for 5 min to activate DNA polymerase, followed by 40 cycles of 94°C for 30 s, 60°C for 40 s, 72°C 30 s. All cyclings were performed with the Roter Gene Q optical 72 well plates (QIAGEN). Three replicates of each reaction were performed, and data were analyzed by Software Version Rotor-Gene 2.0.2.4. Two replicate reactions of non-template controls were included in each run to ensure that reagents were free of contaminants. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that one single PCR product was detected by the SYBR Green dye. Quantities of specific mRNA in the samples were measured according to the corresponding gene specific standard curve.

Results

Cloning and identifying of cDNA genes: PCR amplifications of the two MonoClones with T3/T7 primers led to two products of size approximately 1100 bp (Fig. 1). They share high similarity (>5% similarity in the coding regions) with *CaMs* of *Prunus avium* and *Actinidia kolomikta* in the nucleotide level, and high degree of identity with other CaMs from GenBank data in the amino acid level, thus we propose that the two clones might be full-length CaM cDNAs, termed as *CoCaM1* and *CoCaM2* (GenBank access numbers EU856536 and FJ649316). *CoCaM1* comprises 953 nucleotides, which contains a 77-bp 5'-untranslated region (5'UTR), a 407-bp of 3' UTR, a 19-bp polyadenylation, and an opening reading frame (ORF) of 447 bp. While *CoCaM2* is composed of 1024 nucleotides, which includes an 84-bp 5'UTR, a 406-bp 3'UTR, an 84-bp polyadenylation and an ORF of 447 bp. The 2 cDNAs also contained the polyadenylation signal (AATAA), and one mRNA instability motif (AUUUA) was found followed by a poly (A) tail in the 3' UTR of *CoCaM2* (Fig. 2).

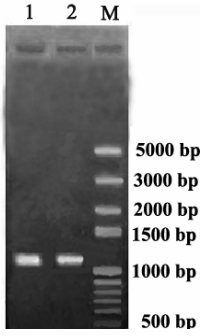

Fig. 1. PCR results of the cDNA clones 1: rzots0_000451.y1.scf , 2: rzots0_002160 .y1.scf, M: 100 bp plus DNA ladder.

