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Calcium Signaling-mediated and Differential Induction of Calmodulin Gene Expression by Stress in *Oryza sativa* L.

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Ca^{2+}/calmodulin transduction pathways have been implicated in mediating stress response and tolerance in plants. Here, three genes encoding calmodulin (*Cam*) members of the EF-hand family of Ca^{2+}-binding proteins were identified from *Oryza sativa* L. databases. Complementary DNA for each of the calmodulin genes, *OsCam1*, *OsCam2*, and *OsCam3* were sequenced. *OsCam1* and *OsCam2* encode a conventional 148-amino acid calmodulin protein that contains four characteristic Ca^{2+}-binding motifs. *OsCam3* encode a similar protein with a 38-amino-acid extension containing a putative prenylation site (CVIL) at the carboxyl terminus. RT-PCR showed that each of the genes is expressed in leaves and roots of 2-week old rice seedlings. By RNA gel blot analysis, *OsCam1* mRNA levels strongly increased in response to NaCl, mannitol and wounding treatments. In contrast, *OsCam2* mRNA levels were relatively unchanged under all conditions investigated. NaCl treatment and wounding also increased the *OsCam3* mRNA level, but in a more transient manner. Our results indicate that although the expression of genes encoding different calmodulin isoforms is ubiquitous, they are differentially regulated by various stress signals. In addition, we have demonstrated that the calcium-channel blocker lanthanum chloride inhibited the induction of *OsCam1* gene expression by both NaCl and mannitol treatments. These results suggest that osmotic stress-induced expression of *OsCam1* gene requires the $[Ca^{2+}]_{cyt}$ elevation that is known to occur in response to these stimuli.

Keywords: Calcium signaling, Calmodulin, *Oryza sativa* L., Stress signals

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Introduction

A wide range of stimuli trigger rapid and transient increases in intracellular Ca^{2+} concentration of plant cells. These stimuli include environmental signals produced by drought, salinity, cold, mechanical perturbation, hormonal signals, symbiotic and pathogenic microorganisms (Knight *et al.*, 1991, 1992, 1997; Kiegle *et al.*, 2000). Accumulating evidence has implicated Ca^{2+} signaling in transducing signals from environmental changes into adaptive responses (reviewed in Bressan and Hasegewa, 1998; Knight and Knight, 2001). Within cells, changes in the cytosolic Ca^{2+} concentration are perceived by the EF-hand families of Ca^{2+}-modulated proteins. Three major groups of Ca^{2+}-modulated proteins that have been characterized in plants are calmodulin (CaM), Ca^{2+}-dependent protein kinase (CDPK), and calcineurin B-like protein (CBL) (Zielinski, 1998; Harmon *et al.*, 2000; Luan *et al.*, 2002). CaM is probably the most well characterized Ca^{2+} sensor among these groups of protein. It is a small (148 residues) multifunctional protein that transduces the signal of increased Ca^{2+} concentration by binding to and altering the activities of a variety of target proteins. The activities of these proteins affect physiological responses to the vast array of specific stimuli received by plant cells (Yang and Poovaiah, 2003).

Primary structures of CaM are generally conserved throughout evolution among various organisms. However, in plants, one striking difference is that numerous isoforms of CaM may occur within a single plant species. A large family of genes encoding CaM isoforms from several plants has been identified including *Arabidopsis* (*Arabidopsis thaliana*) (Ling *et al.*, 1991; Gawienowski *et al.*, 1993; Zielinski, 2001), potato (*Solanum tuberosum*) (Takezawa *et al.*, 1995), soybean (*Glycine max*) (Lee *et al.*, 1995) and petunia (*Petunia hybrida*) (Rodriguez-Concepcion *et al.*, 1999). The broad significance of multiple CaM isoforms is not clearly understood. However, it is frequently proposed that diverged CaM isoforms may activate selected subsets of target proteins involved in Ca^{2+}-mediated signal transduction (Liao *et al.*,

1996; Heo *et al.*, 1999). In addition, differential expression of CaM isoforms (Gawienowski *et al.*, 1993; Zielinski, 2001) and differential Ca^{2+} sensitivity CaM confers to CaM/target protein binding (reviewed in Buaboocha and Zielinski, 2001) may play essential roles in selective target activation in different tissues and under different stimuli.

