Molecular Characterization of Three cDNA Clones Encoding Calmodulin Isoforms of Rice

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Three cDNA clones encoding rice calmodulin (CaM) isoforms (*OsCaM-1*, *OsCaM-2*, and *OsCaM-3*) were isolated from a rice cDNA library constructed from suspension-cultured rice cells treated with fungal elicitor. The coding regions of *OsCaM-1* and *OsCaM-2* were 89% homologous at DNA level, whereas the 5' and 3' untranslated regions were highly divergent. The polypeptides encoded by *OsCaM-1* and *OsCaM-2* was identical except two conservative substitution at position 8 and 75. The coding region of *OsCaM-3* was consist of a typical conserved CaM domain and an additional C-terminal extension. The amino acid sequence of conserved CaM domain of *OsCaM-3* shared only 86% identity with that of *OsCaM-1*. The *OsCaM-3* cDNA is belongs to a novel group of calmodulin gene due to its C-terminal extension of 38 amino acids, a large number of which are positively charged. The extension also contains a C-terminal CaaX-box prenylation site (CVIL). Genomic Southern analysis revealed at least six copies of CaM or CaM-related genes, suggesting that calmodulin may be represented by a small multigene family in the rice genome. Expression of *OsCaM* gene was examined through Northern blot analysis. Transcript level of *OsCaM-3* was increased by treatment with a fungal elicitor, whereas the *OsCaM-1* and *OsCaM-2* genes did not respond to the fungal elicitor. The expression of *OsCaM-3* gene was remarkably inhibited in the rice cells treated with cyclosporine A, calcinurin inhibitor.

Key words: *signal transduction, Ca^{2+} chelator, calmodulin, fungal elicitor.*

Calcium plays a vital role as a second messenger in the regulation of many intracellular processes such as growth, development, and physiology of plant cells.[1,2] Recently, tremendous progress has been made on the role of Ca^{2+} in signal transduction pathways of plants.[3] Cytosolic calcium controls many cellular processes through calcium-modulated proteins such as CaM,[1,4] a highly conserved and ubiquitous calcium-binding protein, which transduces Ca^{2+} signals to various target proteins. In regulating biochemical and molecular functions and ultimately the physiological processes, the CaM actions are involved in modulations of enzymatic activity and various functions of interactive proteins such as NAD kinase, Ca^{2+}-ATPase, protein kinases, NTPase, and glutamate decarboxylase.[1,5,6] Furthermore, in *Saccharomyces cerevisiae*, CaM was found to perform yet unidentified Ca^{2+}-independent functions.[7]

Several CaM and CaM-related genes have been isolated from *Arabidopsis thaliana*,[8-11] potato,[12] soybean,[13] wheat,[14] and rice.[15] Interestingly, the plant CaM genes encode multiple isoforms of CaM in a single organisms, whereas there exists only a single form of CaM in animal systems even though the CaM is encoded by multiple genes.[16,17] The plant CaM isoforms perform different functions via variations in their calcium-binding abilities and activation of CaM-dependent enzymes.[13,18] Recently, it has been proposed that specific CaM isoforms are components of the salicylic acid-independent signal transduction chain that leads to plant disease resistance.[19]

cDNA clones encoding plant CaMs of various plant species have been isolated and characterized. These cDNA clones generally encode polypeptides of 148 amino acids with about 90% homology. Although CaM is a ubiquitous protein, its level of expression varies between different tissues and under different environmental conditions. The expression of CaM gene is thus regulated in a developmental and organ-specific manner during plant growth and in terms of response to physical and chemical stimuli.[12,20-23]

In this report, we describe the isolation and characterization of three rice CaM cDNAs obtained from suspension-cultured rice cells. We also studied the expression of the rice CaMs in response to various chemical

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Abbreviations: ABA, Abscisic acid; CaM, calmodulin; SA, salicylic acid.

The nucleotide sequence data reported will appear in the EMBL, NCBI, and DDJB Nucleotide sequence Databases under the accession numbers AF042840 (OsCaM-1), AF042839 (OsCaM-2), and AF090687 (OsCaM-3).

signals and calcium concentrations in suspension-cultured cells

Materials and Methods

Plant materials. Rice seeds (*Oryza sativa* L. cv. Cheongcheongbyeo) were surface-sterilized in 70% ethanol for 1 min and in a 2% sodium hypochlorite solution containing a drop of Triton X-100 for 30 min and then rinsed thoroughly with sterilized water. Rice plants were grown for 3 weeks in a growth chamber at 28°C under a 16 h light period. Shoots and roots were then harvested and stored at -70°C until used.