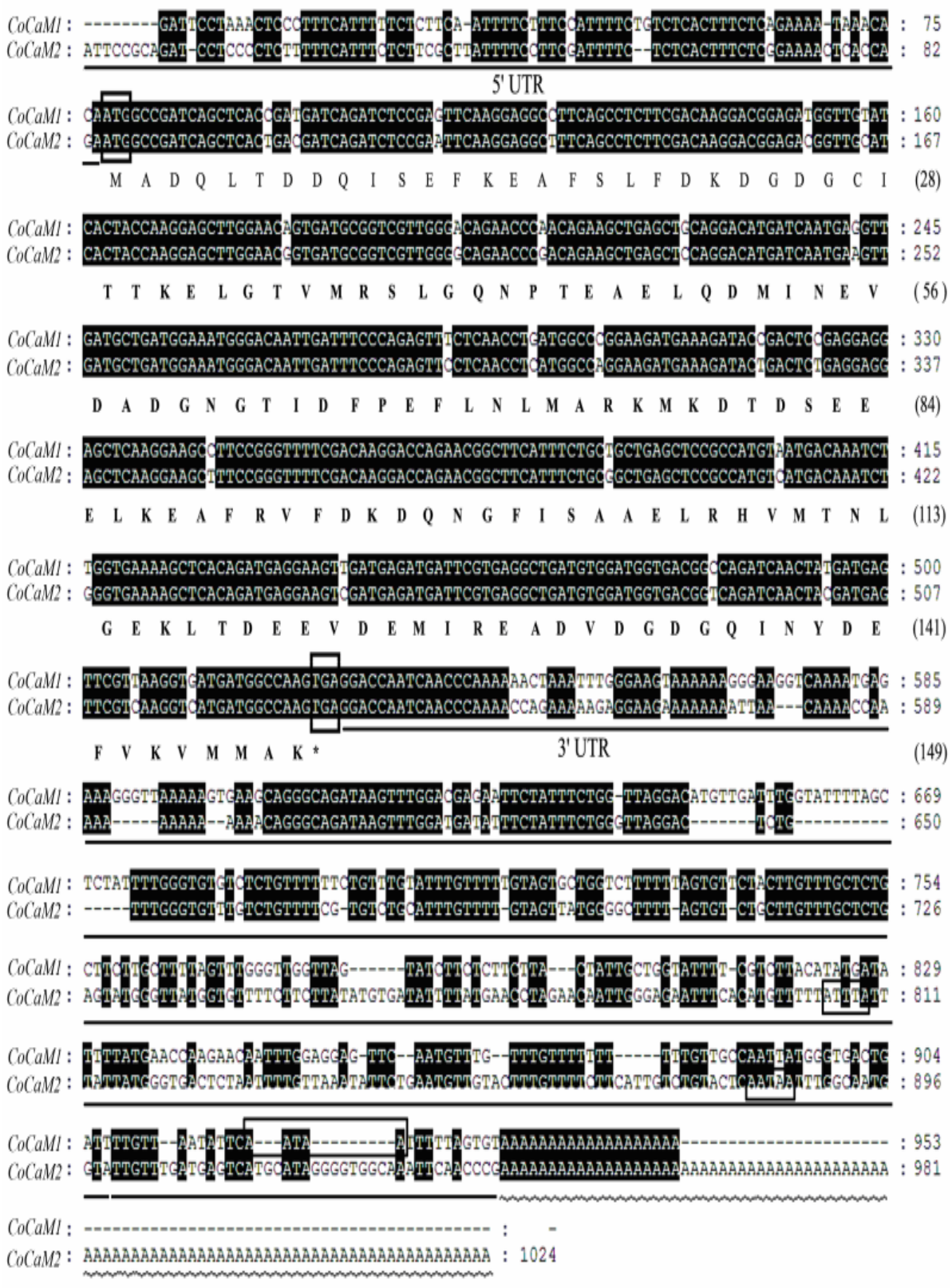

Fig. 2. The nucleotide sequences and the deduced amino acid sequence of full-length *CoCaM1* and *CoCaM2* cDNAs of *C. oleifera*. The identical nucleotides are highlighted, and hyphens indicate gaps. The initiated nucleotide sequences, the translational termination codon, the polyadenylation signal AATAA and the mRNA instability motif (AUUUA) are boxed. The 5'-UTR and the 3'-UTR are underlined. The waving line present polyadenylation, and the stop codon is indicated by asterisk (*).

The most striking feature concerns usage of purine bases at third codon position, in *CoCaM1* 49 (out of 149) end with G only 20 with A; and in *CoCaM2*, 49 (out of 149) end with G only 15 with A. The two *CoCaMs* (83% identity) share surprising identity of 94.4% within their coding regions, of 78% in 5′UTR, and of 70% in 3′UTR. And they present the nucleotide substitutions of C-T, T-C, C-A, A-G, G-C and C-A. Of the 24 substitutions between the two clones, 23 occur at the third codon, and only one is present at first position (CGG versus AGG) (Fig. 2).

Analysis of the deduced amino acid sequence and homology: The analyzed result of the CoCaM protein by ProtParam TMpred and SignalP 3.0 sever showed that the protein contained 149 amino acids with the calculated molecular weight of 16.83 kDa and theoretical pI of 4.10, and that no Trp residue, signal peptide or transmembrane region had been found in the putative polypeptide. Acidic residues and basic residues account for 25.5% and 12.25%, respectively; and hydrophobic residues, charged residues come to 28.90% and 18.89%, respectively. The analyzed result using DNAStar Protean indicated that the central alpha of the CoCaM protein was composed of 18 residues (66-93), in which residues Arg74-Glu84 connecting the 2 lobes of CaM possessed higher flexibility, and made the most contribution to hydrophilicity, and it might bridge the lobes and were entirely expose to solvent. And the solved exposed region in the central helix functions as a flexible tether that is necessary to form functional active enzyme complexs (VanBerkum *et al*., 1990). In addition, nine Met residues (Met-1, Met-37, Met-52, Met-73, Met-77, Met-110, Met-125, Met-146 and Met-147) with hydrophobic side chains were also found in the CoCaM protein.