Even though the explanation of gene redundancy still cannot be ruled out for the presence of multiple CaM isoforms, accumulating evidence suggests that each of *Cam* genes may have distinct and significant functions. Expression of some but not all of the multiple CaM isoforms in a single plant species have been reported to be induced by particular stress signals including signals produced by stimuli such as cold, wind, wounding and pathogenic attack (Botella and Arteca, 1994; van der Luit *et al.*, 1999; Yamakawa *et al.*, 2001). As seen in one example from soybean, SCaM4 and SCaM5 are induced by a fungal elicitor or pathogen while other genes encoding highly conserved CaM are not. Further, constitutive expression of SCaM4 in transgenic plants was reported to trigger the formation of spontaneous lesions reminiscent of hypersensitive cell death on leaves and induced the expression of several resistance-associated genes (Heo *et al.*, 1999). Coupled with the importance of Ca^{2+} signaling in meditating stress response, stress-induced modulation of *Cam* gene expression suggests that different sets of CaM isoforms in a single plant species probably transduce Ca^{2+}-mediated signals produced by different stimuli into physiological responses that result in proper adjustments to their surroundings. In this study, to determine the CaM involvement in mediating stress responses in *Oryza sativa* L., *Cam* gene expression under various stress signals has been monitored. Expression of *Cam* genes encoding different rice CaM isoforms has been investigated in order to suggest possible differential roles in mediating stress signals and to determine which isoforms may participate in transducing specific stimuli. In addition, whether the modulated expression of calmodulin genes depends on the $[Ca^{2+}]_{cyt}$ elevation will be examined in order to investigate the signaling pathways involved. Even though the significance of Ca^{2+}/CaM transduction pathways in several stress responses has been documented, downstream elements remain unclear. Therefore, identifying CaM isoforms that play important roles in mediating response to particular stress will lead to a more directive approach in revealing their respective downstream elements.

Materials and Methods

Materials Enzymes used for manipulating recombinant DNA were obtained from Fermentas (Hanover, USA) and Life Technologies (Gaithersburg, USA). Kits for purifying and gel extracting plasmid DNA were purchased from Qiagen (Chatsworth, USA). pGEM®-T Easy vector system was from Promega (Madison, USA). Radioactive chemicals were purchased from Amersham Biosciences (Piscataway, USA). Synthetic oligonucleotides for reverse transcription-polymerase chain reaction (RT-PCR) were obtained from Bioservice Unit of the National Science and Technology Development Agency (Bangkok, Thailand). Seed of *Oryza sativa* L. cultivar KDML105 was provided by the Thailand Rice Research Institute (Patumtani, Thailand). All cDNA clones were provided by NIAS DNA Bank of the National Institute of Agrobiological Science (Ibaraki, Japan). The GenBank accession numbers for the sequences corresponding to *OsCam1*, *OsCam2*, and *OsCam3* are AU081299, C73257, and AU066154, respectively.

Sequence analysis Nucleotide and protein sequences of different calmodulin isoforms were obtained from GenBank databases via the National Center for Biotechnology Information (http://www.ncbi.mlm.nih.gov) and rice database via the National Institute of Agrobiological Science (http://www.dna.affrc.go.jp). Sequencing of cDNA inserts was carried out at Bioservice Unit of the National Science and Technology Development Agency (Bangkok, Thailand). Multiple sequence alignment was performed using ClustalW (http://www.ebi.ac.uk/clustalw/).

Stress treatments Rice seedlings were hydroponically grown in Limpinuntana's nutrient solution (Limpinuntana, 1978) under a 12-hr light/12-hr dark photoperiod. All stresses were initiated 2 hours after the start of the light period by transferring 2-week old seedlings to the growth medium containing 150 mM NaCl or mannitol to imitate salinity or drought stress, respectively. For wounding treatment, leaves were wounded with a pair of forceps pressing across the midribs 3-4 times along the leaf. To test the effect of lanthanum, lanthanum chloride (Sigma, St. Louis, USA) was added to the final concentration of 10 mM in the nutrient solution 2 hours before stress treatments. Samples were collected, immersed in liquid nitrogen at time points indicated in Figure 3 and 4 and stored at −70°C.

Analysis by Reverse transcription polymerase chain reaction (RT-PCR) *Oryza sativa* L. tissues were ground in liquid nitrogen using chilled mortars and pestles. Total RNA was isolated according to Verwoerd *et al.* (1989) and used in reverse transcription. Reverse transcription was primed by random hexamer synthetic oligonucleotides, and PCR was carried out using: 5'-CTGCCGATGATGGCATAGTA-3' and 5'-GCAAGCCTTAACAGATTCAC-3' for *OsCam1*; 5'-GAGGAGGGTTCCCATTAAAT -3' and 5'-CGCAAGATAAGCATCACAAT-3' for *OsCam2*; and 5'-GAGGATAGAGGAGAAGAGGG-3' and 5'-GCCAGTGTTATTTCTCGATC-3' for *OsCam3* as the sense and antisense primers, respectively. PCR amplification by Taq polymerase consisting of 30 cycles was conducted using a program of 94°C for 2 minutes, 50°C for 1 minute, and 72°C for 1 minute for *OsCam1* and *OsCam2*; and a program of 94°C for 3 minutes, 52°C for 1 minute, and 72°C for 1 minute for *OsCam3*. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining and UV fluorescencing.

