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Protein Note

Evidence for the plasma membrane localization of a putative voltage-dependent Ca^{2+} channel, OsTPC1, in rice

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Abstract In conjunction with Ca^{2+} signaling, the subcellular localization of Ca^{2+} channels in various plant cells has been under intense scrutiny. The present study focused on the subcellular localization of OsTPC1, a putative voltage-dependent calcium channel in rice. A newly developed antibody to the linker domain of OsTPC1 was shown to bind this protein *in vitro*. Immunoblotting of a plasma membrane (PM)-enriched fraction obtained by aqueous two-phase partitioning, as well as indirect immunofluorescence confocal microscopy of rice protoplasts, supported the idea that OsTPC1 is predominantly localizes in the PM, at least of suspension-cultured rice cells.

Key words: Immunocytochemistry, *Oryza sativa*, plasma membrane, voltage-dependent calcium channel.

Changes in cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_c$) are essential for the transduction of various abiotic and biotic signals in plants (Sanders et al. 2002). Ca^{2+} is thought to be mobilized into the cytosol by influx from outside the cell or from intracellular Ca^{2+} stores, such as the vacuole and endoplasmic reticulum, via Ca^{2+} channels. However, the molecular bases for these dynamics are only just becoming apparent.

In recent years, candidates for the Ca^{2+} channel-encoding gene family, TPCs (two-pore channels) have been identified: *AtTPC1* from *Arabidopsis thaliana* (Furuichi et al. 2001), *OsTPC1* from rice (Hashimoto et al. 2004; Kurusu et al. 2004; Kurusu et al. 2005) and *NtTPC1A/B* from tobacco BY-2 cells (Kadota et al. 2004). The presence of its homologs in several other plants was also reported (White et al. 2002). The deduced TPCs proteins are structurally similar to voltage-dependent Ca^{2+} channels (VDCCs) of animal cells. Heterologous expression experiments in a yeast mutant lacking endogenous Ca^{2+} channel activity showed that the role of TPCs is to provide passages for Ca^{2+} entering from outside the cell. Consequently TPCs were expected to be localized in the PM, and AtTPC1-GFP expressed in tobacco BY-2 cells and GFP-OsTPC1 introduced into onion epidermal cells were indeed found to be localized in the PM (Kawano et al. 2004; Kurusu et al. 2005). In contrast, AtTPC1 and AtTPC1-GFP were reported to predominantly locate in the vacuolar membrane (VM) rather than in the PM, and to play a role in Ca^{2+}-activated Ca^{2+} release from intracellular Ca^{2+} stores (Peiter et al. 2005). Proteomic analyses of *Arabidopsis* suggested that AtTPC1 is relatively abundant in vacuoles (Szponarski et al. 2004; Carter et al. 2004). These contradictory observations prompted us to unambiguously determine the localization of OsTPC1 in a homologous system by using specific anti-OsTPC1 antibodies and cultured rice cells.

To perform this investigation, we prepared suspension-cultured rice cells (*Oryza sativa* L. japonica cv. Nipponbare) according to the method described by Saito et al. (2003). Rabbit polyclonal anti-OsTPC1 antibodies were generated against a fusion protein consisting of the linker domain of OsTPC1 (I359-S403) (Figure 1A) fused to a histidine-tag in the pET-32a(+) vector (Novagen, WI, USA). The recombinant protein synthesized in *Escherichia coli* was purified by affinity column chromatography using His·Bind resin (Novagen) and used for immunization. Anti-OsTPC1 was purified using an affinity column, Hitrap NHS-activated HP (Amersham Biosciences K. K., Tokyo, Japan) conjugated to histidine-tagged OsTPC1 (I359-S403), according to the manufacturer's protocol. The antibody fraction thus obtained was further purified to remove anti-histidine-tag antibodies by using another Hitrap NHS-activated HP column that was conjugated with recombinant histidine-tag alone.