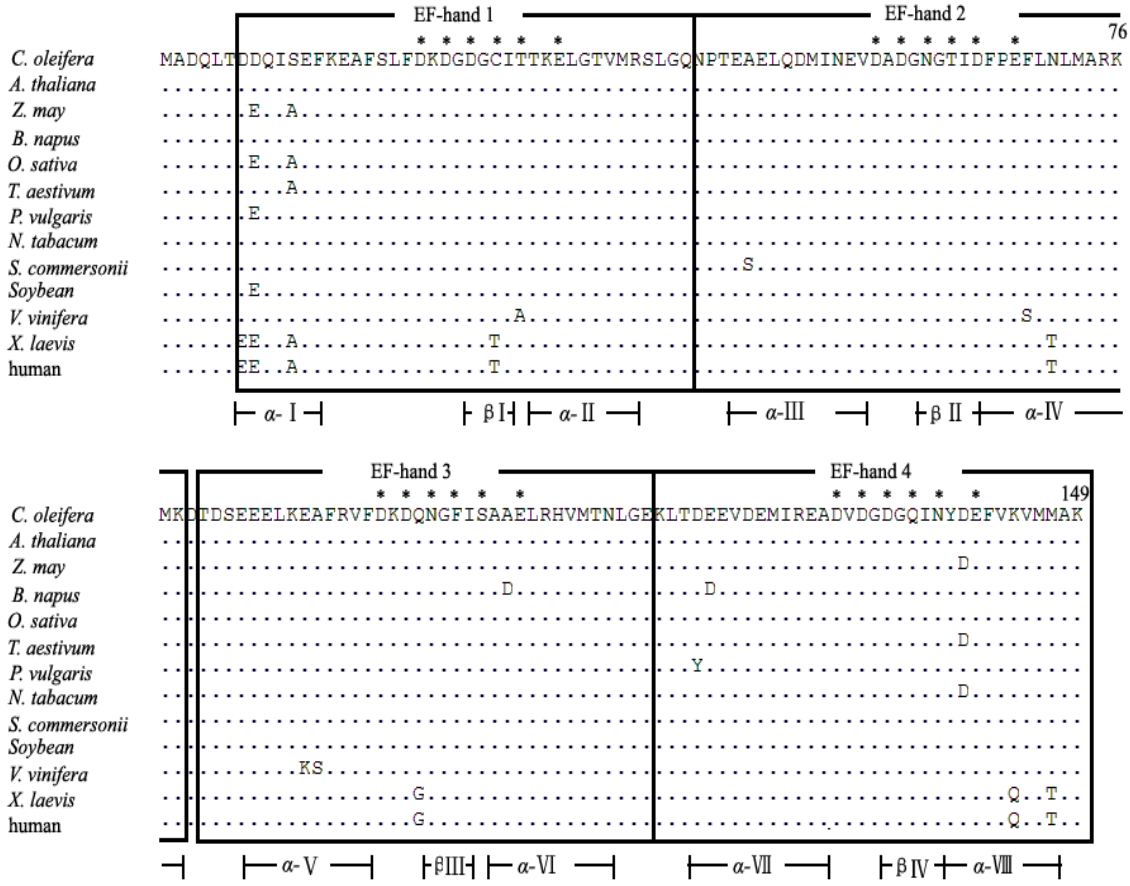

Fig. 3. Alignment of the deduced amino acid sequences of *Camellia oleifera* CaM with those of other species CaMs. Abbreviations and corresponding accession numbers are: *C. olerifera* (EU856536 and FJ649316, this study); *A. thaliana*, AtCaM-2, 3 (AY065179 and AY091301); *Z. mays* (X773397); *B. napus* (AF150059); *O. sativa* (AF042839); *T. aestivum*, TaCaM2-2 (U48691); *P. vulgaris* (AF030033); *N. tabacum*, NtCaM9 (AB050845); *S. commersonii* (AJ318521); Soybean, SCaM-1,3 (L01430 and L01432); *V. vinifera* (AY070232); *X. laevis,* cDNA clones of 71 and 11G2 (K01945 and K01944); human (J04046, M19311 and M27319). Multiple sequence alignment was carried out by Vector NTI 10.0, and displayed by GeneDoc. Amino acid residues identical to that of the *C. oleifera* CaM are indicated by dots; asterisks above residues are indicative of the positions of amino acid involved in Ca^{2+}-binding. The domains of α-helices and β-sheets below the alignment present the predicted secondary structure of human CaM (P62158).

