Regulation of a Proteinaceous Elicitor-induced Ca\(^{2+}\) Influx and Production of Phytoalexins by a Putative Voltage-gated Cation Channel, OsTPC1, in Cultured Rice Cells*\(^S\)\(^S\)

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**Background:** Molecular mechanisms for elicitor-induced changes in cytosolic Ca\(^{2+}\) concentration and its molecular link with regulation of phytoalexin biosynthesis in plant immunity remain mostly unknown.

**Results:** TvX-induced Ca\(^{2+}\) influx and the phytoalexin accumulations were suppressed in Ostpc1 knock-out cells.

**Conclusion:** OsTPC1 plays a role in TvX-induced Ca\(^{2+}\) influx consequently required for the regulation of phytoalexin biosynthesis.

**Significance:** Voltage-dependent plasma membrane Ca\(^{2+}\)-permeable channel activity of the plant TPC1 was shown for the first time.

Pathogen/microbe- or plant-derived signaling molecules (PAMPs/MAMPs/DAMPs) or elicitors induce increases in the cytosolic concentration of free Ca\(^{2+}\) followed by a series of defense responses including biosynthesis of antimicrobial secondary metabolites called phytoalexins; however, the molecular links and regulatory mechanisms of the phytoalexin biosynthesis remains largely unknown. A putative voltage-gated cation channel, OsTPC1 has been shown to play a critical role in hypersensitive cell death induced by a fungal xylanase protein (TvX) in suspension-cultured rice cells. Here we show that TvX induced a prolonged increase in cytosolic Ca\(^{2+}\), mainly due to a Ca\(^{2+}\) influx through the plasma membrane. Membrane fractionation by two-phase partitioning and immunoblot analyses revealed that OsTPC1 is localized predominantly at the plasma membrane. In retrotransposon-insertional Ostpc1 knock-out cell lines harboring a Ca\(^{2+}\)-sensitive photoproteor, aquorin, TvX-induced Ca\(^{2+}\) elevation was significantly impaired, which was restored by expression of OsTPC1. TvX-induced production of major diterpenoid phytoalexins and the expression of a series of diterpene cyclase genes involved in phytoalexin biosynthesis were also impaired in the Ostpc1 cells. Whole cell patch clamp analyses of OsTPC1 heterologously expressed in HEK293T cells showed its voltage-dependent Ca\(^{2+}\)-permeability. These results suggest that OsTPC1 plays a crucial role in TvX-induced Ca\(^{2+}\) influx as a plasma membrane Ca\(^{2+}\)-permeable channel consequently required for the regulation of phytoalexin biosynthesis in cultured rice cells.

Calcium ions are firmly established as a ubiquitous second messenger in plants. Upon recognition of pathogen/microbe- or plant-derived signaling molecules (pathogen/microbe/damage-associated molecular patterns; PAMPs/MAMPs/DAMPs) or elicitors, plant cells induce changes in the cytosolic free calcium concentration ([Ca\(^{2+}\)]\(_{cyt}\)). The change in [Ca\(^{2+}\)]\(_{cyt}\) is critical for activating a variety of defense responses, including production of reactive oxygen species (ROS), activation of mitogen-activated protein kinases (MAPK), and expression of pathogenesis-related genes, often followed by programmed cell death known as a hypersensitive response (HR) (1–6).

Plant defense reactions against pathogen infection include synthesis and accumulation of low-molecular antimicrobial

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3. The abbreviations used are: PAMP/MAMP/DAMP, pathogen/microbe/damage-associated molecular patterns; BAPTA, 1,2-bis (2-aminophenoxy) ethane-N,N,N’,N’-tetraacetic acid; bZIP, basic leucine zipper; CBL, calcineurin B-like protein; [Ca\(^{2+}\)]\(_{cyt}\), cytosolic free Ca\(^{2+}\) concentration; CNGC, cyclic nucleotide-gated Ca\(^{2+}\)-permeable channel; GUS, ß-glucuronidase; HR, hypersensitive response; TGA, TGACG-sequence-specific-binding protein; TPC, two-pore channel; TvX, xylanase from Trichoderma viride; PM, plasma membrane; VM, vacuolar membrane.
Roles of Rice TPC1 in Phytoalexin Biosynthesis

substances, known as phytoalexins. In rice, fourteen diterpenoid phytoalexins have been identified and can be classified into four groups, based on the structure of their hydrocarbon precursors: phytocassanes A–E, oryzalexins A–F, momilactones A and B, and oryzalexin S. Biosynthesis of these phytoalexins is induced by various elicitors including chitin fragments, cerebroside and xylanase protein from Trichoderma viride (TvX)/ethylene-inducing xylanase (EIX) in rice-cultured cells, along with a variety of defense responses (7–9). Ca\(^{2+}\) channel blockers inhibit cerebroside-induced phytoalexin production (8), suggesting possible involvement of Ca\(^{2+}\)-permeable channels in the regulation of elicitor-induced phytoalexin biosynthesis. However, the molecular identity of the Ca\(^{2+}\) channel(s) involved remains unknown.

Electrophysiological studies have characterized the activity of Ca\(^{2+}\) channels localized at the plasma membrane (PM) and vacuolar membrane (VM) in many plant species (10–11). The two-pore channel (TPC) family, originally isolated from rat, is homologous to the α1 subunit of vertebrate voltage-dependent Ca\(^{2+}\) channels (12). Human TPCs mediate nicotinic acid adenine dinucleotide phosphate-induced Ca\(^{2+}\) release from acidic organelles in HEK293 cells (13). Plant TPC family members have been characterized in several plant species. Arabidopsis AtTPC1 has been reported to show a slow-activating vacuolar cation channel activity (14), and the stomatal response to extracellular Ca\(^{2+}\) changes (14, 17). NtTPC1s have roles in increasing Ca\(^{2+}\) concentrations, defense-related gene expression, and regulation of programmed cell death triggered by cryptogein, an elicitor from an oomycete, in tobacco BY-2 cells (18). Characterization of the retrotransposon-insertional knock-out mutant of rice OsTPC1 revealed that OsTPC1 affects the sensitivity to TvX and plays a role in the regulation of TvX-induced activation of a MAP kinase and hypersensitive cell death in cultured rice cells (4). TPC1 has been suggested to amplify the elicitor-induced [Ca\(^{2+}\)]\(_{cyt}\) increase (19). In contrast, increase in Ca\(^{2+}\) concentrations, ROS generation, and gene expression induced by two MAMPs, elf18 and flg22, in the T-DNA insertional mutant of AtTPC1, attpc1–2, were comparable to the wild-type (15). A physiological role of the TPC family in plant innate immunity remains undefined.