Calli were initiated from mature seeds of rice on NB medium[24] supplemented with 2.0 mg/l 2,4-D. After 3 weeks of culture, embryogenic calli were transferred to 25 ml liquid R2S medium[25] supplemented with 2.0 mg/l 2,4-D and 3% sucrose in 100 ml Erlenmeyer flask and cultured on a gyratory shaker (120 rpm) at 28°C. Rice suspension cell cultures were subcultured at weekly intervals. To examine the inducibility of the OsCaM genes, suspension-cultured cells were treated with 2.0 mg/l 2,4-D (2 μg/ml), 50 μM salicylic acid, 100 μM calcium chelator (EDTA), 200 μM CaM-antagonist (W-7), or 0.1% glycol chitin for 12 h. The treatments of suspension cultures occurred 4 days after the suspension cultured cells were transferred into fresh medium.

Preparation of fungal elicitor. The preparation of fungal elicitor followed the method of Doke and Furuichi.[26] Mycelia of *Magnaporthe grisea* (rice blast fungus) were cultured on potato dextrose broth (PDB) at 25°C for 7 days. The mycelia were then chopped up and homogenized in 50 mM sodium acetate buffer (pH 4.5), sonicated, and centrifuged. The pellet was again homogenized in 0.1 M borate buffer (pH 8.8). The homogenate was then autoclaved at 121°C for 10 min and centrifuged. The resulting supernatant was extensively dialyzed against distilled water at 4°C and then freeze-dried.

Screening of cDNA library. A ZAPII cDNA library was constructed from poly(A)$^+$ RNA isolated from 4-day old suspension-cultured rice cells treated with 50 μg/ml fungal elicitor for 30 min. The CaM cDNAs were isolated by screening the rice cDNA library with a randomly primed ^{32}P-labeled RCaM-1 gene probe.[15] Screening of the cDNA library was performed according to standard procedures.[27] Approximately 3×10^5 recombinant plaques were screened by plaque hybridization. The nitrocellulose filters were prehybridized for 2 h and then incubated with the probe overnight at 65°C in a solution containing 6× SSC [1× SSC is 150 mM NaCl, 15 mM trisodium citrate, pH 7.0], 5× Denhardts solution [1× Denhardts solution is 0.02% (w/v) Ficoll 400, 0.02% (w/v) polyvinylpyrrolidone], 0.5% (w/v) SDS, 10 mM EDTA, 100 μg/ml denatured salmon sperm DNA, and pH 8.0. The membrane was washed three times for 15 min in 2× SSC, 0.5% SDS at 65°C and three times in 0.2× SSC, 0.1% SDS at 65°C for 15 min. The blots were exposed to Kodak XAR-5 X-ray film overnight at -70°C.

DNA sequence analysis. DNA was sequenced by the dideoxy chain termination method[28] using a Taq Dye primer cycle sequencing kit in conjunction with an Applied Biosystem 373A automatic DNA sequencer. A series of overlapping deletion subclones in both orientations were generated using exonuclease III and S1 nuclease.[29] Nucleotide sequence analysis was carried out with the PC/GENE program package, and searches for sequence similarities were done using the BLAST algorithm[30] as implemented on the National Center for Biotechnology Information (NLM, NIH, Bethesda, MD) server.

Genomic DNA isolation and Southern blotting. Genomic DNA was extracted from 2-week old rice plants using the CTAB precipitation method.[31] Ten microgram aliquots of rice genomic DNA was digested with *Eco*RI, *Bam*HI, or *Hin*dIII, fractionated on a 0.8% agarose gel, denatured, and transferred onto a nylon membrane (GeneScreen Plus, Dupont) by capillary transfer using 10× SSC. After UV cross-linking, the membrane was pre-hybridized in a solution containing 6× SSC, 5× Denhardts reagent, 0.5% SDS, and 100 /ml denatured salmon sperm DNA for 2 h at 65°C and then hybridized in the same solution to the ^{32}P-labeled RCaM-1 probe. The filter was washed at room temperature in 2× SSC containing 0.5% SDS, followed by washing in 1× SSC and 0.1% SDS at 68°C for 30 to 60 min. Final washes were carried out in 0.1× SSC and 0.1% SDS at 68°C for 1 h.