The analysis result using SOPMA showed that the polypeptide included alpha helix of 64.43%, beta sheet of 3.36%, beta turn of 8.72% and coil of 23.49%. It contained eight alpha helices, of which six alpha helices II, III, IV, V, VII, and VIII possessed 11 amino acid residues. The analysis result using helical wheel analysis indicated that a tendency to form amphipathic helices with hydrophobic and charged/polar residues distributed

on different faces of the helices II(residues 30-40) and VII (residues 119-129). The predicted results by ScanProsite demonstrated that the protein included four EF hand conformations (residues 8-43, 44-79, 81-116 and 117-149) with Ca^{2+} binding regions (residues 21-32, 57-68, 94-105 and 130-141), termed as domain1 to 4. And the result was identical to those of human and other species CaMs (Fig. 3). Moreover, it was also found that the domain 1 and 3 shared similarity of 59%, and the domain 2 and 4 shared similarity of 45%. Its deduced amino acid sequence was identical to that of peanut CaM (PCaM-1, AAR99409) in domain1 and 2, and merely existed one substitute of L93F in domain 3 and E140D in domain 4, respectively (Meng *et al.*, 2004). In addition, the positions of the ligand were illustrated in Fig. 3, and a 12-residue segment containing 6 coordination ligands was flanked by helical regions (Lukas *et al.*, 1984).

The amino acid sequence of CoCaM shared above 89% identities with those of animal CaMs, and more than 97% with corresponding parts of plant CaMs, respectively (Fig. 3). And it only existed the differences of one amino acid residue with *Arabidopsis thaliana* CaM, At CaM-2 (AY065179) and CaM-3 (AY091301), of two residues with *Triticum aestivum* TaCaM2-2 (U48691), *Zea may* CaM (X773397), *Solanum commersonii* CaM (AJ318521) and soybean, SCaM-1(L01430) and SCaM-3 (L01432), of three residues with *Brassica napus* CaM (AF150059) and *Phaseolus vulgaris* CaM (AF030033), and of five residues with *Vitis vinifera* CaM (AY070232). CoCaM shared amino acid identify of 100% with that of *Nicotiana tabacum* NtCaM9 (AB050845). However, it possessed seven-residue difference in comparison with CaMs of human (J04046, M19311 and M27319) and *Xenopus laevis* cDNA clones of 71 (K01945) and 11G2 (K01944). The results indicated that CoCaM was phylogenetically related to those of the other plants, and shared the closest genetic relationship with *Nicotiana tabacum* NtCaM9 CaM. However, it had the far phylogenetic relationship with CaMs of human and *X. laevis*.

Real-time quantitative PCR to analyze the expression levels of *CoCaM1* and *CoCaM2*: The cDNA standards of *CoCaM1* and *CoCaM2* were obtained from RT-PCR reactions. They are 292-bp fragments encoding for proteins with a deduced peptide sequence of 97 amino acids. Sequencing of the amplified fragments confirmed the identity of the sequences to the previously assembled *CoCaM1* and *CoCaM2* from *C. oleifera*. Fig. 4 A and B show the standard curves generated by serial dilutions from the cDNAs of *CoCaM1* and *CoCaM2* in the range of 10^3-10^8 copy numbers. All of them produced a single peak in the melting curve analyses performed following the qPCR. No amplification was detectable in the absence of template. The observed expression of *CoCaM1* and *CoCaM2* by the qPCR were ubiquitous expressed with different levels and there were distinct differences in different *C. oleifera* matured seeds. The observed expression levels of *CaM2* in 6 varieties *C. oleifera* seeds were higher than those of *CoCaM1,* of which the expression levels of *CoCaM1* and *CoCaM2* in 'Hengdong 17' were the highest. Furthermore, there also displayed a tendency that both *CaM* genes were coordinately expressed in these matured seeds (Figs. 5 &6).

