

## Isolation and Characterization of Calmodulin 2 (*CICAM2*) Gene from *Codonopsis lanceolata*

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### ABSTRACT

Calmodulin, a Ca<sup>2+</sup>-binding protein, has no enzyme activity. It combines with Ca<sup>2+</sup> and makes variable proteins to an active form. Calmodulin 2 is a ubiquitous protein in plants. To investigate the defense mechanism against various stresses, a clone encoding a calmodulin 2 protein was isolated from a cDNA library prepared from taproot mRNAs of *Codonopsis lanceolata*. The cDNA, designated *CICAM2*, is 719 nucleotides long and has an open reading frame of 450 bp with a deduced amino acid sequence of 149 residues. The deduced amino acid sequence of *CICAM2* showed a high similarity with calmodulins of *P. x hybrida* (P27163) 97%, *N. tabacum* (BAB61908) 97%, *S. tuberosum* (AAA74405) 96%, *Z. mays* (CAA74307) 92%, *C. richardii* (AF510075) 93%, *M. truncatula* (AAM81203) 91%, and *G. max* (P62163) 91%. The transcriptional expression of the *CICAM2* gene, was gradually increased by the CaCl<sub>2</sub> treatment. Whereas its expression And it was gradually decreased in the cold stress treatment.

**Key words** : abiotic stress, *Codonopsis lanceolata*, RT-PCR, calmodulin 2

### INTRODUCTION

Plants sense and respond to various environmental stimuli, which often enables them to endure at the conditions of their local environment. For example, in response to low temperature, *Arabidopsis* plants undergo changes that enable them to survive subsequent freezing temperatures (Gilmour *et al.*, 1988; Thomashow, 1994). Environmental stimuli such as

cold, wind, or light might be transduced into plant cells as second messenger signals, which control cellular responses.

Calmodulin (CaM) is a Ca<sup>2+</sup> binding protein involved in numerous Ca<sup>2+</sup> dependent signaling pathways in eukaryotic cells (Roberts and Harmon, 1992). In plants, CaM is implicated in many Ca<sup>2+</sup> -dependent responses, including phytochrome action (Neuhaus *et al.*, 1993), gravitropism (Stinemetz *et al.*, 1987), and

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thigmomorphogenesis (Braam and Davis, 1990). In plant cells, transient influxes of  $\text{Ca}^{2+}$  show different kinetics depending on the type of stimulus applied (Knight *et al.*, 1991). Plant cells respond to a stimuli by uptake of  $\text{Ca}^{2+}$  ion inside of cell.  $\text{Ca}^{2+}$  signals can control variable reactions via interaction with  $\text{Ca}^{2+}$ -specific binding protein. The amount of calmodulin appears to correlate with the level of expressed calmodulin mRNA (Means *et al.*, 1985). Plants contain multiple genes that code for calmodulin (Perera and Zielinski, 1992; Reddy *et al.*, 1991; Poovaiah *et al.*, 1992; Botella and Arteca, 1994; Takezawa *et al.*, 1995). The role of these multiple genes in  $\text{Ca}^{2+}$ /calmodulin-mediated signal transduction is not clearly understood. Studies have shown that several calmodulin and calmodulin-related genes are responsive to signals. Various physical and chemical signals have been shown to induce mRNAs corresponding to calmodulin and calmodulin-related genes. In the case of potato calmodulin cDNA, the effect of auxin and light on calmodulin gene expression was investigated. Exposure of dark grown Merit corn root tips to light increased the calmodulin mRNA level (Jena *et al.*, 1989). Takezawa *et al.* (1995) tested the effect of touch stimulation. Lee *et al.* (1995) have isolated a calmodulin isoform from soybean that activates calmodulin-dependent enzymes in a differential manner. In *Arabidopsis*, Braam and Davis (1990) have identified four cDNAs in a rapid response to a variety of stimuli such as touch, wind, rain and wounding. In this study, we isolated the calmodulin 2 (*CICAM2*) gene from *C. lanceolata* and investigated the correlation between the *CICAM2* expression and abiotic stress by quantitative RT-PCR.

