Structure and expression analysis of the OsCam1-1 calmodulin gene from Oryza sativa L.

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INTRODUCTION

All eukaryote cells utilize changes in Ca\(^{2+}\) concentration as a second messenger to generate cellular responses to extracellular stimuli. In plants, Ca\(^{2+}\) signals are utilized in response to a diverse array of stimuli and have been implicated in transducing signals from the environmental changes into adaptive responses (1, 2). These intracellular Ca\(^{2+}\) signals are not only transient, but they also vary temporally and spatially with different organelles or cytoplasmic regions acting as distinct compartments. Thus, within cells a diverse array of changes in the cytosolic Ca\(^{2+}\) concentration must be correctly perceived and discriminated so as to elicit the correct subsequent cellular response, a task performed in part by the EF-hand family of Ca\(^{2+}\)-modulated proteins. Calmodulin (Cam) proteins, members of the EF-hand family, are small multifunctional proteins that transduce 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 the physiological responses to a vast array of specific stimuli received by plant cells (3, 4).

A large family of Cam genes from several plants has been identified including from the two model plants, Arabidopsis (Arabidopsis thaliana (L.) Heynh) and Rice (Oryza sativa L.) (5, 6), in which Cam and Cam-like (CML) genes have been extensively identified in their genomes. Although the broad significance of multiple CaM isoforms can be postulated to be important in distinguishing between the Ca\(^{2+}\) signals from different stimuli and thus aid in eliciting the correct response, the actual significance is, however, not clearly understood. Nevertheless, accumulating evidence suggests that each of the different Cam genes may have distinct and significant functions. Until now, there is no detailed information on Cam gene functions in response to any particular stress in rice, which is considered a model plant for monocots (7). In this study, the OsCam1-1 gene along with its promoter was isolated from Oryza sativa L. cv. Khao Dok Ma Li 105 (KDMI105), and its expression was examined using lines of transgenic rice plants that harbor the OsCam1-1 promoter fused to a β-glucuronidase (gus) reporter gene (8). To further examine OsCam1-1, the protein encoded by OsCam1-1 was produced in E. coli, purified to apparent homogeneity, and assessed for its functional properties.

RESULTS AND DISCUSSION

Isolation of the OsCam1-1 gene from Oryza sativa L. cv. KDMI105

Through extensive analyses of the rice genome, five OsCam genes that encode three closely related CaM proteins were identified (6). Here, we isolated the OsCam1-1 gene from the KDMI105 rice cultivar by PCR amplification, attaining a product of 1,528 base pairs. Following determination of its sequence and assembly with its upstream region, which was isolated as described below, the gene structure and its predicted mRNA was attained (Fig. 1). Similar to the homologous OsCam1-1 sequence from the Nipponbare rice cultivar (9), it contains a predicted open reading frame of 450 base pairs interrupted by a single intron of 828 base pairs at the location corresponding to the glycine codon at position 26. This arrangement has been found in numerous plant Cam and CML...
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Fig. 1. Organization of the OsCam1-1 gene from Oryza sativa L. cv. KDML105. (a) Schematic diagram displaying OsCam1-1 genomic DNA and mRNA sequences. Exons are indicated by boxed regions; intron, 5' and 3' untranslated regions are indicated by solid lines. The predicted promoter is indicated by a grey arrow, and the sequences encoding the EF hands are depicted by black rectangles. (b) Nucleotide sequence of the OsCam1-1 gene and deduced amino acid sequence of its predicted open reading frame excluding the intron. The predicted TATA box is in bold and double underlined, and the potential regulatory sites for the Sp1 transcription factor are shown in bold and underlined letters; Adh1 GC and GT elements are shown in bold and italic, and potential binding sequences of TGA1 and Krox-24 transcription factors are underlined and double underlined, respectively. The predicted transcription start site is boxed and designated as +1, and the resulting upstream nucleotide positions are shown on the left. The closed triangle represents the location of the single intron. Likely start and stop codons are shown in bold. Residues in the EF-hand Ca2+-binding loops are highlighted in grey and residues serving as likely Ca2+ -binding ligands are underlined and correspond to those in typical CaM proteins. Nucleotides underlined with thick lines are the ones that differ from those of the Nipponbare OsCam1-1 sequence.

