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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]

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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 photoprotein, aequorin, 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)[3] 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, β-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.

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 are induced by various elicitors including chitin fragments, cerebrosides 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 (14–15) and be involved in the sucrose-induced Ca^{2+} rise (16), abscisic acid-induced repression of germination (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*, a*ttpc1–2,* 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 apoaequorin (21), and *Ostpc1* cells (22) were suspension-cultured at 25 °C in a liquid broth L medium (23) and AA medium (24) containing 2,4-D (0.5 mg l^{-1}) and subcultured in fresh medium every 7 days. Cells at 5 days after subculture were used for experiments on defense responses. Xylanase from *Trichoderma viride* was obtained from Sigma. *N*-acetylchitooligosaccharides were provided by Prof. Naoto Shibuya (Meiji University).

Monitoring of Cytosolic Calcium Concentration—Measurement of changes in cytosolic Ca^{2+} concentration was performed essentially as described by Kurusu *et al.* (21). Briefly, apoaequorin-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 aerated 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 M $CaCl_2$ and 20% ethanol after each experiment, and chemiluminescence data transformed into $[Ca^{2+}]_{cyt}$ using the equation established by Mithöfer *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∼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, *apoaequorin* cDNA (26) were cloned into a Ti-based vector pIG121-Hm, and *Agrobacterium*-mediated transformation of *Ostpc1* rice calli was performed. Transformed calli were screened and transgenic plants regenerated. Transgenic cell lines derived from T_1 plants were used for various analyses.

Complementation Analysis—We transformed transgenic *Ostpc1* cell lines expressing wild-type *OsTPC1* and *GUS* (control) cDNA (4). The *apoaequorin* 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 aqueous 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′-CACCATTGATGCTACTGGTCAGGGTTATCT-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.

For immunoblotting analyses, protein samples were separated by 7.5% SDS-PAGE gel and blotted on to a PVDF membrane. The membrane was blocked in 1× TTBS buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.5) with 5% fat free milk overnight at 4 °C. Blots were incubated with the affinity-purified anti-OsTPC1 and then with HRP-linked anti-rabbit IgG (GE Healthcare). Bands were detected using the chemiluminescent HRP substrate (Millipore) and a chemiluminescent analyzer, LAS3000 (GE Healthcare).

RT-PCR Analysis—First-strand cDNA was synthesized from 3 μg of total RNA. PCR amplification was performed with gene-specific primers (supplemental Table S1). *Actin* was used as a quantitative control. Aliquots of individual PCR products were resolved by agarose gel electrophoresis and visualized with ethidium bromide under UV light.

Real-time RT-PCR Quantification—First-strand cDNA was synthesized from 3 μg of total RNA. Real-time PCR was performed using an ABI PRISM 7300 sequence detection system (Applied Biosystems Instruments) with SYBR Green real-time PCR Master Mix (TOYOBO) and gene-specific primers (supplemental Table S1). Relative mRNA abundances were calculated using the standard curve method and normalized to the corresponding *OsActin1* mRNA levels. Standard samples of known template amounts were used to quantify the PCR products.

Phytoalexin Measurements—Phytoalexins were extracted from suspension-cultured rice cells after elicitation and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described previously (33).

Electrophysiology—To generate HEK293T cells expressing *GFP-OsTPC1*, the coding region was amplified using a previously described GFP- OsTPC1 plasmid (4), and sequence specific primers: GFP-OsTPC1F, 5′-CTAGTCTAGAGCCGCCACCATGGTGAGCAAGGGCGAGGA-3′ and GFP-OsTPC1R, 5′-ATGCATCCAGTGTGGTGGCTATTGGTCACGGTTTTGAGATCC-3′, cloned into the pcDNA3.1(−) vector (Invitrogen). The pEGFP-N3 vector (Clontech) was used for GFP control. HEK293T cells were transiently transfected with GeneJuice transfection reagent according to the manufacturer's protocol (Novagen) (34).

