Calcium ions regulate diverse cellular processes in plants as a ubiquitous internal second messenger, conveying signals received at the cell surface to the inside of the cell through spatial and temporal concentration changes that are decoded by an array of Ca\(^{2+}\) sensors (Reddy, 2001; Sanders et al., 2002; Yang and Pooovahia, 2003). Several families of Ca\(^{2+}\) sensors have been identified in higher plants. The best known are calmodulins (CaMs) and CaM-related proteins, which typically contain four EF-hand domains for Ca\(^{2+}\) binding (Zielinski, 1998). Unlike mammals, which possess single molecular species of CaM, plants have at least three distinct molecular species of CaM playing diverse physiological functions and whose expression is differently regulated (Yamakawa et al., 2001; Luan et al., 2002; Karita et al., 2004; Takabatake et al., 2007). The second major class is exemplified by the Ca\(^{2+}\)-dependent protein kinases, which contain CaM-like Ca\(^{2+}\)-binding domains and a kinase domain in a single protein (Harmon et al., 2000). In addition, a new family of Ca\(^{2+}\) sensors was identified as calcineurin B-like (CBLs) proteins, which consists of proteins similar to CaM-related proteins.
both the regulatory β-subunit of calcineurin and the neuronal Ca\(^{2+}\) sensor in animals (Liu and Zhu, 1998; Kudla et al., 1999).

Unlike CaMs, which interact with a large variety of target proteins, CBLs specifically target a family of protein kinases referred to as CBL-interacting protein kinases (CIPKs) or SnRK3s (for sucrose nonfermenting 1-related protein kinases type 3), which are most similar to the SNF family protein kinases in yeast (Luan et al., 2002). A database search of the Arabidopsis (Arabidopsis thaliana) genome sequence revealed 10 CBL and 25 CIPK homologues (Luan et al., 2002). Expression patterns of these Ca\(^{2+}\) sensors and protein kinases suggest their diverse functions in different signaling processes, including light, hormone, sugar, and stress responses (Batistic and Kudla, 2004). AtCBL4/Salt Overly Sensitive3 (SO3) and AtCIPK24/SOS2 have been shown to play a key role in Ca\(^{2+}\)-mediated salt stress adaptation (Zhu, 2002). The CBL-CIPK system has been shown to be involved in signaling pathways of abscisic acid (Kim et al., 2003a), sugar (Gong et al., 2002a), gibberellins (Hwang et al., 2005), salicylic acid (Mahajan et al., 2006), and K\(^+\) channel regulation (Li et al., 2006; Lee et al., 2007; for review, see Luan, 2009; Batistic and Kudla, 2009). However, physiological functions of most of the family members still remain largely unknown.

Plants respond to pathogen attack by activating a variety of defense responses, including the generation of reactive oxygen species (ROS), synthesis of phytoalexins, expression of pathogenesis-related (PR) genes, cell cycle arrest, and mitochondrial dysfunction followed by a form of hypersensitive cell death known as the hypersensitive response (Nürnberger and Scheel, 2001; Greenberg and Yao, 2004; Kadota et al., 2004b). Transient membrane potential changes and Ca\(^{2+}\) influx are involved at the initial stage of defense responses (Kuchitsu et al., 1993; Pugin et al., 1997; Blume et al., 2000; Kadota et al., 2004a). Many kinds of defense responses are prevented when Ca\(^{2+}\) influx is compromised by Ca\(^{2+}\) chelators (Nürnberger and Scheel, 2001; Lecourieux et al., 2002). Since complex spatiotemporal patterns of cytosolic free Ca\(^{2+}\) concentration have been suggested to play pivotal roles in defense signaling (Nürnberger and Scheel, 2001; Sanders et al., 2002), multiple Ca\(^{2+}\) sensor proteins and their effectors should function in the defense signaling pathways. Although possible involvement of some CaM isoforms (Heo et al., 1999; Yamakawa et al., 2001), Ca\(^{2+}\)-dependent protein kinases (Romeis et al., 2000, 2001; Ludwig et al., 2005; Kobayashi et al., 2007; Yoshioka et al., 2009), as well as Ca\(^{2+}\) regulation of EF-hand-containing enzymes such as ROS-generating NADPH oxidase (Ogasawara et al., 2008) have been suggested, other Ca\(^{2+}\)-regulated signaling components still remain to be identified. No CBLs or CIPKs have so far been implicated as signaling components in defense signaling.

N-Acetylchitoooligosaccharides, chitin fragments, are microbe-associated molecular patterns (MAMPs) that are recognized by plasma membrane receptors (Kaku et al., 2006; Miya et al., 2007) and induce a variety of defense responses, such as membrane depolarization (Kuchitsu et al., 1993; Kikuyama et al., 1997), ion fluxes (Kuchitsu et al., 1997), ROS production (Kuchitsu et al., 1995), phytoalexin biosynthesis (Yamada et al., 1993), and induction of PR genes (Nishizawa et al., 1999), without hypersensitive cell death in rice (Oryza sativa) cells. In contrast, a fungal proteinaceous elicitor, xylanase from Trichoderma viride (TvX)/ethylene-inducing xylanase (EIX), which is recognized by two putative plasma membrane receptors, LeEix1 and LeEix2 (Ron and Avni, 2004), triggers hypersensitive cell death along with different kinetics of ROS production and activation of a mitogen-activated protein kinase, OsMPK6, previously named as OsMPK2 or OsMAPK6, in rice cells (Kurusu et al., 2005). These two fungal MAMPs thus provide excellent model systems to study innate immunity in rice cells.

This study identified two CIPKs involved in various MAMP-induced layers of defense responses, including PR gene expression, phytoalexin biosynthesis, mitochondrial dysfunction, and cell death, in rice. Molecular characterization of these CIPKs, including interaction with the putative Ca\(^{2+}\) sensors as well as their physiological functions, is discussed.