The sequence upstream of the OsCam1-1 coding region was isolated by PCR amplification, yielding a product of 1,370 base pairs. The sequence was determined and the contig assembled with the coding region as shown in Fig. 1. The upstream sequence was bioinformatically analyzed, which revealed a likely promoter region with a TATA box (sequence TATAAA) and a predicted transcription start site within a few base pairs from the postulated start site of the proposed OsCam1-1 gene obtained from the KDML105 rice. The nucleotide sequence obtained confirmed the predicted sequence of the deduced ORF and mRNA derived from the genomic DNA sequence depicted in Fig. 1. Within the KDML105 OsCam1-1 coding region, only one synonymous nucleotide difference from that of the Nipponbare cultivar of japonica rice (9) was found, and thus no differences exist in the predicted amino acid sequences between the two rice subspecies. More differences are seen within the introns between Nipponbare and KDML105, in which seven-nucleotide substitutions or deletions occur (data known not shown). Similarly, we have isolated from the KDML105 rice the OsCML1 gene, which encodes a CaM-like protein that shares the highest degree of amino acid identity among different OsCML proteins with the OsCaM proteins (6). Its coding region has nine nucleotide differences from that of Nipponbare, which result in four pre-
dicted amino acid differences (data not shown). Considering the similarity in their sizes (450 and 564 nucleotides and 149 and 187 amino acids for OsCam1-1 and OsCML1 genes, respectively), the OsCam1-1 gene exhibits a very high degree of conservation between the two subspecies both at the nucleotide and the amino acid sequence levels.

**OsCam1-1 promoter analysis in OsCam1-1::gus transgenic rice plants**

To determine whether the isolated OsCam1-1 upstream sequence can function as a promoter, lines of transgenic rice plants that harbor the OsCam1-1::gus construct were generated. Rice plants from three independent transgenic lines were obtained, which showed no abnormal morphological characteristics, and were analyzed for expression of the reporter gene product. GUS staining patterns, detected using X-Gluc as a substrate, were consistent among all transgenic lines and their representative images are shown in Fig. 2 and 3. Under normal growth conditions, GUS activity was histochemically observed in whole organs and tissues including leaf blades, leaf sheaths, roots, lateral roots, and several floral parts including stigmas, anthers, and pollen (Fig. 2a-g). To visualize the localization of the GUS activity in leaves and roots, they were sectioned (70-100 μm) and stained with X-Gluc. GUS staining was observed throughout the cross-sections of leaf blades, but staining was strongly detected in the large and small vascular bundles, specifically in the phloem and the bundle sheath cells (Fig. 3a-d). Some epidermal cells were stained stronger than others, but hair cells were always stained. It should be noted that GUS staining was also found in the guard cells. Similar to leaf blades, GUS staining was predominantly observed in the vascular bundles in cross-sections of leaf sheaths (Fig. 3e-h), in the vascular cylinder of roots (Fig. 3i-k), and during the development of lateral roots. In agreement with our results, expression in vascular tissues was reported in cherry rootstock transformed with the apple Cam::gus construct (17), although higher levels of expression and more cell types were observed in our experiments. Overall, gus was found to be expressed in all organs and many tissues throughout the OsCam1-1::gus transgenic rice plants. Assuming under these conditions that the OsCam1-1 promoter is driving the gus gene expression in the same manner as it drives endogenous OsCam1-1 gene expression, this data suggests a likely and important role for OsCam1-1 under normal growing conditions. Moreover, the OsCam1-1 driven gus expression in the outer parts of the plant body, such as the epidermis (including hair cells and guard cells), suggests that OsCam1-1 may contribute to Ca²⁺-mediated responses to both
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Fig. 3. Localization of gus activity in cross-sections of leaves and roots of transgenic rice plants harboring OsCam1-1:gus. GUS staining was observed in cross-sections of a)-d) leaf blades, e)-h) leaf sheaths, and i)-k) roots. Inner regions in the sections of the leaf blade (a), leaf sheath (e), and root (i) were magnified and shown in b)-d), f)-g), and j)-k), respectively. Representative staining images of the three transgenic rice plants are shown.