RNA gel blot analysis *Oryza sativa* L. leaves were ground in liquid nitrogen using chilled mortars and pestles. Total RNA was isolated according to Verwoerd *et al.* (1989), fractionated by formaldehyde agarose gel electrophoresis, transferred to positively charged nylon membranes (Boehringer Mannheim, Indianapolis, USA) and immobilized by UV crosslinking in a Bio-Rad GS Gene

Linker™ UV chamber (Hercules, USA). The membranes were hybridized to probes made from the 3'-untranslated regions of the *Cam* genes in solution containing 50% (v/v) deionized formamide, 5XSSPE (20XSSPE is 2.98 M NaCl, 0.2 M $NaH_2PO_4 \cdot H_2O$)/NaOH (pH 7.4), and 20 mM $Na_2 \cdot$ N,N,N',N'-ethylenediamine tetraacetic acid (EDTA)$\cdot 2H_2O$), 1X Denhardt's solution (100X Denhardt's solution consists of BSA, Ficoll, and polyvinylpyrrolidone (PVP) at 2% [w/v] each), and 0.2% (w/v) sodium dodecyl sulfate (SDS) at 40ºC for 16 hours. The blots were washed in 2X SSPE, 0.1% SDS twice and then once in 1X SSPE, 0.1% SDS at room temperature. For some hybridization, the blots were further washed in 0.1X SSPE, 0.1% SDS at 45ºC. Hybridization probes were labeled with [α-^{32}P]-dCTP by random priming (Hodgson and Fisk, 1987).

Results

Computational analysis of *Cam* nucleotide and protein sequences from *Oryza sativa* L. databases Unlike yeast and animals, plants express several isoforms of CaM. BLAST (Altschul *et al.*, 1997) searching of nucleotide and protein databases using nucleotide sequence and amino acid sequence of *Arabidopsis* CaM2, respectively revealed several isoforms of CaM from *Oryza sativa* L. Via Entrez, three large UniGene clusters of rice *Cam* genes comprising of several mRNA and EST sequences were selected for these studies. They are UniGene clusters Os.2226, Os.10290, and Os.2216 and CaM proteins deduced from these clusters will be referred as OsCaM1, OsCaM2, and OsCaM3, respectively. cDNA clones encoding these sequences were obtained from NIAS DNA Bank of the National Institute of Agrobiological Science (Ibaraki, Japan) and their nucleotide sequences were determined.

The deduced amino acid sequences of genes comprising these three clusters are shown in Fig. 1 compared with CaM sequences from *Arabidopsis*, vertebrate, and yeast. *OsCam1* and *OsCam2* encode a typical plant CaM of 148-amino-acid residues that contains four characteristic Ca^{2+}-binding motifs. Deduced amino acid sequences derived from *OsCam1* and *OsCam2* differ by 2 and 4 residues, respectively from *Arabidopsis* CaM2 (Ling *et al.*, 1991). Both share about 90% and 60% identity with those from vertebrates and yeast, respectively. OsCaM1 sequence differs by 2 amino acid residues, an Asp to Glu substitution at residue 8 and an Arg to Lys substitution at residue 75, from OsCaM2. Despite the conservative substitutions, the difference between OsCaM1 and OsCaM2 may be functionally significant as further discussed below.

OsCaM3 encodes a similar protein with a 38-amino-acid extension containing a basic domain and a prenylation site (CVIL) at the carboxyl terminus. Excluding the carboxyl extension, OsCaM3 shares 86% amino acid identity to OsCaM1 and OsCaM2. OsCaM3, known as OsCaM61 was identified as a novel CaM-like protein by Xiao and colleagues (Xiao *et al.*, 1999). This CaM isoform was reported to be membrane-associated when it is prenylated and localized in the nucleus when it is unprenylated (Dong *et al.*, 2002). A