In the present study, we characterized OsTPC1 and its knock-out mutant in cultured rice cells. Evidence presented here suggests that OsTPC1 is predominantly localized at the plasma membrane and has a role in the regulation of TvX-induced increases in [Ca\(^{2+}\)]\(_{cyt}\) as well as phytoalexin biosynthesis.

**EXPERIMENTAL PROCEDURES**

Plant Materials and Growth Conditions—Surface-sterilized seeds of rice, Oryza sativa L. cv. Nipponbare, were germinated on Murashige and Skoog medium (20) containing 0.8% agar and grown for 10 days in a growth chamber under long day conditions (16 h light/8 h darkness, 28 °C). Seedlings were transplanted into soil and grown in a greenhouse (16 h light/8 h darkness, 28 °C and 60% humidity). To generate cultured cells, seeds were placed onto callus-inducing medium. Rice cells expressing apop Quantum—Measurement of changes in cytosolic Ca\(^{2+}\) concentration was performed essentially as described by Kurisu et al. (21). Briefly, apop Quantum—expressing rice cells (7 days after subculture) were incubated with 1 μM coelenterazine for at least 12 h at 25 °C. The cell suspension was transferred to a culture tube, set in a luminometer (Lumicounter 2500, Microtech Nition) and atherated by rotation (18). Ca\(^{2+}\)-dependent aequorin chemiluminescence was measured after incubation for 15 min to stabilize the cells. To estimate Ca\(^{2+}\)\(_{cyt}\) changes in the cells, all remaining aequorin was discharged with 2 mM CaCl\(_2\) and 20% ethanol after each experiment, and chemiluminescence data transformed into [Ca\(^{2+}\)]\(_{cyt}\) using the equation established by Mithofer et al. (25). To quantify the effects of inhibitors on [Ca\(^{2+}\)]\(_{cyt}\) changes, total [Ca\(^{2+}\)]\(_{cyt}\) was calculated by subtracting the mean of basal [Ca\(^{2+}\)]\(_{cyt}\) before elicitor application (−10 to 0 s) from the sum of [Ca\(^{2+}\)]\(_{cyt}\) between 0 and 10 min. The total [Ca\(^{2+}\)]\(_{cyt}\) in the control was standardized as 100%. To express aequorin in the cytosol of Ostpc1 cells, apop Quantum cDNA (26) were cloned into a Ti-based vector pLG121-Hm, and Agrobacterium-mediated transformation of Ostpc1 rice calli was performed. Transformed calli were screened and transgenic plants regenerated. Transgenic cell lines derived from T1 plants were used for various analyses.

Complementation Analysis—We transformed transgenic Ostpc1 cell lines expressing wild-type Ostpc1 and Glus (control) cDNA (4). The apop Quantum cDNA was cloned into the Ti-based vector pSMAB704 (27) and Agrobacterium-mediated transformation of the transgenic rice calli was performed. Transformed calli were screened and used for the complementation analysis.

Subcellular Membrane Fractionation and Immunoblot Analyses—PM and VM were isolated from cultured rice cells using an aequorin two-phase partition method comprising PEG/dextran (28–29) and the sucrose/sorbitol method (30–31), respectively. Rabbit polyclonal anti-OsTPC1 antibody was generated as described previously (32). The coding region of the linker domain of OsTPC1 (I359–S403) was amplified using sequence specific primers: I359–S403F, 5′-CACCATTTGA-TGCTACTGGTCAGGTTATCT-3′ and I359–S403R, 5′-TCAACTCTGATCAAGCTCGGCAAAAATTAA-3′. A fusion protein consisting of the domain fused to a histidine-tag in the pDEST17 vector (Invitrogen) and transformed into Escherichia coli BL21-AI (Invitrogen). Inclusion bodies with the recombinant protein were obtained after induction at 37 °C for 6 h and resolved using a preparative 15% SDS-PAGE gel. A ground polyacrylamide gel slice of the fusion protein was checked using MS/MS analysis and used to immunize rabbits by intradermal injections.
Roles of Rice TPC1 in Phytoalexin Biosynthesis

RESULTS

TvX Triggered a Prolonged Increase in Cytosolic Ca2+ in Suspension-cultured Rice Cells—TvX triggers a variety of defense responses in plants (4, 36–38). In rice cell culture, external Ca2+ was required for TvX-induced hypersensitive cell death, suggesting that Ca2+ influx through the PM is indispensable for TvX-induced defense responses (4). However, the effect of TvX on changes in [Ca2+]cyt has never been characterized. To analyze the involvement of Ca2+ flux in TvX-induced defense responses, we measured TvX-induced changes in [Ca2+]cyt using transgenic rice cell lines expressing apoaequorin, a Ca2+-sensitive photoprotein (21) (supplemental Fig. S1). As shown in Fig. 1A, TvX triggered a prolonged increase in [Ca2+]cyt in a dose-dependent manner.

