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Note

Regulation of xylanase elicitor-induced expression of defense-related genes involved in phytoalexin biosynthesis by a cation channel OsTPC1 in suspension-cultured rice cells

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Abstract Signal molecules derived from pathogens/microbes or plants (pathogen/microbe/damage-associated molecular patterns; PAMPs/MAMPs/DAMPs), elicitors, trigger changes in cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$) to activate plant immune responses. A rice two-pore channel 1 (OsTPC1) has been suggested to be involved in fungal xylanase elicitor (TvX)-induced defense responses including $[Ca^{2+}]_{cyt}$ increase, phytoalexin production and hypersensitive cell death in suspension-cultured rice cells. However, little is known on the molecular links between $[Ca^{2+}]_{cyt}$ rise and elicitor-induced gene expression. To gain insights on the possible roles of OsTPC1 in TvX-induced gene expression, we performed DNA microarray analysis using a rice 44K oligo-microarray system, and revealed that TvX induce expression of thousands of genes including WRKY-type transcription factors, a serine hydrolase involved in hypersensitive cell death, and diterpene cyclases required for phytoalexin biosynthesis, which are suppressed in the *Ostpc1* knockout mutant. TvX-induced expression of genes involved in the methylerythritol phosphate (MEP) pathway, which is located upstream of the phytoalexin biosynthesis pathway, was also suppressed in *Ostpc1* cells. Possible involvement of OsTPC1 in the regulation of gene expression and metabolism in cultured-rice cells is discussed.

Key words: DNA microarray, phytoalexin, rice suspension-cultured cells, two-pore channel 1 (TPC1), xylanase elicitor.

Ethylene-inducing xylanase (EIX) is a potent elicitor to induce immune responses including ethylene biosynthesis, electrolyte leakage, alkalinization of extracellular media, expression of pathogenesis-related (PR) proteins and hypersensitive cell death in a variety of plant species such as tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*), Arabidopsis as well as rice (Avni et al. 1994; Bailey et al. 1990; Bar et al. 2010; Benschop et al. 2007; Kurusu et al. 2005; Suzuki et al. 1999). Along with the induction of the defense responses, it also induces retardation in root growth (Kawaguchi et al. 2012).

A fungal EIX from *Trichoderma viride* (TvX) induces increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$), leading to the activation of phytoalexin biosynthesis and synchronous hypersensitive cell death in suspension-cultured rice cells (Hamada et al. 2012). Xylanase activity itself does not appear to be required for the induction of the immune responses (Kurusu et al. 2005). Pharmacological analyses with Ca^{2+} channel inhibitors suggest importance of channel-mediated changes in $[Ca^{2+}]_{cyt}$ in the induction of TvX-induced responses (Hamada et al. 2012; Kurusu et al. 2005). Thus TvX-triggered immune responses in rice cultured cells provide a model system for understanding the roles of Ca^{2+} signals/channels in defense responses including hypersensitive cell death.

Activated Ca^{2+}-permeable channels localized at the plasma membrane (PM) and endomembranes such as the tonoplast induce cytosolic Ca^{2+} rise, and

Abbreviations: $[Ca^{2+}]_{cyt}$, cytosolic free Ca^{2+} concentration; CPS, *ent*-copalyl diphosphate synthase; Cy3, cyanin-3; Cy5, cyanin-5; DXR, DXP reductoisomerase; DXS, DXP synthase; EIX, ethylene-inducing xylanase; GGDP, geranylgeranyl diphosphate; GGPS, GGDP synthase; HDR, HMBDP reductase; HDS, HMBDP synthase; KSL, *ent*-kaurene synthase-like; MCS, MECDP synthase; MEP, methylerythritol phosphate; PAMPs/MAMPs/DAMPs, pathogen/microbe/damage-associated molecular patterns; PM, plasma membrane; TPC, two pore channel; TvX, EIX from *Trichoderma viride*.

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consequently form the spatiotemporal patterns of $[Ca^{2+}]_{cyt}$ (Jammes et al. 2011). While homologs of many major animal Ca^{2+}-permeable channels have not been found in plants, the two-pore channel (TPC) family, which are homologous to the α1 subunit of vertebrate voltage-dependent Ca^{2+} channels, are found in most plant species as well as in animals (Jammes et al. 2011; Kuchitsu et al. 2005). AtTPC1 from Arabidopsis shows a slow-activating vacuolar cation channel activity (Dadacz-Narloch et al. 2011; Hedrich and Marten 2011; Peiter et al. 2005), and has been shown to be involved in sucrose-induced Ca^{2+} rise (Furuichi et al. 2001), abscisic acid-induced repression of germination (Islam et al. 2010) and stomatal response to extracellular Ca^{2+} changes (Islam et al. 2010; Peiter et al. 2005). Tobacco NtTPC1s have roles in $[Ca^{2+}]_{cyt}$ rise and regulation of defense-related gene expression as well as hypersensitive cell death triggered by cryptogein, an elicitor from an oomycete, in tobacco BY-2 cells (Kadota et al. 2004; Kadota et al. 2005). Monocot TPC1s from rice and wheat appear to function in responses to abiotic stresses (Kurusu et al. 2004; Wang et al. 2005). They exhibit slow-activating vacuolar cation channel activity and are localized at least in part to the tonoplast (Dadacz-Narloch et al. 2013; Kurusu et al. 2012). In contrast, membrane fractionation by two-phase partitioning and immunoblot analyses revealed that OsTPC1 is localized predominantly at the PM in rice cultured cells (Hamada et al. 2012; Hashimoto et al. 2005). OsTPC1 expressed in HEK293T cells shows Ca^{2+}-permeable channel activity (Hamada et al. 2012). Unknown components that interact with TPC channels may affect their intracellular localization and properties.