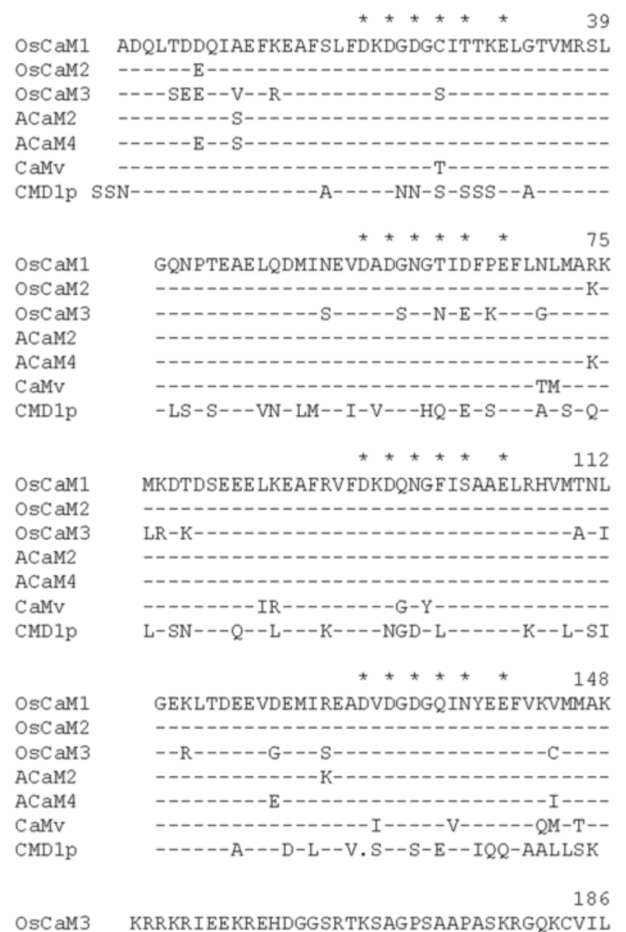

Fig. 1. Amino acid sequence alignment of *Oryza sativa* L. CaM isoforms and CaM proteins from *Arabidopsis* (ACaM2 and ACaM4), vertebrates (CaMv) and *Saccharomyces cerevisiae* (CMD1p). The sequences are compared with OsCaM1 as a standard; identical residues in other sequences are indicated by a dash (-), and gaps introduced for alignment purposes are indicated by dots (.). The sequences are arranged to show the relationships among the four Ca^{2+}-binding domains of the molecules. Residues serving as Ca^{2+}-binding ligands are marked with asterisks (*). OsCaM1, OsCaM2, and OsCaM3 amino acid sequences were deduced from mRNA sequences composing the UniGene clusters Os.2226, Os.10290, and Os.2216, respectively. GenBank accession numbers for the sequences used in the alignment are: ACaM2 (M38380); ACaM4 (Z12022); CaMv (M65156); and CMD1p (M14760).

similar CaM protein called CaM53 previously found in petunia also contains an extended C-terminal basic domain and a CAAX (C is Cys, A is aliphatic, and X is a variety of amino acids) motif which are required for efficient prenylation of protein (Rodriguez-Concepcion *et al.*, 1999). Similar subcellular localization depending on its prenylation state was reported.

As part of the rice genome project, genomic DNA sequences encoding OsCaM1, OsCaM2, and OsCaM3 were recently deposited in GenBank (Accession numbers AC119748, AC129718, AP003260, respectively). The genes encoding

OsCaM isoforms described in this study are dispersed in the rice genome (*OsCam1* in chromosome 3, *OsCam2* in chromosome 5, *OsCam3* in chromosome 1). All the genes are interrupted by an intron immediately after the codon encoding Gly25, a typical rearrangement of all plant *Cam* genes. On the contrary, *OsCaM3* has an additional intron at the codon corresponding to the last residue in *OsCam1* and *OsCam2*. The position of this intron reflects the separation of functional domains within the OsCaM3 protein and suggests that the sequence encoding the carboxyl extension of OsCaM3 have arose later in the evolution by fusion of an existing *Cam* gene to the additional exon.

Three rice *Cam* mRNAs are ubiquitously expressed in leaf and root To experimentally determine whether the expression of these genes is restricted to specific organs in rice, total RNA was isolated form leaves and roots of 2-week old seedlings and used to perform reverse transcription and PCR amplification reactions. Primers selected by computer analysis of the EST sequences corresponding to these genes are given in the Materials and Methods section. A control RT-PCR reaction without adding reverse transcriptase was done in parallel with each experimental reaction using total RNA to ensure that the product obtained could be attributed to the product of the reverse transcriptase reaction. Figure 2A shows that a band of the expected size based on each of the *Cam* gene sequences was detected in all samples. These results indicate that all *Cam* mRNAs are expressed in both organs of the 2-week old rice seedlings. The relative abundance of *Cam* mRNAs was not determined by this method.