To elucidate the origin of Ca2+ flux, we first performed pharmacological analyses. The addition of a Ca2+ chelator, BAPTA, into the extracellular medium or the substitution of Ca2+ free medium inhibited the TvX-induced increase in [Ca2+]cyt (Fig. 1B and supplemental Fig. S2). Similarly, a Ca2+ channel blocker, La3+ or Gd3+, and a voltage-dependent Ca2+ channel inhibitor, nifedipine (11), suppressed the TvX-induced increase in [Ca2+]cyt (Fig. 1B and supplemental Fig. S2). A phospholipase C inhibitor, neomycin, and a potential endomembrane Ca2+-permeable channel inhibitor, ruthenium red, has been reported to suppress Ca2+ release from intracellular Ca2+ stores (39–40). In contrast to BAPTA and La3+, neomycin, and ruthenium red scarcely inhibited the TvX-induced increase in [Ca2+]cyt (Fig. 1B), suggesting that TvX-induced increase in [Ca2+]cyt is predominantly due to the influx of extracellular Ca2+ through voltage-dependent Ca2+-permeable channels.

Involvement of OsTPC1 in the TvX-induced Changes in Cytosolic Ca2+ Concentration—A putative voltage-dependent cation channel, OsTPC1 has been suggested to be involved in TvX-induced defense responses, including activation of a MAPK and hypersensitive cell death (4). To test the possible involvement of OsTPC1 in the regulation of TvX-induced Ca2+ rise, we generated OsTPC1 knock-out cell lines harboring apoaequorin. We confirmed the expression of apoaequorin mRNA in transgenic rice cells by RT-PCR. Several lines expressing similar levels of apoaequorin mRNA were selected for further experiments (supplemental Fig. S1).

As shown in Fig. 1C, the TvX-induced increases in [Ca2+]cyt were partially but significantly suppressed in Ostpc1 knock-out cell lines in comparison with that of the wild-type. To confirm that this phenotype was due to the functional knock-out of OsTPC1, we performed a complementation analysis using Ostpc1 cells expressing both wild-type OsTPC1 and apoaequorin cDNA (supplemental Fig. S1). Transformation of the mutant with a control vector that carried GUS had no effect on the TvX-induced Ca2+ increase (Fig. 1D). In contrast, expression of OsTPC1 recovered the TvX-induced Ca2+ increase (Fig. 1D), indicating that the observed mutant phenotype was attributable to OsTPC1. These results suggest that OsTPC1 participates in TvX-induced Ca2+ rise in cultured rice cells.

Intracellular Localization of OsTPC1—Molecular and electrophysiological studies have shown that Arabidopsis TPC1 was mainly localized in the VM (14, 41). In contrast, TPCs in
monocots including OsTPC1 have been suggested to be localized in the PM (4, 32, 42). To confirm the intracellular localization of OsTPC1, we prepared an affinity-purified specific rabbit anti-OsTPC1 antibody, performed membrane fractionation using an aqueous two-phase partitioning method, and analyzed the intracellular localization of OsTPC1. Immunoblot analyses of crude extracts from suspension-cultured rice cells using the anti-OsTPC1 antibody detected a protein migrating with an apparent molecular mass of 87 kDa (Fig. 2A). This band was absent when the antibodies were incubated with the recombinant antigen as a competitor, but not the recombinant GUS fused histidine tag (Fig. 2A), indicating that the affinity-purified anti-OsTPC1 could detect OsTPC1 protein, specifically.

We fractionated total protein extracts into PM and VM fractions. Each fraction was obtained with little cross-contamination, as determined by immunoblot analyses using specific marker proteins (Fig. 2B). As shown in Fig. 2B, we detected OsTPC1 predominantly in the PM. This result is consistent with the previous reports and confirm that OsTPC1 is predominantly localized at the PM in cultured rice cells (32).

Involvement of OsTPC1 in the Regulation of Phytoalexin Biosynthesis—Pharmacological evidence suggests that the Ca\(^{2+}\) influx in elicitor-induced phytoalexin biosynthesis is important in rice (8, 43). In rice, ent-copalyl diphosphate synthase 4 (OsCPS4) and ent-kaurene synthase like-4 (OsKSL4); and ent-copalyl diphosphate synthase 2 (OsCPS2) and ent-kaurene synthase-like 7 (OsKSL7) are responsible for the biosynthesis of momilactones and phytocassanes, respectively (44–46). The expression of all these cyclase genes was induced by TvX, which was significantly suppressed by pre-treatment with BAPTA or La\(^{3+}\), suggesting that the influx of extracellular Ca\(^{2+}\) through the Ca\(^{2+}\)-permeable channels is required for the expression of these cyclase genes upon recognition of the elicitor (Fig. 3A and supplemental Fig. S3). Similarly, TvX-induced induction of these genes was suppressed in Ostpc1 knock-out
Roles of Rice TPC1 in Phytoalexin Biosynthesis

Immediately after the recognition of several elicitors, plant cells induce $[\text{Ca}^{2+}]_{\text{cyt}}$ changes, which are important for activating various defense responses including phytoalexin accumulation. A diverse range of PAMPs/MAMPs/DAMPs derived from fungi and bacteria or plants themselves have been reported to induce various spatiotemporal changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ (1, 3, 6, 43). Here, we showed that TvX triggered a prolonged change in $[\text{Ca}^{2+}]_{\text{cyt}}$, mainly due to $\text{Ca}^{2+}$ influx from the extracellular space (Fig. 1, A and B). A sustained increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ is postulated to have a key role in the induction of HR cell death (47–48). $\text{Ca}^{2+}$ influx is required for TvX-induced HR cell death (4). Hence, a prolonged change in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to TvX may be a prerequisite to activate HR cell death in rice cultured cells.