## MATERIALS AND METHODS

### Plant materials

Four-year taproot of *Codonopsis lanceolata* cultivated in field were used for the cDNA library

construction and the gene expression analysis.

### RNA isolation and construction of a cDNA library

Total RNA was isolated from four-year taproot from *C. lanceolata* by an aqueous phenol extraction procedure as described by Morris *et al.* (1990). A commercial cDNA synthesis kit was used to construct a library according to the manufacture's instruction manual (Clontech, PT3000-1, USA). Fractions containing cDNA greater than 500 bp were recovered. The library was amplified to yield a final titer of  $2 \times 10^9$  pfu  $\cdot$  ml<sup>-1</sup>. Individual colonies were propagated and saved at -80 °C until further use.

### Nucleotide sequencing and sequence analysis

pTriplEx phagemids were excised from the Uni-ZAP XR library and used as templates for sequence analysis. The 5' ends of randomly selected cDNA inserts were sequenced by an automatic DNA sequencer (ABI prism 3700). Nucleotide and amino acid sequence analyses were performed using DNASIS program (Hitachi). Comparison of sequences to DNA and protein databases at NCBI was performed using the blast algorithm of Altschul *et al.* (1990).

The functional classification of EST clone was based on the results of a comparison to the non-redundant protein database of GenBank using the blastx algorithm. EST clone was annotated manually following the Munich Information Center for Protein Sequences (MIPS) role categorization (Frishman *et al.*, 2001). ExPasy (<http://www.expasy.org/tools/>) and PSORT (<http://psort.ims.u-tokyo.ac.jp>) were used for the prediction of pI, MW and signal peptide of protein.

### Stress treatments

For wounding- and cold-stress treatments, root were damaged by cutting them into slices and floating them on MS media for various intervals at either 26 °C or 4 °C. The treatment for  $\text{Ca}^{2+}$  ion induction involved floating

the root slices on MS media containing 0.5, 1, 2, and 2.5 mM CaCl<sub>2</sub> for 1 h. After the treatments, the tissues were immediately frozen in liquid N<sub>2</sub> and stored at -80 °C.

**Quantitative RT-PCR analysis**

The gene-specific primers of *CICAM2* was designed and used for RT-PCR analysis. Specific primers for coding region of each gene included the following: (*CICAM2*-forward) 5'-CTA GGA TCC AAG ATG GCA GAA C-3'; (*CICAM2*-reverse) 5'-GTG CAG GTA CCC TAT CAC TTA G-3'. As a control, we used the primers specific to *C. lanceolata* actin gene *ClAct1*, 5'-CGA GAA GAG CTA CGA GCT ACC CGA TGG-3'(forward) and 5'-CTC GGT GCT AGG GCA GTG ATC TCT TTG CT-3'(reverse). Ten micrograms of total RNA were used for the RT-PCR analysis,

according to the method of Takakura et al. (2000). The PCR cycles numbered 36 for *CICAM2* and 33 for *ClAct1*.

**RESULTS AND DISCUSSION**

The EST clones homologous to calmodulin genes were obtained from a cDNA library constructed with 4-year root of *C. lanceolata* and named *CICAM2*. The *CICAM2* clone was 719 nucleotides long and contains an open reading frame of 450 bp encoding a deduced 149 amino acid polypeptide (Fig. 1). The deduced amino acid sequences had a strong similarity to the previously described calmodulins of other plants (Fig. 2). Identical amino acids are shown in white against black. The amino acid sequences of some phylogenetically conserved calmodulin are aligned with

