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Isolation and Characterization of Calmodulin Gene from *Panax ginseng* C. A. Meyer

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Abstract : Ca^{2+} and calmodulin (CaM), a key Ca^{2+} sensor in all eukaryotes, have been implicated for defense responses of plants. Eukaryotic CaM contains four structurally and functionally similar Ca^{2+} domains named I, II, III and IV. Each Ca^{2+} binding loop consists of 12 amino acid residues with ligands arranged spatially to satisfy the octahedral symmetry of Ca^{2+} binding. To investigate the altered gene expression and the role of CaM in ginseng plant defense system, cDNA clone containing a CaM gene, designated *PgCaM* was isolated and sequenced from *Panax ginseng*. *PgCaM*, which has open reading frame of 450 nucleotides predicted to encode a precursor protein of 150 amino acid residues. Its sequence shows high homologies with a number of other CaMs, with more similarity to CaM of *Daucus carota* (AAQ63461). The expression of *PgCaM* in different *P. ginseng* organs was analyzed using real time PCR. The results showed that *PgCaM* expressed at different levels in young leaves, shoots, and roots of 3-week-old *P. ginseng*. In addition, the expressions of *PgCaM* under different abiotic stresses were analyzed at different time intervals.

Key words : Abiotic stress, calmodulin, *Panax ginseng,* real-time PCR, stress

INTRODUCTION

Calmodulin (CaM), a highly conserved, well characterized Ca^{2+} sensor in eukaryotes, is a small protein with four EF-hands that each binds to Ca^{2+}. The EF-hands are paired in two globular domains connected by a central helix. CaM is encoded by multiple genes that have been shown to be expressed differentially. In addition, different CaM isoforms are known to interact differentially with the target proteins. Binding of Ca^{2+} to CaM results in a conformational change that exposes hydrophobic pockets that can then interact with target proteins.[1)]

CaM regulates the activity/function of diverse proteins in a Ca^{2+} dependent manner.[2)] The physiological response that is elicited by the elevated cytosolic Ca^{2+} signal is derived, to some extent, from the expression patterns and activities of the proteins regulated by CaM.[3)] Ca^{2+} binding proteins such as CaM detect Ca^{2+} signals and regulate downstream targets as part of a coordinated cellular response to a given stimulus.[4)] It has no catalytic activity of its own but, upon binding Ca^{2+}, functionally activates numerous downstream target proteins.[5)] One of the calmodulin dependent protein, CaM kinase II is first demonstrated in 1980 by gel filtration.[6)]

In plants, CaM was discovered by Anderson and Cormier (1980)[2)] and Spinach CaM was first sequenced in 1984.[3)] Accumulating evidence suggests that one of the earliest events that occur during plant pathogen interaction is the raise in cytoplasmic free calcium levels, which in turn thought to activate defense responses through CaM.[1)]

This is the first report on isolation and characterization of full length CaM gene in *P. ginseng*. Korean ginseng saponins (ginsenosides) have been reported to have various biological properties.[7)] Ginseng is a perennial herb in the family Araliaceae and is cultivated for its highly valued root used for medicinal purposes.[8)] In the present study, we analyzed the expression profiles of CaM genes in various organs and also under various abiotic and biotic stress conditions. Based on morphological studies with CaM antagonists, it has been proposed that CaM, a

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major Ca^{2+} signal transducer in both animals and plants[9], is involved. The present study demonstrated that the genes showed differential expression patterns under environmental stress and in a variety of organs. Collectively, our data support a role of CaM as important mediators of Ca^{2+} dependent signals during the plant immune response to various stress conditions.

