Rice C2-Domain Proteins Are Induced and Translocated to the Plasma Membrane in Response to a Fungal Elicitor†

Cha Young Kim,[‡,§,||] Yoon Duck Koo,[‡,§] Jing Bo Jin,[§,⊥] Byeong Cheol Moon,[‡] Chang Ho Kang,[‡] Sun Tae Kim,[‡] Byung Ouk Park,[‡] So Young Lee,[‡] Man Lyang Kim,[‡] Inhwan Hwang,[⊥] Kyu Young Kang,[‡] Jeong Dong Bahk,[‡,@] Sang Yeol Lee,*[,‡,@] and Moo Je Cho*[,‡,@]

Division of Applied Life Science and Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, Jinju 660-701, and Division of Molecular and Life Sciences and Center for Plant Intracellular Trafficking, Pohang University of Science and Technology, Pohang 790-784, Korea

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ABSTRACT: Hundreds of proteins involved in signaling pathways contain a Ca^{2+}-dependent membrane-binding motif called the C2-domain. However, no small C2-domain proteins consisting of a single C2-domain have been reported in animal cells. We have isolated two cDNA clones, *OsERG1a* and *OsERG1b*, that encode two small C2-domain proteins of 156 and 159 amino acids, respectively, from a fungal elicitor-treated rice cDNA library. The clones are believed to have originated from a single gene by alternative splicing. Transcript levels of the *OsERG1* gene are dramatically elevated by a fungal elicitor prepared from *Magnaporthe grisea* or by Ca^{2+} ions. The OsERG1 protein produced in *Escherichia coli* binds to phospholipid vesicles in a Ca^{2+}-dependent manner and is translocated to the plasma membrane of plant cells by treatment with either a fungal elicitor or a Ca^{2+} ionophore. These results suggest that OsERG1 proteins containing a single C2-domain are involved in plant defense signaling systems.

Plants protect themselves against pathogen attack by launching both localized and systemic defense responses (*1*, *2*). When a pathogen attacks plant tissues, receptors on the plasma membrane recognize signals called elicitors that result from combined reactions of the pathogen and host cells, and they activate defense systems through diverse intracellular signaling cascades (*1*−*5*). However, the molecular mechanisms of elicitor-mediated signal transduction pathways in plants remain to be elucidated.

In the coordination of a wide range of cellular signaling processes in all higher eukaryotes (*6*, *7*), Ca^{2+} ions play an important role as intracellular second messengers in response to a variety of stimuli, including hormones, light, oxidative stress, drought, cold, and pathogen elicitors (*8*−*13*). Changes in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), including transient increases, sustained changes, or oscillations, trigger key cellular signaling processes in plants (*8*, *14*−*16*). A variety of Ca^{2+}-binding proteins are required to transduce the $[Ca^{2+}]_i$ signals to downstream signaling pathways. Most Ca^{2+}-binding proteins have one of the following three Ca^{2+} regulatory motifs: EF-hands (*17*, *18*), annexin folds (*19*, *20*), or C2-domains (*21*, *22*). Among them, the C2-domain first identified from the α, β, and γ isoforms of mammalian Ca^{2+}-dependent PKC[1] (*23*−*26*) contains approximately 130−145 amino acid residues that form a conserved eight-β-strand antiparallel sandwich connected by variable loops. Whereas the C2-domain found in a wide variety of animal proteins is implicated in eukaryotic signal transduction and membrane trafficking processes (*11*, *27*−*31*), only a few C2-domain proteins have been reported in plants. In particular, no functional characterization of proteins containing a single C2-domain, which are designated small C2-domain proteins in this paper and which have only been identified in plant cells, has previously been performed.

In this study, we report the isolation and characterization of two small C2-domain rice proteins, named *OsERG1a* and *OsERG1b* for rice (*Oryzae sativa*) elicitor-responsive genes. These proteins are significantly induced by a fungal elicitor, interact with phospholipids in a Ca^{2+}-dependent manner, and are translocated to the plasma membrane in response to pathogen signals. Thus, we propose that small C2-domain proteins play a functional role in defense signaling systems in plant cells.

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* To whom correspondence should be addressed. Division of Applied Life Science (BK21 program), Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, 900 Gazwadong, Jinju 660-701, Korea. Telephone: +82-55-751-5957. Fax: +82-55-759-9363. E-mail: mjcho@nongae.gsnu.ac.kr.

‡ Division of Applied Life Science, Gyeongsang National University.

§ These authors contributed equally to this work.

|| Current address: Department of Biology, Pennsylvania State University, University Park, PA 16802.

⊥ Pohang University of Science and Technology.

@ Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University.

[1] Abbreviations: BSA, bovine serum albumin; DTT, DL-dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GST, glutathione *S*-transferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene fluoride; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PKC, protein kinase C; RT-PCR, reverse transcription polymerase chain reaction; GFP, green fluorescence protein; ER, endoplasmic reticulum.

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EXPERIMENTAL PROCEDURES

Culture of Rice Suspension Cells. For suspension cell cultures, sterilized rice seeds (*O. sativa* L. Milyang 117) were plated on NB medium (N6 macroelements, B5 microelements, B5 vitamin, MS medium Fe-EDTA, 0.5 mg/L proline, 0.5 mg/L glutamine, 0.3 mg/L casein enzyme hydrolysate, and 0.25% Phytagel) supplemented with 2.0 mg/L 2,4-D and 3% sucrose and cultured in the dark at 28 °C for 4 weeks. To establish embryogenic culture cells, the induced calli from embryos were transferred into 100 mL flasks containing liquid R2 medium supplemented with 2.0 mg/L 2,4-D and 3% sucrose (*32*). Liquid cultures were maintained by a weekly subculture with shaking at 90−100 rpm. Log phase culture cells were used for RNA or protein analysis.