RNA gel blot analysis of the three *Cam* mRNA accumulations was also carried out to detect their expression in leaf and would be further used to determine their expression under stress below. Even though the coding regions of these genes are highly conserved, the 5' and 3' untranslated regions (UTR) are highly diverged therefore the probes made from these sequences can be used to differentiate their respective mRNAs. From sequence comparison, while their nucleotide sequences share more than 85% identity in the coding regions, the identity among the sequences in the 3' UTR is relatively low (less than 40%). Therefore, DNA fragments made from the 3' UTR were used to prepare isoform-specific probes. Figure 2B shows that all mRNAs of the expected size were observed in the blots prepared from total RNA isolated from the leaf further confirming that all *Cam* sequences are expressed in this organ. It should be noted that the blots shown here were not resulted from the same condition and exposure time. Hence they are not a representation of their relative level of steady state mRNAs. Nevertheless, consistent with RT-PCR, results from the RNA gel blot analysis suggest that the three *Cam* mRNAs concurrently accumulate in the 2-week old rice seedlings.

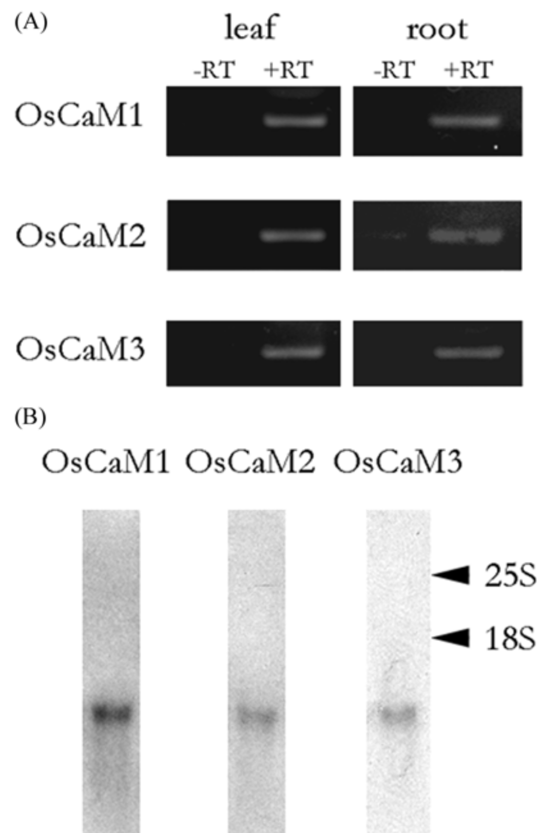

Fig. 2. *Cam* mRNA is expressed in leaves and roots of 2-week old rice seedlings. A. Total RNA isolated from leaves and roots was used in RT-PCR assays either without (-RT) or with (+RT) the addition of M-MLV reverse transcriptase. The cDNAs were amplified by PCR using gene-specific primers. The products derived from 100 ng of total RNA were separated in agarose gels and visualized by ethidium-bromide staining. B. RNA gel blot analyses of *Cam* mRNA accumulation in rice leaves. Twenty micrograms of total RNA were fractionated in formaldehyde agarose gels, transferred to charged nylon membranes, and hybridized with the respective 3'-untranslated regions of the *Cam* mRNAs at 40ºC for 16 hours. The blots were washed in 2X SSPE, 0.1% SDS twice and then once in 1X SSPE, 0.1% SDS at room temperature. For OsCaM2 and OsCaM3, the blots were further washed in 0.1X SSPE, 0.1% SDS at 45ºC. Positive hybridizing bands were detected by autoradiography. The approximate sizes of *Cam* mRNAs were deduced from the synthetic RNA markers (Fermentas) run in parallel lanes of the gel, which was stained with ethidium bromide.