Production in E. coli and properties of the rOsCaM1-1 protein
To examine the molecular properties of OsCaM1-1, its coding sequence was engineered by PCR amplification and cloned into the T7-based expression plasmid, pET21a. The resulting recombinant expression plasmid encoding rOsCaM1-1 was introduced into the E. coli (K12) strain BL21(DE3) and used to produce the recombinant protein following IPTG induction. SDS-PAGE based analysis of the protein product induced by 0.2 mM IPTG displayed a distinct band of the expected size at 17.6 kDa in the soluble fraction. The protein was purified by Ca2+-dependent hydrophobic chromatography on phenyl-Sepharose and the purity of CaM was judged by SDS-PAGE (Fig. 4a). One of the characteristics of CaM is its ability to bind Ca2+ in the presence of SDS, which increases its electrophoretic mobility relative to CaM in the absence of Ca2+. Fig. 4a shows that rOsCaM1-1 displayed this characteristic electrophoretic mobility shift when incubated with 1 mM Ca2+ prior to electrophoresis. This result indicates that the rOsCaM1-1 protein produced in E. coli and purified by these methods is likely to be a functional Ca2+-binding protein. To further examine the properties of the OsCaM1-1 protein, its ability to bind the peptide derived from CaM kinase II (CaMKII) was assessed by gel mobility shift assay. Incubation of 100 picomoles of rOsCaM1-1 protein in the presence of 1 mM Ca2+, with different molar equivalents of the peptide (Fig. 4b) prior to PAGE-4 M urea resolution, showed a clear dose dependent band shift consistent with the notion that rOsCaM1-1 binds the CaMKII with a 1:1 stoichiometry and suggesting its mechanisms of action are likely to be similar to those from known CaMs.

In conclusion, this work has verified that OsCam1-1 encodes a functional Ca2+-binding calmodulin protein and identified its promoter region from the KDML105 rice. Analyses of the OsCam1-1::gus transgenic rice plants suggest that OsCam1-1 is highly expressed in vascular tissues and during the emergence of lateral roots. These expression patterns highlight the tissues and developmental stages worth assaying by techniques such as immunocytochemistry in the future. Knowledge of the expression patterns and properties of OsCam1-1 obtained in this study will help facilitate further investigations into its roles.
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Fig. 4. Recombinant OsCaM1-1 possesses Ca\(^{2+}\) and peptide-binding properties in vitro. (a) Purification and Ca\(^{2+}\)-induced electrophoretic mobility shift of the rOsCaM1-1. Separation by 12% (w/v) SDS-PAGE of the protein extracted from E. coli harboring pET21a expression plasmids following phenyl-Sepharose hydrophobic chromatography. To analyze calcium-induced electrophoretic mobility of the rOsCaM1-1, three micrograms of the eluted rOsCaM1-1 and 1 mM of either EGTA (lane +EGTA) or CaCl\(_2\) (lane +CaCl\(_2\)) were resolved. Protein bands were detected by Coomassie blue staining. Lane marked M contained molecular mass standard proteins (Fermentas). (b) Gel mobility shift analysis of rOsCaM1-1 interaction with a peptide from CaMKII. Different molar equivalents of peptide (indicated) mixed with 100 picomoles of rOsCaM1-1 were fractionated in a 12% (w/v) PAGE-4 M urea gel and detected by Coomassie blue staining to reveal band shifts.