Treatment of Stress Signals and Fungal Infections. To examine the inducibility of the *OsERG1* gene in suspension-cultured cells, the fungal elicitor (50 μg of glucose equivalents/mL), 1 mM salicylic acid, 2 mM hydrogen peroxide (H_2O_2), 200 mM sodium chloride (NaCl), or a calcium ionophore (10 mM Ca^{2+} and 20 μM A23187) was added to the cells. The fungal elicitor was prepared from mycelia of rice leaf blast fungus (*Magnaporthe grisea* KJ301) as described previously (*33*).

RNA Gel Blotting. Total RNA was isolated by phenol/chloroform extraction, followed by lithium chloride precipitation (*34*). The RNA (20 μg) was denatured and separated by electrophoresis on a 1.5% (w/v) agarose−formaldehyde gel, and transferred onto a nylon membrane (GeneScreen Plus, NEN, Boston, MA). The filter was prehybridized for 30 min and incubated for 16 h at 60−65 °C in Church's hybridization solution according to the method of Church and Gilbert (*35*). The membrane was washed three times for 15 min in 2× SSC with 0.1% SDS and three times for 15 min in 0.2× SSC with 0.1% SDS at 60−65 °C. Equal sample loading was confirmed by ethidium bromide staining of the samples in the gel.

RT-PCR Analysis. Total RNA was extracted from rice cells treated with the fungal elicitor and reverse transcribed to cDNA using an oligo$(dT)_{18}$ primer and Moloney murine leukemia virus reverse transcriptase for 1 h at 37 °C under conditions recommended by the manufacturer (Promega). PCR was then performed in the presence of [α-^{32}P]dATP using 10 pmol of each primer for 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C. An N-terminal OsERG1 primer with the sequence 5′-AAAGATGGCGGG-GAGCGGTGT-3′ and an oligo$(dT)_{18}$ primer were used. Control amplifications of *OsERG1a* and *OsERG1b* were performed from each cDNA template under identical conditions. The resulting PCR products were digested with *Bsi*WI and analyzed on a 6% denaturing polyacrylamide gel as described previously (*33*). The gel was then dried and exposed to X-ray film.

Expression of Recombinant Protein in E. coli. For recombinant protein expression of OsERG1 in *E. coli*, each of the two *OsERG1* cDNAs was subcloned into *Eco*RI−*Sal*I restriction sites of the pGEX-5X-3 bacterial expression vector (Amersham Pharmacia Biotech), in which the insert was fused in frame to the C-terminus of GST. The resulting construct was introduced into protease-deficient *E. coli* strain BL21(DE3)pLysS (Novagen). The bacterial cultures (at an OD_{600} of 0.6) were induced with 0.2 mM IPTG for 3 h at 30 °C. The cells were then lysed by sonication in PBS containing 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, and 150 mM NaCl (pH 7.4) in the presence of a protease inhibitor cocktail (100 μg/mL PMSF, 19 μg/mL aprotinin, and 25 μg/mL leupeptin). The soluble fraction of the lysate was incubated with glutathione (GSH)−Sepharose CL-4B beads (Amersham Pharmacia Biotech) for 30 min at 4 °C. The beads were washed with PBS buffer, and the proteins were eluted with 10 mM GSH in 50 mM Tris-HCl (pH 8.0). The eluates were desalted and concentrated with a speed vacuum drier (Savant, AS290). SDS−PAGE was performed according to the Laemmli method (*36*).

Antibody Production and Immunoblot Analysis. After removal of GST from the GST−OsERG1 fusion proteins using protease factor Xa, polyclonal antibodies were generated by immunizing rabbits with each of the purified OsERG1 proteins. For Western blot analysis, protein samples were subjected to 13% SDS−PAGE and electrotransferred to a PVDF membrane by wet electroblotting (Hoefer Scientific Instruments). The membrane was blocked in TBS-T buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20] with 6% (w/v) nonfat dried milk (Carnation, Glendale, CA) for 2−4 h and probed with an anti-OsERG1 polyclonal antibody (1:2000). Horseradish peroxidase-conjugated mouse anti-rabbit IgG (1:5000 dilution, Sigma) was used as a secondary antibody, and the reaction was visualized using the enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech).

Phospholipid Binding Assays. Phospholipids were prepared as described by Davletov *et al.* (*37*). Forty micrograms of recombinant OsERG1 protein bound to GSH−Sepharose beads was equilibrated with binding buffer and then added to a reaction mixture containing either 1 mM $CaCl_2$ or 2 mM EGTA, 50 mM HEPES (pH 7.2), 100 mM NaCl, and ^{3}H-labeled liposomes (17.5 mg of phospholipids) in a total volume of 400 μL. The mixture was incubated at room temperature for 15 min with vigorous shaking and then briefly centrifuged in a microcentrifuge. After the reaction, beads were washed three times with 1 mL of binding buffer and the amount of radioisotope bound to OsERG1 beads was quantified with a liquid scintillation counter. The free Ca^{2+} concentration was adjusted with a Ca^{2+}/EGTA buffer calculated with computer software developed by Fabiato (*38*). The binding of the GST−OsERG1 protein to phospholipid vesicles was also verified by a centrifugation binding assay (*39*). To perform the assay, 4 μg of the fusion protein was incubated with 200 μL of the reaction mixture containing 50 mM HEPES (pH 7.2), 100 mM NaCl, 150 μM phospholipid vesicle, and either 1 mM $CaCl_2$ or 2 mM EGTA. The reaction was carried out at 30 °C for 15 min. Free and bound proteins were separated by sedimentation at 12000*g* for 30 min. The supernatant and pellet fractions were resolved with 13% SDS−PAGE.

Subcellular Fractionation. Rice cells treated with a fungal elicitor were harvested at various time points and frozen in liquid nitrogen. Frozen cells were ground to a fine powder with a mortar in homogenization buffer [25 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 2.5 mM EGTA, 2.5 mM EDTA, 4 mM DTT, and 2 mM PMSF] with a protease inhibitor cocktail. After filtration through two layers of Miracloth (Calbiochem), the homogenate was centrifuged at 12000*g*

for 10 min at 4 °C. The supernatant was collected as a total protein fraction and centrifuged again at 100000*g* for 1 h at 4 °C. The supernatant was used as a cytosolic soluble fraction. After the pellet had been completely washed with homogenization buffer, the final precipitate was resuspended in the same buffer using a glass homogenizer and was used as a microsomal fraction. It was applied onto a continuous sucrose gradient (from 12 to 45%, w/v) of ultracentrifugation buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 2 mM PMSF, and a protease inhibitor cocktail and centrifuged at 110000*g* for 18 h. Fractions (0.8 mL) were collected from the top, immediately frozen in liquid nitrogen, and stored at −80 °C until they were used.