RESULTS

Identification of CIPKs Induced by MAMPs

A database search of the rice genome sequence revealed 30 CIPK homologues (Kolukisaoglu et al., 2004). As a first step toward elucidating the molecular mechanisms of CBL-CIPKs in defense signaling, the expression patterns of several CIPK genes were surveyed in suspension-cultured rice cells in response to several MAMPs by semiquantitative reverse transcription (RT)-PCR analyses. The expression levels of two CIPK family members, OsCIPK14 and OsCIPK15, were greatly enhanced by N-acetylchitoheptaose and TvX/EIX, while those of other CIPKs, such as OsCIPK2, OsCIPK10, OsCIPK11, OsCIPK12, and OsCIPK19 (accession nos. AK072868, AK066541, AK103032, AK101442, and AK069486, respectively), did not show significant changes (Supplemental Fig. S1).

The full-length cDNAs of OsCIPK14 (GenBank accession no. AB264036) and OsCIPK15 (accession no. AB264037), which encoded polypeptides of 439 and 434 amino acids, respectively, were obtained using RACE-PCR (Supplemental Fig. S2). OsCIPK14 and OsCIPK15 did not have introns and were located on different chromosomes (OsCIPK14 on chromosome 12 and OsCIPK15 on chromosome 11). They had over 95% nucleotide sequence identity even in the surrounding noncoding regions of the genome. The predicted amino acid sequences of OsCIPK14 and OsCIPK15 were almost identical and differed in only a single amino acid substitution except for the five additional amino acids (Supplemental Fig. S2). These results...
suggest that OsCIPK14 and OsCIPK15 are duplicated genes.

OsCIPK14/15 showed 44% homology with AtCIPK24/SOS2 at the amino acid level (Liu et al., 2000). Amino acid sequences of the N terminus of the kinase domain and the FISL/NAF motif (Albrecht et al., 2001) were especially well conserved (Supplemental Fig. S2).

Spatiotemporal Expression Patterns of OsCIPK14/15

MAMP-induced accumulation of OsCIPK14 and OsCIPK15 mRNAs was analyzed by real-time RT-PCR analyses. Because OsCIPK14 and OsCIPK15 are so similar, their expression pattern could not be discriminated by RT-PCR analyses. The expression of OsCIPK14/15 was up-regulated at 2 h by both TvX/EIX and N-acetylchitoheptaose (Fig. 1A). The time course of induction of OsCIPK14/15 was more rapid and transient than that of a PR gene, Probenazole-Inducible Gene1 (PBZ1)/PR10a (accession no. D38170; Midoh and Iwata, 1996; Fig. 1B). These results were confirmed by northern-blot analyses (Supplemental Fig. S3A).

Tissue-specific expression of OsCIPK14/15 genes in shoots and roots of 7-d-old seedlings, mature leaves, and cultured cells was analyzed by northern hybridization. The OsCIPK14/15 mRNAs were expressed throughout the plants in seedlings as well as in cultured cells and were most abundant in aerial parts of seedlings (Supplemental Fig. S3B).

Interaction of OsCIPK14/15 with OsCBLs

A database search of the rice genome sequence revealed 10 CBL homologues, which are categorized as several subgroups by phylogenetic analyses (Kolukisaoglu et al., 2004). We picked up six OsCBLs from several subgroups (OsCBL2, accession no. J033068L17; OsCBL3, J033133I20; OsCBL4, J033035L13; OsCBL5, J013071C24; OsCBL6, J013001C11; OsCBL9, J023045O14) and tested possible interaction with OsCIPK14/15 by the yeast two-hybrid method. The OsCIPK14 and OsCIPK15 genes were fused to the GAL4-binding domain, and six OsCBLs were fused to the GAL4 activation domain. Figure 2, A and C, shows the growth of yeast cells on the selection medium and the corresponding β-galactosidase assay when OsCIPK14/15 and OsCBLs were used as bait and prey, respectively. OsCIPK14 and OsCIPK15 interacted with several OsCBLs (OsCBL2, OsCBL3, OsCBL4, OsCBL5, and OsCBL6) but not with OsCBL9 and showed the strongest interaction with OsCBL4 (Fig. 2, A, C, and D). Interestingly, the expression of the OsCBL4 gene was also induced upon elicitation, while OsCBL9 was not (Supplemental Fig. S5).

The FISL/NAF motif is highly conserved in both OsCIPK14 and OsCIPK15 proteins (Supplemental Fig. S2). This domain of the CIPK family has been shown to interact with CBLs (Albrecht et al., 2001) and to possess autoinhibitory function (Guo et al., 2001; Gong et al., 2002a). Mutant proteins (OsCIPK14/15DF) deficient in the FISL/NAF motif were generated (Supplemental Fig. S6). OsCIPK14DF and OsCIPK15DF failed to interact with OsCBLs in yeast (Figure 2, B–D), suggesting that the FISL/NAF motif in OsCIPK14/15 is crucial for interaction with OsCBLs, and this interaction mechanism between CIPKs and CBLs is highly conserved among plant species.

The presence of conserved EF-hand motifs in OsCBLs suggests that they may bind Ca²⁺. To test the Ca²⁺-binding activity of OsCBLs, Ca²⁺-induced electrophoretic mobility shift analyses were carried out.

Figure 1. Induction of OsCIPK14/15 genes in response to MAMPs. mRNA accumulations of OsCIPK14/15 (A) and PBZ1 (B) in TvX/EIX-treated (circles) or N-acetylchitoheptaose-treated (triangles) cells. Total RNA was isolated from rice cells harvested at the indicated time points of TvX/EIX (60 μg mL⁻¹) or N-acetylchitoheptaose (1 μM) treatment. The amount of each mRNA was calculated from the threshold point located in the log-linear range of RT-PCR. The relative level of each gene in the control cells at time 0 was standardized as 1. The error bars indicate SD of four experiments.
Ca\textsuperscript{2+}-binding proteins such as CaM bind Ca\textsuperscript{2+} in the presence of SDS and show higher electrophoretic mobility than in the absence of free Ca\textsuperscript{2+} (Zielinski, 2002). Recombinant glutathione S-transferase (GST)-OsCBL4 fusion proteins, which showed the strongest interaction with OsCIPK14/15, were expressed in *Escherichia coli* and partially purified using glutathione-Sepharose columns. OsCBL4 displayed a characteristic electrophoretic mobility shift when incubated with 2 mM CaCl\textsubscript{2} prior to electrophoresis, while the mobility shift was not observed for GST protein as a control (Fig. 2F). These results suggest that OsCBL4 binds Ca\textsuperscript{2+} to function as a putative Ca\textsuperscript{2+} sensor.