MATERIALS AND METHODS

1. Plant materials

Korean ginseng (*P. ginseng* C. A. Meyer) var. "Hwang-Sook Jong" seeds (obtained from Ginseng Genetic Resource Bank, Korea) were immersed in 70% ethanol for 1 min, surface-sterilized using 2% NaOCl for 15 min, rinsed three times with sterilized distilled water, and then the inner zygotic embryos were dissected out. Intact zygotic embryos were placed on Murashige and Skoog (1962)[10] basal medium containing 3% sucrose and 0.7% agar to elongate. Cultured plantlets were planted in glass bottles that contained a 70-ml MS medium with 10 mg/l giberrellic acid, 3% (w/v) sucrose and 0.7% plant agar under controlled conditions of 25/18°C and a 16-h photoperiod from white fluorescent lamps. Healthy, 3-week-old plants were used for the treatments and for analysis of gene expression in different organs, leaves and stems of *P. ginseng* plantlets at 6-week-old plantlets of *P. ginseng* were used for the analysis of gene expression in different organs, leaves, and stems. Hairy root, adventitious roots and fresh root samples were provided by Ginseng bank.

2. RNA purification and construction of a cDNA library

Total RNA was isolated from a 4-year-old ginseng root by using the aqueous phenol extraction method. Poly (A)+ RNA was isolated by oligo (dT) cellulose column using the Poly (A) Quick mRNA isolation kit (Stratagene, US). A commercial cDNA synthesis kit was used to construct library according to the manufacturer's instruction manual (Clontech, US). Size-selected cDNA was ligated into λTriplEx2 vector and was packaged in vitro using Gigapack III Gold Packaging Extract kits (Stratagene, US).

3. Nucleotide sequencing and sequence analysis

pTriplEx phagemids were excised from the ëpTriplEx2 and used as templates for sequence analysis. The 5 ends of cDNA inserts was sequenced by an automatic DNA sequencer (ABI prism 3700 DNA sequencer, Perkin-Elmer, USA). Homologous sequences of CaM EST are searched against the GenBank nr databases using a BLASTX algorithm. A pTriplEx phagemid for CaM cDNA was excised from the λpTriplEx2 and used as templates for sequence analysis. Nucleotide and amino acid sequence analyses were performed using DNASIS program (Hitachi, Japan).

These deduced amino acid sequences were searched for homologous proteins in the databases, using BLAST network services at the National Center for Biotechnology Information.[11] We used ClustalX with default gap penalties to perform multiple alignment of CaM isolated in ginseng and previously registered in other species.[12] Based on this alignment, a phylogenetic tree was constructed according to the neighbor-joining method, using the MEGA3 programs.[13] Bootstrap analysis with 1,000 replicates was also conducted in order to obtain confidence levels for the branches.[14] Secondary structure was determined by SOPMA,[15] and the protein properties are estimated using ProtParam.[16]

4. Abiotic stress treatments

To investigate the response of the *P. ginseng* CaM gene to various stresses, the *P. ginseng* plantlets were treated with abiotic stimuli for about 3 weeks after zygotic embryo germination. For chemical stress or plant hormone treatments, the plantlets were placed in MS media containing indicated concentrations of chemicals; 11% Mannitol, 100 μM abscisic acid (ABA), 10 mM H_2O_2 and 500 μM copper sulphate (CuSO4). Stress treated plantlet samples were collected at 1, 2, 8, 12, 24 and 48 hrs. In all cases, stress treatments were carried out on the MS media. Control plants held in a growth room at 25°C under a 16-hrs photoperiod. The stressed plant materials from all completed treatments were immediately frozen in liquid nitrogen and stored at 70°C until required.

5. Real time quantitative RT-PCR

Total RNA was extracted from seedlings of *P. ginseng* using RNeasy mini kit (Qiagen, Valencia, CA, USA). For RT-PCR, 2 ng of total RNA was used as a template for reverse transcription using oligo(dT)15 primer (0.2 mM) and AMV reverse transcriptase (10 U/ìl) (INTRON Biotechnology, Inc., South Korea) according to the manufacturer's instructions. Real-time quantitative PCR was performed using 3-ul of cDNA in a 10 ul reaction volume using SYBR® Green SensimixPlus Master Mix (Quantace, Watford, England). Specific primers for *PgCaM*, 5'- GAT CAG ATC TCT GAG TTC AAG GAA GC -3 and 5'- TGA GAT TAA GGA ACT CAG GAA AGT CG -3' were used

to perform real time-PCR. The thermal cycler conditions recommended by the manufacturer were used as follow: 10 min at 95°C, followed 40 cycles of 95°C for 10 s, 58°C for 10 s, and 72°C 20 s. The fluorescent product was detected at the last step of each cycle. Amplification, detection, and data analysis were carried out with a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Sydney, Australia). Threshold cycle (Ct) represents the number of cycles at which the fluorescence intensity was significantly higher than the background fluorescence at the initial exponential phase of PCR amplification. To determine the relative fold differences in template abundance for each sample the Ct value for *PgCaM* was normalized to the Ct value for β-actin and calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$. Three independent experiments were performed.