Transient Expression of the OsERG1::smGFP in Arabidopsis. OsERG1b cDNA was fused individually in frame to the 5′-untranslated region of smGFP (soluble modified GFP) driven by the *CaMV* 35S promoter (OsERG1::smGFP) (*40*). The DNA construct was transformed into *Arabidopsis* leaf protoplasts prepared from 3−4-week-old whole seedlings grown in liquid MS medium at 20 °C with a modified polyethylene glycol method as described previously (*41*). Typically, 0.3 mL of a protoplast suspension (5×10^6 cells/mL) was used for transformation of 20 μg of plasmid DNA of either smGFP (control) or OsERG1:smGFP. The transformed protoplasts were incubated in the dark at 22 °C overnight and treated with a fungal elicitor (10 μg of glucose equivalents/mL) or calcium ionophore (5 mM Ca^{2+} and 10 μM A23187). The fluorescence was monitored 6−12 h after stimulation using fluorescence microscopy (Axioplan 2, Zeiss, Jena, Germany), and the images were processed using an automatic imaging system (FISH, Carl Zeiss). All transient expression assays were repeated at least three times.

RESULTS

Cloning of Rice OsERG1a and OsERG1b. To identify early inducible genes that respond to rice blast fungus (*M. grisea*) and that may play a potential upstream regulatory role in the activation of plant defense systems, we employed a combined method of mRNA differential display and cDNA library screening (*32*). Through the screening of fungal elicitor-responsive genes from rice suspension-cultured cells treated with fungal elicitor of rice blast fungus, we have isolated two elicitor-responsive genes, *OsERG1a* and *OsERG1b*. The cDNA clones have open reading frames of 480 and 471 bp encoding 159 and 156 amino acids, respectively (Figure 1). The amino acid sequences of OsERG1a and OsERG1b are significantly similar with those of the mammalian Ca^{2+}-dependent lipid binding (C2) domains that exist in human PKCα and cPLA2 and in rat synaptotagmin (Figure 1A). Proteins containing a single C2-domain have only been identified from plant sources as sequence files in the GenBank database and include proteins from maize (ZmC2-1, U64437), pumpkin (Cmpp 16-1 and -2, AF079170 and AF079171, respectively), and *Arabidopsis* (AtC2−1, AAG52148) (Figure 1B). Although more than 100 proteins containing multiple C2-domains have been identified in mammalian systems, no small C2-domain proteins consisting of a single C2-domain have been found in mammalian cells. Furthermore, there has been no functional characterization of the plant small C2-domain proteins, except for the pumpkin Cmpp16-1 and -2 phloem proteins, which have been proposed to mediate RNA delivery for long-distance translocation (*42*).

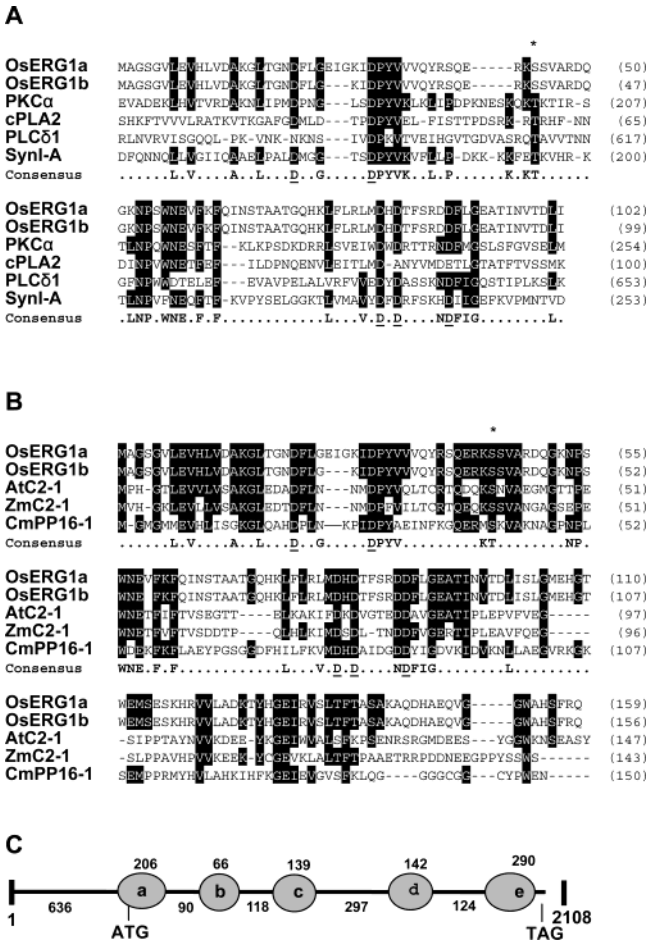

FIGURE 1: Comparison of the deduced amino acid sequences of OsERG1 with representative C2-domain proteins and their genomic organization. (A) Alignment of the protein sequences derived from OsERG1a and OsERG1b with C2-domains from human PKCα (amino acid residues 167−267, P17252), human cPLA2 (residues 17−113, M72393), bovine PLCδ1 (residues 569−666, P10895), and rat Synl-A, synaptotagmin I C2A (residues 152−251, P21707) proteins. (B) Comparison of the deduced amino acid sequences of OsERG1 with the plant C2-domain proteins of *Arabidopsis*, AtC2-1 (AAG52148), maize, ZmC2-1 (U64437), and pumpkin, CmPP16-1 (AF079170). Amino acid residues identical to the consensus C2-domain sequences are shown in black. Dashes mark gaps introduced to maximize homology. A conserved threoine residue in animal C2-domain proteins that may serve as a phosphorylation site for serine/threonine kinases is substituted with a serine residue in plant C2-domain proteins (asterisk). The putative Ca^{2+}-binding aspartates are underlined in the consensus sequences. The Wisconsin Package Version 8.1 (Genetics Computer Group) was used for data analysis. (C) Genomic structure of the *OsERG1* gene. On the basis of a comparison to OsERG1a cDNA, the coding regions of *OsERG1* are divided into five exons (1−5) interrupted by four introns.