OsTPC1 plays a role in the regulation of TvX-induced $[Ca^{2+}]_{cyt}$ rise leading to the activation of phytoalexin biosynthesis and hypersensitive cell death in cultured cells (Hamada et al. 2012; Kurusu et al. 2005). On the other hand, $[Ca^{2+}]_{cyt}$ rise induced by chitin fragments (*N*-acetylchitooligosaccharides) shows distinct kinetics from that induced by TvX, and was not affected in the *Ostpc1*-knockout mutant cells (Hamada et al. 2012). Similarly, flg22-induced $[Ca^{2+}]_{cyt}$ rise was not affected in the *attpc1*-knockout mutant in Arabidopsis (Ranf et al. 2008). These imply that the TPC family may not be important in the signal transduction of general pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) such as chitin fragments and flg22, but may be one of multiple Ca^{2+}-permeable channels activated by some specific elicitors. Little is known, however, on the molecular links between OsTPC1 and Ca^{2+}-mediated signaling and TvX-induced gene expression. A series of microarray analyses have been performed in rice and revealed some key regulators to activate immune responses (Kaneda et al. 2009; Shimono et al. 2007). To investigate the involvement of OsTPC1 in the TvX-induced gene expression at various stages, we performed comprehensive DNA microarray analysis using the rice 44K oligo-microarrays (Agilent Technologies, Palo Alto, CA, USA), and identified a number of TvX-induced genes controlled by OsTPC1.

Total RNA was isolated from the wild-type and *Ostpc1* mutant (Kurusu et al. 2004) rice cultured cells treated with TvX (30 μg ml^{-1}; Sigma, St. Louis, MO, USA) for 0, 0.5, 2, 6, and 12 h using the RNeasy Plant Mini Kit (QIAGEN, Maryland, USA) according to the manufacturer's protocol. Cyanin-3 (Cy3) or cyanin-5 (Cy5)-labeled cRNAs were prepared using the Quick Amp Labeling Kit, Two-Color (Agilent Technologies) according to the manufacturer's protocol, and purified by the RNeasy Plant Mini Kit (QIAGEN). A total of 825 ng Cy3 or Cy5-labeled cRNA was fragmented and hybridized on a slide of rice 4×44K microarray (Agilent Technologies) at 65°C for 17 h. After hybridization, glass slides were washed in 6×SSC/0.005% Triton X-102 for 10 min at 24°C in the dark, followed by washing in 0.5×SSC/0.005% Triton X-102 for 5 min at 4°C. Slides were scanned on an Agilent G2505B DNA microarray scanner, and background correction of Cy3 and Cy5 raw signals was performed using the FEATURE EXTRACTION 10.5.1.1 (Agilent Technologies). To confirm that the mutant phenotype was due to the functional knockout of OsTPC1, we performed a comparative transcriptomic analysis as a complementation test using total RNA isolated from rice cultured cells of TvX-treated *Ostpc1*/*OsTPC1* and *Ostpc1*/*GUS* control lines (Kurusu et al. 2005) for 0, 2, 6, and 12 h. Each value represents the ratio of TvX-treated wild-type cells or complementation cell line (*Ostpc1*/*OsTPC1*) at 0 h.

For real-time RT-PCR analysis, total RNA was isolated from rice cultured cells using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized from 3 μg RNA with an oligo-dT primer and reverse transcriptase (Invitrogen). Real-time PCR was performed using an ABI PRISM 7300 sequence detection system (Applied Biosystems Instruments, Foster City, CA, USA) with SYBR Green real-time PCR Master Mix (Toyobo, Osaka, Japan) and gene specific primers; *OsActin1*-F, 5′-TGC ACA GGA AAT GCT TCT AAT TCT T-3′ and *OsActin1*-R, 5′-ACG GCG ATA ACA GCT CCT CTT-3′; *OsDXS3*-F, 5′-GCT TCC AAA TGC AAA ATG ATA ATG-3′ and *OsDXS3*-R, 5′-GCA TGC CCC ATC TTC ATG TC-3′; *OsDXR*-F, 5′-TTG CAA GCT GGG TTC ACT GA-3′ and *OsDXR*-R, 5′-GAT CCA TCG ACG GGT ATT TCA-3′; *OsMCS*-F, 5′-GCT GAT TTC GGA TAC GTT GGA-3′ and *OsMCS*-R, 5′-GAC GGT CTT GCC AAC ATA AAG G-3′; *OsHDS*-F, 5′-TCA TCT GCC AGG CGT CTC TA-3′ and *OsHDS*-R, 5′-TCC CCT GGC CCA TTG AC-3′; *OsHDR*-F, 5′-CTG ATG GCT TGG TGA AGG TT-3′ and *OsHDR*-R, 5′-CAG CAC ATG CCG

Table 1. Representative data of differentially expressed transcripts between the wild-type and *Ostpc1* knockout cells after TvX treatment.

