Rice Small C2-Domain Proteins Are Phosphorylated by Calcium-Dependent Protein Kinase

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We previously reported that *OsERG1* and *OsERG3* encode rice small C2-domain proteins with different biochemical properties in Ca^{2+} - and phospholipid-binding assays. *OsERG1* exhibited Ca^{2+} -dependent phospholipid binding, which was not observed with *OsERG3*. In the present study, we show that both *OsERG1* and *OsERG3* proteins exhibit oligomerization properties as determined by native polyacrylamide gel electrophoresis (PAGE) and glutaraldehyde cross-linking experiments. Furthermore, *in vitro* phosphorylation assays reveal the phosphorylation of *OsERG1* and *OsERG3* by a rice calcium-dependent protein kinase, *OsCDPK5*. Our mutation analysis on putative serine phosphorylation sites shows that the first serine (Ser) at position 41 of *OsERG1* may be an essential residue for phosphorylation by *OsCDPK5*. Mutation of Ser41 to alanine (*OsERG1S41A*) and aspartate (*OsERG1S41D*) abolishes the ability of *OsERG1* to bind phospholipids regardless of the presence or absence of Ca^{2+} ions. In addition, unlike the *OsERG1* wild-type form, the mutant *OsERG1* (*S41A*)::smGFP construct lost the ability to translocate from the cytosol to the plasma membrane in response to calcium ions or fungal elicitor. These results indicate that Ser41 may be essential for the function of *OsERG1*.

INTRODUCTION

A number of lipid-binding domains in proteins have been identified and characterized (Meijer and Munnik, 2003). Among them, the C2 domain, also known as CalB (from calcium and lipid binding), is one of the well-characterized lipid-binding modules that have an affinity for both Ca^{2+} and lipids. The C2 domain was originally identified as the second of two conserved domains (C1-C2) in the α , β , and γ isoforms of mammalian Ca^{2+} -dependent protein kinase C (PKC), which contains approximately 130–145 amino acid residues that form a conserved eight-stranded anti-parallel β -sandwich connected by variable

loops (Coussens et al., 1986; Knopf et al., 1986; Newton and Johnson, 1998; Parker et al., 1986). In yeast and animals, more than 150 C2 domain-containing proteins have been identified as various signaling molecules (Gallagher et al., 2006), and they possess the C2-domain modules involved in phospholipid binding, protein-protein interaction, membrane and vesicular trafficking, and signal transduction (Chapman et al., 1995; Cho and Stahelin, 2006; Davis et al., 1996; Gallagher et al., 2006; Nalefski and Falke, 1996). Several animal proteins, such as synaptotagmin, rabphilin-3A, Doc2, and N-copine, have two C2 domains and constitute the double C2-domain protein family (Nakayama et al., 1998; Orita et al., 1995; Sudhof and Rizo, 1996). Unlike other well-known C2 domain-containing proteins, the small C2-domain proteins that are only found in plants have a single C2 domain and lack the additional conserved motifs present in multi-domain proteins such as PKC. Previously, we identified three small C2-domain proteins (*OsERG1a*, *OsERG1b*, and *OsERG3*) that were expressed as isolated domains in rice (Kang et al., 2011; Kim et al., 2003). To date, limited information is available on the small C2-domain proteins in plants compared to a variety of reports on the C2 domain-containing proteins found in animals. Detailed roles of the plant small C2-domain proteins are still waiting to be elucidated. Previous reports have suggested that the plant small C2-domain proteins appear to have diverse functions (Meijer and Munnik, 2003; Yang et al., 2008), including mRNA long-distance transport, plant defense, heavy metal stress response, leaf senescence, stress tolerance, and membrane targeting (Kim et al., 2003; Ouelhadj et al., 2006; Xoconostle-Cázares et al., 1999; Yang et al., 2006; Yokotani et al., 2009). We previously reported that *OsERG1* and *OsERG3* are small C2-domain proteins that exhibit different properties in molecular and biochemical studies (Kang et al., 2011; Kim et al., 2003). *OsERG1* showed the general characteristics of Ca^{2+} -dependent lipid binding and membrane translocation similar to most mammalian C2 domain-containing proteins. In contrast, *OsERG3* protein did not interact with phospholipids and failed to translocate to the

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plasma membrane in response to fungal elicitor or Ca^{2+} ions. In addition, OsERG3 protein showed Ca^{2+} -binding activity, while this was not observed with OsERG1 protein.