We generated apoaequorin-expressing Osstpc1 cells and found that TvX-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ changes were partially suppressed in Osstpc1 cells (Fig. 1C). We thus postulate that multiple $\text{Ca}^{2+}$-permeable channels are involved in the elicitor-triggered $[\text{Ca}^{2+}]_{\text{cyt}}$ changes. Osstpc1 heterologously expressed in HEK293T cells showed voltage-dependent $\text{Ca}^{2+}$ permeability through the PM (Fig. 6), suggesting that Osstpc1 functions as a $\text{Ca}^{2+}$-permeable channel. The present results suggest that Osstpc1 plays a role as one of the multiple $\text{Ca}^{2+}$-permeable channels activated by TvX and is involved in the elicitor-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ change.

It has been reported that Attpc1 is not involved in $[\text{Ca}^{2+}]_{\text{cyt}}$ changes triggered by two MAMPs, elf18 and flg22 (15). Interestingly, $[\text{Ca}^{2+}]_{\text{cyt}}$ changes induced by chitin fragments (N-acyethylchitooligosaccharides) in Osstpc1 cells was almost comparable to the wild-type cells (supplemental Fig. S6). This apparent discrepancy may be explained by the differences in signaling pathways among elicitors. In fact, the temporal patterns of increased $[\text{Ca}^{2+}]_{\text{cyt}}$ are significantly different between TvX and chitin fragments: $[\text{Ca}^{2+}]_{\text{cyt}}$ increase triggered by chitin fragments is large and transient (supplemental Fig. S6) (21), while that induced by TvX is much smaller but sustained (Fig. 1C). The differences in the temporal pattern of $[\text{Ca}^{2+}]_{\text{cyt}}$ changes correlates with the induction of programmed cell death; almost no cell death is induced by chitin fragments, while TvX triggers programmed cell death, which is also affected by Osstpc1 (4). These results suggest that Osstpc1 may be one of the multiple $\text{Ca}^{2+}$-permeable channels activated by some specific elicitors but not all to trigger sustained increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ and HR cell death.

The primary structure of Attpc1 and Osstpc1 is similar. According to Wolf PSORT, a protein subcellular localization prediction program (49), both proteins are predicted to be localized at the PM. However, Attpc1 and Osstpc1 are predominantly localized at the VM and PM, respectively (4, 14, 32). A

cells and the suppression was restored after complementation of Ostpc1 (Fig. 3B and supplemental Fig. S4). Quantitative HPLC-tandem mass spectrometry analyses revealed that TvX induces the accumulation of monomiacetates and phytocassanes, which are major phytoalexins in rice (9). The level of monomiacetates continued to increase from the first measurement at 24 h, through to the final measurement at 120 h, and that of phytocassanes reached a maximum at 72 h and gradually decreased thereafter in response to TvX (Fig. 4, A and B).

In Ostpc1 knock-out cells, the accumulation of both monomiacetates and phytocassanes was significantly suppressed (Fig. 5A). The suppression in Ostpc1 knock-out cells was restored by introducing the Osstpc1 gene (Fig. 5B), suggesting that Osstpc1 plays a role in regulation of TvX-induced phytoalexin biosynthesis. The close correlation between TvX-induced changes in cytosolic $\text{Ca}^{2+}$ concentration and phytoalexin biosynthesis in Ostpc1 knock-out cells suggest the significance of the signaling role of $\text{Ca}^{2+}$ regulated by Osstpc1 at least in part in TvX-induced phytoalexin biosynthesis.

$\text{Ca}^{2+}$-permeability of Osstpc1—Possible involvement of Osstpc1 in the TvX-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increase (Fig. 1C) and its localization at the PM (Fig. 2B) led us to hypothesize that Osstpc1 may function as a $\text{Ca}^{2+}$-permeable channel at the PM in rice cells. However, no electrophysiological characterization has so far been reported for Osstpc1. We thus recorded the whole-cell current in HEK293T cells expressing GFP or GFP-Osstpc1 cDNA. Membrane fractionation analysis revealed that heterologously expressed Osstpc1 was localized to the PM at least partially in HEK293T cells (supplemental Fig. S5A). Voltage-dependent currents carried by $\text{Ba}^{2+}$ instead of $\text{Ca}^{2+}$ ($-230 \pm 57$ pA at $-130$ mV, $n = 5$) was observed in GFP-Osstpc1-expressing cells (Fig. 6, A and B and supplemental Fig. S5B). On the other hand, we detected no such currents in GFP-expressing cells ($-59 \pm 7$ pA at $-130$ mV, $n = 5$). Treatment of La$^{3+}$ significantly suppressed the voltage-activated $\text{Ba}^{2+}$ currents in GFP-Osstpc1-expressing cells, but not neomycin (supplemental Fig. S5C), indicating that Osstpc1 functions as a voltage-activated $\text{Ca}^{2+}$-permeable channel.

DISCUSSION

Immediately after the recognition of several elicitors, plant cells induce $[\text{Ca}^{2+}]_{\text{cyt}}$ changes, which are important for activating various defense responses including phytoalexin accumulation. A diverse range of PAMPs/MAMPs/DAMPs derived from fungi and bacteria or plants themselves have been reported to induce various spatiotemporal changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ (1, 3, 6, 43).

Here, we showed that TvX triggered a prolonged change in $[\text{Ca}^{2+}]_{\text{cyt}}$, mainly due to $\text{Ca}^{2+}$ influx from the extracellular space (Fig. 1, A and B). A sustained increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ is postulated to have a key role in the induction of HR cell death (47–48). $\text{Ca}^{2+}$ influx is required for TvX-induced HR cell death (4). Hence, a prolonged change in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to TvX may be a prerequisite to activate HR cell death in rice cultured cells.

We generated apoaequorin-expressing Ostpc1 cells and found that TvX-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ changes were partially suppressed in Ostpc1 cells (Fig. 1C). We thus postulate that multiple $\text{Ca}^{2+}$-permeable channels are involved in the elicitor-triggered $[\text{Ca}^{2+}]_{\text{cyt}}$ changes. Osstpc1 heterologously expressed in HEK293T cells showed voltage-dependent $\text{Ca}^{2+}$ permeability through the PM (Fig. 6), suggesting that Osstpc1 functions as a $\text{Ca}^{2+}$-permeable channel. The present results suggest that Osstpc1 plays a role as one of the multiple $\text{Ca}^{2+}$-permeable channels activated by TvX and is involved in the elicitor-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ change.