Functional expression of membrane currents was investigated using the whole-cell patch clamp technique. Whole-cell currents were recorded with a CEZ-2200 patch clamp amplifier (NIHON KOHDEN). The resulting values were not corrected for liquid junction potential and leak currents were not subtracted. We used pCLAMP 8.2 software (Molecular Devices) for data analysis. The pipette solution contained 120 mM CsCl, 3 mM MgATP, 10 mM EGTA, and 10 mM HEPES (pH 7.1). The bath solution contained 40 mM $BaCl_2$, 80 mM CsCl, and 10 mM HEPES (pH 7.4) (35). A ramp voltage protocol from 0 to −130 mV (holding potential, 0 mV; ramp speed, 130 mV s^{-1}) was used.

Statistical Analysis—Statistical significance was determined using an unpaired Student's *t* test, with a maximum *p* value of < 0.05 required for significance. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

RESULTS

TvX Triggered a Prolonged Increase in Cytosolic Ca^{2+} in Suspension-cultured Rice Cells—TvX triggers a variety of defense responses in plants (4, 36–38). In rice cell culture, external Ca^{2+} was required for TvX-induced hypersensitive cell death, suggesting that Ca^{2+} influx through the PM is indispensable for TvX-induced defense responses (4). However, the effect of TvX on changes in $[Ca^{2+}]_{cyt}$ has never been characterized. To analyze the involvement of Ca^{2+} flux in TvX-induced defense responses, we measured TvX-induced changes in $[Ca^{2+}]_{cyt}$ using transgenic rice cell lines expressing apoaequorin, a Ca^{2+}-sensitive photoprotein (21) (supplemental Fig. S1). As shown in Fig. 1*A*, TvX triggered a prolonged increase in $[Ca^{2+}]_{cyt}$ in a dose-dependent manner.

To elucidate the origin of Ca^{2+} flux, we first performed pharmacological analyses. The addition of a Ca^{2+} chelator, BAPTA, into the extracellular medium or the substitution of Ca^{2+} free medium inhibited the TvX-induced increase in $[Ca^{2+}]_{cyt}$ (Fig. 1*B* and supplemental Fig. S2). Similarly, a Ca^{2+} channel blocker, La^{3+} or Gd^{3+}, and a voltage-dependent Ca^{2+} channel inhibitor, nifedipine (11), suppressed the TvX-induced increase in $[Ca^{2+}]_{cyt}$ (Fig. 1*B* and supplemental Fig. S2). A phospholipase C inhibitor, neomycin, and a potential endomembrane Ca^{2+}-permeable channel inhibitor, ruthenium red, has been reported to suppress Ca^{2+} release from intracellular Ca^{2+} stores (39–40). In contrast to BAPTA and La^{3+}, neomycin, and ruthenium red scarcely inhibited the TvX-induced increase in $[Ca^{2+}]_{cyt}$ (Fig. 1*B*), suggesting that TvX-induced increase in $[Ca^{2+}]_{cyt}$ is predominantly due to the influx of extracellular Ca^{2+} through voltage-dependent Ca^{2+}-permeable channels.

Involvement of OsTPC1 in the TvX-induced Changes in Cytosolic Ca^{2+} 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 Ca^{2+} 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. 1*C*, the TvX-induced increases in $[Ca^{2+}]_{cyt}$ 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 the TvX-induced Ca^{2+} increase (Fig. 1*D*). In contrast, expression of *OsTPC1* recovered the TvX-induced Ca^{2+} increase (Fig. 1*D*), indicating that the observed mutant phenotype was attributable to *OsTPC1*. These results suggest that OsTPC1 participates in TvX-induced Ca^{2+} 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