RESULT AND DISCUSSION

1. Isolation and amino acid analysis of *PgCaM* gene

As part of a genomic project to identify genes of *P. ginseng*, cDNA library prepared with the RNA obtained from the plantlets of *P. ginseng* screened. Clone encoding for the CaM identified from the 20,000 cDNAs and used for further characterization. The representative clone for CaM was given the name, *PgCaM* as shown in Fig. 1

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             10        20        30        40        50        60        70        80
1    CTCATATCTCTTTCTCTCCCTTTGTTCTTATTTTTCAATTTCAAACACCACACCTAAATACAACTAGATAGATATGGCGG 80
                                                                                M  A

             90       100       110       120       130       140       150       160
81   ATCAGCTCACCGACGATCAGATCTCTGAGTTCAAGGAAGCCTTCAGCCTATTCGACAAGGATGGCGATGGTTGCATCACT 160
     D  Q  L  T  D  D  Q  I  S  E  F  K  E  A  F  S  L  F  D  K  D  G  D  G  C  I  T

            170       180       190       200       210       220       230       240
161  ACCAAGGAGCTTGGGACTGTGATGAGGTCGCTAGGGCAGAACCCCACTGAGGCTGAACTCCAGGATATGATAAATGAAGT 240
      T  K  E  L  G  T  V  M  R  S  L  G  Q  N  P  T  E  A  E  L  Q  D  M  I  N  E  V

            250       260       270       280       290       300       310       320
241  GGATGCTGATGGTAATGGAACCATCGACTTTCCTGAGTTCCTTAATCTCATGGCCAGGAAGATGAAGGATACTGACTCCG 320
       D  A  D  G  N  G  T  I  D  F  P  E  F  L  N  L  M  A  R  K  M  K  D  T  D  S

            330       340       350       360       370       380       390       400
321  AAGAGGAGCTGAAAGAGGCATTCCGAGTTTTTGACAAGGATCAGAATGGTTTCATTTCTGCTGCTGAGCTGCGCCATGTC 400
     E  E  E  L  K  E  A  F  R  V  F  D  K  D  Q  N  G  F  I  S  A  A  E  L  R  H  V

            410       420       430       440       450       460       470       480
401  ATGACCAACCTGGGGGAGAAGCTTACTGATGAAGAAGTTGACGAGATGATACGGGAGGCTGATGTTGATGGCGATGGCCA 480
      M  T  N  L  G  E  K  L  T  D  E  E  V  D  E  M  I  R  E  A  D  V  D  G  D  G  Q

            490       500       510       520       530       540       550       560
481  GATCAATTATGAAGAATTTGTTAAGGTGATGATGGCCAAGTGATGAGCTCCTCCACCCTACCAGATCCATAAAGACATTG 560
       I  N  Y  E  E  F  V  K  V  M  M  A  K  *  *  A  P  P  P  Y  Q  I  H  K  D  I

            570       580       590       600
561  AGTATAAAAATGAACTTCAAAAAAAAAAAAAAAAAAAAAAAAA 602
     E  Y  K  N  E  L  Q  K  K  K  K  K  K  K
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of a *PgCaM* cDNA isolated from *P.ginseng*. Numbers on the right represent nucleotide positions. The deduced amino acid sequence is shown in single-letter code below the nucleotide sequence. The arrow denotes the translation stop signal.