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human TPC2 mutant lacking a di-leucine motif in its N-terminal has recently been shown to be localized to the PM instead of the lysosome, suggesting that this motif is required for its localization to intracellular acidic organelles (50). Plant TPC family members have a similar motif in their N-terminal tail, suggesting that they may be localized to the VM. However, previous studies (4, 32), as well as data presented here, show that OsTPC1 is mainly localized at the PM in cultured rice cells as well as in HEK293T cells (Fig. 2B and supplemental Fig. S5A). TaTPC1 from wheat has also been reported to be localized at the PM (42, 51). Other unknown components that interact with OsTPC1 may regulate the intracellular localization of OsTPC1. Intracellular localization of the plant TPC family is an emerging subject that warrants further analysis.

The production of major diterpenoid phytoalexins, momilactones and phytocassanes, is triggered in rice upon recognition of pathogens. TcX-induced expression of diterpene cyclase genes. Diterpenoid phytoalexins (momilactone A, B, and phytocassane A–E) were extracted from culture medium collected at the indicated time points after the addition of TcX. The total amount of momilactones A and B (A) and phytocassanes A–E (B) were quantified by HPLC-ESI-MS/MS as described under “Experimental Procedures.” Circle: 60 μg ml⁻¹, triangle: 30 μg ml⁻¹, square: 15 μg ml⁻¹, diamond: culture medium treatment. Average values and standard errors for three independent samples are shown.

FIGURE 3. TcX-induced expression of diterpene cyclase genes. A, effect of BAPTA on the expression of the diterpene cyclase genes, OsCPS2, OsCPS4, Osksl4, and Osksl7 at 0 h (white bar) and 6 h (gray bar) after TcX treatment. BAPTA (1 mM) was added to the rice cells 30 min prior to TcX treatment (30 μg ml⁻¹). Average values and standard errors of three independent experiments are shown. B, relative mRNA levels of the diterpenoid cyclase genes in wild-type, Ostpc1 and complementation lines at 0 h (white bar) and 6 h (gray bar) after TcX treatment (30 μg ml⁻¹). mRNA levels were determined using real-time quantitative PCR. Average values and standard errors of three or four independent experiments for each line are shown.

FIGURE 4. TcX-induced accumulation of diterpenoid phytoalexins. Diterpenoid phytoalexins (momilactone A, B, and phytocassane A–E) were extracted from culture medium collected at the indicated time points after the addition of TcX. The total amount of momilactones A and B (A) and phytocassanes A–E (B) were quantified by HPLC-ESI-MS/MS as described under “Experimental Procedures.” Circle: 60 μg ml⁻¹, triangle: 30 μg ml⁻¹, square: 15 μg ml⁻¹, diamond: culture medium treatment. Average values and standard errors for three independent samples are shown.
tion of various elicitors (7–8, 33). OsTGAP1, a basic leucine zipper (bZIP) transcription factor induced by chitin fragments, has recently been shown to be involved in the expression of biosynthetic genes of the diterpenoid phytoalexins including the upstream MEP pathway genes, and that overexpression of OsTGAP1 exhibited enhanced expression of those phytoalexin biosynthetic genes leading to hyperaccumulation of the diterpenoid phytoalexins (7). TvX also induced the accumulation of momilactones and phytocassanes in rice cells (Fig. 4) (9). However, the time course of this accumulation appeared to be more prolonged in comparison with chitin fragments (33), and expression of OsTGAP1 was not induced by TvX treatment (data not shown). These results suggest that phytoalexin biosynthesis is regulated by multiple pathways, and the time course, as well as the regulatory pathways, are different, at least in part, between chitin fragments and TvX.

Pharmacological analyses using Ca\(^{2+}\)/H\(_{11001}\) channel blockers, LaCl\(_3\) and GdCl\(_3\), indicate that Ca\(^{2+}\)/H\(_{11001}\) influx via Ca\(^{2+}\)/H\(_{11001}\) channels is associated with production of phytoalexins (8). This is consistent with our findings that a Ca\(^{2+}\) chelator, BAPTA or a Ca\(^{2+}\) channel blocker, La\(^{3+}\), suppressed the TvX-induced expression of diterpene cyclase required for phytoalexin biosynthesis (Fig. 3A and supplemental Fig. S3). Both phytoalexin accumulation and the expression of diterpene cyclase genes were partially suppressed in Ostpc1 cells and complemented by expression of wild-type OsTPC1 (Figs. 3B and 5). These results indicate that OsTPC1 is involved in regulation of TvX-induced sustained increase in [Ca\(^{2+}\)]\(_{cyt}\), and consequently of phytoalexin biosynthesis in rice-cultured cells.

Only the treatment with a Ca\(^{2+}\) ionophore, ionomycin, did not induce the expression of diterpene cyclase genes (supplemental Fig. S7). This is consistent with our previous observation that though ionomycin trigger a rise in [Ca\(^{2+}\)]\(_{cyt}\), it does not necessarily trigger Ca\(^{2+}\)-dependent downstream events such as ROS production in rice cells (21). The regulation of the

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**FIGURE 5. Effects of Ostpc1 disruption and complementation on TvX-induced diterpenoid phytoalexin accumulation.** The total amounts of momilactones and phytocassanes accumulated in the culture medium was quantified. Each cell line was treated with TvX (30 \(\mu\)g ml\(^{-1}\)). A, solid circle: wild-type; open circle: Ostpc1; B, solid square: Ostpc1/OsTPC1, open square: Ostpc1/GUS. Average values and standard errors from four independent experiments are shown.