Comparison of the complete nucleotide sequences of *OsERG1a* and *OsERG1b* cDNA clones reveals identical DNA sequences except for a 9 bp (GAGATAGGC) deletion encoding three amino acid residues, $_{25}$EIG$_{27}$, in OsERG1b compared to OsERG1a (Figure 1B). The 5′- and 3′-untranslated regions of both clones were also identical (GenBank entries U95135 and U95136, respectively). This result implies that both cDNA clones may originate from the same genomic sequence by alternative splicing. To confirm this possibility, the genomic sequence corresponding to the cDNA clones was isolated and its complete nucleotide

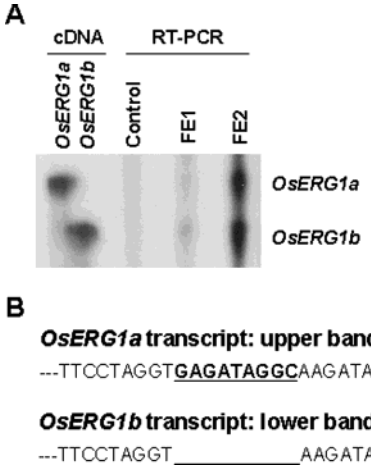

FIGURE 2: RT-PCR analysis of the *OsERG1* transcripts. (A) Denaturing PAGE of RT-PCR products templated on total RNAs isolated from rice cells treated with fungal elicitor for 0 min (Control), 30 min (FE1), and 1 h (FE2) in the presence of [^{32}P]-dATP as described in Experimental Procedures. The resulting PCR products were digested with *Bsi*WI and analyzed on a 6% denaturing polyacrylamide gel. Control amplifications of *OsERG1a* and *OsERG1b* were performed with each cDNA template under identical conditions. The upper band is *OsERG1a*, and the lower band is *OsERG2b*. (B) The two PCR products were sequenced, and the difference in the nucleotide sequences between *OsERG1a* and *OsERG1b* is shown.

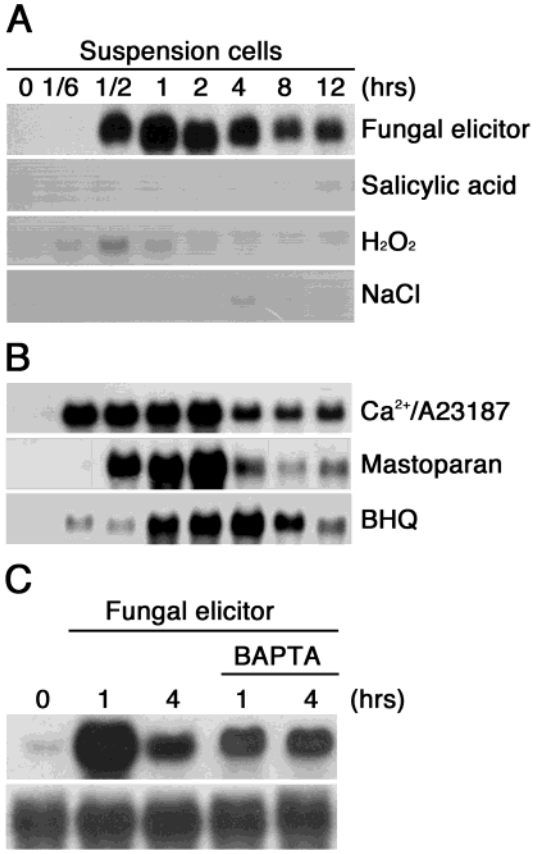

FIGURE 3: Expression patterns of the *OsERG1* transcript in response to various stress signals. Northern blot analysis of *OsERG1* expression in rice cell cultures. Total RNAs were isolated from rice suspension-cultured cells at the indicated time points and probed with a full-length *OsERG1b* cDNA. Rice cells were treated with stress signals, including a fungal elicitor, salicylic acid, H_2O_2, and NaCl (A), and with reagents affecting calcium influx, including Ca^{2+} ionophore (10 mM $CaCl_2$ and 20 μM A23187), mastoparan, and BHQ (B). In panel C, the Ca^{2+} signaling inhibitor BAPTA (5 mM) was pretreated for 1 h prior to addition of a fungal elicitor (50 μg of glucose equivalents/mL) to the rice cells for the indicated times. The same blot was also probed with a rice 28S rDNA fragment as a loading control. The membranes were exposed to X-ray film at −80 °C for 1 day.

sequence was determined (data not shown, GenBank entry AF512505). On the basis of comparison to *OsERG1*a cDNA, the coding region of the *OsERG1* gene is divided into five exons of 206, 66, 139, 142, and 290 bp interrupted by four introns of 90, 118, 297, and 124 bp, respectively (Figure 1C). This intron−exon structure is similar to those of the pumpkin C2-domain proteins (*42*).

Gene Organization. The existence of two adjacent splicing sites in the *OsERG1* genomic sequence is suggestive of how this gene yields two distinct cDNA transcripts. Putative consensus intron−donor Ggt sites are present in nucelotide positions 206−208 and 197−199 of the genomic clone (GenBank entry AF512505). This suggests that alternative splicing at positions 207 and 198 leads to two transcripts, with *OsER1b* being 9 bp smaller than *OsERG1a*. To further examine whether two different splicing variants of *OsERG1a* and *OsERG1b* are produced from the same genomic clone, we amplified cDNAs by RT-PCR templated from total RNAs isolated from fungal elicitor-treated rice cells, and the cDNA products were sequenced. Two transcripts with the same electrophoretic mobility as the PCR products templated from the *OsERG1a* and *OsERG1b* cDNAs were produced from rice cells (Figure 2A), and their nucleotides were sequenced. These two transcripts had identical sequences, except for the same 9 bp deletion as shown in Figure 2B. Southern blot analysis using a full-length *OsERG1* as a probe further confirmed that the rice genome produces a single-copy gene (data not shown). These results together suggest that alternative splicing occurs from a single genomic DNA in rice cells treated with a fungal elicitor, resulting in the production of two highly homologous cDNAs, *OsERG1*a and *OsERG1b*.