1	ACCCTAGACT	GTTGCGCTCC	CTCTCCCTCT	CCCTCTCTAG	GGTTTTCTTC	CTAGTCTGAT		
61	ATTCTAATCC	GTGCGGGTTG	ATCGGGAAGA	TGGCAGAACA	GTTAACCGAG	GAACAGATTG		
				M	A	E	Q	L T E E Q I A 1
121	CTGAGTTCAA	GGAAGCTTTC	AGCCTTTTCG	ACAAGGATGG	CGATGGCTGC	ATCACTACTA		
	E F K	E A F	S L F D	K D G	D G C	I T T K		12
181	AAGAGTTGGG	AACTGTTATG	AGATCGTTGG	GGCAGAATCC	CACCGAGGCT	GAATTGCAAG		
	E L G	T V M	R S L G	Q N P	T E A	E L Q D		32
241	ATATGATCAA	TGAAGTTGAT	GCTGATCAGA	ATGGAACCAT	TGATTTTCCT	GAGTTCCTGA		
	M I N	E V D	A D Q N	G T I	D F P	E F L N		52
301	ACTTGATGGC	TCGAAAAATG	AAGGATACTG	ACTCAGAGGA	GGAACTCAA	GAAGCTTTCA		
	L M A	R K M	K D T D	S E E	E L K	E A F K		72
361	AGGTCTTTGG	CAAGGATCAG	AATGGATATA	TTTCTGCTGC	GGAGCTGCGC	CATGTAATGA		
	V F G	K D Q	N G Y I	S A A	E L R	H V M T		92
421	CAAACCTAGG	GGAGAAGCTG	ACTGATGAAG	AAGTGGATGA	GATGATAAGA	GAAGCTGACA		
	N L G	E K L	T D E E	V D E	M I R	E A D M		112
481	TGGATGGTGA	TGGTCAAGTG	AACTACGAGG	AGTTTGTGAG	GATGATGCTT	GCTAAGTGAT		
	D G D	G Q V	N Y E E	F V R	M M L	A K *		132
541	AGCCATAGTT	TGTTGAATAC	TGTCTAGCAT	CTCACCTTTA	CACTTGTAGC	CTGTAGGCGG		
601	GTAGTTGCTA	TGTTTTCTTG	TCATTACGAC	TGTTCCACGG	TCAATCAACA	TATACTGCTT		
661	TATTGAGATC	ACATATATCT	TTCTTTTTTCT	ATCCGAACAA				

Fig. 1. Nucleotide and deduced amino acid sequence of *CICAM2*, calmodulin 2 cDNA, from *C. lanceolata*. The positions of nucleotides are shown on the left and the positions of amino acids on the right. Asterisk shows the termination codon.