**FIGURE 6. Voltage-dependent Ca\(^{2+}\) current in HEK cells transiently expressing GFP-OsTPC1.** The expression of OsTPC1 enhanced voltage-dependent currents in HEK293T cells. A, representative currents from CMV:GFP or CMV:GFP-OsTPC1 expressing HEK293T cells. A voltage ramp from 0 to \(-130\) mV was used 16 times for each cell. B, current-voltage relationships of voltage-dependent currents from CMV:GFP or CMV:GFP-OsTPC1 expressing HEK293T cells. Data are means of 5 cells as recorded in A. Error bars show S.E.
Roles of Rice TPC1 in Phytoalexin Biosynthesis

phytoalexin biosynthetic pathway may require not only Ca\(^{2+}\) entry from the extracellular space, but also other signaling events triggered by TvX. These results also reinforce the concept that Ca\(^{2+}\) flux and sustained [Ca\(^{2+}\)]\(_{cyt}\) increase triggered by TvX and mediated by OsTPC1 at least in part may have a specific role in defense signaling.

Little is known of the signaling components connecting PAMPs/MAMPs-induced [Ca\(^{2+}\)]\(_{cyt}\) changes and downstream defense responses. OsTPC1 plays a crucial role in TvX-induced activation of a MAP kinase, OsMPK6 (4). Chitin fragment-induced synthesis of diterpenoid phytoalexins has recently been shown to involve the OsMKK4-OsMPK6 MAP kinase cascade (52). OsMPK6 whose activation is regulated by OsTPC1 may also participate in the induction of phytoalexin biosynthesis induced by TvX. OsCIPK14/15 activated by binding of calcineurin B-like Ca\(^{2+}\) sensor proteins are involved in various TvX-induced defense responses including the expression of the same diterpene cycloace gene and phytoalexin production (9, 53). The phenotypes of knockdown cell lines of the CiPKs (9) are similar with those of OsTPC1 (Fig. 5), suggesting the Ca\(^{2+}\)-permeable channel OsTPC1 may act upstream of the Ca\(^{2+}\)-regulated protein kinases, OsCIPK14/15.

In summary, the present results indicate that OsTPC1 has a role in the regulation of TvX-induced sustained increase in [Ca\(^{2+}\)]\(_{cyt}\), leading to phytoalexin biosynthesis in rice cultured cells. Considering that TvX-induced increases in [Ca\(^{2+}\)]\(_{cyt}\) and phytoalexin biosynthesis were impaired only partially in Ostpc1 knock-out cells, multiple Ca\(^{2+}\)-permeable channels may act redundantly to bypass OsTPC1 to regulate TvX-induced defense responses. Cyclic nucleotide-gated Ca\(^{2+}\)-permeable channels (CNGCs) may have recently been implicated in a variety of plant immune responses (54–57). CNGCs may therefore be candidates for such Ca\(^{2+}\)-permeable channels.