MATERIALS AND METHODS

Materials
Enzymes used for manipulating recombinant DNA were from Fermentas (Hanover, MD, USA). Kits for purifying and gel extracting plasmid were purchased from Qagen (Hilden, Germany). pGEM\textsuperscript{R}®-T vector and oligo(dT)\(_{15}\) primers were obtained from Promega (Madison, WI, USA). The expression plasmid pET21a and host E. coli (K12) strain BL21(DE3) were from Novagen (Madison, WI, USA). The E. coli (K12) strain XL1-Blue was from Stratagene (Cedar Creek, TX, USA). Phenyl-Sepharose was purchased from Amersham Biosciences (Piscataway, NJ, USA).

Synthetic oligonucleotides were obtained from Operon Technologies (Cologne, Germany). The synthetic peptide representing the CaM-binding domain of CaMKII was purchased from Sigma (St. Louis, MO, USA). Plasmid pCAMBIA1381Z was obtained from CAMBIA (Canberra, Australia). Seed of Oryza sativa L. cv. KDML105 was provided by the Department of Agriculture, Ministry of Agriculture and Cooperatives (Bangkok, Thailand).

Cloning of the OsCam1-1 gene
Oryza sativa L. cv. KDML105 seedlings were ground in liquid nitrogen using chilled mortars and pestles. Genomic DNA was isolated according to Agrawal et al. (18). To isolate the gene, PCR amplification was conducted using oligonucleotides designed from the homologous OsCam1-1 sequences from the Nipponbare cultivar of Oryza sativa L. ssp. japonica (9), 5'-GA AGCCAGGCTAAGCCCAGC-3' and 5'-GCCAACCTTAACAGATTTCAC-3' as the sense and antisense primers, respectively. PCR utilized Taq polymerase (Fermentas) and 30 cycles of 94°C for 3 minutes, 60°C for 0.5 minutes, and 72°C for 2 minutes with a final elongation phase of 72°C for 10 minutes.

To isolate the upstream promoter region of the presumed OsCam1-1 first exon, PCR amplification was conducted as above except using oligonucleotides designed from the genomic DNA sequence of the 93-11 cultivar of Oryza sativa L. ssp. indica (19): 5'-TCCCAATCCTCCCTGCTGATGTTGC-3' and 5'-CCATGCCGCGGGGCTTAGCCTGGCT-3' as the sense and antisense primers, respectively.

To clone OsCam1-1 cDNA, Oryza sativa L. tissues were ground in liquid nitrogen using chilled mortars and pestles. Total RNA was isolated according to Verwoerd et al. (20) and used as a template for reverse transcription primed by using oligo(dT)\(_{15}\) primers in a 20-μl reaction with 200 units of M-MLV reverse transcriptase (Promega) at 42°C for 1 hour. PCR amplification of the total cDNA was conducted as above, using the same primers as used to isolating the gene, to amplify the OsCam1-1 transcript. PCR amplification was conducted as above except a denaturation time of 2 min and an annealing temperature of 55°C were used.

All PCR products were cloned into the pGEM\textsuperscript{R}®-T vector, transformed into XL1-Blue cells for cloning and propagation of the plasmid, and their sequences were then determined from four independent clones.

Sequence retrieval and analyses
Sequences from Oryza sativa L. ssp. were retrieved from the Rice Annotation Project Database at the NIAS (http://rapdb.dna.affrc.go.jp/) and Rice Information System at the Beijing Genomics Institute (http://rise.genomics.org.cn/rice/index2.jsp).
Alignments were performed using EMBOSS pairwise alignment algorithms at the European Bioinformatics Institute (http://www.ebi.ac.uk/). To identify promoter and transcriptional elements, sequences were analyzed using the computer programs "Promoter Scan" and "Signal Scan" at the Bioinformatics and Molecular Analysis Section (http://bimas.dcter.nih.gov/molbiol/), Computational Bioscience and Engineering Lab, Division of Computational Bioscience, Center for Information Technology, at the National Institute of Health (21).