In this report, we have continued to look for additional characteristics of OsERG1 and OsERG3. We describe the oligomerization properties of OsERG1 and OsERG3 proteins, and show that both OsERG1 and OsERG3 proteins are phosphorylated by OsCDPK5, a calcium-dependent protein kinase (CDPK) from rice.

MATERIALS AND METHODS

Site-directed mutagenesis

Mutagenesis of *OsERG1(S41A)*, *OsERG1(S42A)*, *OsERG1(S41D)*, *OsERG3(S40A)*, and *OsERG3(S41A)* was performed in a DNA thermal cycler using QuickChange Site-Directed Mutagenesis Kit (Stratagene, USA) according to the manufacturer's instructions. In *OsERG1(S41A)*, *OsERG1(S42A)*, and *OsERG1(S41D)*, the *OsERG1b* cDNA was separately amplified by *Pfu-Turbo* DNA polymerase with two pairs of synthetic oligonucleotides [5'-CCAGGAGCGCAAGGCCAGCGTCGCCAG-3' for *OsERG1(S41A)*; 5'-GGAGCGCAAGAGCGCCGTCGCCAGATC-3' for *OsERG1(S42A)*; 5'-CCAGGAGCGCAAGGACAGCGTCGCCAG-3' for *OsERG1(S41D)*]. In *OsERG3(S40A)* and *OsERG3(S41A)*, the *OsERG3* cDNA was separately amplified with two pairs of synthetic oligonucleotides [5'-GCAGGAGCAG AAGGCCAGCGTTGCGTC-3' for *OsERG3(S40A)*; 5'-GGAGCAGAAGAGCGCCGTTGCGTCAGG-3' for *OsERG3(S41A)*]. The resulting PCR products were digested with the methylation-sensitive restriction endonuclease *DpnI* to selectively remove the original plasmid, followed by transformation into *Escherichia coli* cells. The constructs were verified by DNA sequencing.

Expression of recombinant proteins in *Escherichia coli*

Each of the *OsERG1b* and *OsERG3* cDNAs and the (de)phosphorylation mimicking mutants of *OsERG1(S41A)*, *OsERG1(S42A)*, and *OsERG1(S41D)* were cloned into the pGEX-5X-3 vector (Amersham Pharmacia Biotech). The resulting constructs were expressed in *E. coli* BL21(DE3)pLysS (Novagen) and purified using glutathione-Sepharose CL-4B beads (Amersham Pharmacia Biotech) as described previously (Kang et al., 2011; Kim et al., 2003).

Chemical cross-linking assays

For protein cross-linking experiments, the purified proteins (1 μg) with removed GST tags were incubated with different glutaraldehyde concentrations (0–0.02%) in binding buffer (50 mM HEPES, pH 7.2, 100 mM NaCl) in a total volume of 4 μl . Incubations were conducted at room temperature for 10 min as described previously (Hao et al., 1998). The cross-linked products were separated on 13% SDS-PAGE followed by Western blot analysis with either anti-OsERG1 or anti-OsERG3 antibodies as described previously (Kang et al., 2011; Kim et al., 2003).

In vitro phosphorylation assays

Two micrograms of the purified GST-OsERG1, GST-OsERG1(S41A), GST-OsERG1(S42A), and GST-OsERG3 proteins and 1 μg of the purified GST-OsCDPK5 recombinant protein were incubated in 20 μl of kinase buffer (50 mM Tris-HCl, pH 7.0, 1 mM dithiothreitol, and 10 mM MgCl_2) containing 10 μCi of [α - ^{32}P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech) in the absence (2 mM EGTA) or presence (1 mM CaCl_2) of calcium ions. Reactions were incubated for 30 min at 30°C and termi-

nated by the addition of 5 μl of 4 $\times$ SDS sample buffer. Duplicate 13% SDS-PAGE were analyzed for *in vitro* kinase assay and Coomassie brilliant blue staining with identical protein samples in the presence or absence of the ^{32}P -labeled ATP. After electrophoresis, one gel was stained with Coomassie blue and the other one was dried to visualize by autoradiography.