(Genbank FJ82575). *PgCaM* is 602 bp in length, and it has an open reading frame (ORF) of 450 bp nucleotides with a 73-nucleotide upstream sequence and a 79-nucleotide downstream sequence. The ORF of *PgCaM* starts at nucleotide position 74 and ends at position 523. *PgCaM* encodes a precursor protein of 148 amino acids. The calculated molecular mass of the matured protein is approximately 16.7 kDa and isoelectric point is around 4.11, which shows similarity with human antibody CaM.[17] The instability index (II) is computed to be 24.52. This classifies the protein as stable one. The aliphatic index, regarded as a positive factor for increased thermostability, was calculated as 69.40 by using ProtParam. Hamadane *et al*. explained about the thermostability of calmodulin like proteins in his studies of human neuroglobins and cytogobins.[18] In the deduced amino acid sequence of *PgCaM* protein, the total number of negatively charged residues (Asp + Glu) was 38, while the total number of positively charged residues (Arg + Lys) was 15.

2. Homology analysis of *PgCaM*

In order to determine the evolutionary relationships existing among different CaM from a variety of plant species, a phylogenetic tree was compiled using MEGA3 software, and the amino acid sequences used from the GenBank database (Fig. 2A). A GenBank Blast search revealed that *PgCaM* has the highest sequence homology to the wild carrot (*Dacaus carota*) CaM 4 (AAQ63461) with 99.3% identity and 84% similarity. Sequence analysis of BLASTX program in NCBI database also showed high and similar identities with many other species like oak (*Quercus petraea*), 99.3% identity and 81% similarity (CAH57708), sweet potato (*Ipomoea batatas*), 99.3% identity and 84% similarity (ACG63497). Secondary structure analysis and molecular modeling for *PgCaM* were performed by SOPMA. The secondary structure analysis revealed that *PgCaM* consists of 95 α-helices, 15 β-turns joined by 6 extended strands, and 33 random coils. This is exactly similar to that of secondary structure of CaM of carrot (*Daucus carota*).When compared to oak (*Quercus petraea*) CaM, showed 94 α-helices, 14 β-turns jointed by 13 extended strands and 28 random coils and to sweet potato (*Ipomoea batatas)* CaM, which contains 95 α-helices, 13 β-turns jointed by 5 extended strands, and 36 random coils (Fig. 3). Neighbor-joining method was used for construction of phylogenetic tree based on CaM amino acid sequences to show relationships between *PgCaM* and other plant CaM. Phylogenetic analysis of plant CaM has been carried out using the

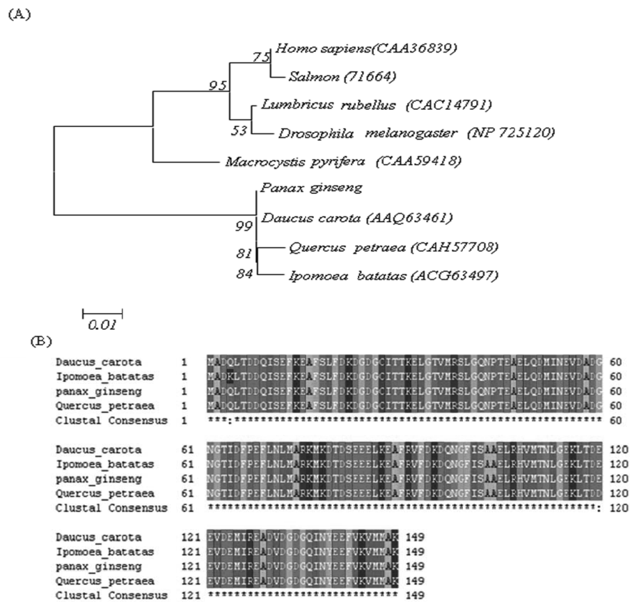

Fig. 2. (A) A phylogenetic tree based on amino acid sequence, showing the phylogenetic relation between *PgCaM* and other plant CaMs. The tree was constructed using the Clustal X method (Neighbor-joining method) and a bar represents 0.1 substitutions per amino acid position. (**B**) Multiple alignment of the deduced amino acid sequence of *PgCaM* with those of CaM genes from other species; *Daucus carota*, (AAQ63461), *Quercus petraea*, (CAH57708) and (d) sweet potato *Ipomoa batatas,* (ACG63497). Sequence data were obtained from GeneBank listed and aligned using DDBJ Clustal Wand GeneDoc.