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REFERENCES

1. Navazio, L., Moscatelli, R., Bellincampi, D., Baldan, B., Meggio, F., Brini, M., Bowler, C., and Mariani, P. (2002) The role of calcium in oligogalacturonide-activated signalling in soybean cells. Plant cell 115, 596–605
2. Lecourieux, D., Ranjeva, R., and Pugin, A. (2006) Calcium in plant defense-signalizing pathways. New Phytol. 171, 249–269
3. Lecourieux, D., Mazars, C., Pauly, N., Ranjeva, R., and Pugin, A. (2002) Analysis and effects of cytosolic free calcium increases in response to elicitors in Nicotiana plumagbagfida cells. Plant Cell 14, 2627–2641
4. Kurusu, T., Kagawa, T., Miyao, A., Hirochika, H., and Kuchitsu, K. (2005) Identification of a putative voltage-gated Ca\(^{2+}\) channel as a key regulator of elicitor-induced hypersensitive cell death and mitogen-activated protein kinase activation in rice. Plant J 42, 798–809
5. Kobayashi, M., Ohara, I., Kawakita, K., Yokota, N., Fujimori, M., Shiomamoto, K., Doke, N., and Yoshioka, H. (2007) Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. Plant Cell 19, 1065–1080
6. Kadota, Y., Goh, T., Tomatsu, H., Tamauchi, R., Higashi, K., Muto, S., and Kuchitsu, K. (2004) Cryptogein-induced initial events in tobacco BY-2 cells: pharmacological characterization of molecular relationship among cytosolic Ca\(^{2+}\) transients, anion efflux, and the production of reactive oxygen species. Plant Cell Physiol. 45, 160–170
7. Okada, A., Okada, K., Miyamoto, K., Koga, J., Shibuya, N., Nojiri, H., and Yamane, H. (2009) OsTPGAP1, a bZIP transcription factor, coordinately regulates the inductive production of diterpenoid phytoalexins in rice. J. Biol. Chem. 284, 26510–26518
8. Umemura, K., Ogawa, N., Koga, J., Iwata, M., and Usami, H. (2002) Elicitor activity of cerebrosides, a sphingolipid elicitor, in cell suspension cultures of rice. Plant Cell Physiol. 43, 778–784
9. Kurusu, T., Hamada, J., Nakajima, H., Kitagawa, Y., Iyokuda, M., Takahashi, A., Hanamata, S., Ohno, R., Hayashi, T., Okada, K., Koga, J., Hirochika, H., Yamane, H., and Kuchitsu, K. (2010) Regulation of microbe-associated molecular pattern-induced hypersensitive cell death, phytoalexin production, and defense gene expression by calcineurin B-like protein-interacting protein kinases, OsCIPK14/15, in rice-cultured cells. Plant Physiol. 153, 678–692
10. Sanders, D., Pelloux, J., Brownlee, C., and Harper, J. F. (2002) Calcium at the crossroads of signaling. Plant Cell 14, Suppl. 5401–5417
11. Gelli, A., Higgins, V. J., and Blumwald, E. (1997) Activation of plant plasma membrane Ca\(^{2+}\)-permeable channels by race-specific fungal elicitors. Plant Physiol. 113, 269–279
12. Ishibashi, K., Suzuki, M., and Imai, M. (2000) Molecular cloning of a novel form (two-repeat) protein related to voltage-gated sodium and calcium channels. Biochem. Biophys. Res. Commun. 270, 370–376
13. Calcraft, P. J., Ruas, M., Pan, Z., Cheng, X., Arredouani, A., Hax, O., Tang, J., Riedt, K., Tekoul, L., Chuang, K. T., Lin, P., Xiao, R., Wang, C., Zhu, Y., Lin, Y., Wyatt, C. N., Parrington, J., Ma, J., Evans, A. M., Galione, A., and Zhu, M. (2009) NADP mobilizes calcium from acidic organelles through two-pore channels. Nature 459, 596–600
14. Peiter, E., Maathuis, F. J., Mills, L. N., Knight, H., Pelloux, J., Hetherington, A. M., and Sanders, D. (2005) The vacuolar Ca\(^{2+}\)-activated channel TPC1 regulates germination and stomatal movement. Nature 434, 404–408
15. Ranf, S., Wünnenberg, P., Lee, J., Becker, D., Dunkel, M., Hedrich, R., Scheel, D., and Dietrich, P. (2008) Loss of the vacuolar cation channel, AtTPC1, does not impair Ca\(^{2+}\) signals induced by abiotic and biotic stresses. Plant J 53, 287–299
16. Fukui, T., Cunningham, K. W., and Muto, S. (2001) A putative two pore channel AtTPC1 mediates Ca\(^{2+}\) influx in Arabidopsis leaf cells. Plant Cell Physiol. 42, 900–905
17. Islam, M. M., Munemasa, S., Hossain, M. A., Nakamura, Y., Mori, I. C., and Murata, Y. (2010) Roles of AtTPC1, vacuolar two pore channel 1, in Arabidopsis stomatal closure. Plant Cell Physiol. 51, 302–311
18. Kadota, Y., Fukui, T., Ogasawara, Y., Goh, T., Higashi, K., Muto, S., and Kuchitsu, K. (2004) Identification of putative voltage-dependent Ca\(^{2+}\)-permeable channels involved in cryptogein-induced Ca\(^{2+}\) transients and defense responses in tobacco BY-2 cells. Biochem. Biophys. Res. Commun. 317, 823–830
19. Ma, W., and Berkowitz, G. A. (2007) The grateful dead: calcium and cell death in plant innate immunity. Cell Microbiol. 9, 2571–2585
20. Murashige, T., and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant 15, 473–497
21. Kurusu, T., Hamada, H., Sugiyama, Y., Yagala, T., Kadota, Y., Fukui, T., Hayashi, T., Umemura, K., Komatsu, S., Miyao, A., Hirochika, H., and Kuchitsu, K. (2011) Negative feedback regulation of microbe-associated molecular pattern-induced cytosolic Ca\(^{2+}\) transients by protein phosphorylation. J. Plant Res. 124, 415–424
22. Kurusu, T., Sakurai, Y., Miyao, A., Hirochika, H., and Kuchitsu, K. (2004) Identification of a putative voltage-gated Ca\(^{2+}\)-permeable channel (OsTPC1) involved in Ca\(^{2+}\) influx and regulation of growth and development in rice. Plant Cell Physiol. 45, 693–702
23. Kuchitsu, K., Kikuyama, M., and Shibuya, N. (1993) N-acetylchitosioligosaccharides, biotic elicitors for phytoalexin production, induce transient membrane depolarization in suspension-cultured rice cells. Protoplasma 174, 79–81
24. Su, R. C., Rudert, M. L., and Hodges, T. K. (1992) Fertile indica and japonica rice plants regenerated from protoplasts isolated from embryogenic haploid suspension cultures. Plant Cell Rep. 12, 45–49
25. Mithöfer, A., and Mazzaros, C. (2002) Aequorin-based measurements of
intracellular Ca\textsuperscript{2+}-signatures in plants cells. *Biol. Proced. Online* **4**, 105–118.
26. Knight, M. R., Campbell, A. K., Smith, S. M., and Treweas, A. J. (1991) Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytosolic calcium. *Nature* **352**, 524–526.
27. Igasaki, T., Ishida, Y., Mohri, T., Ichikawa, H., and Shinohara, K. (2002) Transformation of Populus alba and direct selection of transformants with the herbicide bialaphos. *Bulletin FFPR* **1**, 235–240.
28. Larsson, C., Sommarin, M., and Widell, S. (1994) Isolation of highly purified plant plasma membranes and separation of inside-out and right-side-out vesicles. *Methods Enzymol.* **228**, 451–469.
29. Smallwood, M., Yates, E., Willats, W., Martin, H., and Knox, J. (1996) Immunochemical comparison of membrane-associated and secreted arabo-

---

**Roles of Rice TPC1 in Phytoalexin Biosynthesis**

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36. Ron, M., and Avni, A. (2004) The receptor for the fungal elicitor ethylene-