Induction of OsERG1 Gene Expression by a Fungal Elicitor. To investigate the potential responsiveness of *OsERG1* gene expression to pathogen invasion, rice suspension cells were treated with a fungal elicitor prepared from rice blast fungus. The sequence homology study proved OsERG1b to be more common because of the absence of three additional amino acid residues (EIG), like other plant small C2-domain proteins (Figure 1B), and the initial characterization study provided no difference between two *OsERG1* cDNAs. Thus, our immediate study was focused on *OsERG1b*, and further study of *OsERG1a* is ongoing. The results obtained with the *OsERG1b* clone are described from this point on. The undetectable level of the *OsERG1* transcript in rice suspension cells increased dramatically within 30 min in response to fungal elicitor treatment. The transcript reached a maximal level at 1 h and then declined gradually to basal levels by 24 h. Interestingly, *OsERG1* expression was not induced by any known defense signaling molecules, including salicylic acid, hydrogen peroxide, and sodium chloride (*33*, *43*, *44*) (Figure 3A). Since Ca^{2+} concentration changes have been reported to play important roles in defense signaling in plant cells, we also investigated the effects of Ca^{2+} ion concentration on *OsERG1* gene expression (Figure 3B). We found that *OsERG1* gene expression is also rapidly induced by the Ca^{2+} ionophore

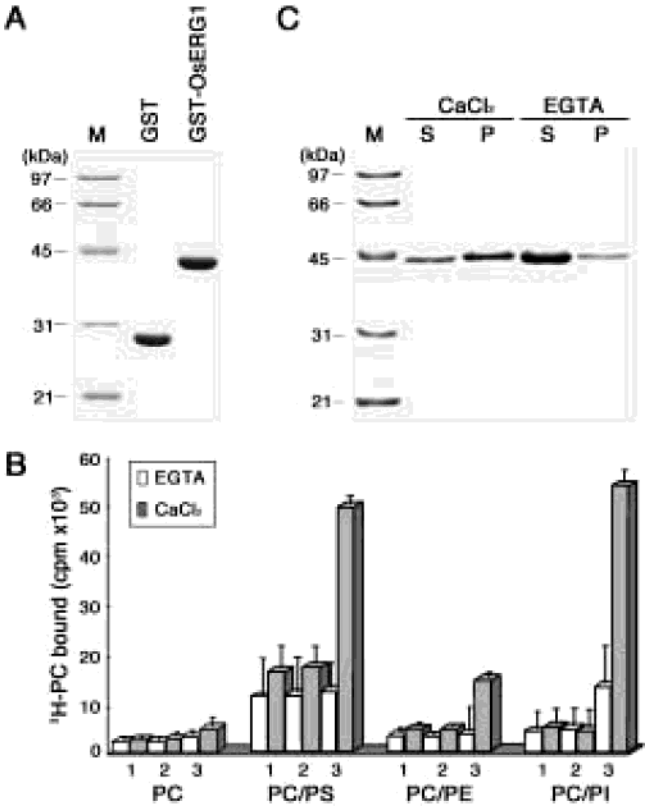

FIGURE 4: Ca^{2+}-dependent binding of OsERG1 to phospholipid vesicles. (A) SDS−PAGE of the purified recombinant GST and GST−OsERG1 proteins. Lane M contained the standard molecular mass markers. (B) Ca^{2+}-dependent binding of the GST−OsERG1 protein to ^{3}H-labeled phospholipid vesicles. Samples (40 μg of protein) were incubated in either 2 mM EGTA (white columns) or 1 mM Ca^{2+} ion (gray columns) in 50 mM HEPES (pH 7.2), 0.1 M NaCl, with [^{3}H]PC-labeled liposomes prepared with PC and PS, PE, or PI in a molar ratio of 2.5:1. GSH−Sepharose beads alone (1), GST bound to GSH−Sepharose beads (2), and the GST−OsERG1 protein bound to GSH−Sepharose beads (3) were used for the binding of the OsERG1 protein to ^{3}H-labeled phospholipid vesicles. [^{3}H]PC bound to beads was quantified with a liquid scintillation counter. (C) Ca^{2+}-dependent binding of the GST−OsERG1 protein to phospholipid vesicles. The GST−OsERG1 fusion protein (4 μg) was incubated at 30 °C for 15 min in 50 mM HEPES (pH 7.2) and 100 mM KCl in the presence of 1 mM Ca^{2+} or 2 mM EGTA, and 2.5:1 PC/PS phospholipid vesicles. After centrifugation of the vesicles at 12000*g* for 30 min, the pellet (P, phospholipid binding fraction) and supernatant (S, nonbinding fraction) were separated. An equal portion of the supernatant and pellet fractions were resolved by 13% SDS−PAGE.

Ca^{2+} and A23187, the Ca^{2+} channel activator mastoparan, and the Ca^{2+}-ATPase inhibitor BHQ, suggesting that Ca^{2+} signals may be involved in regulating *OsERG1* gene expression. Consistent with this idea, the Ca^{2+} channel blocker BAPTA significantly suppressed *OsERG1* mRNA expression to a level that was $\sim^1/_5$ of the amount of its maximal expression obtained 1 h after fungal elicitor treatment (Figure 3C). However, since basal *OsERG1* expression levels were not obtained with BAPTA treatment, there may be additional signaling pathways that mediate the induction of *OsERG1* expression.