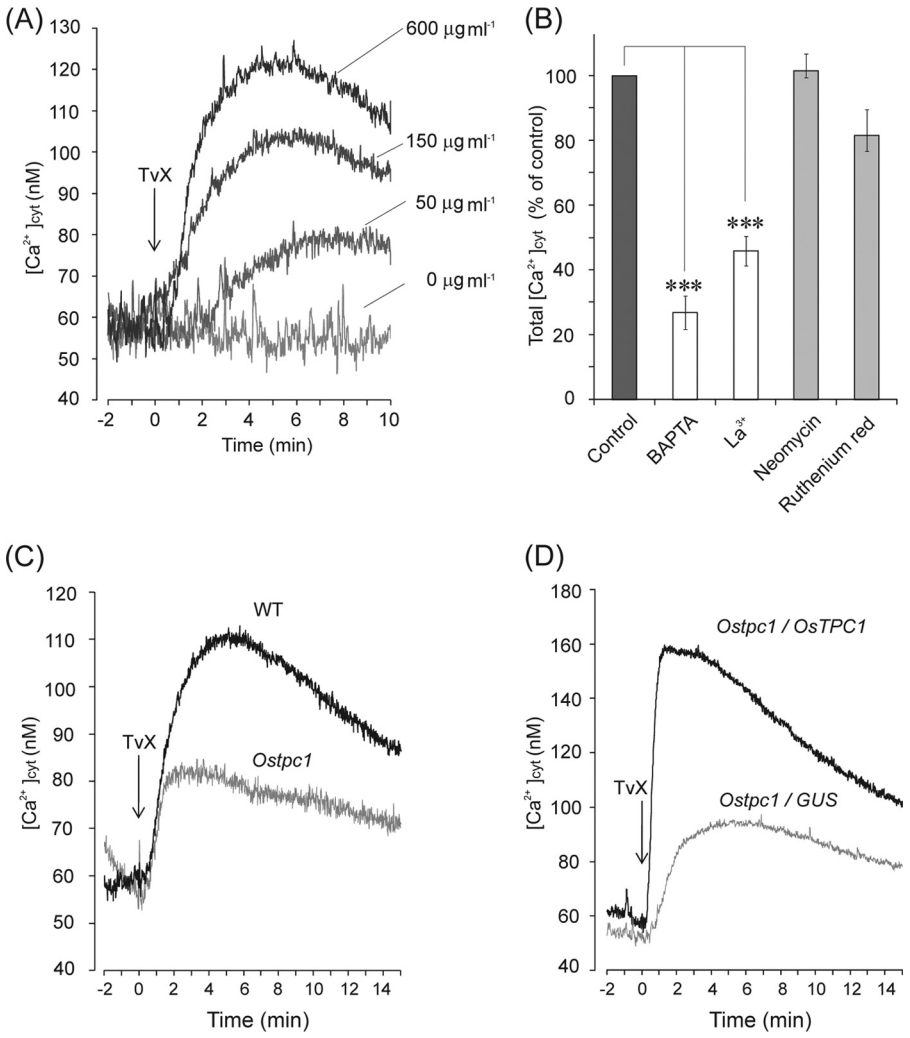

FIGURE 1. **TvX-induced increase in cytolocis Ca^{2+} concentration in apoaequorin-expressing cultured rice cells.** Transformed rice cells were treated with xylanase from *Trichoderma viride* (TvX). *A*, dose-dependent changes in $[Ca^{2+}]_{cyt}$ induced by TvX were monitored continuously in wild-type cells. *B*, effects of several inhibitors on TvX-induced changes in $[Ca^{2+}]_{cyt}$. BAPTA (5 mM), $LaCl_3$ (5 mM), ruthenium red (100 μM), or neomycin (500 μM) were added to the rice cells 15 min prior to TvX (600 μg ml^{-1}). Average values and standard errors of three or four independent experiments are shown. *C*, $[Ca^{2+}]_{cyt}$ changes induced by TvX (600 μg ml^{-1}) were monitored in wild-type and *Ostpc1* cells. Four replicate experiments were performed and average data is shown. *D*, $[Ca^{2+}]_{cyt}$ changes induced by the MAMP in *apoaequorin*-expressing complementation line (*Ostpc1/OsTPC1*) and its control line (*Ostpc1/GUS*). Four replicate experiments were performed and average data is shown.