---

44. Cho, E. M., Okada, A., Kenmoku, H., Otomo, K., Toyomasa, T., Mitsu-

---

51. Song, W. Y., Zhang, Z. B., Shao, H. B., Guo, X. L., Cao, H. X., Zhao, H. B., Fu, Z. Y., and Hu, X. J. (2008) Relationship between calcium-decoding elements and plant abiotic-stress resistance. *Int. J. Biol. Sci.* **4**, 116–125.
52. Kishi-Kaboshi, M., Okada, K., Kurimoto, L., Murakami, S., Umezawa, T., Shibuya, N., Yamane, H., Miyao, A., Takatsuji, H., Takahashi, A., and Hirochika, H. (2010) A rice fungal MAMP-responsive MAPK cascade regulates metabolic flow to antimicrobial metabolite synthesis. *Plant Cell* **15**, 599–612.
53. Kurusu, T., Hamada, J., Hamada, H., Hanamata, S., and Kuchitsu, K. (2010) Roles of calcineurin B-like protein-interacting protein kinases in innate immunity in rice. *Plant Signal Behav.* **5**, 1045–1047.
54. Balagué, C., Lin, B., Alcon, C., Flottes, G., Malmström, S., Köhler, C., Neuhaus, G., Pelletier, G., Gaymard, F., and Roby, D. (2003) HLM1, an essential signaling component in the hypersensitive response, is a member of the cyclic nucleotide-gated channel ion channel family. *Plant Cell* **15**, 365–379.
55. Clough, S. J., Fengler, K. A., Yu, I. C., Lippok, B., Smith, R. K., Jr., and Bent, A. F. (2000) The *Arabidopsis* dnd1 "defense, no death" gene encodes a mutated cyclic nucleotide-gated ion channel. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 9323–9328.
56. Jurkowski, G. I., Smith, R. K., Jr., Yu, I. C., Ham, J. H., Sharma, S. B., Klessig, D. F., Fengler, K. A., and Bent, A. F. (2004) *Arabidopsis* DND2, a second cyclic nucleotide-gated ion channel gene for which mutation causes the "defense, no death" phenotype. *Mol. Plant Microbe Interact.* **17**, 511–520.
57. Yoshioka, K., Moeder, W., Kang, H. G., Kachroo, P., Masmoudi, K., and Mansfield, J. (2004) Proteomics of the rice cell: systematic identification of the protein populations in subcellular compartments. *Mol. Genet. Genomics* **271**, 566–576.
58. Hashimoto, K., Saito, M., Iida, H., and Matsuoka, H. (2005) Evidence for the plasma membrane localization of a putative voltage-dependent Ca\textsuperscript{2+} channel, OsTPC1, in rice. *Plant Biotechnol.* **22**, 235–239.
59. Shimizu, T., Jikumaru, Y., Okada, A., Okada, K., Koga, J., Umemura, K., Minami, E., Shibuya, N., Hasegawa, M., Kodama, O., Noji, H., and Yamane, H. (2008) Effects of a bile acid elicitor, cholic acid, on the biosynthesis of diterpenoid phytoalexins in suspension-cultured rice cells. *Phytochemistry* **69**, 973–981.
60. Ogasawara, Y., Kaya, H., Hiraoka, G., Yamato, F., Kumura, S., Kadota, Y., Hishinuma, H., Senzaki, E., Yamagoe, S., Nagata, K., Wada, M., Suzuiki, K., Tanokura, M., and Kuchitsu, K. (2008) Synergistic activation of the *Arabidopsis* NADPH oxidase AtRbohD by Ca\textsuperscript{2+} and phosphorylation. *J. Biol. Chem.* **283**, 8885–8892.
61. Pérez-García, M. T., Kamp, T. J., and Marbán, E. (1995) Functional properties of cardiac L-type calcium channels transiently expressed in HEK293 cells. Roles of α1 and β subunits. *J. Gen. Physiol.* **105**, 289–305.
62. Ron, M., and Avni, A. (2004) The receptor for the fungal elicitor ethylene-

---

63. Brailoiu, E., Rahman, T., Churamani, D., Prole, D. L., Brailoiu, G. C., Hooper, R., Taylor, C. W., and Patel, S. (2010) An NADP-gated two-pore channel targeted to the plasma membrane uncouples triggering from amplifying Ca\textsuperscript{2+} signals. *J. Biol. Chem.* **285**, 38511–38516.
64. Song, W. Y., Zhang, Z. B., Shao, H. B., Guo, X. L., Cao, H. X., Zhao, H. B., Fu, Z. Y., and Hu, X. J. (2008) Relationship between calcium-decoding elements and plant abiotic-stress resistance. *Int. J. Biol. Sci.* **4**, 116–125.
65. Kishi-Kaboshi, M., Okada, K., Kurimoto, L., Murakami, S., Umezawa, T., Shibuya, N., Yamane, H., Miyao, A., Takatsuji, H., Takahashi, A., and Hirochika, H. (2010) A rice fungal MAMP-responsive MAPK cascade regulates metabolic flow to antimicrobial metabolite synthesis. *Plant Cell* **15**, 599–612.
66. Kurusu, T., Hamada, J., Hamada, H., Hanamata, S., and Kuchitsu, K. (2010) Roles of calcineurin B-like protein-interacting protein kinases in innate immunity in rice. *Plant Signal Behav.* **5**, 1045–1047.
67. Balagué, C., Lin, B., Alcon, C., Flottes, G., Malmström, S., Köhler, C., Neuhaus, G., Pelletier, G., Gaymard, F., and Roby, D. (2003) HLM1, an essential signaling component in the hypersensitive response, is a member of the cyclic nucleotide-gated channel ion channel family. *Plant Cell* **15**, 365–379.
68. Clough, S. J., Fengler, K. A., Yu, I. C., Lippok, B., Smith, R. K., Jr., and Bent, A. F. (2000) The *Arabidopsis* dnd1 "defense, no death" gene encodes a mutated cyclic nucleotide-gated ion channel. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 9323–9328.
69. Jurkowski, G. I., Smith, R. K., Jr., Yu, I. C., Ham, J. H., Sharma, S. B., Klessig, D. F., Fengler, K. A., and Bent, A. F. (2004) *Arabidopsis* DND2, a second cyclic nucleotide-gated ion channel gene for which mutation causes the "defense, no death" phenotype. *Mol. Plant Microbe Interact.* **17**, 511–520.
70. Yoshioka, K., Moeder, W., Kang, H. G., Kachroo, P., Masmoudi, K., Berkowitz, G., and Klessig, D. F. (2006) The chimeric *Arabidopsis* cyclic nucleotide-gated ion channel11/12 activates multiple pathogen resistance responses. *Plant Cell* **18**, 747–763.