Interaction of OsERG1 Protein with Phospholipids. Since most of the mammalian proteins with multiple C2-domains have been reported to interact with phospholipids to modulate diverse cellular signaling processes, we decided to assess the binding ability of OsERG1 protein with phospholipid

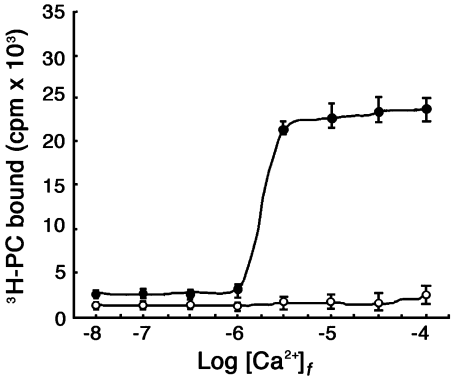

FIGURE 5: Concentration dependency of Ca^{2+} ion for the binding of OsERG1 to phospholipid vesicles. GST (○) or GST−OsERG1 (●) protein bound to GSH−Sepharose beads was incubated with ^{3}H-labeled PS/PC vesicles at various concentrations of free Ca^{2+} ions, and [^{3}H]PC bound to the beads after complete washing of the nonspecifically bound radioisotope was quantified with a liquid scintillation counter. The values reported are the means ± standard deviation (bar) of three independent experiments.

liposomes prepared with various compositions of phospholipids (PC alone, PC and PS, PC and PE, and PC and PI at a molar ratio of 2.5:1). The full-length *OsERG1b* cDNA was expressed in *E. coli* as a GST fusion protein, and the GST−OsERG1 recombinant protein was then homogeneously purified using a GSH−Sepharose column (Figure 4A). In contrast to the GSH−Sepharose or GST proteins that were used as controls, a major portion of the GST−OsERG1 protein interacted with ^{3}H-labeled liposomes in the presence of Ca^{2+} ions, whereas Ca^{2+} depletion by EGTA inhibited this binding (Figure 4B). This result clearly shows that the OsERG1 protein specifically binds to phospholipids in a Ca^{2+}-dependent manner. And lipid polarity may play a critical role in this binding, since anionic phospholipids such as PS and PI bind to the protein more effectively than do cationic phospholipids such as PC and PE.

Ca^{2+}-dependent binding of OsERG1 with liposomes was further confirmed by lipid binding assays following centrifugation with liposomes prepared with PC and PS in a ratio of 2.5:1. Most of the GST−OsERG1 protein tightly bound to liposomes in a Ca^{2+}-dependent fashion, and the binding complex was precipitated by and was detected in the pellet fraction (Figure 4C). In contrast, even in the presence of Ca^{2+} ions, GST alone did not precipitate with the liposomes at all (data not shown). From the data showing the absolute requirement of Ca^{2+} ions in formation of the protein−lipid complex, we analyzed the concentration dependency of Ca^{2+} ions in protein binding to PC/PS liposomes. The amount of GST−OsERG1 protein bound to the liposome increased sharply from a Ca^{2+} concentration of 10^{-6} M and reached a plateau at $\sim 5 \times 10^{-6}$ M (Figure 5). Thus, the half-maximal binding of the GST−OsERG1 protein to the PC/PS vesicles was observed at a Ca^{2+} concentration of $\sim 2.5 \times 10^{-6}$ M.

OsERG1 Translocates from the Cytosol to the Plasma Membrane of Plant Cells following Treatment with a Fungal Elicitor or Ca^{2+} Signals. Binding of OsERG1 to lipid vesicles strongly suggests that OsERG1 can interact with plant membranes in a Ca^{2+}-dependent manner to transduce elicitor signals. We therefore decided to determine the intracellular localization of OsERG1 in rice suspension cells following

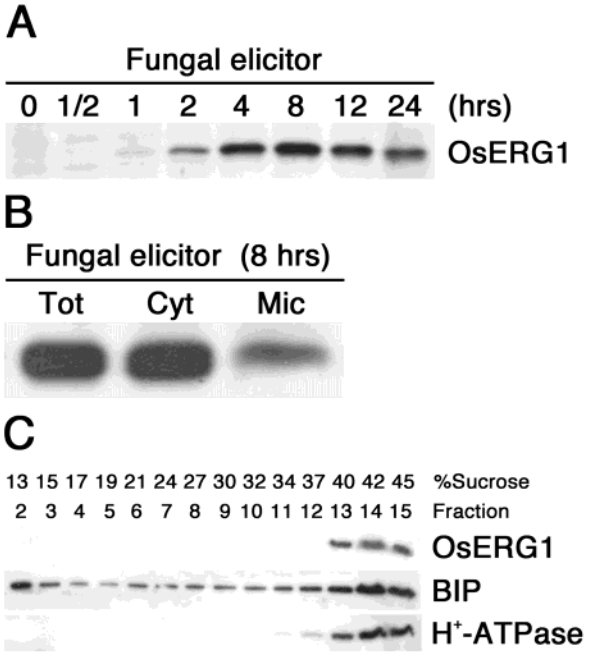

FIGURE 6: Induction of the OsERG1 protein in response to a fungal elicitor and subcellular localization of the protein. (A) Expression kinetics of the OsERG1 protein in response to fungal elicitor treatment was analyzed by Western blot. Total proteins (40 μg) of rice suspension cells treated with a fungal elicitor were separated via SDS−PAGE, transferred to a membrane, and immunoblotted with an anti-OsERG1 antibody diluted 2000-fold. (B) Distribution of the OsERG1 protein in the cytosol and membrane fractions of rice cells. After rice suspension, cells were treated with a fungal elicitor for 8 h, the cytosol and membrane fractions were separated by ultracentrifugation, and each fraction was immunoblotted with an anti-OsERG1 antibody: Tot, total protein fraction; Cyt, cytosolic protein fraction; and Mic, microsomal protein fraction. (C) Subcellular fractionation of OsERG1 in the microsomal fraction by sucrose gradient ultracentrifugation. After treatment with a fungal elicitor, the microsomal membrane fraction was isolated from rice cells and fractionated on a continuous sucrose gradient (from 12 to 45%, w/v) by ultracentrifugation. From the fractionated proteins collected with 0.8 mL in each fraction from the top of centrifugation tube, 10 μL of each fraction was used for immunoblotting with antibodies prepared against OsERG1 (1:2000), BIP (1:3000) as an endoplasmic membrane marker, and H^+-ATPase (1:5000) as a plasma membrane marker.