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. 2*A*). 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. 2*A*), 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. 2*B*). As shown in Fig. 2*B*, 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. 3*A* and supplemental Fig. S3). Similarly, TvX-induced induction of these genes was suppressed in *Ostpc1* knock-out

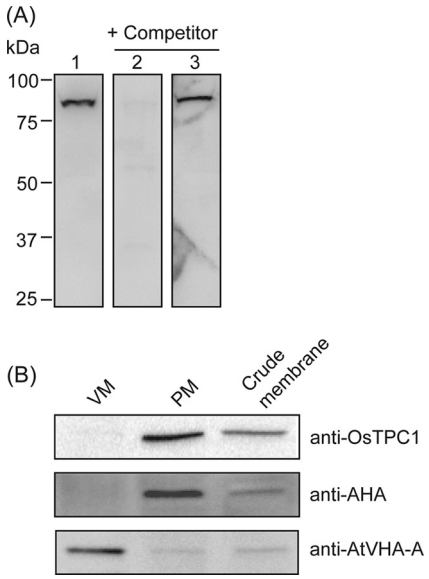

FIGURE 2. **Subcellular localization of OsTPC1.** *A*, protein sample of crude membrane fraction (20 μg per lane) was subjected to SDS-PAGE and immunoblotting analyses. OsTPC1 was detected using an affinity-purified anti-OsTPC1 antibody (*lane 1*). As a competitor, 0.5 mg ml^{-1} histidine-tagged OsTPC1 (I359-S403) was added at 1:1000 (*lane 2*) or 0.5 mg ml^{-1} histidine-tagged GUS was added at 1:1000 dilution (*lane 3*). *B*, subcellular fractions of suspension-cultured rice cells were prepared using an aqueous two-phase system and a sucrose/sorbitol system. Protein samples (10 μg per lane) of each fraction were subjected to SDS-PAGE and immunoblotting analyses. OsTPC1 was detected using an affinity-purified anti-OsTPC1 antibody. The purified fractions were assessed using a polyclonal antibody of plasma membrane H^+-ATPase and vacuolar H^+-ATPase subunit A (COSMO BIO).

cells and the suppression was restored after complementation of *Ostpc1* (Fig. 3*B* and supplemental Fig. S4). Quantitative HPLC-tandem mass spectrometry analyses revealed that TvX induces the accumulation of momilactones and phytocassanes, which are major phytoalexins in rice (9). The level of momilactones 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 momilactones and phytocassanes was significantly suppressed (Fig. 5*A*). The suppression in *Ostpc1* knock-out cells was restored by introducing the *OsTPC1* gene (Fig. 5*B*), suggesting that OsTPC1 plays a role in regulation of TvX-induced phytoalexin biosynthesis. The close correlation between TvX-induced changes in cytosolic Ca^{2+} concentration and phytoalexin biosynthesis in *Ostpc1* knock-out cells suggest the significance of the signaling role of Ca^{2+} regulated by OsTPC1 at least in part in TvX-induced phytoalexin biosynthesis.

Ca^{2+}-permeability of OsTPC1—Possible involvement of OsTPC1 in the TvX-induced $[Ca^{2+}]_{cyt}$ increase (Fig. 1*C*) and its localization at the PM (Fig. 2*B*) led us to hypothesize that OsTPC1 may function as a Ca^{2+}-permeable channel at the PM in rice cells. However, no electrophysiological characterization has so far been reported for OsTPC1. We thus recorded the whole-cell current in HEK293T cells expressing *GFP* or *GFP-OsTPC1* cDNA. Membrane fractionation analysis revealed that heterologously expressed OsTPC1 was localized to the PM at least partially in HEK293T cells (supplemental Fig. S5*A*). Voltage-dependent currents carried by Ba^{2+} instead of Ca^{2+} (-230 ± 57 pA at -130 mV, $n = 5$) was observed in *GFP-OsTPC1*-expressing cells (Fig. 6, *A* and *B* and supplemental Fig. S5*B*). On the other hand, we detected no such currents in GFP-expressing cells (-59 ± 7 pA at -130 mV, $n = 5$). Treatment of La^{3+} significantly suppressed the voltage-activated Ba^{2+} currents in *GFP-OsTPC1*-expressing cells, but not neomycin (supplemental Fig. S5*C*), indicating that OsTPC1 functions as a voltage-activated Ca^{2+}-permeable channel.