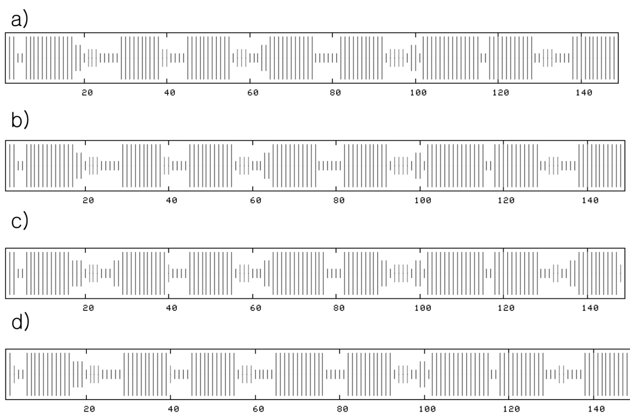

Fig. 3. Comparison of secondary structure of *PgCaM* by SOPMA. (a) *PgCaM*, (b) wild carrot (*Daucus carota*, AAQ63461) (c) oak (*Quercus petraea*, CAH57708) and (d) sweet potato (*Ipomoa batatas,* ACG63497).

Clustal X program (Fig. 2B).

3. Tissue expression pattern analysis of *PgCaM*

To investigate the expression profile of CaM in different

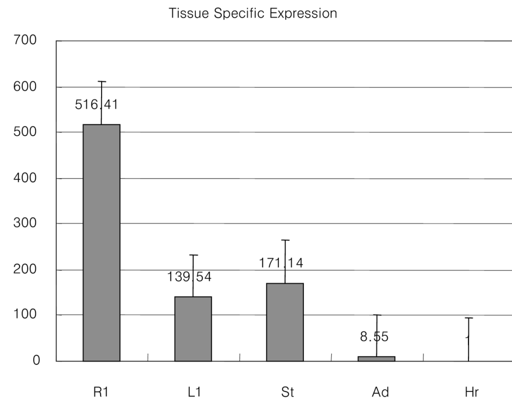

Fig. 4. Relative quantities of *PgCaM* mRNA in different plant organs.

tissues of *P. ginseng*, total RNA was isolated from root (R1), stem (St), leaf (L1) adventitious roots (Ad) and hairy roots (Hr). Tissues subjected to RT-PCR using *PgCaM* primers. The result showed (Fig. 4) that CaM expression could be detected in all tissues but at different expression levels, with the strong expression in roots (510folds), moderate expression in stem (170 folds). Previous studies in common wheat showed the similar results[19]. Whereas the expression found in leaves was 140 folds. Perera *et al.* (1992)[20] also found the similar response of transcripts in leaves. Weak expression in adventitious the hairy roots showed no expression at all. The concomitant expression results suggested that CaM is a subsistent expressing gene in different tissues.

4. Gene expression changes involved in stress response

To check the *PgCaM* response under various stress conditions, *P. ginseng* plantlets were treated with various environmental lifts, such as mannitol, abscisic acid, H_2O_2 and $CuSO_4$. Expression level of mRNA was monitored for 48 hrs at a regular interval using real time PCR. (Fig. 5A) tissue experiencing the mannitol stress exhibited the unique expression profile. Under mannitol stress (11%) treatment, *PgCaM* transcript level was increased 2.11 fold at 2 hrs, and was decreased insignificantly at 8hrs to 1.88 fold. The expression increased to maximum at 12 hrs by 4.8 fold followed by rapid fall in expression at 24 hrs to 0.6 fold. Expression level was improved by 2.02 fold by 48hrs. Tiny transient and inconsistent responses suggest some kind of adaptation must have happened.[21] Exposure to abscisic acid, H_2O_2, and copper caused the biphasic response in *PgCaM* expression. Increased accumulation of *PgCaM* transcripts was found at 2 hrs by 2.83, 2.08, and 2.07 fold for ABA, H_2O_2, and copper respectively