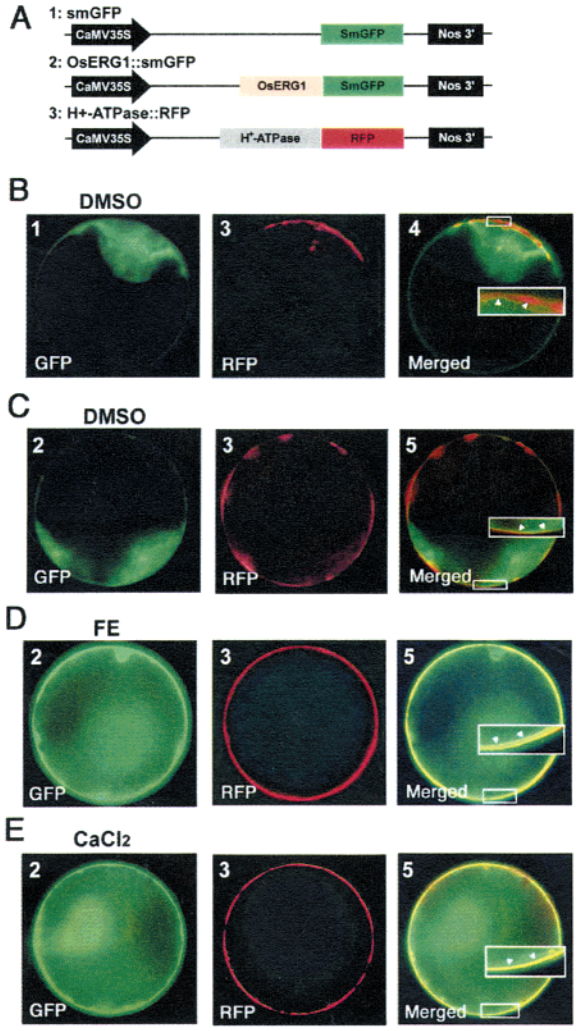

FIGURE 7: Translocation of OsERG1 fused with smGFP to the plasma membrane of *Arabidopsis* leaf protoplasts when stimulated with a fungal elicitor or Ca^{2+} agonist. (A) Schematic representation of DNA constructs of the control vector (1, smGFP), OsERG1 fused with smGFP (2, OsERG1::smGFP), and H^+-ATPase fused with RFP (3, H^+-ATPase::RFP) under the control of the CaMV35S promoter. (B−E) The plasmids were transformed into *Arabidopsis* protoplasts and viewed with green (474 nm) or red (540 nm) light excitation. Merged indicates overlapping images of red and green fluorescent signals (4 and 5). The fluorescence of the cells was analyzed 6−12 h after treatment with DMSO (B and C), fungal elicitor (10 μg of glucose equivalents/mL) (D), and Ca^{2+} ionophore (5 mM $CaCl_2$ and 10 μM A23187) (E). The images in the tiny rectangles in panels B−E were enlarged as larger rectangular boxes (inset), and arrows indicate the plasma membrane.

fungal elicitor treatment. Using Western blot analysis, we were unable to detect basal levels of the OsERG1 protein, which is in agreement with its gene expression pattern. Protein expression was induced 1 h after fungal elicitor treatment and reached a maximal level at 8 h (Figure 6A).

Using the cells that expressed a maximal level of OsERG1, we separated the cytosolic and microsomal fractions by ultracentrifugation and analyzed the protein distribution by Western blotting. As shown in Figure 6B, while most of the OsERG1 protein was present in the cytosol, a significant amount was also detected in the microsomal fractions of elicitor-treated cells. To further identify the suborganellar localization of OsERG1 in these microsomal fractions, we resuspended the microsomal fractions in the homogenization buffer described in Experimental Procedures and fractionated them with a sucrose gradient ultracentrifugation technique. Each fraction was immunoblotted with anti-OsERG1, anti-H^+-ATPase, and anti-BIP antibodies. H^+-ATPase and BIP are commonly used as markers for plasma membrane and ER proteins, respectively (*45*, *46*). OsERG1 was most abundant in fractions with sucrose contents between 40 and 45%, which are quite similar to that of H^+-ATPase (Figure 6C). In contrast, BIP was found in widely dispersed fractions across the sucrose gradient. From these results, it can be proposed that the OsERG1 protein induced by a fungal elicitor is located not only in the cytosol but also in the plasma membrane of rice cells.

To analyze the translocation of the OsERG1 protein by a fungal elicitor, we prepared an smGFP-tagged version of OsERG1 (OsERG1::smGFP). RFP-tagged H^+-ATPase (H^+-ATPase::RFP) and smGFP vector clone were used for plasma membrane and cytosolic protein controls, respectively (Figure 7A). Each DNA construct was transformed individually into *Arabidopsis* leaf protoplasts, and the expression of each fusion protein was controlled under the CaMV35S promoter.

Approximately 12 h after transformation of the DNA constructs into protoplasts, we observed both green and red fluorescence using fluorescence microscopy.

As shown in Figure 7, OsERG1::smGFP green fluorescence was uniformly dispersed throughout the cytosol in the absence of fungal elicitor treatment (Figure 7C, left panel), which was similar to the expression pattern of the smGFP control vector (Figure 7B, left panel). In contrast, H^+-ATPase::RFP red fluorescence was specifically localized to the plasma membrane of protoplast cells (middle panels of Figure 7B and Figure 7C), which is consistent with previous reports of H^+-ATPase localization (*47*). Thus, the green fluorescence of the smGFP and OsERG1::smGFP proteins did not overlap with the red fluorescence of H^+-ATPase in merged images (right panels of Figure 7B and Figure 7C).