**Regulation of the Protein Kinase Activity of OsCIPK14 and OsCIPK15**

To characterize the biochemical properties of OsCIPK14 and OsCIPK15, recombinant GST-OsCIPK14 and GST-OsCIPK15 fusion proteins were expressed in *E. coli* and partially purified using glutathione-Sepharose columns. Proteins of the expected size (78 kD for...
GST-OsCIPK14 and 77.4 kD for GST-OsCIPK15) were detected by SDS-PAGE (top bands in Fig. 3A), both of which showed autophosphorylation activity in the presence of Mn\(^{2+}\) but not of other divalent cations such as Mg\(^{2+}\) or Ca\(^{2+}\) (Fig. 3A), suggesting that Mn\(^{2+}\) functions as a cofactor for OsCIPK14/15. These biochemical properties of the recombinant OsCIPK14/15 are similar to those of other CIPKs in other plant species (Shi et al., 1999; Gong et al., 2002b; Kim et al., 2003b). The bottom bands shown in Figure 3A seemed to correspond to the degraded forms of GST-OsCIPK14/15 proteins.

In Arabidopsis, the FISL/NAF motif of CIPKs possess autoinhibitory function and CIPKs are activated by binding of CBLs via the FISL/NAF motif in a Ca\(^{2+}\)-dependent manner (Halfter et al., 2000; Guo et al., 2001; Gong et al., 2002a). To investigate whether the kinase activity of OsCIPK14/15 was modulated by interaction with OsCBLs, the autophosphorylation activity in the presence or absence of OsCBLs was...
analyzed using an in vitro kinase assay. The autophosphorylation activity of OsCIPK14/15 was enhanced by GST-OsCBL4 in the presence of Mn$^{2+}$ and Ca$^{2+}$ (Fig. 3, B and C) but not in the absence of Ca$^{2+}$ in the reaction buffer (Fig. 3, B–D). The autophosphorylation activity of OsCIPK14/15 was not affected by OsCBL9 (Fig. 3, B and C), which did not show an interaction with OsCIPK14/15 (Fig. 2, A and C).

A CBL from pea (Pisum sativum) has been reported to be phosphorylated by PsCIPK (Mahajan et al., 2006). Phosphorylation of OsCBL4 by OsCIPK14/15 was also detected in the presence of Mn$^{2+}$, Ca$^{2+}$, and a substantial amount of OsCBL4 protein (Fig. 3D).

To assess the roles of the FISL motif of OsCIPK14/15, a recombinant mutant protein of OsCIPK14/15 defective in the FISL/NAF motif, GST-OsCIPK14/15DF (Supplemental Fig. S6), was expressed in E. coli and partially purified (Fig. 3B). The mutant protein showed higher autophosphorylation activity than the wild-type protein (Fig. 3, B and C). Exogenous Ca$^{2+}$ showed no effect on the autophosphorylation activity of either GST-OsCIPK14/15 (Fig. 3A) or GST-OsCIPK15DF (Fig. 3D). These results indicate that the protein kinase activity of OsCIPK14/15 is negatively regulated by its FISL/NAF motif. The autoinhibitory function of OsCIPK14/15 may be canceled by Ca$^{2+}$-dependent binding of OsCBL4 via the FISL/NAF motif.

Suppression of MAMP-Induced Mitochondrial Dysfunction and Hypersensitive Cell Death in the OsCIPK14/15-RNAi Lines

Since OsCIPK14 and OsCIPK15 are duplicated genes, their translated products may be functionally redundant. To reveal the physiological roles of OsCIPK14 and OsCIPK15, transgenic cell lines were generated in which the expression of both OsCIPK14 and OsCIPK15 was suppressed by RNA interference (RNAi) using gene-specific sequences (340 bp of the 3’ untranslated region of OsCIPK14). Five independent transgenic cell lines were generated by means of Agrobacterium tumefaciens-mediated transformation. Nontransgenic cells were investigated simultaneously as a control, whose transduced genes were removed by heterozygous segregation. RT-PCR and real-time RT-PCR analyses revealed significant reductions in OsCIPK14/15 mRNA levels in comparison with the control (Fig. 4, A and B). The expression of the most closely related homolog, OsCIPK11 (Kolukisaoglu et al., 2004), was not affected (Fig. 4A), indicating that only the transcripts of OsCIPK14/15 were specifically reduced.

A series of TvX/EIX-induced defense responses were compared between the wild-type and OsCIPK14/15-RNAi lines (Fig. 4–7). Evans blue staining was applied to quantify the levels of cell death. Hypersensitive cell death was observed in the control lines after treatment with the TvX/EIX elicitor (Fig. 4B–D). In contrast, the OsCIPK14/15-RNAi lines showed significantly reduced levels of cell death, indicating that OsCIPK14/15 plays a role in the regulation of mitochondrial dysfunction and hypersensitive cell death.
death was significantly suppressed in the OsCIPK14/15-RNAi lines compared with the control, and the levels of cell death corresponded with the expression levels of OsCIPK14/15 (Fig. 4, B–D).

Increasing evidence suggests the involvement of mitochondrial dysfunction in the induction of hypersensitive cell death in plants as well as in animals (Lam et al., 2001). Reductase activity of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) was monitored as a marker for mitochondrial function (Ikegawa et al., 1998; Yamamoto et al., 2002). TxV/EIX induced a rapid reduction in MTT reductase activity in rice cells (Fig. 5A). Cycloporeine A (CsA) is a potent inhibitor for mitochondrial permeability transition and mitochondria-mediated apoptosis in animals (Marchetti et al., 1996). CsA also inhibits protoporphyrin IX-induced mitochondrial membrane depolarization and cell death in Arabidopsis (Yao et al., 2004). CsA inhibited TxV/EIX-induced reduction in MTT reductase activity (Fig. 5B) and cell death in rice cells (Fig. 5C), suggesting that mitochondrial dysfunction is involved in TxV/EIX-induced hypersensitive cell death.