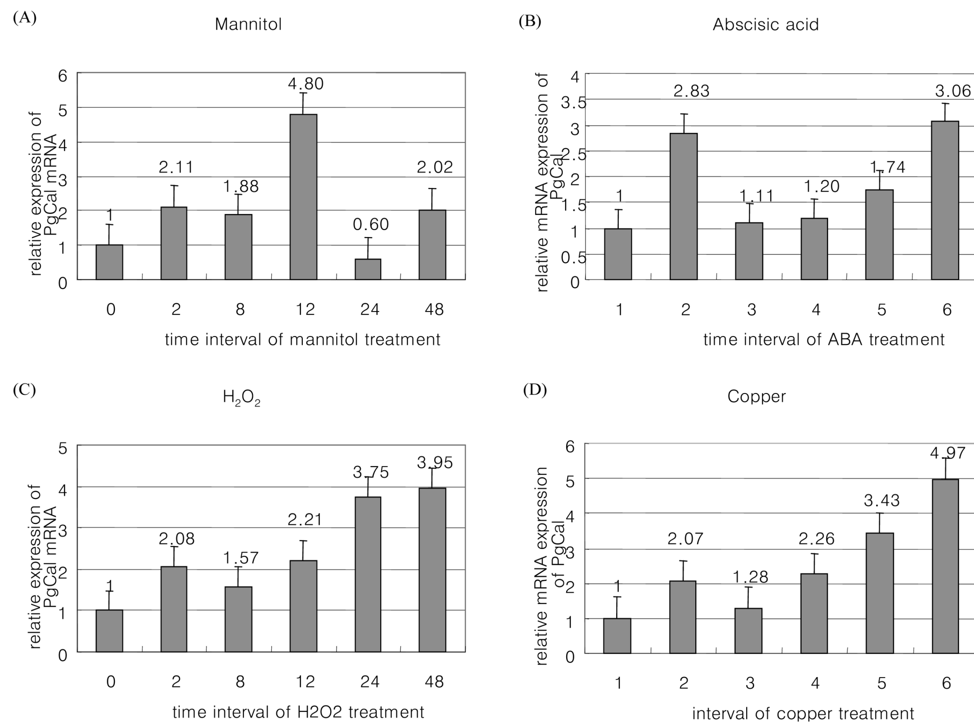

Fig. 5. Relative quantities of *PgCaM* mRNA at various time points (h) post-treatment with various stresses. (A) Mannitol, (B) Abscisic acid, (C) H_2O_2, (D) $CuSO_4$. The error bars represent the standard error of the means of three independent replicates.

(Fig. 5B,C,D respectively). The expression was decreased after 2hrs and then again showed steady and unceasing aggregation of mRNA till 48hrs. After 48hrs of treatment with ABA, H_2O_2 and copper the content of CaM reached maximum values, being 3.06-fold and 3.95-fold and 4.97-fold respectively, higher than the control values. Xiuli Hu *et al.* (2007)[5)] showed that both ABA and H_2O_2 induces the increase in calcium level and expression of CaM. The disconnection in above response can be due to transient elevations of Ca^{2+} levels[22)] and the information encoded in transient Ca^{2+} signals which is deciphered by CaM .[23,24)] Under copper stress (500 μM) treatment, *PgCaM* transcript level was 2.07-fold at 2 hrs, declined to 1.28-fold at 8 hrs, and then again continuously increased to 2.26-fold at 12 hrs followed by maximum expression at 48 hrs by 4.97-fold (Fig. 5D). Heavy metals are considered to substitute for Ca2+ effectively in CaM. Activation of CaM by these metals could upset its normal regulation by the cellular flux of Ca^{2+}. Perhaps this could constitute in part a basis for the toxicity of these metals in cell physiology.[24)] Multiple CaM isoforms are present in plants. Since these isoforms exhibit a reciprocal activation and inhibition of some CaM target enzymes, a particular arm of the Ca^{2+}/CaM signal transduction pathway might be activated, while other arms are inhibited, when a particular isoform expressed. The deviating and contrasting results suggest that in some system the Ca^{2+} fluxes are apparently sufficient whereas in others additional elements seem to be required for the induction of downstream effects. All data shown here indicate that these CaM genes are differentially regulated by stress signals under investigation.

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