To confirm that the affinity-purified anti-OsTPC1 rabbit IgG could detect endogenous OsTPC1, whole cell protein extracts of suspension-cultured rice cells were subjected to immunoblot analysis. Twenty μg of the

Abbreviations: ; $[Ca^{2+}]_c$, cytosolic free calcium ion concentration; TPC, Two-pore channel; VDCC, voltage-dependent Ca^{2+} channel.

protein sample was separated by SDS-PAGE and blotted on a PVDF membrane. The blots were then incubated with a 1:50 dilution of the affinity-purified anti-OsTPC1. After washing, the blots were incubated with the second antibody, a 1:10000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences K. K.). To develop the immunoblots, the ECL Plus Western blotting detection system (Amersham Biosciences K. K.) was used. The resulting single band was found at approximately 87 kDa, which agreed with the molecular weight estimated from the deduced amino acid sequence of OsTPC1 (Figure 1B, lane 1). When the antibodies were incubated with the recombinant antigen as a competitor, the band could be abolished by increasing the amount of competitor (Figure 1B, lane 2–4). Detection of the band was not abolished by the recombinant histidine-tag alone (Figure 1B, lane 5). These results indicate that the affinity-purified anti-OsTPC1 can detect OsTPC1 specifically.

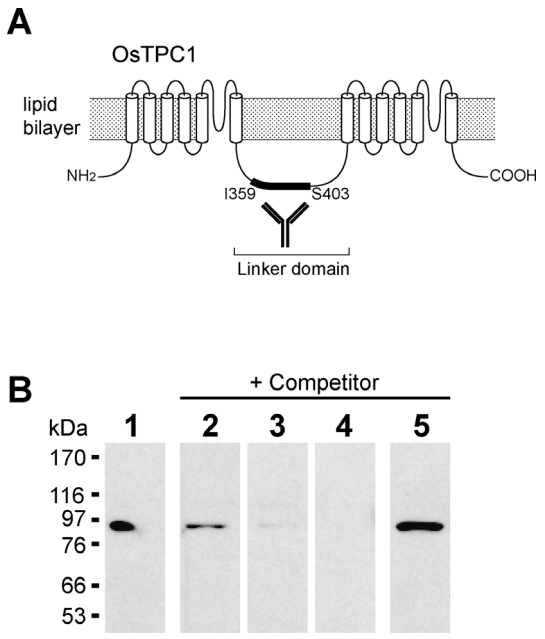

Figure 1. Polyclonal antibodies against OsTPC1. A, Structural model of OsTPC1 and the position of a polypeptide used for the preparation of anti-OsTPC1 antibodies. The position (I359-S403) was drawn in bold line. B, Detection of OsTPC1 protein by immunoblot analysis. Protein sample (20 μg) was separated by SDS-PAGE and blotted to a PVDF membrane, followed by detection with the affinity-purified anti-OsTPC1 (lane 1). As a competitor, 1 mg ml^{-1} histidine-tagged OsTPC1 (I359-S403) was added at 1:5000 (lane 2), 1:2500 (lane 3), and 1:1250 (lane 4) dilution or 1 mg ml^{-1} histidine-tag alone was added at 1:1250 dilution (lane 5). The blot signals were detected with BioMax XAR films (Eastman Kodak, NY, USA).