Northern blot analysis. Total RNA was extracted from the shoots, roots, and suspension-cultured cells treated with various chemicals using the guanidium isothiocyanate method with subsequent ultracentrifugation.[27] Twenty microgram RNA was fractionated on a 1.5% (w/v) agarose gel with formaldehyde, blotted onto a nylon membrane, and incubated at 65°C for 1 h in hybridization buffer (6× SSC pH7.2, 5× Denhahardt's solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA). The conserved OsCaM or specific OsCaM-3 probe was radiolabeled with ^{32}P-dATP through random priming according to the manufacturer's instructions (Pharmacia) and purified through spin chromatography.[27] The probe was heat-denatured, added to the hybridization buffer, and incubated with the blocked membrane overnight at 65°C. The membrane was washed 2× SSC for 20 min at room temperature, 2× SSC for 30 min at 65°C, and 1× SSC and 0.1% SDS at 65°C for 20 min before exposing overnight on Kodak XAR-5 X-ray film.

Results and Discussion

Isolation of rice CaM cDNA clones. To understand the possible role of CaM in Ca^{2+}-mediated signaling pathways, three different cDNA clones encoding CaM isoforms were isolated by screening a rice cDNA library constructed from rice suspension-cultured cells treated with fungal elicitor.

After screening about 3×10^5 phage plaques, 8 positive clones were isolated. The positive clones were grouped into three different CaM cDNAs which were designated as *OsCaM-1, -2,* and *-3* (Fig. 1A, B, C). The *OsCaM-1* and *-2* encoded calmodulins which were very similar to other plant calmodulins such as alfalfa, barley, soybean, potato, and *Arabidopsis.*[1,13] However, OsCaM-3 encodes a calmodulin isoform which differs substantially in its C-terminal (residues 150-187) from from other reported calmodulins. Its sequence comprises 971 bp, including 105 bp of 5'-untranslated, 561 bp of coding, and 273 bp of 3'-untranslated sequence. The ORF of OsCaM-3 is 187 amino acids which contains additional 38 amino acid at the C-terminal region. While OsCaM-1 and OsCaM-2 lack a consensus polyadenylation signal, the OsCaM-3 has a putative polyadenylation signal at the 3' end which may account for

OsCaM1 [A]

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cattctctccgcgacggtctcgtcttccccaccctcgcctcctcgcgcgctcggtgagagaagcgaagaag      72
aagaagaagaggaggaggaagaagccaggctaagcccagcggcATGGCGGACCAGCTCACCGACGACCAGAT     144
                                          M  A  D  Q  L  T  D  D  Q  I    (10)
CGCCGAGTTCAAGGAGGCCTTCAGCCTCTTCGACAAGGACGGCGATGGTTGCATCACAACCAAGGAGCTGGG     216
  A  E  F  K  E  A  F  S  L  F  D  K  D  G  D  G  C  I  T  T  K  E  L  G    (34)
AACCGTGATGCGTTCGCTGGGGCAGAACCCAACGGAGGCCGAGCTCCAGGACATGATCAACGAGGTCGACGC     288
  T  V  M  R  S  L  G  Q  N  P  T  E  A  E  L  Q  D  M  I  N  E  V  D  A    (58)
GGACGGCAACGGCACCATCGACTTCCCGGAGTTCCTCAACCTGATGGCACGCAAGATGAAGGACACCGACTC     360
  D  G  N  G  T  I  D  F  P  E  F  L  N  L  M  A  R  K  M  K  D  T  D  S    (82)
GGAGGAGGAGCTCAAGGAGGCGTTCAGGGTGTTCGACAAAGACCAGAACGGCTTCATCTCCGCCGCCGAGCT     432
  E  E  E  L  K  E  A  F  R  V  F  D  K  D  Q  N  G  F  I  S  A  A  E  L    (106)
CCGCCACGTCATGACCAACCTCGGCGAGAAGCTGACCGACGAGGAGGTCGACGAGATGATCCGCGAAGCCGA     504
  R  H  V  M  T  N  L  G  E  K  L  T  D  E  E  V  D  E  M  I  R  E  A  D    (130)
CGTCGACGGTGACGGCCAGATCAACTACGAGGAGTTCGTCAAGGTCATGATGGCCAAGTGAggcaccacttc     576
  V  D  G  D  G  Q  I  N  Y  E  E  F  V  K  V  M  M  A  K  *                (149)
ccctgccgatgatggcatagtaccctgggaggaggaaaccgtgcattgccgtattagtaaggggatgcaaac     648
actggtttcagtcgtcttccctgatgaagaaaaccgaaccgtactagttgtagttgctgaacatttttctat     720
ctctccagtctctccggtgtgccatggaacttcttgcttgatttttctgtgtgaatctgttaaggcttgctc     792
tgatctctccgaaaaaaaaaaaaaaaaaaaa                                             824
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OsCaM2 [B]