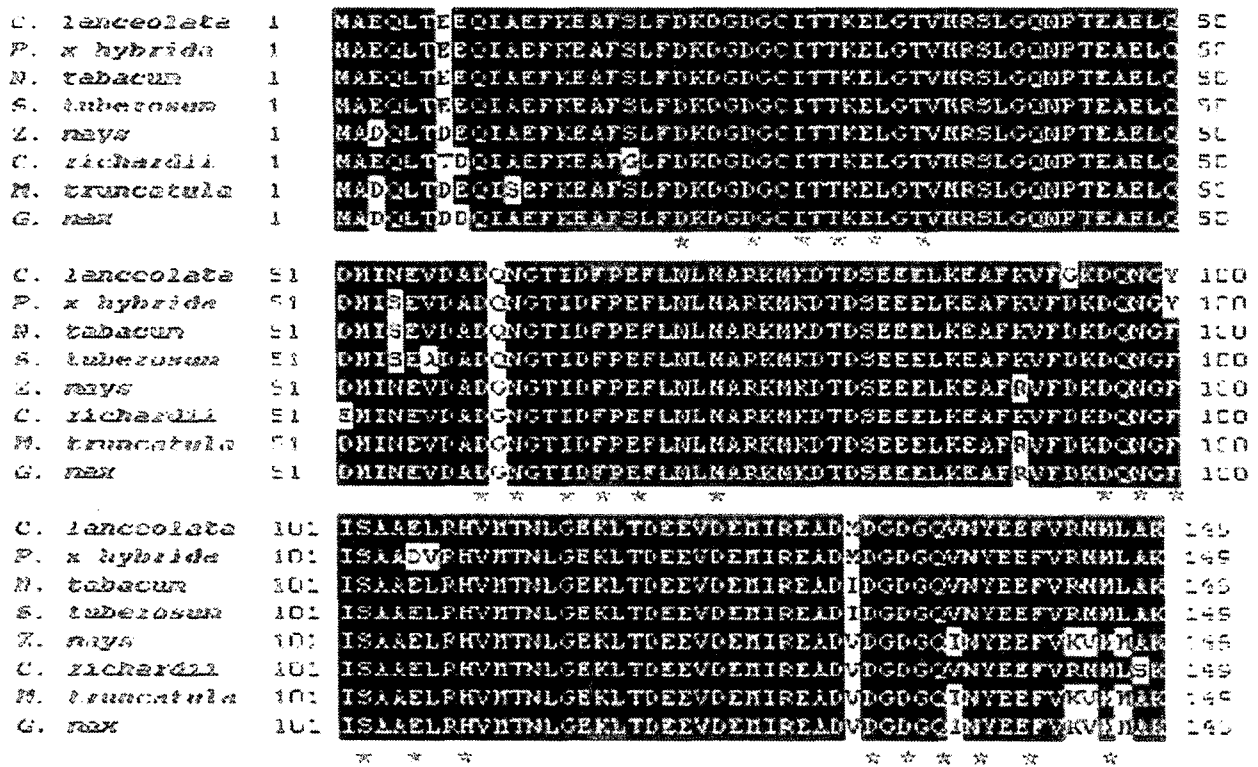


Fig. 2. Comparison of the amino acid residues among *CICAM2* isolated in other species; *P. tremuloides x hybrida* (P27163), *N. tabacum* (BAB61908), *S. tuberosum* (AAA74405), *Z. mays* (CAA74307), *C. richardii* (AF570075), *M. truncatula* (AAM81203) and *G. max* (AAL51087). Identical amino acid residues are shown in white against black. The amino acid sequences of some phylogenetically conserved calmodulin are aligned with respect to the four Ca<sup>2+</sup> binding sites (asterisks).

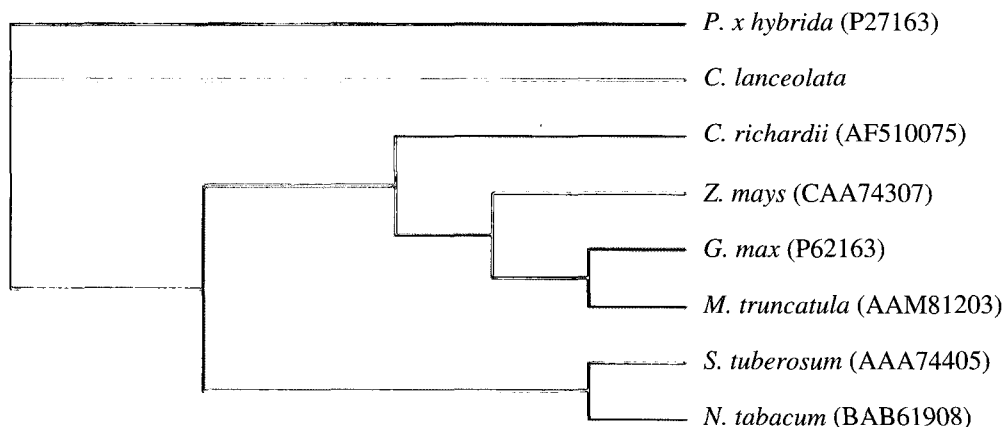


Fig. 3. Phylogenetic relationship of *CICAM2* to other calmodulins.

Table 1. List of CICAM 2 registered in other plants.

Species	Gene	Amino acid residue	Nucleotide identity(%)	GeneBank accession No.	Lineage
<i>Codonopsis lanceolata</i>	<i>CICAM 2</i>	149			Plant
<i>Petunia x hybrida</i>	<i>CAM72</i>	149	97%	P27163	Plant
<i>Nicotiana tabacum</i>	<i>NiCaM2</i>	149	97%	BAB61908	Plant
<i>Solanum tuberosum</i>		149	96%	AAA74405	Plant
<i>Zea mays</i>	<i>CaM2</i>	149	92%	CAA74307	Plant
<i>Ceratopteris richardii</i>	<i>CrCaM1</i>	149	93%	AF510075	Plant
<i>Medicago truncatula</i>		149	91%	AAM81203	Plant
<i>Glycine max</i>	<i>CAM-2</i>	149	91%	P62163	Plant