For the determination of OsTPC1 protein localization using the affinity-purified anti-OsTPC1, we first performed subcellular fractionation. Suspension-cultured rice cells were homogenized in an ice-cold homogenization buffer (0.25 M sorbitol, 50 mM Tris/acetate [pH 7.5], 1 mM EDTA, 2 mM DTT, 1% (w/v) PVP, 10 mM PMSF), filtered through four layers of gauze, and centrifuged for 10 min at 3,600×g at 4°C. The supernatant, designated whole cell protein extract, was centrifuged again for 30 min at 120,000×g at 4°C. The supernatant from this step was obtained as the soluble protein fraction, while the pellet was resuspended in a suspension buffer (0.5 M sucrose, 10 mM Tris/acetate [pH 7.5], 1 mM EDTA, 2 mM DTT) to obtain the crude membrane fraction. From 303 mg of whole cell protein extract, 91.8 mg of crude membrane fraction was obtained. This fraction was divided into 2 aliquots: 17.8 mg and 74 mg. From the 17.8 mg aliquot, the PM-enriched fraction (2.4 mg) and the PM-depleted fraction (11.3 mg) were obtained by using an aqueous two-phase system (Larsson et al. 1994; Smallwood et al. 1996). The VM-enriched fraction (0.47 mg) and the VM-depleted fraction (71 mg) were obtained from the 74 mg aliquot by using a sucrose/sorbitol system (Maeshima and Yoshida 1989; Tanaka et al. 2004). The purity of the fractions was evaluated by assaying for H^+-ATPase activity (Tanaka et al. 2004). Na_3VO_4 and KNO_3 are used as the specific inhibitors of P- and V-type H^+-ATPases, which are specifically located in the PM and the VM respectively (Sze 1985). This sensitive assay showed that both the PM-enriched fraction and the VM-enriched fraction were obtained in high purity (Table 1). Protein samples extracted from these subcellular fractions were subjected to immunoblot analysis as described above. The 87 kDa band was detected in the PM-enriched fraction and the VM-depleted fraction (Figure 2, lane 4, 7), but not in the PM-depleted fraction nor the VM-enriched fraction (Figure 2, lane 5, 6). These results indicate that OsTPC1 is predominantly localized in the plasma membrane.

We assume that the relative concentrations of PM in

Table 1. Effects of specific inhibitors on the activity of H^+-ATPase in the subcellular fractions of suspension-cultured rice cells.

Treatment	Crude membrane fraction	Plasma membrane		Vacuolar membrane	
		enriched fraction	depleted fraction	enriched fraction	depleted fraction
Control	0.321 (100)	0.399 (100)	0.482 (100)	0.137 (100)	0.247 (100)
+vanadate	0.200 (62)	0.110 (28)	0.412 (85)	0.135 (99)	0.162 (66)
+nitrate	0.301 (94)	0.425 (107)	0.440 (91)	0.050 (36)	0.246 (100)

Assays were performed in the presence of 0.02% (w/v) Triton X-100. The activity was expressed as μmol of Pi liberated per mg of protein per min. Vanadate (0.1 mM Na_3VO_4) or nitrate (50 mM KNO_3) was added in the assay sample as indicated. The values in parentheses indicate the percentage of the control.

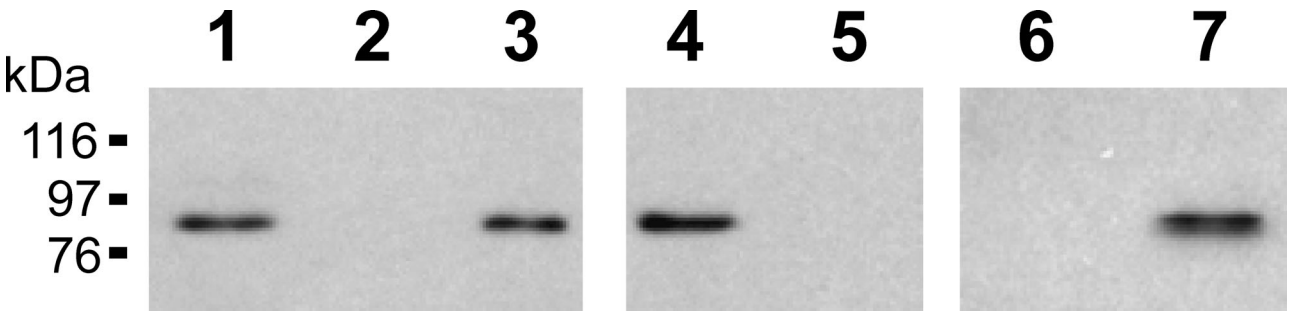

Figure 2. Subcellular localization of OsTPC1. Subcellular fractions of suspension-cultured rice cells were prepared by aqueous two-phase system: lane 1, the whole cell extracts; lane 2, the soluble protein fraction; lane 3, the crude membrane fraction; lane 4, the PM-enriched fraction; lane 5, the PM-depleted fraction; lane 6, the VM-enriched fraction; lane 7, the VM-depleted fraction. Protein samples (10 μg per lane) of each fraction were subjected to SDS-PAGE and immunoblot analysis. OsTPC1 was detected by the affinity-purified anti-OsTPC1.