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gccactcgttccccttccttcctctcctcctctcgcggaaccttctcgaagcttccacacccccaacctcgc      72
ctccaccaccaaccccccATGGCGGACCAGCTCACCGACGAGCAGATCGCCGAGTTCAAGGAGGCGTTCAGC     144
                    M  A  D  Q  L  T  D  E  Q  I  A  E  F  K  E  A  F  S    (18)
CTCTTCGACAAGGACGGCGACGGTTGCATCACTACTAAGGAGCTTGGAACCGTGATGCGGTCCCTTGGTCAG     216
 L  F  D  K  D  G  D  G  C  I  T  T  K  E  L  G  T  V  M  R  S  L  G  Q     (42)
AACCCAACTGAGGCGGAGCTGCAGGACATGATCAACGAGGTTGATGCTGATGGCAATGGGACCATTGACTTC     288
 N  P  T  E  A  E  L  Q  D  M  I  N  E  V  D  A  D  G  N  G  T  I  D  F     (66)
CCAGAGTTCCTGAACCTGATGGCGAAGAAGATGAAGGATACCGACTCTGAGGAGGAGCTCAAGGAGGCCTTC     360
 P  E  F  L  N  L  M  A  K  K  M  K  D  T  D  S  E  E  E  L  K  E  A  F     (90)
CGTGTGTTTGACAAGGACCAGAACGGTTTCATCTCGGCTGCTGAGCTCCGCCACGTCATGACCAACCTTGGT     432
 R  V  F  D  K  D  Q  N  G  F  I  S  A  A  E  L  R  H  V  M  T  N  L  G     (114)
GAGAAGCTGACCGACGAGGAAGTCGACGAGATGATCCGTGAGGCTGACGTCGATGGCGATGGCCAGATCAAC     504
 E  K  L  T  D  E  E  V  D  E  M  I  R  E  A  D  V  D  G  D  G  Q  I  N     (138)
TACGAGGAGTTCGTTAAGGTCATGATGGCCAAGTGAggagggttcccattaaataagttctgtctgaagtga     576
 Y  E  E  F  V  K  V  M  M  A  K  *                                         (149)
actaaaactgtcagggcctacaacaaagctgtactttgtgatgttccttttaagtacctatctttgctggtg     648
gtaaaaaaaaaaaaaaaaaaaaa                                                     671
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OsCaM3 [C]

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                                        gctctatatcatcctcctctccgacacagcccgcg   35
cacctccacaccattagccatcaacgaccagcatctcggctttgctcgccttctcgaagcttctgctgccAT   107
                                                                      M     (1)
GGCGGACCAGCTCTCCGAAGAGCAGATTGTAGAGTTCAGGGAGGCCTTCAGCCTCTTCGACAAGGACGGCGA   179
  A  D  Q  L  S  E  E  Q  I  V  E  F  R  E  A  F  S  L  F  D  K  D  G  D    (25)
CGGTTCTATCACCACCAAGGAGCTAGGAACCGTGATGCGAAGTCTGGGGCAGAACCCAACAGAGGCGGAGCT   251
  G  S  I  T  T  K  E  L  G  T  V  M  R  S  L  G  Q  N  P  T  E  A  E  L    (49)
GCAGGACATGATCAGCGAGGTGGACGCGGACAGCAACGGCAACATCGAATTCAAGGAGTTCCTGGGCCTGAT   323
  Q  D  M  I  S  E  V  D  A  D  S  N  G  N  I  E  F  K  E  F  L  G  L  M    (73)
GGCGCGCAAGCTGAGGGACAAGGACTCCGAGGAGGAGCTGAAGGAGGCGTTCCGCGTCTTCGACAAGGACCA   395
  A  R  K  L  R  D  K  D  S  E  E  E  L  K  E  A  F  R  V  F  D  K  D  Q    (97)
GAACGGCTTCATCTCCGCCGCCGAGCTCCGCCACGTGATGGCCAACATCGGGGAGCGGCTCACCGACGAGGA   476
  N  G  F  I  S  A  A  E  L  R  H  V  M  A  N  I  G  E  R  L  T  D  E  E  (121)
GGTCGGCGAGATGATCAGCGAGGCCGACGTCGACGGCGACGGGCAGATCAACTACGAGGAGTTCGTCAAGTG   539
  V  G  E  M  I  S  E  A  D  V  D  G  D  G  Q  I  N  Y  E  E  F  V  K  C  (145)
CATGATGGCCAAGAAGAGGAGGAAGAGGATAGAGGAGAAGAGGGAGCACGACGGCGGCAGCAGGACGAAGAG   611
  M  M  A  K  K  R  R  K  R  I  E  E  K  R  E  H  D  G  G  S  R  T  K  S  (169)
TGCAGGGCCCTCCGCCGCGCCGGCGAGCAAGCGTGGCCAGAAGTGCGTGATCCTGTAAtaattgagccagca   683
  A  G  P  S  A  A  P  A  S  K  R  G  Q  K  C  V  I  L  *                 (187)
ctgagattctcatgagtcaatgagctacacgaatgatgtgtgttgttgtaaaatggcaaaacaattgtgtgg   755
gcgtgtagtatgatgctccactgaaagatcgagaaataacactggccagtgcaagttcacagccgtagtccg   827
tagtccgtacgctgtgggcccgtgccggcccatgggcaacagaccaaaacttactctttatatcctcgtgtt   899
tcctaaatttggcagtggtaaatttcatagctttaaaggggccaaaaaaaaaaaaaaaaaaa             971
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Fig. 1. Nucleotide and deduced amino acid sequences of the rice OsCaM-1 [A], OsCaM-2 [B], and OsCaM-3 [C] cDNAs. Nucleotides and amino acid residues in parentheses are numbered on the right side of the figure. C-terminal extension sequences of OsCaM-3 clone are indicated by underline. The putative polyadenylation site identified from the sequence of the OsCaM-3 cDNA is dot-underlined.