DISCUSSION

Immediately after the recognition of several elicitors, plant cells induce $[Ca^{2+}]_{cyt}$ 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 $[Ca^{2+}]_{cyt}$ (1, 3, 6, 43). Here, we showed that TvX triggered a prolonged change in $[Ca^{2+}]_{cyt}$, mainly due to Ca^{2+} influx from the extracellular space (Fig. 1, *A* and *B*). A sustained increase in $[Ca^{2+}]_{cyt}$ is postulated to have a key role in the induction of HR cell death (47–48). Ca^{2+} influx is required for TvX-induced HR cell death (4). Hence, a prolonged change in $[Ca^{2+}]_{cyt}$ 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 $[Ca^{2+}]_{cyt}$ changes were partially suppressed in *Ostpc1* cells (Fig. 1*C*). We thus postulate that multiple Ca^{2+}-permeable channels are involved in the elicitor-triggered $[Ca^{2+}]_{cyt}$ changes. OsTPC1 heterologously expressed in HEK293T cells showed voltage-dependent Ca^{2+} permeability through the PM (Fig. 6), suggesting that OsTPC1 functions as a Ca^{2+}- permeable channel. The present results suggest that OsTPC1 plays a role as one of the multiple Ca^{2+}-permeable channels activated by TvX and is involved in the elicitor-induced $[Ca^{2+}]_{cyt}$ change.

It has been reported that AtTPC1 is not involved in $[Ca^{2+}]_{cyt}$ changes triggered by two MAMPs, elf18 and flg22 (15). Interestingly, $[Ca^{2+}]_{cyt}$ changes induced by chitin fragments (*N*-acetylchitooligosaccharides) in *Ostpc1* 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 $[Ca^{2+}]_{cyt}$ are significantly different between TvX and chitin fragments: $[Ca^{2+}]_{cyt}$ increase triggered by chitin fragments is large and transient (supplemental Fig. S6) (21), while that induced by TvX is much smaller but sustained (Fig. 1*C*). The differences in the temporal pattern of $[Ca^{2+}]_{cyt}$ 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 *OsTPC1* (4). These results suggest that OsTPC1 may be one of the multiple Ca^{2+} permeable channels activated by some specific elicitors but not all to trigger sustained increase in $[Ca^{2+}]_{cyt}$ and HR cell death.

The primary structure of AtTPC1 and OsTPC1 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 OsTPC1 are predominantly localized at the VM and PM, respectively (4, 14, 32). A