However, fungal elicitor treatment induced translocation of a part of the green fluorescent signal emitted from OsERG1::smGFP from the cytosol to the cell periphery (Figure 7D), although some of the GFP signal remained in the cytosol. The peripheral green fluorescence of OsERG1 overlapped with the red fluorescent signal of H^+-ATPase, which produced yellow fluorescent lines at the plasma membrane (right panel of Figure 7D), whereas no movement of GFP fluorescence from the smGFP vector control was detected following the same treatment (data not shown). In addition, we investigated the effects of Ca^{2+} ions on the cellular distribution of the OsERG1 protein using the Ca^{2+}-mobilizing agonist A23187, since the binding of OsERG1 to liposomes was significantly affected by Ca^{2+} ions (Figures 4 and 5). The cellular distribution of OsERG1::smGFP following treatment with this Ca^{2+} ionophore was analyzed using conditions similar to those used in the fungal elicitor treatment. The OsERG1::smGFP fluorescence detected in the cytosol of unstimulated protoplasts was also moved to the plasma membrane by Ca^{2+} ions (Figure 7E). These results strongly suggest that a portion of the OsERG1 protein in the cytosol translocates to the plasma membrane of plant cells in response to both fungal elicitor and Ca^{2+} signals.

DISCUSSION

In recent years, several transgenic plants with enhanced resistance to a variety of pathogens have been created by manipulating a single gene that is active in the early stages of the defense signaling process (*48*). To create blast-resistant transgenic rice, we decided to isolate genes that play pivotal roles in the upstream signaling system for defense against the rice blast fungus. Employing an mRNA differential display technique, we found two highly homologous *OsERG1* clones among several cDNAs that are upregulated early in response to fungal elicitor treatment. Sequence analysis of *OsERG1* suggests that these proteins contain a C2-domain known as a Ca^{2+}- and lipid-binding motif, which appears in a variety of mammalian proteins such as protein kinases, phospholipases, and many regulatory proteins. Whereas most of the mammalian proteins contain multiple C2-domains with other functional motifs, the small C2-domain proteins that consist of only a single C2-domain, such as AtC2-1, ZmC2-1, CmPP16-1, and OsERG1, are only found in plants.

Interestingly, as judged by RT-PCR analysis (Figure 3), two *OsERG1* transcripts, *OsERG1a* and *OsERG1b*, are produced from a single *OsERG1* genomic DNA by alternative intron processing. Accumulating evidence suggests that alternative splicing can delete or modify protein activity, alter protein localization within a cell, and/or affect RNA stability and translational efficiency (*49*). Furthermore, alternative splicing in most plant species yields two polypeptides of different sizes that are identical except for additional amino acids (*50*). Dinesh-Kumar and Baker reported that the N gene of tobacco is alternatively spliced to produce short and long transcripts, both of which are required for complete resistance against tobacco mosaic virus (*51*). In the case of the OsERG1 proteins, we could not detect the functional differences between the two alternatively spliced products that may be important for the determination of their *in vivo* roles or the regulatory modes of these genes.

It has been shown that mammalian C2 proteins are involved in Ca^{2+} signaling or membrane trafficking processes mediated by binding of C2-domains to membrane phospholipids (*22*, *52*). In response to Ca^{2+} being imported, the C2-domains of phospholipases, synaptotagmin I, ubiquitin protein ligase Nedd4, and protein kinase C were shown to bind Ca^{2+} and migrate from the cytosol to the plasma membrane, thereby transducing the foreign signal into the cells (*53*−*60*). Using phospholipid binding and smGFP-mediated transient expression assays, we also found that OsERG1 proteins rapidly overexpressed by fungal elicitor treatment potentially relayed elicitor signals through the binding of proteins to membrane phospholipids in a Ca^{2+}-dependent manner. However, in contrast to mammalian C2 proteins, there is only one report on the functional role of plant small C2-domain proteins. The sequence of the pumpkin CmPP16-1 protein is highly homologous to that of OsERG1 and has been reported to increase the size of mesophyll plasmodesmata to transport cellular materials, including RNA molecules, from cell to cell (*42*). In addition, immunocytochemical experiments showed that CmPP16-1 was associated with the plasma membrane. Structural analyses of the C2-domains of synaptotagmin, cPLA2, PLC-δ1, and PKCβ show that the structure consists of a compact β-sandwich composed of two four-stranded β-sheets. The predicted three-dimensional structure of OsERG1 is similar to those of PKCβ and synaptotagmin I, indicating that the C2-domain of OsERG1 belongs to topology group I, depending on the topology of the β-strand connections (*27*). These analyses have suggested that the interaction between the C2-domain and Ca^{2+} occurs via five conserved aspartate (Asp) residues located in bipartite loops within the C2-domain (*22*, *61*−*63*). The five Ca^{2+}-binding Asp residues are also conserved in the OsERG1 proteins (Figures 1 and 2). NMR studies of the C2-domain of synatotagmin 1A (*27*, *64*, *65*) showed that charge neutralization of the Asp residues by Ca^{2+} binding allowed positively charged side chains in the loops to interact with negatively charged phospholipids, and that the ternary structure thus formed primarily supported electrostatic interactions between the C2-domain and the plasma membrane. In addition to phospholipids, binding of C2-domains to proteins has also been reported. Whereas the synaptotagmin C2-A and C2-B domains have been shown to interact with other proteins (*50*) and the C2-domain (CaLB) of $p120^{GAP}$ directly interacts with annexin VII (*66*), no interacting partner protein of the plant C2-domain has been identified. The identification of target proteins of

OsERG1 should help us elucidate the functions of OsERG1 in plant defense signaling systems.

From our results presented here, we can postulate about which OsERG1 proteins that are induced upon pathogen infection migrate to the plasma membrane, where they may recruit several target proteins to activate the defense response signaling pathways. This study lays the foundation for further investigation of the complex mechanisms of membrane targeting and the roles of OsERG1 proteins in plant defense responses, which involve interactions with calcium, phospholipids, and other proteins.

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