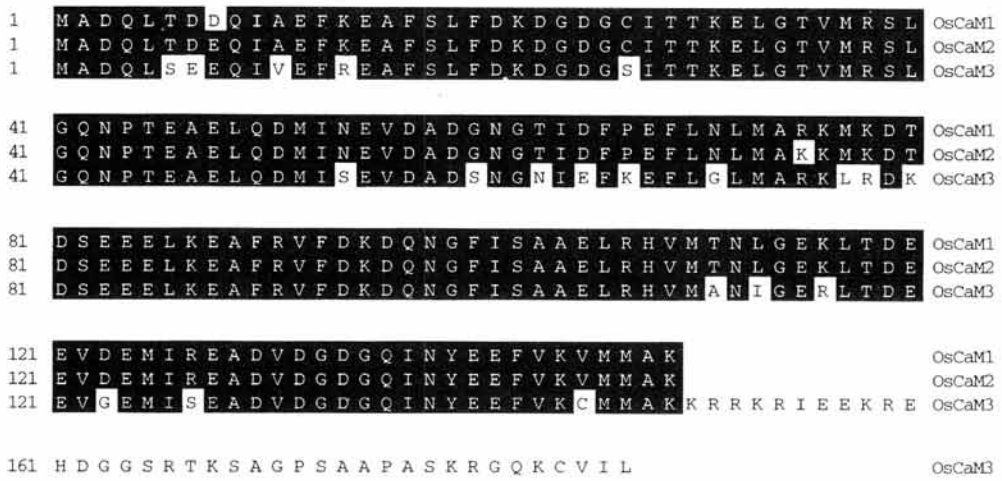

Fig. 2. Sequence alignment of the deduced amino acid sequence of rice OsCaM-1, OsCaM-2, and OsCaM-3. Amino acid residues are numbered on the left of the figure. Shaded boxes indicate same amino acids.

the six extra bases before the poly(A)$^+$ tail. The coding regions of OsCaM-1 and OsCaM-2 revealed 89% identity at the nucleotide level, while the 5'- and 3'-untranslated regions are highly divergent. The deduced amino acid sequences of both OsCaM-1 and OsCaM-2 showed 20 mismatches with the N-terminal 150 amino acids of OsCaM-3. The CaM domain of OsCaM-3 was also very similar to other CaMs in petunia CaM53[39] and *Arabidopsis* AtCaM2.[9]