In addition to the suppression of TxV/EIX-induced cell death (Fig. 4, C and D), reduction in MTT reductase activity was also significantly suppressed in the OsCIPK14/15-RNAi lines compared with the control (Fig. 5A). Taken together, these results suggested that OsCIPK14/15 may be involved in the regulation of the MAMP-induced mitochondria-mediated hypersensitive cell death.

Involvement of OsCIPK14/15 in the Regulation of Phytoalexin Biosynthesis

Pharmacological evidence suggests the importance of Ca2+ in MAMP-induced phytoalexin biosynthesis in rice (Umemura et al., 2002). Quantitative HPLC-tandem mass spectrometry analyses (Shimizu et al., 2008) revealed that TxV/EIX induces the accumulation of momilactones and phytocassanes, which are major phytoalexins in rice (Fig. 6, A and B; Koga et al., 1995, 1997). In the OsCIPK14/15-RNAi cells, the accumulation of both momilactones and phytocassanes was impaired significantly (Fig. 6, A and B).

In rice, ent-copalyl diphosphate synthase 4 (OsCPS4/OsCyc1) and ent-kaurene synthase-like 4 (OsKSL4/OsKS4) are responsible for the biosynthesis of momilactones and ent-copalyl diphosphate synthase 2 (OsCPS2/OsCyc2) and ent-kaurene synthase-like 7 (OsKSL7/OsDTC1) are responsible for the biosynthesis of phytocassanes (accession nos. AB066270, AB126934, AB066271, and AB089272, respectively; Cho et al., 2004; Otomo et al., 2004a, 2004b; Shimizu et al., 2008). Slow and prolonged expression of all these cyclase genes was induced by TxV/EIX, which was significantly suppressed in the OsCIPK14/15-RNAi lines (Fig. 6C). Taken together, these results suggest the possible involvement of OsCIPK14/15 in the regulation of TxV/EIX-induced phytoalexin biosynthesis.

Elicitor Sensitivity in Transgenic Cultured Cells Overexpressing OsCIPK15 and OsCIPK15DF

Because these results suggested that the FISL/NAF motif in OsCIPK14/15 is an important element to interact with native rice CBLs that have Ca2+-binding activity (Fig. 2, B and F), it was tested whether the FISL/NAF motif in OsCIPK14/15 is involved in elicitor-induced defense signaling. Transgenic cultured cells overexpressing OsCIPK15 mRNA were treated with the elicitor, and the resulting hypersensitive cell death and phytoalexin accumulation were studied. The elicitor-induced cell death was much more evident in OsCIPK15-overexpressing cells than in GUS-expressing control cells, and their cell death corresponded to the OsCIPK15 expression level in the transgenic cells (Fig. 8, A and B). Moreover, in the OsCIPK15-overexpressing line, the accumulation of both momilactones and phytocassanes was much more abundant than in the control line at a low concentration of TxV (Fig. 8C). In contrast to the OsCIPK15 overexpressors, the levels of TxV-induced hypersensitive cell death in the OsCIPK15DF overexpressors were comparable to those in the GUS control (Fig. 8, A and B). These results indicate that the overexpression of OsCIPK15 enhanced sensitivity to the TxV elicitor and that the FISL/NAF motif is an essential component in the MAMP-triggered signal transduction pathway.
DISCUSSION

This study identified and characterized the two MAMP-induced CIPKs, OsCIPK14/15, which interact with Ca\(^{2+}\) sensor proteins, OsCBLs. Functional characterization of the OsCIPK14/15-RNAi lines as well as overexpressors suggested that the CIPKs are involved in various TvX/EIX-induced layers of defense responses, including hypersensitive cell death, mitochondrial dysfunction, phytoalexin biosynthesis, and PR gene expression.

Biochemical Properties of OsCIPK14/15 and Their Interaction with Rice CBLs

Our results suggest that the FISL/NAF motif in OsCIPK14/15 is an important element to interact with native rice CBLs that have Ca\(^{2+}\)-binding activity (Fig. 2, B and F) and that the interaction with OsCBL4 is necessary for cancellation of the autoinhibitory function of OsCIPK14/15 (Fig. 3, B–D). The binding of AtCBL1 to AtCIPK1 is highly dependent on external Ca\(^{2+}\) (Shi et al., 1999). The protein kinase activity of AtCIPK24/SOS2 also depends on both AtCBL4/SOS3 and external Ca\(^{2+}\) (Halfter et al., 2000). OsCIPK14 and OsCIPK15 may be inactivated by autoinhibition at a resting cytosolic Ca\(^{2+}\) level and could be activated upon elicitation by MAMPs through the binding of a Ca\(^{2+}\)-bound form of OsCBLs via their FISL/NAF motif. The common machinery for the regulation of CBL-CIPK seems to be highly conserved in a variety of plant species.

Although each CIPK family member has been assumed to interact specifically with a subset of CBLs to transmit Ca\(^{2+}\) signals to downstream components, interaction between OsCBLs and OsCIPKs has not yet been reported except for the interaction between OsCBL4 and OsCIPK24 (Martinez-Atienza et al., 2007). We here showed that OsCIPK14 and OsCIPK15 interact with at least five OsCBLs (OsCBL2, OsCBL3, OsCBL4, OsCBL5, and OsCBL6) but not with OsCBL9 in yeast cells (Fig. 2, A–D). Among the OsCBLs, the interaction was strongest with OsCBL4, which was shown to bind Ca\(^{2+}\) (Fig. 2F) and to be transcriptionally induced by TvX elicitor (Supplemental Fig. S5). Since overexpression of the mutant form of OsCIPK15 defective in the FISL/NAF motif had no effect on the TvX/EIX-induced hypersensitive cell death (Fig. 8B), some OsCBLs or other unknown factors that interact with OsCIPK15 via the FISL/NAF motif may regulate the activity and localization of OsCIPK14/15 in the MAMP-triggered signal transduction pathway.