Phospholipid-binding assays

Phospholipid-binding assays of the purified GST-OsERG1, GST-OsERG1(S41A), and GST-OsERG3 proteins were carried out using ^3H -labeled liposomes of phosphatidylcholine (PC) and phosphatidylserine (PS) or phosphatidylinositol (PI) as previously reported (Kang et al., 2011).

Transient expression of the green fluorescent protein constructs in *Arabidopsis*

OsERG1b cDNA and its mutant *OsERG1(S41A)* were fused in frame to the 5'-untranslated region of smGFP driven by the *CaMV 35S* promoter. Transient expression of *OsERG1::smGFP* and *OsERG1(S41A)::smGFP* was conducted in *Arabidopsis* leaf protoplasts as described previously (Kang et al., 2011; Kim et al., 2003).

RESULTS

OsERG1 and OsERG3 are small C2-domain proteins with oligomerization properties

We previously reported that *OsERG1* and *OsERG3* encode small C2-domain proteins in rice (Kang et al., 2011; Kim et al., 2003). To examine the biochemical characteristics of OsERG1 and OsERG3 proteins, *OsERG1b* and *OsERG3* cDNAs were expressed in bacteria and purified as GST-fusion proteins (Fig. 1 and Supplementary Fig. 1). It was shown that the second C2 (C2B) domain of synaptotagmin mediates Ca^{2+} -triggered synaptotagmin oligomerization (Chapman et al., 1996; 1998). In this context, we also have examined the oligomerization properties of OsERG1 and OsERG3. The purified GST-OsERG1 and GST-OsERG3 fusion proteins were each analyzed in the presence or absence of calcium ions by native PAGE. Interestingly, both proteins formed oligomeric complexes on native PAGE, irrespective of the presence of calcium ions (Supplementary Fig. 2). This result suggests the possibility that OsERG1 and OsERG3 proteins may form oligomeric complexes by self-association as observed in the C2B domain of synaptotagmin. Mobility shift patterns observed in the presence of calcium, using soybean calmodulin (SCaM1) as a positive control, were not detected with the GST-OsERG1 and GST-OsERG3. In addition, calcium ions did not affect the oligomerization state of OsERG proteins. SCaM1 and GST proteins were unable to oligomerize in native PAGE analysis under the same conditions. To eliminate the possibility that GST might influence the oligomerization of GST-OsERG recombinant proteins, the N-terminal GST tag was removed by Factor Xa digestion and the purified OsERG proteins lacking GST were used in glutaraldehyde cross-linking experiments. Glutaraldehyde cross-linking followed by SDS-PAGE has been used as a tool for analysis of the association of oligomeric proteins (Jaenicke and Rudolph, 1986). Figure 2 shows oligomeric complexes formed during the cross-linking of OsERG1 and OsERG3 proteins by glutaraldehyde on denaturing SDS-PAGE gels. Dimers of OsERG1 and OsERG3 were preferentially formed at very low glutaraldehyde concentrations (0.0003125–0.00125%). In contrast, bands corresponding to trimers, tetramers, and higher-order oligomers were detected at high glutaraldehyde concentrations (0.0025–

Fig. 1. Purification of GST-OsERG1 and OsERG3 fusion proteins. The purified GST and GST-OsERG recombinant proteins (10 μ g) after elution from glutathione beads were subjected to 13% SDS-PAGE and stained with Coomassie brilliant blue. Lane M, protein molecular mass markers.

0.02%). The oligomerization increased proportionally to the amount of cross-linker. Interestingly, in the case of OsERG3, there was formation of stable dimers even without the addition of glutaraldehyde cross-linker (Fig. 2B). This suggests that although OsERG3 may preferentially exist as a dimer in solution, both proteins were most commonly present as monomers.