Amino acid sequence comparisons of the rice CaMs. The deduced amino acid sequences of the three rice CaM cDNAs were compared to protein sequences deposited in the public databases. OsCaM-1 and OsCaM-2 showed a high degree of amino acid sequence homology to other plant CaMs from barley, soybean, potato, petunia, and *Arabidopsis*. The deduced amino acid sequences of OsCaM-1 and OsCaM-2 were identical except for two amino acid substitutions at positions 8 and 75 (Asp vs. Glu, Arg vs. Lys) resulting in 98.6% homology at the amino acid level. However, the N-terminal 149 amino acids of the OsCaM-3 exhibited only 86% identity to the corresponding sequences of OsCaM-1 and OsCaM2 (Fig. 2). The comparisons of the deduced amino acid sequences of the three OsCaMs to those of other plant CaMs revealed only minor differences. The amino acid sequence of OsCaM-1 was completely identical to that of barley[32] and carrot,[33] and most of the amino acid sequence was also identical to other plant CaMs, with only two residue substitutions in comparison to *Arabidopsis* AtCaM-2[9] and alfalfa CaM[34] and 9 residue changes in comparison to the CaM of potato.[35] These amino acid changes did not occur within the highly conserved Ca^{2+}-binding loop regions.

Interestingly, OsCaM-3 has a C-terminal extension of 38 amino acids rich in lysine and arginine (Fig. 1C). The last four amino acids in the C-terminal domain of OsCaM-3 are CVIL. This sequence comprises a functional CaaX-box substrate for yeast geranylgeranyl transferase I, the enzyme that catalyzes prenylation reactions in animals and yeast.[36-38] *Petunia hybrida* CaM53 also contains a similar lysine/arginine-rich C-terminal extension of 34 amino acid ending in the CaaX-box sequence CTIL.[39] CaM53 binds Ca^{2+}, activates glutamate decaboxylase (a known CaM-binding protein in plant), and complements the lethal yeast *cmd1* mutant, confirming that this protein functions as a typical CaM. The presence of the polybasic C-terminal domain in CaM53 is intriguing because it establishes a novel mechanism by which this CaM can be relocated to the nucleus in the absence of the prenyl group.[39] Our novel type of rice OsCaM-3 as well as the petunia CaM53 probably function in specific Ca^{2+}-activated pathways either at the plasma membrane or in the nucleus, depending on the metabolic status of the cell and the prenylation status of the protein.

Genomic organization of the rice CaM. In order to determine the copy number and genomic structure of the CaM genes present in the rice genome, a genomic Southern blot analysis was performed using a rice CaM genomic DNA clone (RCaM-1) as a probe. The RCaM-1 probe was a 1.2 kb *Bam*HI fragment containing exon-2 and 3'-noncoding region of pGA1076.[15] As shown in Fig. 3, four intense and a few weaker bands were observed in the restriction fragments. Most restriction fragments showed high intensity hybridization signals under stringent conditions, suggesting the presence of a small multigene family for CaM in the rice genome as in other plants including *Arabidopsis*,[10,11] soybean,[13] potato,[12] and wheat.[23] Overall, the results from Southern blot analysis revealed that the gene family encoding rice CaM or CaM-related proteins consisted of six to eight numbers.

Expression of OsCaM genes. To get an insight into the regulation of the OsCaMs, we looked for tissue specificity of their expression using total RNA isolated from shoots and roots with the conserved OsCaM probe. Figure 4 shows that OsCaM mRNA accumulated to higher levels in the roots than shoots. This suggests that OsCaM gene expression is

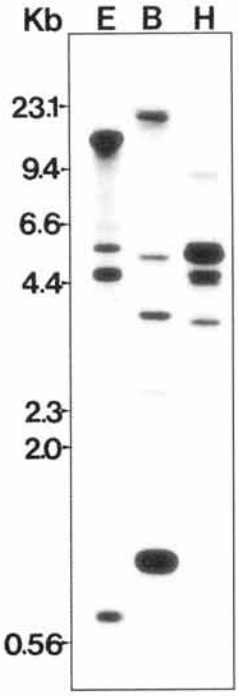

Fig. 3. Southern blot analysis of the OsCaM gene in the rice genome. Ten microgram aliquots of genomic DNA were digested with *Eco*RI (E), *Bam*HI (B), or *Hin*dIII (H) and electrophoresed in a 0.8% agarose gel. The DNA was transferred onto a nylon membrane and probed with the *Bam*HI fragment of the rice genomic CaM (RcaM-1) labeled by random priming with ^{32}P-dATP. The DNA size markers are shown on the left.