Figure 5. Effects of mitochondrial dysfunction and the suppression of OsCIPK14/15 on TvX/EIX-induced hypersensitive cell death. A, Effects of the suppression of OsCIPK14/15 on the TvX/EIX (120 \(\mu\)g mL\(^{-1}\))-induced reduction in MTT reductase activity. MTT reductase activity is a putative marker for mitochondrial dysfunction. Black triangles, control line; light gray squares, RNAi-1; white circles, RNAi-2. B, Effects of a potent inhibitor for mitochondrial permeability transition (CsA) on mitochondrial dysfunction in rice cells treated with the TvX/EIX elicitor (120 \(\mu\)g mL\(^{-1}\)). C, Effects of CsA on the TvX/EIX elicitor-induced cell death in rice cells. Rice cells were tested at 72 h after the addition of the TvX/EIX elicitor. CsA (5 \(\mu\)M) or ethanol (EtOH) was added to rice cells 30 min prior to the elicitor treatment. Average values and SE of three independent experiments are shown. Ethanol was used as a control. D.W., Distilled water as a control.
The Role of OsCIPK14 and OsCIPK15 in MAMP-Triggered Innate Immune Responses

Phytoalexin production is commonly associated with defense responses in plants. Although MAMP-triggered cytosolic free Ca$^{2+}$ concentration increases have been proposed to be involved in their regulation (Mithöfer et al., 1999; Blume et al., 2000), Ca$^{2+}$-regulated signaling components still remain mostly unknown. We have shown that TvX/EIX-induced accumulation of momilactones and phytocassanes as well as induction of the cyclase genes, the key enzymes responsible for the biosynthesis of these phytoalexins (Otomo et al., 2004a), are impaired in the OsCIPK14/15-RNAi lines (Fig. 6, A and B). These results suggest that OsCIPK14/15 is involved in the regulation of phytoalexin production through transcriptional activation of key enzymes, including diterpene cyclase.

Figure 6. Involvement of OsCIPK14/15 in the regulation of TvX/EIX-induced phytoalexin biosynthesis. A and B, TvX/EIX-induced phytoalexin accumulation in the culture medium. Amounts of momilactones (A) and phytocassanes (B) 72 h after the addition of TvX/EIX (120 μg mL$^{-1}$) or water were quantified by HPLC as described in “Materials and Methods.” Average values and se of six independent experiments for the control line (black bars) and RNAi-2 line (white bars) are shown. C, Quantification of the expression levels of the genes involved in biosynthesis of the phytoalexins OsCyc1, OsKs4, OsCyc2, and OsDTC1 in the control line (black triangles) and RNAi-2 line (white circles) by real-time quantitative PCR. Total RNA was isolated from the cells harvested at the indicated time points of TvX/EIX treatment (120 μg mL$^{-1}$). The amount of each mRNA was calculated from the threshold point located in the log-linear range of RT-PCR. The relative level of each gene in control cells at time 0 was standardized as 1. The error bars indicate se of three experiments. D.W., Distilled water as a control.

Figure 7. Involvement of OsCIPK14/15 in TvX/EIX-induced PR gene expression. mRNA accumulations of PBZ1, Cht-3, EL2, EL3, and PAL in the control line (black triangles) and RNAi-2 line (white circles) are shown. Total RNA was isolated from rice cells harvested at the indicated time points of TvX/EIX treatments (120 μg mL$^{-1}$). The amount of each mRNA was calculated from the threshold point located in the log-linear range of RT-PCR. The relative level of each gene in control cells at time 0 was standardized as 1. The error bars indicate se of three experiments.
Expression of OsCIPK14/15 is also reported to be up-regulated by salt stress, and overexpression of OsCIPK15 confers improved salt tolerance (Xiang et al., 2007). Since recent studies reveal that alternative CBL/CIPK complexes at different membranes create a dual function in salt stress responses in Arabidopsis (Qiu et al., 2002; Kim et al., 2007; Waadt et al., 2008), OsCBL/OsCIPK14/15 complexes may be involved in multiple defense responses against biotic and abiotic stress. Alternatively, other components that interact...
with OsCIPK15 via the FISL/NAF motif may function as a limiting factor for the MAMP-triggered signal transduction pathway.

Increasing evidence suggests the importance of mitochondria in the induction of hypersensitive cell death in defense signaling pathways (Lam et al., 2001; Yao et al., 2004). TvX/EIX-induced cell death (Fig. 5C) and the reduction in putative mitochondrial reductase activity (Fig. 5B) were inhibited by CsA, an inhibitor of mitochondrial permeability transition, suggesting the involvement of mitochondrial dysfunction on TvX/EIX-induced hypersensitive cell death in rice.

TvX/EIX-induced mitochondrial dysfunction as well as hypersensitive cell death requires extracellular Ca$^{2+}$ and a putative Ca$^{2+}$-permeable channel, OsTPC1 (Kurusu et al., 2005). Our results here indicate that OsCIPK14/15 is also crucial for TvX/EIX-induced mitochondrial dysfunction as well as hypersensitive cell death (Figs. 4, C and D, and 5A), suggesting that OsCIPK14/15 may be involved in transducing MAMP-triggered Ca$^{2+}$ signals to regulate the mitochondria-mediated hypersensitive cell death pathway. OsPDCD5, which is involved in programmed cell death during leaf and root senescence, has been suggested to be phosphorylated by OsCIPK23 (Su et al., 2006). Interestingly, the basal level of the cell death in the OsCIPK15-overexpressing lines was slightly higher than in GUS-expressing control lines (Fig. 8B). OsCIPK15 may control the activation of cell death regulators. Searches for the in vivo substrates of OsCIPK14 and OsCIPK15 are currently under way to further understand the relationship between Ca$^{2+}$ signaling and the regulation of hypersensitive cell death in innate immunity.