**Generation of **OsCam1-1::gus** transgenic plants**

Rice seeds were dehusked and sterilized with 70% (v/v) ethanol for two minutes and then with 2% (v/v) sodium hypochlorite for 20 minutes. The seeds were rinsed three times with sterile water and placed on NB medium (22) containing 2 mg/L 2,4-dinitrophenoxy acetic acid (2,4-D) and incubated in the dark at 28°C for two weeks. Before transformation, the growing calli were subcultured on fresh medium and incubated under the same conditions for four days. To generate an OsCam1-1::gus construct, BamHI and NcoI recognition sequences (bold) were introduced into the promoter by PCR using the oligonucleotides 5'-GGATCCCAATCTCTCCTGCTGATG-3' and 5'-CCATGGCCGGGGGCTAGCCGGC3' as the sense and antisense primers, respectively. PCR by Taq polymerase (Fermentas) consisted of 30 cycles of 94°C for 3 minutes, 58°C for 0.5 minutes and 72°C for 1.5 minutes with a final elongation phase of 72°C for 10 minutes. The PCR product was ligated into pGEM-T prior to subsequent ligation into a BamHI-NcoI-cleaved pCAMBIA1301 vector. The sequence of the resulting OsCam1-1::gus was then determined to confirm its insertion and integrity. The recombinant plasmid was introduced into the Agrobacterium tumefaciens strain EHA105 by electroporation. When plants were ready for transformation, A. tumefaciens cells were streaked on solid AB medium (23) containing 25 μg/ml rifampicin and 50 μg/ml kanamycin. The cells were incubated at 28°C for 2-3 days, collected by scraping with a loop and resuspended in AAM medium (24) supplemented with 100 μM acetosyringone. The optical density of the bacterial suspension at 600 nm was adjusted to 1.0 by addition of fresh medium as above for another round of selection and then transferred to NB medium containing 250 μg/ml cefotaxime and incubated at 25°C for 2 days. The cells were then washed with sterile water and resuspended in AAM medium (24) supplemented with 100 μg/ml cefotaxime and incubated at 28°C for 2-3 days, collected by scraping with a loop and resuspended in AAM medium as above for another round of selection and then transferred to NB medium containing 250 μg/ml cefotaxime and 50 μg/ml hygromycin. After incubation at 28°C for 4 weeks, the hygromycin-resistant calli so obtained were subcultured on fresh medium as above for another round of selection and then transferred to NB medium containing 4 mg/L 6-benzylaminopurine and incubated at 28°C under a 16/8 hours light/dark photoperiod for 3-4 weeks. When the green shoots reached 2-3 cm in height, they were cut and transferred to NB medium to stimulate root and stem elongation.

**GUS histochemical assays**

Staining solution [100 mM phosphate buffer, pH 7.5, 0.5 mM potassium ferrocyanide, 0.5 mM ferricyanide, 0.1% (v/v) Triton X-100, 10 mM EDTA, and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc)] was added in a tightly capped tube to cover the tissues to be stained. To remove air trapped in the tissues and allow for maximum stain penetration, a 20-mmHg vacuum was applied to the tissues twice for five minutes. The tube then was capped and placed in a 37°C incubator. After 1 to 2 days the stain solution was removed and the sample was decolorized with several changes of 70% (v/v) ethanol over 1 to 2 days at 37°C.

**Recombinant protein production and gel-mobility shift analysis**

Recombinant pET21a plasmids were introduced into the E. coli (K12) strain BL21(DE3) to produce recombinant proteins. Protein production and purification were carried out using methods employed previously for recombinant plant CaM (25). To examine the Ca2+-binding ability, 1 mM (final concentration) of either CaCl2 or EGTA was added to three micrograms of the protein and mixed. The samples were then resolved through a 12% (w/v) SDS-polyacrylamide gel and detected by Coomassie blue staining. To examine the peptide binding ability, 100 picomoles of the recombinant protein was mixed with the peptide derived from CaMKII (Sigma) at different molar equivalents and then fractionated in a 12% (w/v) polyacrylamide gel containing 4 M urea and detected by Coomassie blue staining.

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