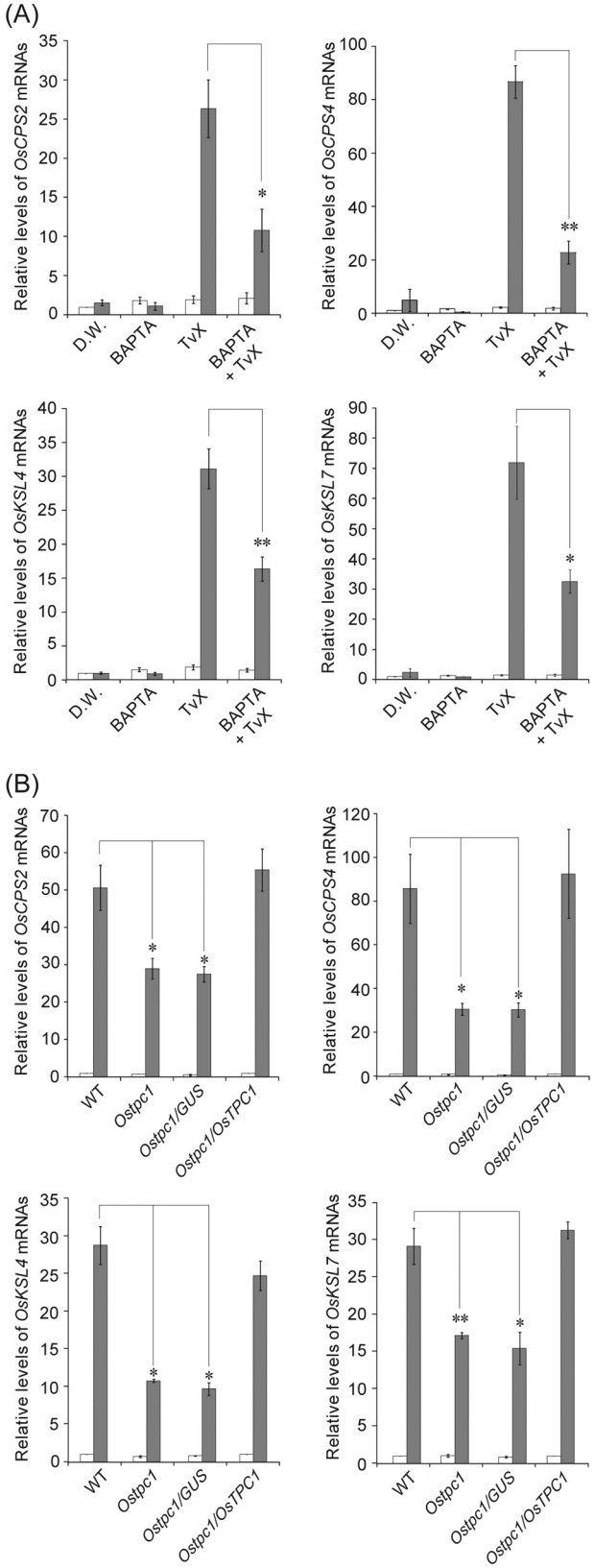

FIGURE 3. **TvX-induced expression of diterpene cyclase genes.** *A*, effect of BAPTA on the expression of the diterpene cyclase genes, *OsCPS2*, *OsCPS4*, *OsKSL4*, and *OsKSL7* at 0 h (*white bar*) and 6 h (*gray bar*) after TvX treatment. BAPTA (1 mM) was added to the rice cells 30 min prior to TvX 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 TvX 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.

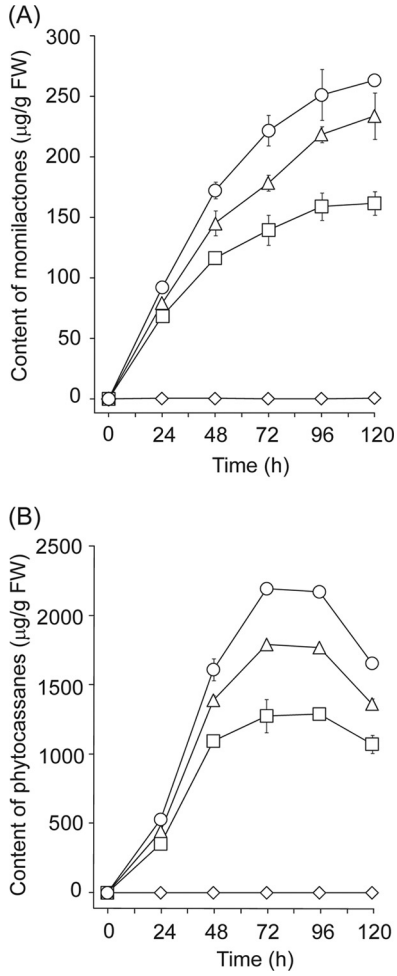

FIGURE 4. **TvX-induced accumulation of diterpenoid phytoalexins.** Diterpenoid phytoalexins (momilactone A, B, and phytocassane A–E) were extracted from culture medium collected at the indicated time points after the addition of TvX. The total amount of momilactones A and B (*A*) and phytocassanes A–E (*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.

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. 2*B* and supplemental Fig. S5*A*). 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 recogni-