Finally, to test whether OsCIPK14/15 has a role in disease resistance in adult plants, we inoculated the OsCIPK14/15-RNAi and OsCIPK15-overexpressing plants with the rice blast fungus Magnaporthe grisea. Overexpression of OsCIPK15 and suppression of OsCIPK14/15 by RNAi did not show any significant effect on the growth of an incompatible and a compatible fungus (Supplemental Fig. S7). In adult plants, the contribution of OsCIPK14/15 to resistance against rice blast fungus may be limited. Future infection assays with various kinds of pathogens as well as detailed elucidation of the defense signaling network in the OsCIPK14/15 mutants would reveal the roles of the CIPKs in vivo in intact plants.

In summary, this study indicates that two CBL-activated protein kinases, OsCIPK14 and OsCIPK15, are involved in the regulation of the defense signaling pathway in rice cultured cells. These findings shed light on our understanding of defense signaling pathways.

**MATERIALS AND METHODS**

**Plant Materials, Cell Cultures, and MAMPs**

Surface-sterilized seeds of rice (Oryza sativa 'Nipponbare') were germinated on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 0.8% agar and grown for 10 d in a growth chamber under long-day conditions (16 h of light/8 h of darkness, 28°C). Seedlings were transplanted into soil and grown in a greenhouse (16 h of light/8 h of darkness, 28°C, 60% humidity). To generate cultured cells, including the OsCIPK14/15-RNAi lines, seeds were placed onto callus-inducing medium (Murashige and Skoog, 1962) containing 2,4-dichlorophenoxyacetic acid (0.25 mg L$^{-1}$) and subcultured in fresh medium every 7 d. Cells at 5 d after subculture were used for experiments with MAMPs. TvX/EIX was obtained from Sigma. N-Acetyl-chitooligosaccharide was kindly provided by Prof. Naoto Shibuya (Meiji University).

**Isolation of OsCIPK14 and OsCIPK15 cDNA**

The estimated coding region of OsCIPK14 and OsCIPK15 was amplified by PCR using two primers each, OsCIPK14FW1, 5'-ATGGAGAGTAGGAGGAAGATA-3'; OsCIPK14RV1, 5'-CTATCACATCCTCAGGAGCAGG-3'; OsCIPK15FW1, 5'-ATGGAGAGTAGGAGGAAGATT-3'; OsCIPK15RV1, 5'-CTATTCATCTCCATG-3'. Total RNA was isolated from leaves of rice with Trizol (Invitrogen) in accordance with the manufacturer’s protocol and quantified spectrophotometrically. First-strand cDNA was synthesized from 3 μg of total RNA with the oligo(dT) primer and reverse transcriptase (Invitrogen). To obtain full-length cDNAs for OsCIPK14 and OsCIPK15 and to define the open reading frame, 3'-RACE-PCR and 5'-RACE-PCR were performed with a 3 full RACE core kit (TaKaRa) and a 5'-RACE system (Invitrogen) in accordance with the manufacturer’s protocol.

**Plasmid Construction**

The coding regions of OsCIPK14 and OsCIPK15 and OsCBL4 and OsCBL9 were amplified by PCR with primers harboring additional sequence (the CACC sequences are underlined) for use with the Gateway system, subcloned into the pENTR/D-TOPO cloning vector (Invitrogen) to yield entry vectors, and cloned into pDEST15 vector (Invitrogen) using LR clonase reaction (Invitrogen). The PCR primers were OsCIPK14FW2, 5'-CACCATGAGAGTAGGAGGAAGATA-3'; OsCIPK14RV2, 5'-CTATCACATCCTCAGGAGCAGG-3'; OsCIPK15FW2, 5'-CACCATGAGAGTAGGAGGAAGATT-3'; OsCIPK15RV2, 5'-CTATTCATCTCCATG-3'. OsCIPK15FW2 was amplified by PCR with primers harboring restriction sites and cloned in frame into the pGBKT7 cloning vector, and then cloned into a pDEST15 vector using the LR clonase I site (Stratagene) of the pGBKT7 vector. The following primers were used for PCR amplification: the forward primers OsCIPK14DF-FW1, 5'-GGATCCATGGAGAGTAGGAGGAAGATT-3' and OsCIPK15DF-FW1, 5'-GGATCCATGGAGAGTAGGAGGAAGATT-3' (the BamHI site is underlined) in each primer; OsCIPK14DF-FW2, 5'-ATGTTGAGTTGAATGGAGAAATT-3'; OsCIPK15DF-FW2, 5'-ATGTTGAGTTGAATGGAGAAATT-3'; OsCIPK14DF-RV1, 5'-TAACTTGTGTTTACAATCTGCTGATCCGCTCT-3'; OsCIPK15DF-RV1, 5'-TAACTTGTGTTTACAATCTGCTGATCCGCTCT-3'; OsCIPK14DF-RV2, 5'-TCTAGACTATTTTATCTCCTGATCGCC-3'; OsCIPK15DF-RV2, 5'-TCTAGACTATTTTATCTCCTGATCGCC-3'. To make the deletion mutants (OsCIPK14DF and OsCIPK15DF), the following primer pairs were used for PCR amplification: the forward primers OsCIPK14DF-FW1, 5'-GGATCCATGGAGAGTAGGAGGAAGATT-3' and OsCIPK15DF-FW1, 5'-GGATCCATGGAGAGTAGGAGGAAGATT-3' (the BamHI site is underlined) in each primer; OsCIPK14DF-FW2, 5'-ATGTTGAGTTGAATGGAGAAATT-3'; OsCIPK15DF-FW2, 5'-ATGTTGAGTTGAATGGAGAAATT-3'; OsCIPK14DF-RV1, 5'-TAACTTGTGTTTACAATCTGCTGATCCGCTCT-3'; OsCIPK15DF-RV1, 5'-TAACTTGTGTTTACAATCTGCTGATCCGCTCT-3'; OsCIPK14DF-RV2, 5'-TCTAGACTATTTTATCTCCTGATCGCC-3'; OsCIPK15DF-RV2, 5'-TCTAGACTATTTTATCTCCTGATCGCC-3'. The deletion mutants OsCIPK14DF and OsCIPK15DF were confirmed by sequencing.