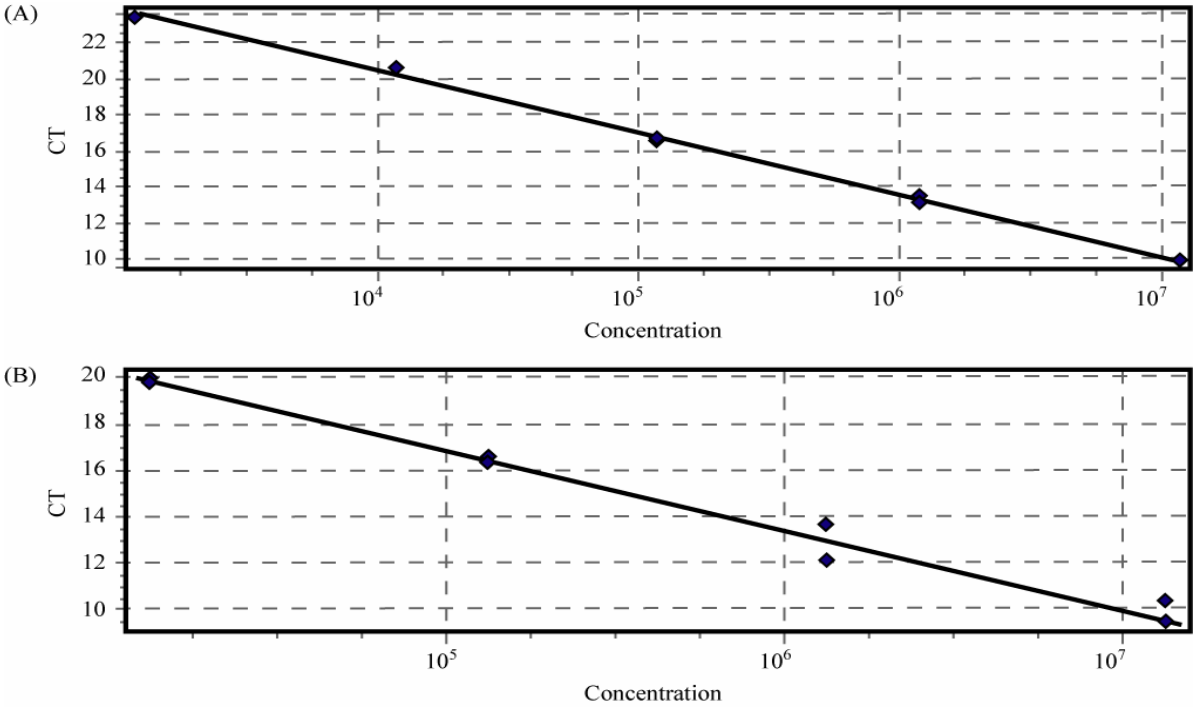

Fig. 4. (A) Concentration-dependent amplification of a *CoCaM1* cDNA standard, serially diluted by factors of 10, was amplified by real-time PCR. A linear relationship was consistently observed with cDNA ranging from 10^3 to 10^8 copies.
(B) Concentration-dependent amplification of a *CoCaM2* cDNA standard, serially diluted by factors of 10, was amplified by real-time PCR. A linear relationship was consistently observed with cDNA ranging from 10^4 to 10^8copies.