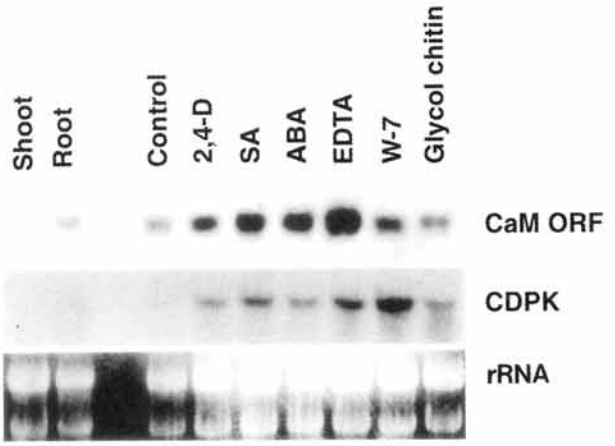

Fig. 4. Northern blot analysis of OsCaM transcripts in rice seedling and suspension-cultured cells. Effects of various environmental stresses on the expression of OsCaM gene. Rice suspension-cultured cells was treated with 2,4-D (2 μg/ml), salicylic acid (50 μM), abscisic acid (50 μM), EGTA (100 μM), W-7 (200 μM), and glycol-chitin (0.1%) for 12 h. OsCaM mRNA and Ca^{2+}-dependent protein kinase (CDPK) mRNA levels were analyzed using cDNA probes.

differentially regulated depending on cell type or organ. Abundant expression of soybean CaM genes has also been observed in the roots of mature soybean plants analyzed with Northern blot, especially in the root apical meristem as detected by immunolocalization experiments.[40]

To investigate the effects of various chemical signals treatments on the expression of CaM gene expression, suspension-cultured rice cells were treated with 2,4-D, salicylic acid (SA), abscisic acid (ABA), calcium chelator (EDTA), CaM-antagonist (W-7), and glycol chitin for 12 h (Fig. 4). The OsCaM-1 clone was used as a probe to study the expression of CaM mRNA in various chemical signals. Expression of the two conserved genes, OsCaM-1 and OsCaM-2, was induced uniformly by all of the above chemical stimuli, and their expression patterns were similar to that of the soybean CaM isoform SCaM-1.[19] Both OsCaM-1 and -2 were also induced by treatment of a 2,4-D, SA, and ABA. The high levels of OsCaM and CDPK mRNA were detected in the rice cells treated with calcium chelator (100 M EDTA) and w-7 (200 M CaM antagoist), respectively. Ca^{2+} directly or indirectly controls the expression of the genes that encode CaM and CaM-related proteins, thus ensuring that enough Ca^{2+}-binding proteins are produced to chelate available Ca^{2+}. However, EGTA, a Ca^{2+}-specific chelator, has no effect on the expression of CaM-related TCH 1 genes of *Arabidopsis*.[20] These results reveal that an additional Ca^{2+}-independent mechanism may act to regulate CaM-related gene expression.

In order to investigate whether rice CaM is involved in any plant defense signaling, we examined the expression of the OsCaM-3 gene after treatment of the cells with fungal elicitor, SA, or H_2O_2 using a gene-specific probe for OsCaM-3 consisting of 38 additional amino acids (Fig. 5). The OsCaM-3 transcript was induced in rice cell suspension-cultured cells treated with the nonspecific fungal elicitor prepared from *Magnaporthe grisea*. The OsCaM-3 mRNA level peaked after 1 h and then slowly declined over the span of 24 h. However, application of exogenous SA or H_2O_2, substances known as defense stimulating molecules, did not induce the expression of the OsCaM-3 gene during a 24 h time course (Fig. 5A). Interestingly, the induction of the OsCaM-3 gene expression preceded that of the class II rice chitinase gene, whose mRNA level began to increase 2 h after treatment (Fig. 5A). OsCaM-3 thus resembles immediate early gene products such as the soybean CaM isoforms, SCaM-4 and SCaM-5.[19] The SCaM-4 and SCaM-5 mRNA levels also peaked after 1 h and then slowly decreased to basal level over 12 h. The expression of OsCaM-3 gene was slightly induced in Ca^{2+}-ionophore A23187-treated cultured cells during a 12-h time course (Fig. 5B). In *Arabidopsis* cultured cells, TCH2, -3, and -4 transcripts begin to accumulate within 10 min after Ca^{2+} addition to the medium.[20] The results suggest that the CaM isoform OsCaM-3 may be specifically activated by the pathogen-derived elicitor and may thus participate in the Ca^{2+}-mediated induction of plant disease resistance responses. While the expression patterns of the OsCaM-1 and OsCaM-2 isoforms are similar to other conserved plant CaMs such as the soybean SCaM-1,[18] mung bean MBCaM-