The coding regions of OsCIPK14 and OsCIPK15 were amplified by PCR with primers harboring restriction sites and cloned in frame into the pGK7K vector (Clontech). The following PCR primers were used: OsCIPK14FW3, 5'-GCTACGTATGAGGATGAGGAGGAAGATT-3' (the BamHI site is underlined); OsCIPK14RV3, 5'-CTGCAATTTTATCCTGATGAGGAGGAAGATT-3' (the PstI site is underlined); OsCIPK14FW5, 5'-GCTACGTATGAGGATGAGGAGGAAGATT-3' (the BamHI site is underlined); and OsCIPK15FW5, 5'-CTGCAATTTTATCCTGATGAGGAGGAAGATT-3' (the BamHI site is underlined). The OsCIPK14FW5 and OsCIPK15FW5 constructs were used as PCR templates to make pGBKT7-OsCIPK14DF and pGBKT7-OsCIPK15DF constructs, respectively. The coding region of OsCBL2, OsCBL3, OsCBL4, OsCBL5, OsCBL6, and OsCBL9 was amplified by PCR with primers harboring restriction sites and cloned in frame into the pGK7K vector.
Capillary was set at 3.0 kV, and the source temperature was 400°C. Other parameters were optimized using spectrometer software (Applied Biosystems Instruments). The diterpenoid phytoalexin levels were determined with combinations of mass-to-charge ratio (m/z) 315/271 (precursor/product ions) for momilactone A, m/z 331/269 for momilactone B, m/z 317/299 for phytoalexanones A, D, and E, and m/z 319/301 for phytoalexane C in the multiple reaction monitoring mode.

Expression and Purification of OsCIPK and OsCBL Proteins

The plasmids pDEST14-OsCIPK14, -OsCIPK15, -OsCIPK14DF, and -OsCIPK15DF and pDEST15-OsCBL4 and -OsCBL9 were transformed separately into E. coli BL21-AI (Invitrogen). Fresh cultures of E. coli carrying the foreign genes were cultured at 37°C until the A600 of the culture medium reached 0.5. To induce GST fusion protein expression, Ara was added to a final concentration of 0.2%. After induction of the cultures at 20°C, the cells were harvested by centrifugation, resuspended in cold phosphate-buffered saline (PBS) buffer, and lysed by the freeze-thaw method in accordance with the supplier’s instructions. The cell lysate was centrifuged at 10,000g for 10 min at 4°C, and the supernatant was applied onto a glutathione-Sepharose 4B column (GE Healthcare Bio-Sciences). After washing the column with PBS buffer, GST fusion proteins were eluted with 5 mM glutathione in PBS buffer and used for both the kinase activity and Ca2+ mobility shift assays.

Protein Kinase Assay

For autophosphorylation activity and cofactor dependence of OsCIPK14/15, aliquots of GST-OsCIPK14/15 fusion proteins (100 ng) were incubated in kinase assay buffer (10 mM Tris-HCl, pH 8.0, 1 mM diithiothreitol [DTT], and either 5 mM MgCl2, MnCl2, CaCl2, or EDTA) for 10 min at 37°C. For autophosphorylation activity of OsCIPK14/15 in the presence of OsCBLs, aliquots of GST-OsCIPK14/15 or GST-OsCIPK14DF/15DF fusion proteins in the presence or absence of GST-OsCBL4 and GST-OsCBL9 fusion proteins were incubated in the kinase assay buffer (100 mM of [γ-32P]ATP, 20 mM Tris-HCl, pH 8.0, 1 mM DTT, and 5 mM MnCl2, with or without 1 mM CaCl2) for 10 min at 37°C. The reaction was stopped by the addition of 4× SDS sample buffer, boiled immediately for 5 min, and analyzed by 7.5% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue and dried, and their signals were obtained quantitatively using Typhoon 9210 with ImageQuant software and normalized by the intensity of Coomassie Brilliant Blue staining with the corresponding protein.

Yeast Two-Hybrid Analyses

A Matchmaker Gal4-based Two-Hybrid System 3 was used as described by the manufacturer (Clontech). Yeast strain AH109 was cotransformed with each pBD-OsCIPK (OsCIPK14, OsCIPK15, OsCIPK14DF, and OsCIPK15DF) and with each pAD-OsCBL (OsCBL4, OsCBL5, OsCBL6, OsCBL16, and OsCBL9) by the lithium acetate method. Cotransformants were first screened on synthetic medium lacking Leu and Trp (SD/-Leu/-Trp). The yeast cells were then streaked onto the medium lacking Leu, Trp, and His (SD/-Leu/-Trp/-His) containing X-gal (40 μg/mL).

For quantitative assays, yeast strain Y187 was cotransformed with each pBD-OsCIPK (OsCIPK14, OsCIPK15, OsCIPK14DF, and OsCIPK15DF) and with each pAD-OsCBL (OsCBL4, OsCBL5, OsCBL6, OsCBL16, and OsCBL9) by the lithium acetate method. Assays of β-galactosidase activity were performed in triplicate using chlorophenol red (picric-acetyl)-phenyl-β-D-galactoside as the substrate in accordance with the manufacturer’s protocol (Clontech). β-Galactosidase activity was calculated using the following equation: units of β-galactosidase activity = 1,000 × A600/(T × V × A000), where V is the volume of the culture (mL), T is the reaction time (min), and A600 is the optical density of the yeast cells at 600 nm.

Transgenic Rice Cells

For generating the RNA-silencing-triggered inverted repeat constructs, the region (340 bp of the 3′ untranslated region of OsCIPK14) was amplified using RNAiFW, 5′-CCACCAATGTTGTAAGGAGAATGCAAC-3′ and RNAiRV, 5′-CTCCAGATCAGAACATGATGCTC-3′ as the specific primers, subcloned into the pENTR/D-TOPO cloning vector, and then cloned into the Ti-based vector pGreen II.