Differential expression pattern of *Cam* genes in response to stress signals Modulation of gene expression in response to stress signals may reflect the function of the corresponding gene product. Members of the *Cam* gene family may possess differential roles in mediating responses to various stresses, therefore this experiment was conducted to determine whether steady state mRNA levels of any of these three rice *Cam* genes were modulated under various stress conditions including treatments with NaCl, mannitol and wounding. To perform RNA gel blot analysis, two-week old rice seedlings containing about 4 leaves were treated as described in the

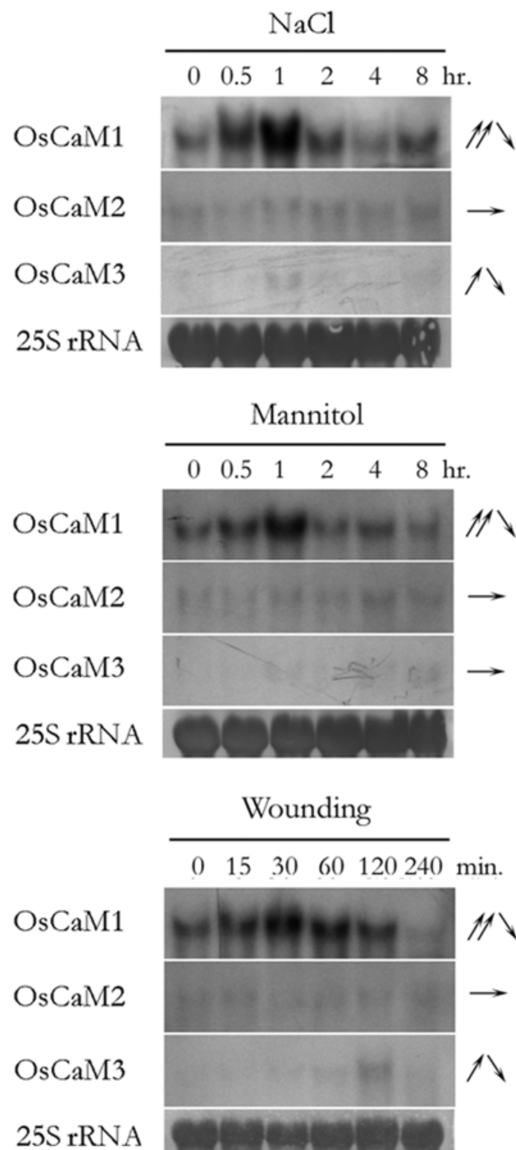

Fig. 3. Expression pattern of three rice CaM-encoding genes under various stress signals. Twenty micrograms of total RNA isolated from leaves harvested at time points indicated after each treatment were loaded into each lane in formaldehyde agarose gels, transferred to charged nylon membranes, and hybridized with the respective 3'-untranslated regions of the *Cam* mRNAs at using the same hybridization conditions as above. Positive hybridizing bands were detected by autoradiography. Blots showing the staining by methylene blue of 25S rRNA are used as a loading control.

Materials and Methods section. The leaf was collected and stored in liquid nitrogen at the time points indicated in Fig. 3. Total RNA was extracted and twenty micrograms of RNA was separated by formaldehyde gel eletrophoresis, transferred to positively charged nylon membranes, and hybridized with the same probes used in the experiment described in Fig. 2B.

As shown by the autoradiographs in Fig. 3, treatments with NaCl and mannitol as well as wounding strongly increased mRNA level of *OsCam1* as early as 30 min after NaCl and mannitol treatments and 15 min after wounding. The level of *OsCam1* transcript peaked at 1 h after NaCl and mannitol treatments and 30 min after wounding and their levels were maintained higher than that of controls for 1-2 h before decreased to their original levels. On the contrary, the level of *OsCam2* mRNA appeared relatively unchanged throughout the period monitored under any stress conditions. Figure 3 also demonstrates that the RNA gel blot analysis detected a low level of *OsCam3* mRNA which did not change in response to mannitol treatment but transiently increased at 1 h after NaCl treatment. In addition, under wounding stress, the mRNA level significantly increased at 2 h after treatment in wounded leaves and after 4 h of treatment, its level decreased to the original level. All data shown here indicates that these three rice *Cam* genes are differentially regulated by stress signals under investigation.

Induction under osmotic stress of *OsCam1* gene expression is mediated by Ca^{2+} signal The previous experiment has shown that the expression level of *OsCam1* gene is significantly increased in response to osmotic stress. In order to investigate the signaling pathways possibly involved in this induction, preincubation of rice seedlings with 10 mM lanthanum chloride prior to stress treatments was conducted. The calcium-channel blocker lanthanum chloride was shown to cause a significant reduction in the magnitude of the $[Ca^{2+}]_{cyt}$ elevation in response to salt and mannitol in *Arabidopsis* (Knight *et al*., 1997). The authors showed that lanthanum inhibited the expression of the gene *p5cs*, which encodes a protein with a protective role in the response to drought. By monitoring ATP levels in both treated and non-treated plants, they concluded that the inhibition by lanthanum was for the most part, or entirely, due to their effects on calcium signaling, not simply as a result of general toxic effects. To examine the involvement of the $[Ca^{2+}]_{cyt}$ elevation in response to salt and mannitol on the expression of *OsCam1* gene, levels of *OsCam1* mRNA from lanthanum-treated rice seedlings and non-treated seedlings were compared by RNA gel blot analysis. From our results, the expression of *OsCam1* mRNA reached maximal levels after 1 hour for both salt and mannitol-treated seedlings so this time point was used for assessing the effect of lanthanum. Figure 4 shows that preincubation in 10 mM lanthanum chloride for 2 hours prior to salt stress significantly reduced the level of *OsCam1* expression. Similar result was obtained when seedlings were treated with mannitol treatment. These results clearly indicate that calcium signaling is required for the induction of *OsCam1* gene in response to osmotic stress.