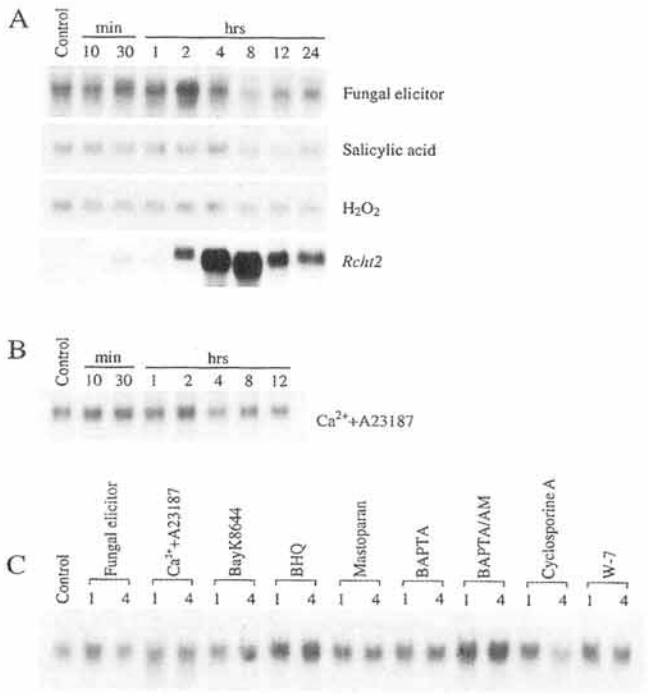

Fig. 5. Northern blot analysis of OsCaM-3 transcript in rice suspension- cultured cells. A. Effects of defense signaling molecules on the expression of OsCaM-3 gene. Rice cells were treated with fungal elicitor (50 mg/ml of glucose equivalent), salicylic acid (1 mM), and H_2O_2 (2 mM) for the times indicated. B. Effects of the Ca^{2+}-ionophore A23187 (10 mM Ca^{2+}/20 μM A23187) on the expression of OsCaM-3 transcript. C. Effects of fungal elicitor or Ca^{2+}-mobilizing chemicals on the expression of OsCaM-3 transcript. Total RNA was isolated from rice cells 1 or 4 h after the treatment with fungal elicitor (50 mg/ml glucose equivalent), Ca^{2+}+A23187 (10 mM Ca^{2+}/20 μM A23187), BayK8644 (100 μM), BHQ (100 μM), Mastoparan (10 μM), BAPTA (2 mM), BAPTA/AM (100 μM), Cyclosporine A (10 mg/ml), and W-7 (200 μM) and the level of transcript of OsCaM-3 gene was analyzed as described in "Materials and Methods".

2,[21] and the potato PCaM-5 and PCaM-6,[12] the expression of OsCaM-3 is similar to those of the SCaM-4 and SCaM-5 isoforms of soybean.[19] The expression of OsCaM-3 transcript was also examined for effects of Ca^{2+}-mobilizing chemical treatments (Fig. 5C). Rice suspension cells were treated with compounds or chemicals such as fungal elicitor (50 mg/ml glucose equivalent), Ca^{2+}+A23187 (10 mM Ca^{2+}/20 μM A23187), BayK8644 (100 μM), BHQ (100 μM), Mastoparan (10 μM), BAPTA (2 mM), BAPTA/AM (100 μM), Cyclosporine A (10 mg/ml), and w-7 (200 μM) for 1 or 4 h. The induction of OsCaM-3 transcript expression was observed in treatment of potent calcium chelators (BHQ and BAPTA/AM). These results raise the possibility that CaM-like protein does not act as a Ca^{2+} modulator during growth, but instead performs its essential function without binding Ca^{2+}. In particular, the expression of OsCaM-3 gene was induced in the cultured rice cells treated with CaM antagonist, w-7. The regulation of CaM-related gene expression by CaM antagonist has not yet been reported for any organism. Further studies are needed to identify whether CaM functions are required for OsCaM-3 gene regulation in response to [Ca^{2+}] increases and w-7. The reduction of OsCaM-3 transcript expression was observed in the rice cells 4 h after cyclosporine A (10 mg/ml), a calcinurin inhibitor, treatment.

However, at present, it is not yet known what the exact physiological functions of the OsCaM isoforms are in the various plant responses including the pathogen defense pathways. Identification of the CaM and CaM-related proteins' functions will undoubtedly yield insight on the role of Ca^{2+} and its variations in signaling characteristics in regulating gene expression.

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