RNA Isolation, RT-PCR, and Northern-Blot Analyses

Total RNA was isolated from rice cells using Trizol reagent in accordance with the manufacturer’s protocol and quantified using a spectrophotometer. First-strand cDNA was synthesized from 3 μg of total RNA with an oligo(dT) primer and reverse transcriptase. PCR amplification was performed with an initial denaturation step at 94°C for 3 min followed by the indicated number of cycles of incubation at 94°C for 30 s, 55°C for 90 s, and 72°C for 1 min using specific primers (Supplemental Table S1). Unigain was used as a quantitative control. Aliquots of individual PCR products were resolved by agarose gel electrophoresis and visualized using ethidium bromide staining by exposure to UV light. Because both OsCIPK14 and OsCIPK15 have no introns, we confirmed that the PCR products from RT-PCR were derived from cDNA and not from genomic DNA by performing control experiments comparing samples with or without reverse transcriptase treatment. We only detected PCR products for OsCIPK14 and OsCIPK15 in the presence of reverse transcriptase (Supplemental Fig. S4), indicating that the PCR products were derived from cDNA.

For Northern-blots analyses, 20 μg of total RNA was resolved by electrophoresis on a 2% agarose gel containing 5.5% formaldehyde and transferred to a nylon membrane filter (Hybond-N+; GE Healthcare Bio-Sciences). Phoresis on a 1% agarose gel containing 5.5% formaldehyde and transferred to a nylon membrane filter (Hybond-N+; GE Healthcare Bio-Sciences). Proteins were then stained with the corresponding protein.

Real-Time RT-PCR Quantification

First-strand cDNA was synthesized from 3 μg of total RNA with an oligo(dT) primer and reverse transcriptase. Real-time PCR was performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems) with SYBR Green real-time PCR Master Mix (Toyobo) and the gene-specific primers (Supplemental Table S1). Relative mRNA abundances were calculated using the standard curve method and normalized to the corresponding OsActin1 gene levels. Standard samples of known template amounts were used to quantify the PCR products.

Phytoalexin Measurements

The amounts of induced diterpenoid phytoalexins (momilactones and phytoalexanones) in the culture medium were analyzed by HPLC. An Agilent 1100 separation module (Agilent Technologies) equipped with a Pegasus C18 column (150 mm × 2.1 mm in diameter; Senshu Scientific) was used for the HPLC analysis. For diterpenoid phytoalexins, elution was conducted in 70% aqueous acetonitrile containing 0.1% acetic acid at a flow rate of 0.02 mL min⁻¹. The respective authentic samples of diterpenoid phytoalexins were dissolved in 79% aqueous ethanol containing 7% aqueous acetonitrile and 0.01% acetic acid, which was used as a standard solution. For the selection of diagnostic precursor-to-product ion transitions, the standard solutions were directly infused at a flow rate of 5 mL min⁻¹ into a quadrupole tandem mass spectrometer (API-3000; Applied Biosystems Instruments) fitted with an electrospray ion source. All diterpenoid phytoalexins were analyzed in positive-ion mode. Nitrogen was used as the collision gas. The electrospray
DNAi vector pANDA (Miki and Shimamoto, 2004) using the LR clonase reaction. The construct was introduced into rice calli by means of Agrobacterium tumefaciens-mediated transformation in accordance with the method of Tanaka et al. (2001). Transformed calli were screened by hygromycin selection (50 μg mL⁻¹), followed by regeneration of transgenic plants. Transgenic cell lines derived from T2 plants were used for various analyses.

To overexpress OsCIPK15, OsCIPK15Δ5, and GUS cDNAs, the sequences were cloned into the Ti-based vector pZIPH-lac (Fuse et al., 2001) downstream of the maize (Zea mays) ubiquitin promoter, and Agrobacterium-mediated transformation of rice calli was performed. Transformed calli were screened by hygromycin selection (50 μg mL⁻¹), followed by regeneration of transgenic plants.

Cell Death Assay with Evans Blue
An aliquot of the cell suspension (50 mg fresh weight in 0.5 mL) was incubated with 0.05% Evans blue (Sigma) for 10 min and washed to remove unabsorbed dye. The selective staining of dead cells with Evans blue depends on extrusion of the dye from living cells via an intact plasma membrane (Turner and Novacky, 1974). Dye that had been absorbed by dead cells was extracted with 50% methanol plus 1% SDS for 1 h at 60°C and quantified by A₅₉₀.

MTT Reductase Assay
An aliquot of the suspension (50 mg fresh weight in 0.5 mL) was incubated with 0.2 mL of 12 mM MTT solution (Dojindo) for 0.5 h at 28°C. The supernatant was removed by centrifugation, and 1 mL of acidic isopropanol (40 mM HCl) was added to the stained cells, followed by incubation at 60°C for 15 min to elute the dye from the cells. A₅₉₀ was determined to measure the reductase activity.

Statistical Analysis
Statistical significance was determined using an unpaired Student's t test with P < 0.05 required for significance.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AB264036 and AB264037.

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure S1. Comparative expression analysis of several OsCIPKs in response to MAMPs.

Supplemental Figure S2. Alignment of the predicted amino acid sequences of OsCIPK14, OsCIPK15, and SOS2.

Supplemental Figure S3. Expression of OsCIPK14/15 genes in rice.

Supplemental Figure S4. Confirmation of PCR products of OsCIPK14 and OsCIPK15 with or without reverse transcriptase treatment.

Supplemental Figure S5. Induction of OsCBLA genes in response to MAMPs.

Supplemental Figure S6. Schematic diagrams of OsCIPK14 and OsCIPK15 mutants (OsCIPK14/15Δ5).

Supplemental Figure S7. Effects of the expression levels of OsCIPK14/15 on disease resistance against rice blast fungus.

Supplemental Table S1. Sequences of RT-PCR primers used in this experiment.

Supplemental Materials and Methods S1. Quantification of Magnaporthe grisea growth in rice leaves.

ACKNOWLEDGMENTS
We thank Mr. Kazuki Iwabata and Dr. Hidetaka Kaya for helpful technical suggestions and discussion, Mr. Yobei Iwasaki for technical assistance, Drs. Daisuke Miki and Ko Shimamoto for the RNAi plasmid (pANDA vector), Dr. Naoto Shibuya for the gift of N-acetylcysteineheptasone, and Drs. Morifumi Hasegawa and Osamu Kodama for the gift of momilactones.

Received December 9, 2009; accepted March 29, 2010; published March 31, 2010.

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