Discussion

With the completion of the genomic DNA sequencing project in *Oryza sativa* L., sequences belong to a multigene family

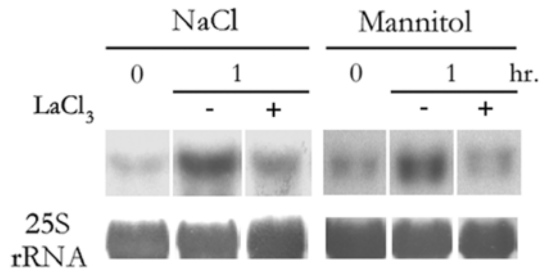

Fig. 4. Effect of lanthanum on the induction of *OsCam1* gene expression by osmotic stress. Two hours prior to stress treatments, lanthanum chloride was added to the nutrient medium to the final concentration of 10 mM. Twenty micrograms of total RNA isolated from leaves harvested at 1 hr after each treatment (collected before each treatment for controls as indicated by 0 hr) were loaded into each lane in formaldehyde agarose gels, transferred to charged nylon membranes, and hybridized with the 3'-untranslated region of the *OsCam1* mRNA using the same hybridization conditions as above. Positive hybridizing bands were detected by autoradiography. Blots showing the staining by methylene blue of 25S rRNA are used as a loading control.

such as CaM can be extensively studied. By searching *Oryza sativa* L. databases using the amino acid and nucleotide sequences of *Arabidopsis Cam2* as query sequences in the programs BLASTp and BLASTn, respectively, numerous genomic and cDNA sequences whose nucleotide sequences share significant identity with those of plant or animal *Cam* sequences were identified. Via the bioinformatics tool called UniGene developed by the National Center for Biotechnology Information at GenBank in which mRNA and EST sequences are clustered into sets of related sequences, a large *Cam* gene family was revealed. The cDNA sequences in the family retrieved belong to at least nine UniGene clusters (data not shown). Three of these clusters consist of numerous EST clones isolated from various rice tissues suggesting that they are ubiquitously expressed. In this study, we confirmed this observation by experimentally detecting the expression of three *Oryza sativa* L. *Cam* genes both in the shoot and the root of 2-week old rice seedlings by RT-PCR. To exclude the possibility that these cDNA sequences represent partial copies of much longer mRNAs, RNA gel blot analysis was also carried out. The size of *OsCam1*, *2*, and *3* mRNAs observed in the blots shown in Figure 2B was about 1000 nucleotides which is consistent with the nucleotide sequence data of rice *Cam* transcripts, therefore, these sequences are unlikely resulted from larger mRNAs.

The ubiquitous expression of several *Cam* genes in a single organ was not surprising because of several similar occurrences in other plant species. In tobacco, all 13 *Cam* genes were expressed in almost all organs examined with a few exceptions, notably *NtCaM13*, which exclusively expressed in the root (Yamakawa *et al.*, 2001). Similarly in *Arabidopsis*, most *Cam* genes are ubiquitously expressed even the highly diverged members of the gene family such as CaM8 and CaM9 (Zielinski, 2001). Although CaM isoforms are ubiquitously expressed, their tissue-specific function can be achieved through its interacting proteins which accumulate in tissue-specific patterns (Reddy *et al.*, 2002). In addition, different CaM-target protein complexes may have distinct affinities and kinetics for Ca^{2+} binding (Persechini *et al.*, 1996; Peersen *et al.*, 1997). Therefore, selective activation of different CaM/target protein complexes can be accomplished by CaM acting in concert with target proteins in transducing Ca^{2+} signals whose characters can be different according to stimuli perceived. In plants, the matter becomes more complicated due to the existence of a large *Cam* gene family. This defining characteristics of CaM in plants may actually equip them with a more fine-tune mechanism to perceive and transduce enormous amount of signals that constantly bombard them.