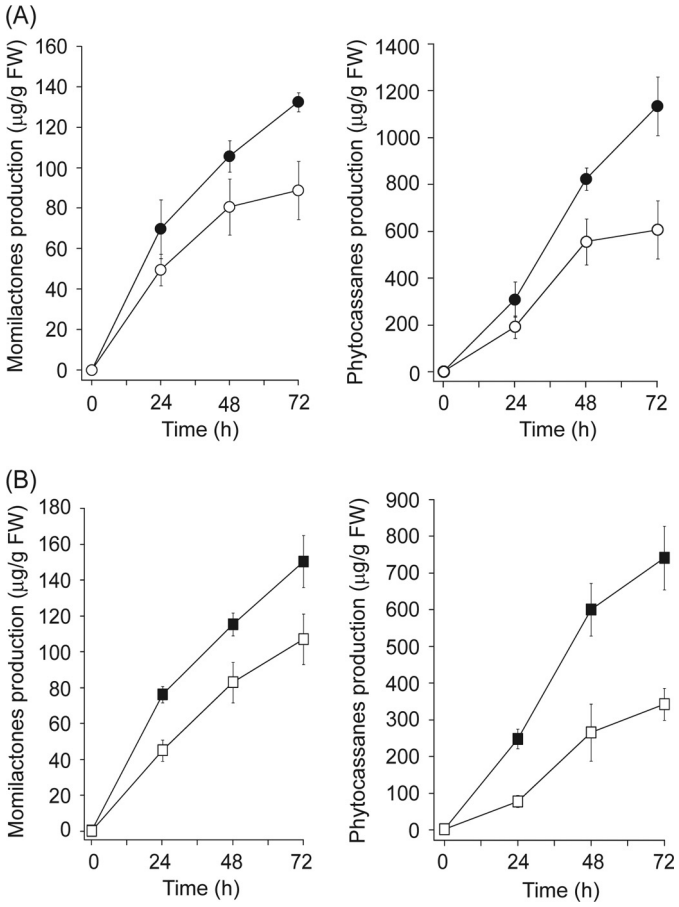

FIGURE 5. **Effects of *Ostpc1* disruption and complementation on TvX-induced diterpenoid phytoalexin accumulation.** The total amounts of momilactones and phytocassanes accumulated in the culture medium was quantified. Each cell line was treated with TvX (30 μg ml^{-1}). *A*, *solid circle*: wild-type;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

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+} channel blockers, $LaCl_3$ and $GdCl_3$, indicate that Ca^{2+} influx via Ca^{2+} 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. 3*A* 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. 3*B* 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

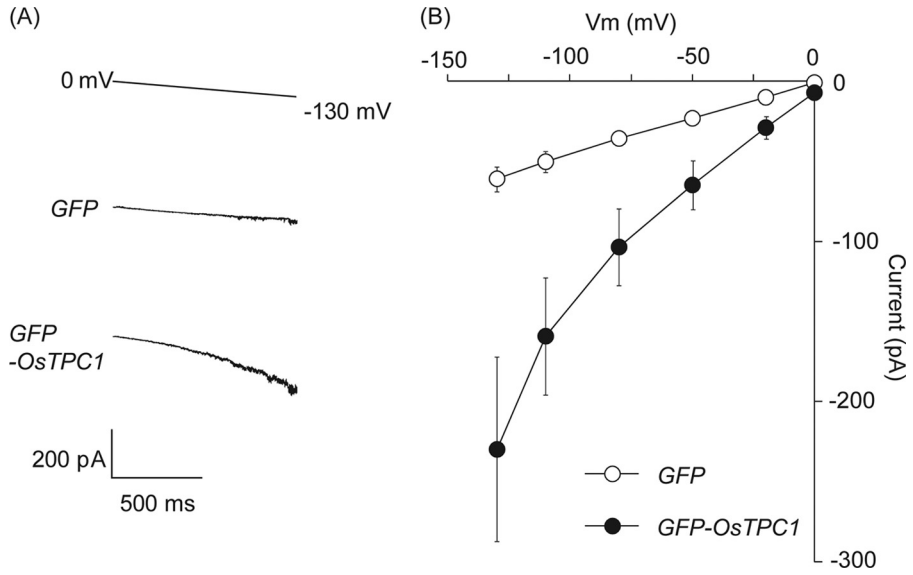

FIGURE 6. **Voltage-dependent Ca^{2+} current in HEK cells transiently expressing GFP-OsTPC1.** The expression of OsTPC1 enhanced voltage-dependent currents in HEK293T cells. *A*, representative currents from *CMV:GFP* or *CMV:GFP-OsTPC1* expressing HEK293T cells. A voltage ramp from 0 to −130 mV was 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.

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 layers of TvX-induced defense responses including the expression of the same diterpene cyclase genes 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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