OsERG1 and OsERG3 are phosphorylated by OsCDPK5

C2-domain-containing proteins including PKC and synaptotagmin are known to be phosphorylated in the region that contains the C2 domain (Pepio et al., 2001; Roggero et al., 2005). Hence, we investigated if OsERG1 and OsERG3 could be phosphorylated by serine/threonine protein kinases. First, we chose a family of rice CDPKs for *in vitro* kinase assays because OsERG1 and OsERG3 proteins have a Ca^{2+} /phospholipid-binding motif and their gene expression is up-regulated in response to calcium ionophore treatment (Kang et al., 2011; Kim et al., 2003). Among serine/threonine protein kinases, we found that OsCDPK5 (accession no. AF194414; Os07g0409900) from rice was able to phosphorylate GST-OsERG1 and GST-OsERG3 proteins (Fig. 3A). The higher degree of phosphorylation activity by OsCDK5 was observed in OsERG1 than in OsERG3. However, a rice mitogen-activated protein kinase (OsBWMK1) had no effect on the phosphorylation of these C2-domain proteins (data not shown). The GST control was also not phosphorylated. Our *in vitro* kinase assays imply that OsERG1 and OsERG3 proteins are probably regulated through phosphorylation by calcium-dependent protein kinases. Our result is the first report that small C2-domain proteins specific to plants are phosphorylated by a serine/threonine protein kinase of the CDPK family. In addition, the purified GST-OsERG1 and GST-OsERG3 proteins were phosphorylated by GST-CDPK5 in a Ca^{2+} -dependent manner (Fig. 3B). These results indicate that plant small C2-domain proteins of OsERG1 and OsERG3

Fig. 2. Chemical cross-linking assays for oligomerization of OsERG1 and OsERG3 proteins. Purified OsERG1 (A) and OsERG3 (B) proteins (1 μ g) without GST tags were chemically cross-linked at room temperature for 10 min by glutaraldehyde at various concentrations: 0% (lane 1), 0.0003125% (lane 2), 0.000625% (lane 3), 0.00125% (lane 4), 0.0025% (lane 5), 0.005% (lane 6), 0.01% (lane 7), and 0.02% (lane 8). The samples were separated by SDS-PAGE followed by Western blot analysis with each anti-OsERG1 or anti-OsERG3 antibody (1:2,000). Triangles and arrowheads indicate the formation of monomers, dimers, and trimers.

may be regulated by phosphorylation that is linked with Ca^{2+} -mediated signaling pathways.

OsERG1 is phosphorylated on the first serine at position 41 by OsCDPK5

To determine which amino acid residues in OsERG1 and OsERG3 are involved in the phosphorylation mediated by OsCDPK5, we examined OsERG protein sequences together with plant small C2-domain proteins, which can serve as putative phosphorylation sites for serine/threonine protein kinases. Roggero et al. (2005) showed that synaptotagmin VI is phosphorylated by PKC β II on the conserved threonine (Thr or T) residue in the

Fig. 3. Calcium-dependent phosphorylation of OsERG1 and OsERG3 proteins by OsCDPK5. (A) Purified proteins (2 μ g) of GST (lane G), GST-OsERG1 (lane 1), or GST-OsERG3 (lane 3) were incubated for 30 min at 30°C with GST-CDPK5 recombinant protein (1 μ g) in 25 μ l of kinase buffer containing 10 μ Ci of [γ -³²P]ATP in the presence (1 mM CaCl₂) of calcium ions and incubated at 30°C for 30 min. (B) Purified fusion proteins (2 μ g) of GST-OsERG1 and GST-OsERG3 were incubated for 30 min at 30°C with (+) or without (-) GST-OsCDPK5 (1 μ g) in 25 μ l of kinase buffer containing 10 μ Ci of [γ -³²P]ATP in the absence (2 mM EGTA) or presence (1 mM CaCl₂) of calcium ions. Reactions were stopped by the addition of SDS-PAGE sample buffer. Samples were subjected to 13% SDS-PAGE, the gel was dried, and radioactivity was visualized (left panel in Fig. 3A or upper panel in Fig. 3B). Identical samples were separated by 13% SDS-PAGE and stained with Coomassie brilliant blue (right panel in Fig. 3A or lower panel in Fig. 3B).