In several plant species, *Cam* genes encoding different CaM isoforms have been reported to be differentially expressed in different developmental stages or in response to external stimuli such as salinity, wind, cold, wounding and pathogen attack (van der Luit *et al.*, 1999; Delumeau *et al.*, 2000; Yamakawa *et al.*, 2001; Duval *et al.*, 2002). To examine the possibility that these three members of the rice *Cam* gene family express differently in response to stress signals therefore have distinct functions, their expression under various stresses was determined by RNA gel blot analysis. The level of steady state mRNA corresponding to *OsCam1* gene strongly increased under salt and mannitol treatments as early as 30 min and peaked at 1 h after treatment. Consistent with this result, the expression of *OsCam1* in two rice varieties, Pokkali and IR29 has been reported to increase during the initial phase of salt stress determined by microarray technique (Kawasaki *et al.*, 2001). Even though the effect of the ionic stress component of NaCl on *OsCam1* gene expression can not be ruled out, its osmotic stress component appears to be an important factor that causes its steady state mRNA level to increase as seen in both NaCl and mannitol treatments in Fig. 3. In addition, NaCl treatment also increased the *OsCam3* mRNA level (1 h) but its increase exhibited a more transient modulation. Similar transient increase was observed when leaves were wounded, however the induction did not appear until 2 h after the treatment. These results suggest possible roles of both OsCaM1 and OsCaM3 in transducing salinity and wounding; however different signaling initiated by these stimuli leading to the accumulation of their mRNAs and downstream elements under their regulation are possible.

The expression pattern of genes encoding OsCaM1 and OsCaM2 was evidently different under all stress treatments. All stimuli that strongly increased the level of steady state mRNA corresponding to *OsCam1* did not affect that of *OsCam2*. Even though the two genes encode proteins containing only two-residue difference, their differential expression pattern was not unexpected. Previous reports have shown that highly conserved CaM isoforms may actually modulate their target proteins differently. In *Arabidopsis*,

CaM2 was shown to stimulate NAD kinase more effectively than CaM4 and CaM6 (Liao *et al.*, 1996). By amino acid sequence comparison, OsCaM1 is more similar to ACaM2, while OsCaM2 is more similar to ACaM4. Glu for Asp substitution at position 7 similar to OsCaM2 may actually affect target binding because of their longer side chain as hypothesized by Duval *et al.* (2002). In addition, competition among CaM isoforms for target proteins may be present therefore different transcriptional regulation can significantly affect their ration of CaM isoforms meaning that more "active" CaM isoform may exist in a cell that responds to a particular stimulus. By this interpretation, OsCaM1 possibly function in mediate stress response under salinity and drought by interacting with a subset of target proteins that might not act as *vivo* targets of OsCaM2.

Because stress-induced modulation of gene expression often reflects the function of the corresponding gene product in signaling, the results observed in this study suggest that these rice CaM isoforms probably function in Ca^{2+}-mediated response to stress signals in distinct pathways. At least two rice *Cam* genes (*OsCam1 and OsCam3*) may be functionally important in mediating response under drought, salinity and wounding stress. In addition, we have demonstrated that the calcium-channel blocker lanthanum chloride inhibited the induction of *OsCam1* gene expression by both NaCl and mannitol treatments. These results indicate that osmotic stress-induced expression of *OsCam1* gene requires the $[Ca^{2+}]_{cyt}$ elevation that occurs in response to these stimuli. The requirement for calcium signal further supports the speculation of possible function for *OsCam1* gene in mediating osmotic stress response which involves calcium signal as an early step. Increased level of specific calmodulin isoforms mediated by stress-induced $[Ca^{2+}]_{cyt}$ elevation would ensure that plant cells have a lasting capacity to transduce stress signals into specific downstream elements. However, *in vivo* studies of their physiological significance and downstream target elements remain to be investigated. Currently, some plant CaM-binding proteins have been characterized and others remained to be identified. How most of them work in conjunction with CaM in the physiological context is poorly understood. In plants, more diversified CaM-binding proteins were observed than those in animals exist (Reddy *et al.*, 2002). Coupled with the presence of numerous CaM isoforms, this observation reflects the complexity of Ca^{2+} signaling pathways involving CaM/target protein activation and underlines the evolutionary necessity for plants to continuously adjust to a variety of environmental cues. By determining which CaM isoform is potentially involved in transducing a particular stress exemplified by our result of *OsCam1* gene, its physiological function and downstream elements will further be characterized in a more defined manner.

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