PM-enriched and PM-depleted fractions are in proportion to the relative intensity of vanadate-sensitive inhibition. In Table 1, they are 72% and 15%, respectively. Therefore the relative concentration of PM in the PM-depleted fraction is estimated as approximately 1/5 (=15/72) of the PM-enriched fraction. Such a low concentration of OsTPC1 cannot be detected under the present immunoblotting conditions (Figure 2, lane 5). On the other hand, OsTPC1 was not detected in the VM-enriched fraction (Figure 2, lane 6). Therefore, the concentration of OsTPC1 in this fraction is no higher than 1/5 of the PM-enriched fraction. Moreover, the total amount of OsTPC1 in VM was estimated based on the relative amount of PM and VM; it was $<$0.5% of total OsTPC1 in the PM.

To confirm OsTPC1 localization in the PM, we employed a different approach, i.e. indirect immunofluorescence confocal microscopy. Rice protoplasts were prepared as described in Saito et al. 2003 and fixed by incubation with 4% (v/v) formaldehyde at room temperature for 1 h and then at 4°C overnight. Fixed protoplasts were washed three times with PBS (1.37 M NaCl, 2.68 mM KCl, 3.18 mM Na_2HPO_4, 1.45 mM KH_2PO_4, pH 7.3), incubated for 5 min in 0.1% Triton X-100 and placed onto 35 mm diameter glass-base dishes (Iwaki, Funabashi, Japan). The protoplasts were incubated for 1 h in PBS containing 10 mg ml^{-1} bovine serum albumin and then with appropriately diluted primary antibody for another 1 h. After washing 5 times with PBS, the protoplasts were incubated with a secondary antibody, Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) (Molecular Probes, Leiden, Netherlands), at a 1:400 dilution in PBS for 1 h, and washed 5 times with PBS. Confocal fluorescence microscopy was performed using a Bio-Rad (Reinach, Switzerland) μRadiance 2000.

When the protoplasts were treated with the affinity-purified anti-OsTPC1 followed by the Alexa Fluor 488-labeled secondary antibodies, no appreciable fluorescent image was obtained (Figure 3A, B). The protoplasts were further treated with Alexa Fluor 488-conjugated donkey anti-goat IgG (H+L) (Molecular Probes) as the third antibody to amplify the fluorescent signal. Results showed a ring-shaped fluorescent image along the outline of the cell (Figure 3C, D), Where as no fluorescent image was observed when protoplasts were treated with the secondary or tertiary antibodies alone (data not shown). When the same treatment as in Figure 3C was performed in the presence of a competitive inhibitor of the anti-OsTPC1, no fluorescent image was observed (Figure 3E, F). These findings indicate that the fluorescent image shown in Figure 3C can be attributed to OsTPC1. A monoclonal IgM antibody, LM2, which specifically recognizes a membrane-associated arabinogalactan-protein (mAGP) of rice (Smallwood et al. 1996; Yates et al. 1996) was used as a marker of the PM, also visualized with primary antibody and Alexa Fluor 488-conjugated goat anti-rat IgG (H+L) (Molecular Probes). This revealed a ring-shaped pattern (Figure 3G, H) that was essentially the same as that seen in Figure 3C, although fluorescence intensity differed between them.