Fig. 4. *In vitro* phosphorylation on serine 41 of OsERG1 by OsCDPK5. (A) Prediction of a putative phosphorylation site on C2 domains by serine/threonine protein kinases. Serine (S) or threonine (T) residues that are conserved in C2-domain proteins, which could serve as phosphorylation sites for serine/threonine protein kinases, are indicated with the asterisk. Amino acid residues identical to the consensus sequence are shown in dark. (B) Mutational analysis for phosphorylation of GST-OsERG1 by OsCDPK5. Wild-type and mutant proteins of GST-OsERG1 (WT), GST-OsERG1 (S41A), GST-OsERG1(S42A), and GST-OsERG1(S41D) constructs and GST-OsCDPK5 recombinant proteins (1 μ g) were incubated for 30 min at 30°C in 25 μ l kinase buffer containing 10 μ Ci of [α -³²P]ATP in the presence of 1 mM calcium ions. Identical samples were separated by 13% SDS-PAGE and stained with Coomassie brilliant blue (right panel).

polybasic region of the C2 domain. The phosphorylation site (T) present in the C2 domains of mammalian proteins is found to be a conserved serine (S) residue in the plant small C2 domains, as shown in Fig. 4A. Thus, potentially two consecutive serine residues, which are expected to serve as substrate phosphorylation sites by serine/threonine protein kinases, were mutated into alanine (A) or aspartate (D) by site-directed mutagenesis. As a result, three mutants of OsERG1, OsERG1 (S41A), OsERG1(S42A), and OsERG1(S41D), were prepared as GST-fusion proteins for an *in vitro* kinase assay by OsCDPK5. As shown in Fig. 4B, mutation at Ser41 (S41A and S41D) greatly reduced phosphorylation of the mutant OsERG1(S41A) by OsCDPK5, while the mutant OsERG1(S42A) was still phos-

phorylated by OsCDPK5, as observed in wild-type OsERG1 (WT). These data suggest that the first Ser at position 41 of OsERG1 may be an essential residue for phosphorylation by serine/threonine protein kinases of CDPK.

Mutation of Ser41 to Ala or Asp abolished their ability of phospholipid binding to OsERG1

Previously, we reported that OsERG1 interacts with phospholipid vesicles in a Ca²⁺-dependent manner (Kim et al., 2003). To further determine if mutation of the OsERG1 Ser41 phosphorylation site can influence the phospholipid-binding property of OsERG1, we prepared two mutants in which Ser41 was replaced by Ala (S41A) or Asp (S41D) and purified as GST-fusion

Fig. 5. Phospholipid-binding assay of OsERG1 and its mutant proteins (OsERG1S41A and OsERG1S41D). (A) Purification of GST-OsERG1 and its mutant proteins (GST-OsERG1-S41A and GST-OsERG1S41D). Purified proteins (1 μ g) were subjected to 13% SDS-PAGE. (B) Phospholipid-binding assay. The purified proteins (40 μ g) were incubated in either 2 mM EGTA or 1 mM CaCl₂ with [³H]PC-labeled liposomes (PC/PS and PC/PI) of the indicated composition (2.5:1). Values are the means from three repeated experiments.

Fig. 6. Cellular localization of OsERG1 mutant::smGFP fusion proteins in transiently transfected *Arabidopsis* leaf protoplasts. (A) Schematic representation of DNA constructs of the control vector (1, OsERG1::smGFP) and OsERG1 mutants fused with smGFP (2, OsERG1S41A::smGFP) under the control of the *CaMV* 35S promoter. (B) The plasmids were transformed into *Arabidopsis* leaf protoplasts and viewed with green (474 nm) or red (540 nm) light excitation. The fluorescence of the cells was analyzed 6–12 h after treatment with Ca²⁺-ionophore (5 mM CaCl₂

and 10 μ M A23187: CaCl₂), fungal elicitor (10 μ g of glucose equivalent/ml: FE), or DMSO as a control.

proteins (Fig. 5A). Phospholipid-binding assays were performed in the presence of either Ca²⁺ or EGTA to examine the interaction between OsERG1 mutant proteins and ³H-labeled phospholipid vesicles comprised of PC/PS and PC/PI (Fig. 5B). Unlike OsERG1 (WT) protein, GST-OsERG1(S41A) and GST-OsERG1(S41D) did not show phospholipid-binding properties, and this lack of phospholipid binding was independent of the presence or absence of Ca²⁺ ions. This suggests that mutation of Ser41 to Ala or Asp inhibited the ability of phospholipid-binding to OsERG1 *in vitro*, indicating the importance of the Ser41 residue for the phospholipid-binding ability of the OsERG1 protein.