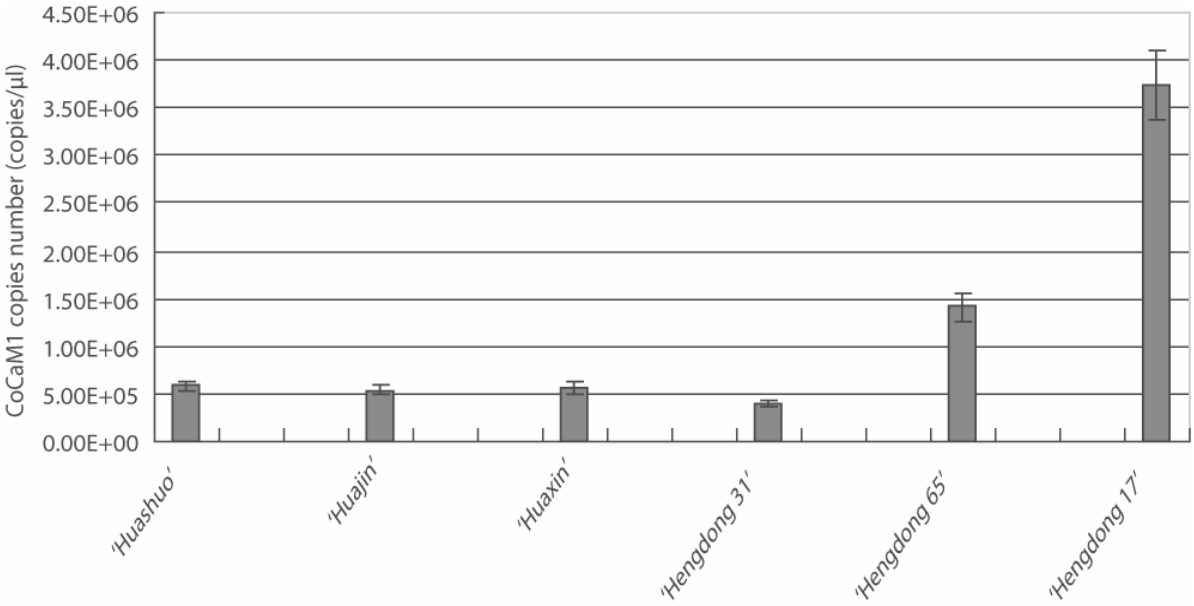

Fig. 5. The observed expression of *CoCaM1* by real-time quantitative PCR in six varieties of *C. oleifera* matured seeds.

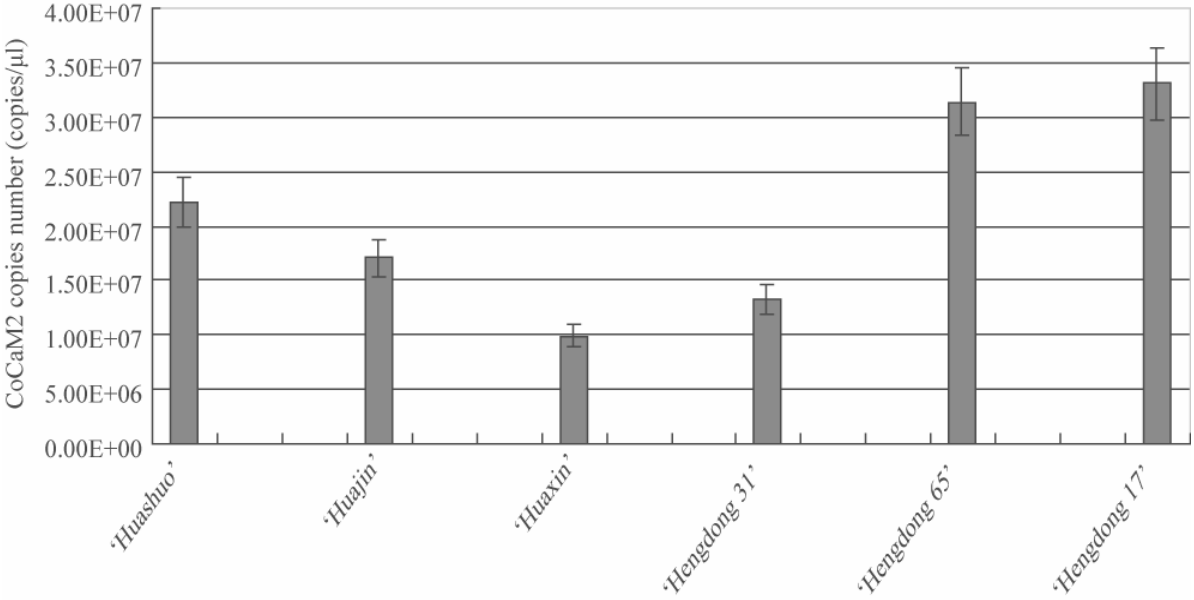

Fig. 6. The observed expression of *CoCaM2* by real-time quantitative PCR in six varieties of *C. oleifera* matured seeds.

Discussion

The two full-length cDNAs for CaM genes encoding the identical protein have been isolated from the cDNA library of *C. oleifera*, supporting the hypothesis "Multigenes possess an identical amino acid sequence" (Xia *et al*., 1999). Moreover, the surprising identity is found in the two CaM-coding sequences (5% nucleotide substitutions), and codon usage, further reflects their evolution relationships (Fig. 2). It is noteworthy that one of the most striking features of the CaM described in this study is the function conservation of hydrophobic residues; and Ca^{2+} binding to CaM induces significant conformational changes exposing Met-rich hydrophobic surfaces that might form critical van der Waals interactions with the hydrophobic face of the target recognition site (Ikura *et al.,* 1992; Crivici & Ikura, 1995; Zielinski, 2002). Met residues might be important for stabilization of the CaM-target peptide complex by hydrophobic interaction and binding target enzymes (Ikura *et al*., 1992; Lee *et al*., 1997; Ma *et al*., 1999). In addition, the central helical region of CaM, enables CaM to bind classical recognition sites, may impart conformation flexibility and also interact with target proteins (VanBerkum *et al*. 1990; Camas *et al*., 2002).