In conclusion, our study has clearly demonstrated that OsTPC1 is exclusively present in the PM in suspension-cultured rice cells. This is the first report on the subcellular localization of intact OsTPC1 in a homologous system. Our finding is consistent with the observation by Kurusu et al. (2005) that GFP-OsTPC1 is localized in the PM of onion epidermal cells. OsTPC1 should be responsible for Ca^{2+} influx into the cytosol from outside the cell, as expected from Ca^{2+} uptake experiments using a heterologous expression system of yeast *cch1* mutant cells (Hashimoto et al. 2004). It may be hypothesized that OsTPC1 and AtTPC1 are differentially targeted to cellular membranes and have different roles in the corresponding organisms. Another possibility is that growth conditions for plants or cultured cells affect the subcellular localization of the plant TPCs family. An *in silico* analysis using SignalP (http://www.cbs.dtu.dk/services/SignalP/) showed that neither AtTPC1 nor OsTPC1 has any localization signal sequences in their N-terminal region. Therefore, further study on the localization of this channel family in

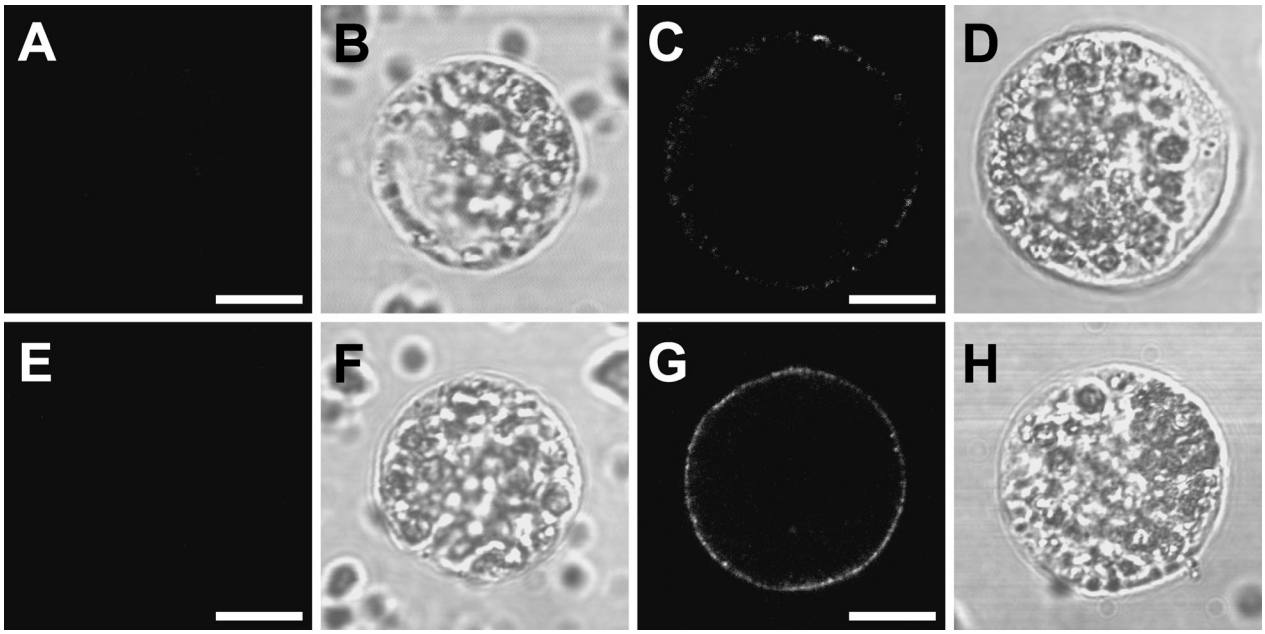

Figure 3. Immunocytochemical localization of OsTPC1 in rice protoplasts. The rice protoplasts were observed by indirect immunofluorescence confocal microscopy (A, C, E, and G) or transmission microscopy (B, D, F, and H). A, a protoplast stained with the affinity-purified anti-OsTPC1 and then with the secondary antibody; C, a protoplast stained as in A and further with the tertiary antibody; E, a protoplast stained as in C in the presence of the specific competitor of OsTPC1; G, a protoplast stained with an anti-PM marker, LM2. The protoplasts shown here are representatives of at least 30 protoplasts showing essentially the same fluorescence and transmission images. Bars = 10 μm.

various species grown under well-defined conditions is necessary to unravel relationships between localization and function.

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