Mutation of Ser41 to Ala impaired the plasma membrane localization of OsERG1 in response to elicitor signals

We previously reported that cytosolic OsERG1 translocates to the plasma membrane of plant cells in response to calcium or fungal elicitor (Kim et al., 2003). Here, in comparison with OsERG1 wild-type protein, we examined the localization of the OsERG1(S41A) mutant protein after transfection of the OsERG1(S41A)::smGFP construct in *Arabidopsis* leaf protoplasts (Fig. 6). The control smGFP was uniformly distributed throughout the cytosol (data not shown). Fluorescence microscopy of leaf tissues revealed that the OsERG1::smGFP fusion protein was predominantly translocated from the cytosol to the plasma membrane (PM) after treatment with Ca²⁺-ionophore (Fig. 6B,

top panel). By contrast, the OsERG1(S41A)::smGFP mutant was not localized in the PM, but was observed throughout the cytosol of leaf protoplasts even in the presence of calcium ions or fungal elicitor (FE) (Fig. 6B, middle panel). In translocation assays with the OsERG1::smGFP and OsERG1(S41A)::smGFP constructs in response to calcium ions and FE in *Arabidopsis* protoplasts, we observed that the OsERG1::smGFP protein appeared to localize to PM at 6–11 h after treatment with calcium ions or FE in more than 60% of the cells. However, no PM-localized cells were detected with the OsERG1(S41A)::smGFP construct under the same treatments (Supplementary Table 1). This agrees well with our result from the *in vitro* phospholipid-binding assay shown in Fig. 5, indicating that the Ser41 residue in OsERG1 is essential for the phospholipid-binding or membrane translocation of OsERG1 protein.

DISCUSSION

We previously isolated two genes (*OsERG1* and *OsERG3*) encoding small C2-domain proteins from rice. They displayed different molecular and biochemical properties (Kang et al., 2011; Kim et al., 2003). Both *OsERG1* and *OsERG3* genes showed early responses to treatments with calcium and fungal elicitor, but the transcript levels of *OsERG1* were much higher than those of *OsERG3*. In spite of the inability of OsERG1 to bind Ca²⁺ ions, OsERG1 protein showed Ca²⁺-dependent lipid-

binding and membrane translocation properties, which are general characteristics of most C2-domain-containing proteins. By contrast, OsERG3 protein had a Ca^{2+} -binding module but lacked phospholipid-binding properties. In this report, we describe the biochemical properties of *in vitro* oligomerization and *in vitro* phosphorylation by OsCDPK5 for OsERG1 and OsERG3 proteins. Furthermore, substitution of the Ser at position 41 of OsERG1 to Ala (OsERG1S41A) abolished the ability of OsERG1 to bind phospholipids and to translocate to the membrane in response to Ca^{2+} signals.

Rice small C2-domain proteins exist as oligomerized forms

To examine the mobility shift patterns that might occur in OsERG proteins with Ca^{2+} -binding modules in response to the presence of Ca^{2+} ions, the purified GST-OsERG1 and GST-OsERG3 proteins were analyzed in the presence or absence of calcium ions by native polyacrylamide gel electrophoresis (PAGE). However, we found that OsERG proteins do not show the mobility shift patterns observed in Ca^{2+} -binding proteins (SCaM1). From this experiment, we noted that both OsERG proteins could form oligomeric complexes on native PAGE (Supplementary Fig. 1). Furthermore, through the glutaraldehyde cross-linking experiment, we confirmed that OsERG proteins could exist in oligomeric states. To our knowledge, this is the first report showing that small C2-domain proteins can form oligomeric complexes by self-association in solution (Fig. 2 and Supplemental Fig. 1). These findings increase the possibility that other C2-domain proteins in plants can also form oligomeric complexes. Chapman et al. (1996) reported that the second C2 domain (C2B) of synaptotagmin is involved in its Ca^{2+} -dependent oligomerization, primarily forming dimers. Self-association of synaptotagmin dimers *via* the C2B domain was Ca^{2+} -dependent, but OsERGs displayed oligomeric patterns irrespective of the presence of calcium ions. Although our present data suggest the oligomerization properties of OsERG proteins, their functional significance in plants remains to be determined. It is also possible that the OsERG C2 domains mediate protein-protein interactions with target proteins, as indicated by studies with other C2-domain proteins (Chapman et al., 1995; Davis et al., 1996). However, very little is known about the interaction partners of small C2-domain proteins in plants. The *Arabidopsis* small C2-domain protein, BAP1 (BON1 associated protein 1), was demonstrated to be a functional partner of BON1, a negative regulator in *R* gene-mediated defense responses. The BAP1/BON1 complex negatively regulated both basal and *R* gene-mediated defense responses in *Arabidopsis* (Yang et al., 2006). Work is currently in progress to identify target proteins interacting with OsERG proteins by yeast two-hybrid screening. The identification of interaction partners of OsERGs *via* the C2 domain will provide valuable clues to elucidate the cellular function of OsERG proteins in plants.