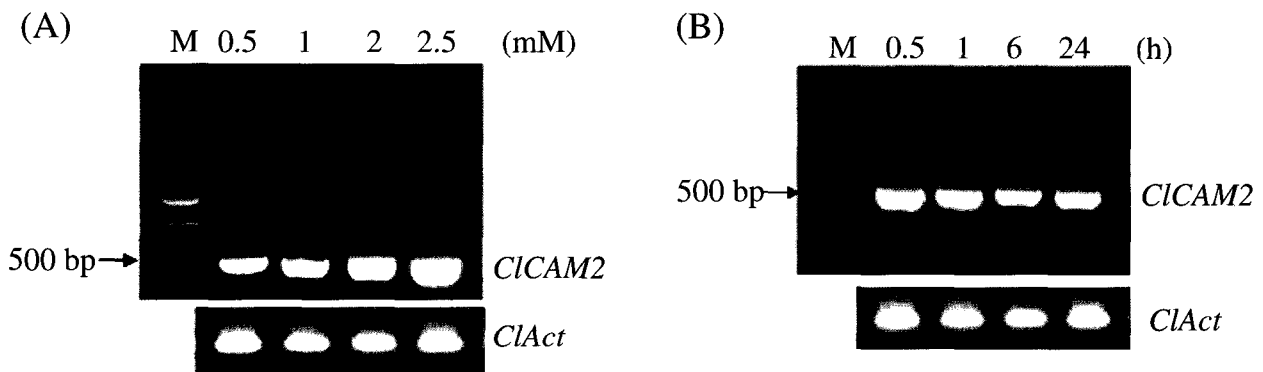


Fig. 4. Stress-specific expression of *CICAM2* in *C. lanceolata*. (A)  $\text{CaCl}_2$ -specific and (B) cold stress-specific expression. Total RNA from stress-treated samples served as templates for quantitative RT-PCR with gene-specific primers. A *ClAct* gene was used for PCR control.

respect to the four  $\text{Ca}^{2+}$  binding sites (asterisks).

Phylogenetic analysis (Fig. 3) is based on the deduced amino acid sequences of calmodulin 2 from various plant species mentioned in Fig. 2. The tree was generated by TreeView Ver.1.6.1 (Hitachi software). Of them, *P. tremuloides x hybrida* (P27163) and *N. tabacum* (BAB61908) had the highest homology (Table 1).

We performed semi-quantitative RT-PCR (QPCR) analysis to examine whether the expression of the *CICAM2* gene related with abiotic stresses. Identity of the PCR bands was confirmed by DNA sequence

analysis. Expression of the *Codonopsis* actin gene, *ClAct1*, served as an internal control for PCR. The transcriptional expression of *CICAM2* gene, encoding calmodulin, was gradually increased by concentration of  $\text{CaCl}_2$  (Fig. 4A).

As a result of stimulus-induced increase in cytosolic free  $[\text{Ca}^{2+}]$ , *CICAM2* is activated to interact with and influence the activity of a variety of target enzymes (Klee and Vanaman, 1982; Roberts *et al.*, 1986; Cohen and Klee, 1988). In this way, *CICAM2* acts as a major  $\text{Ca}^{2+}$  receptor in cells and mediates a response to this second messenger.

Cold-stress-induced *CICAM2* gene response was fast and transient. *CICAM2* mRNAs increased within 15 min of 4°C stimulation and decreased within 1 h and maintained the constant expression (Fig. 4B). It is possible that increase in cytosolic [Ca<sup>2+</sup>] mediate plant response to cold temperatures (Minorsky and Spanswick, 1989). Cold shock results in transient increase of [Ca<sup>2+</sup>] in tobacco seedlings (Knight *et al.*, 1991), and chilling causes an influx of Ca<sup>2+</sup> into *Chara* (Reid and Smith, 1992). Therefore, cold shock results in transient increase of calmodulin expression. We will further investigate the relations between *CICAM2* and another abiotic stresses and then produce the abiotic stress-tolerant transformants by re-introduction of *CICAM2* into *C. lanceolata*.

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