CaMs are composed of four homologous EF-hand domains, and possess all conserved critical features for Ca^{2+}-binding motif. The structure may be the result of ancient gene duplications and fusion, suggesting that CaMs have evolved by two successive duplications (Chiou *et al.*, 2008; Baba *et al.*, 1984). And this suggestion is supported by the facts that protein domains 1 and 3, 2 and 4 are more homologous to each other than to the other pair. Analysis of the two *C. oleiera* cDNAs supports this result. Moreover, the amino acid sequences of the CoCaM protein showed an overall homology of more than 97% and 89% with those of plant and animal species, respectively; and the high degree of homology found in calcium-binding domain of CaMs in phylogenetically divergent species indicates (1) that they play a fundamental role in all living organisms, and this essential role might be the reason for strict conservation of the primary structure of CaM during evolution (2) the high conservativeness is very significant for maintaining the binding with many Ca^{2+}-binding families (Jena *et al.*, 1989; Crivici & Ikura, 1995; Lee *et al.*, 1997).

Accumulation evidences have implicated that CaMs are crucial in numerous development processes and stress responses by regulating the activity of target molecules, *e.g.* Ca^{2+} activation of CaM stimulates NAD kinase and activates NADPH oxidase to produce ROS (reactive oxygen species), and thus activates cell death and defense gene expression (Heo, *et al.*, 1999). Thus we tentatively put forward to the hypothesis that the *C. oleifera* CaM might participate in clearing the caused harmful products in the processes of seed development and lipid biosynthesis via activating NADPH oxidase to produce ROS.

Noticeably, amphipathic helices of CaM might be a structural motif that binds and alters the associated plasma or organelle membranes and present inexchangeable plasma lipoproteins, can stabilize membranes and possess lipid affinity, thus we hypothesize that the amphipathic helices TKELGTVMRSL (residues 30-40) and DEEVDEMIREA (residues 119-129) found in the CoCaM protein may be crucial for lipid biosynthesis during the peak of lipid biosynthesis (Epand *et al.*, 1995). *CoCaM1* and *CoCaM2* found in the constructed cDNA and EST library also are abundantly expressed in nearly matured *C. oleifera* seeds, suggesting that they may participate in lipid synthesis of seeds (Hu *et al*., 2004; Tan *et al*., 2006).

RT-PCR approach has been used to detect the expression of regulatory genes in many plants (Jamil *et al.,* 2010; Xu *et al.*, 2011). In this study, the expression levels of *CaM1* and *CaM2* in six *C. oleifera* matured seeds are compared using qPCR, which demonstrates that there are similar variations of the *CaM1* and *CaM2* component mRNAs, and thus certain regulatory mechanisms of the two CaM genes seem to mediate similar expression in different *C. oleifera* matured seeds. Moreover, the highly conserved CaM isoforms might actually regulate their target proteins differentially. Competition among CaM isoforms for target proteins may occur, and therefore different transcription can significantly affect the ratio of CaM isoforms. It means that more 'active' CaM isoforms may exist in a cell responding to a particular stimulus (Maghuly *et al.,* 2009). The advantage of several expressible CaM genes for an identical protein in an organism is clear; one gene may be of the housekeeping type providing the cell with a basal level of CaM, while the "extra" gene(s) may be differentially regulated in response to various external stimuli (Chien & Dawid, 1984; Fischer *et al*., 1988).

Acknowledgements

This work was supported by National Natural Science Foundation of China (Grant No. 31070603), Hunan Provincial Innovation Foundation For Postgraduate (Grant No. CX2011B313) and the Graduate's Scientific Research Fundation of Central South University of Forestry and Technology (Grant No. 2009bx01).

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(Received for publication 3 January 2011)