Rice small C2-domain proteins are phosphorylated by OsCDPK5

Protein phosphorylation constitutes an important process in transmembrane signal transduction in eukaryotic cells. Accumulating evidence has addressed the C2-domain-specific phosphorylation, the phosphorylating enzymes involved, and the diverse effects on membrane localization (Ng et al., 1999; Pepio and Sossin, 2001; Roggero et al., 2005). However, very little is known about the regulation of C2-domain functions that are affected by phosphorylation. Only a few C2 domain-containing proteins such as PKC and synaptotagmin have been

shown to be regulated by phosphorylation in the region of their C2 domains. A phosphorylation site was identified in the C2 domain of vertebrate PKC α that is important for PKC activation (Ng et al., 1999). PKC Apl II contains two autophosphorylation sites (Ser2 and Ser36) in the C2-domain region. C2-domain phosphorylation at Ser36 of PKC Apl II appeared to promote its membrane interaction (Pepio and Sossin, 2001). Moreover, mutation of Ser36 to Ala significantly reduced membrane translocation of PKC Apl II. It is possible that C2-domain phosphorylation induces a conformational change that is involved in lipid binding. Our *in vitro* kinase assays demonstrated the phosphorylation of OsERG proteins by OsCDPK5 in a Ca^{2+} -dependent manner (Fig. 3). This suggests that plant small C2-domain proteins of the OsERG-type are likely regulated *via* phosphorylation by CDPKs. Furthermore, the first Ser residue (Ser41) of OsERG1 was identified as a phosphorylation site by OsCDPK5. However, the mutation at Ser41 (OsERG1S41A) completely did not abolish phosphorylation, indicating the existence of an additional phosphorylation site (Fig. 4). We previously reported that OsERG1 possesses Ca^{2+} -dependent phospholipid-binding activity and membrane translocation (Kim et al., 2003). However, substitution of Ser41 with Ala (OsERG1S41A) disrupted the ability of OsERG1 to bind phospholipids and translocate to the membrane in a Ca^{2+} -dependent manner (Figs. 5 and 6). Unexpectedly, the phosphorylation-mimicking mutant form of OsERG1, OsERG1(S41D), also lost its phospholipid-binding activity. It is possible that substitution of Ser by an acidic Asp residue may alter the protein structure of OsERG1, affecting the Ca^{2+} -binding or lipid-binding properties. However, further studies are needed to find other possible clues. CDPKs are a large gene family of plant protein kinases and they are implicated as important Ca^{2+} sensors in plants. Genome-wide analyses revealed 34 CDPK genes in *Arabidopsis* (Cheng et al., 2002; Hrabak et al., 2003) and 29 CDPK genes in rice (Asano et al., 2005). It has been reported that plant CDPKs are induced by various stimuli, such as hormones, stresses, and elicitors (Botella et al., 1996; Chico et al., 2002; Patharkar et al., 2000; Romeis et al., 2001). The biochemical data in the present report provides new insights into the regulation of small C2-domain proteins in plants, suggesting that OsCDPK5-mediated phosphorylation of the OsERGs is a regulatory mechanism that can promote or alter the interaction of these proteins with lipids, membranes, or other effectors. However, future studies will be necessary to determine if endogenous OsCDPK5 can phosphorylate and regulate the OsERG proteins in response to Ca^{2+} ions or elicitor signals in plants.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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