Accession	Description	Symbol	Wild-type					*Ostpc1*					*Ostpc1*/*OsTPC1*				*Ostpc1*/*GUS*			
			0	0.5	2	6	12	0	0.5	2	6	12 (h)	0	2	6	12	0	2	6	12 (h)
MEP pathway																				
AK100909	1-deoxy-D-xylulose 5-phosphate synthase 2 precursor	*OsDXS3*	1.00	1.12	2.01	29.10	33.87	1.41	0.93	2.02	16.13	11.64	1.00	2.35	53.67	55.51	0.82	2.93	34.17	31.98
AK105762	Isoprenoid biosynthesis-like protein	*OsHDS*	1.00	1.18	2.33	14.22	12.49	1.33	1.19	2.15	10.01	5.23	1.00	3.93	18.21	12.12	0.61	3.76	10.37	6.68
AK059692	1-deoxy-D-xylulose 5-phosphate reductoisomerase	*OsDXR*	1.00	0.96	3.37	23.95	21.65	1.43	1.96	3.37	15.26	14.16	1.00	4.54	23.31	13.77	1.52	4.65	18.69	12.83
AK060238	2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase	*OsMCS*	1.00	1.05	4.11	9.73	10.58	1.13	1.33	3.15	5.35	6.18	1.00	4.54	8.91	7.87	0.61	3.21	6.83	6.16
AK062113	Isopentenyl/dimethylallyl diphosphate synthase	*OsHDR*	1.00	0.85	2.38	9.26	9.17	1.30	1.25	2.45	6.30	5.68	1.00	3.07	11.34	7.33	0.85	3.19	8.34	6.35
Phytoalexin biosynthesis																				
AK120768	GGDP synthase	*GGPS*	1.00	0.79	4.71	14.88	11.15	1.38	0.93	3.36	8.11	4.67	1.00	6.37	27.00	16.15	0.61	5.04	12.65	9.04
AY347880	Terpene synthase	*OsKSL4*	1.00	1.43	15.80	79.94	57.71	2.43	1.92	21.94	51.48	19.32	1.00	21.30	79.16	44.53	0.26	11.10	43.97	22.51
AK071546	Cytochrome P450	*CYP99A2*	1.00	1.02	6.99	49.70	28.72	2.59	1.59	6.31	24.94	12.45	1.00	14.34	162.30	74.31	0.86	15.79	97.07	47.58
AK120852	Momilactone A synthase	*OsMAS*	1.00	1.26	7.83	52.26	36.07	2.03	2.69	8.78	25.49	17.54	1.00	8.18	36.16	19.26	0.31	6.24	20.85	13.21
AK100631	Copalyl diphosphate synthetase	*OsCPS4*	1.00	1.15	3.80	12.72	12.03	1.28	1.12	3.33	11.19	8.84	1.00	5.99	16.86	15.18	0.69	4.69	9.76	9.24
AK068310	Terpene synthase, metal-binding domain containing protein	*OsKSL7*	1.00	0.97	4.17	50.57	63.77	0.96	1.30	3.06	29.22	26.10	1.00	2.79	60.75	47.26	0.81	3.13	39.12	44.65
Defense-related																				
AK063697	WRKY transcription factor 45	*OsWRKY45*	1.00	4.12	2.43	3.28	1.60	0.51	2.01	1.14	1.83	2.35	1.00	1.38	2.71	0.92	0.41	0.90	0.83	1.04
AK121190	WRKY transcription factor 53 (Transcription factor WRKY12)	*OsWRKY53*	1.00	6.47	5.41	2.79	1.87	0.77	1.47	2.52	2.15	0.84	1.00	5.76	3.53	2.95	0.82	3.40	2.09	1.51
AY676927	WRKY transcription factor 71 (Transcription factor WRKY09)	*OsWRKY71*	1.00	4.69	2.92	1.80	1.30	0.84	1.88	1.92	1.39	1.20	1.00	2.43	2.00	1.39	0.85	1.50	1.46	1.16
AK100934	Hydrolase	*hsr203J-like*	1.00	3.11	5.91	1.46	1.19	0.88	1.21	1.94	1.24	0.78	1.00	7.31	2.70	1.76	0.57	3.04	0.79	0.75

The relative expression level of each gene in the wild-type or the complementation line (*Ostpc1*/*OsTPC1*) at time 0 was standardized as 1.

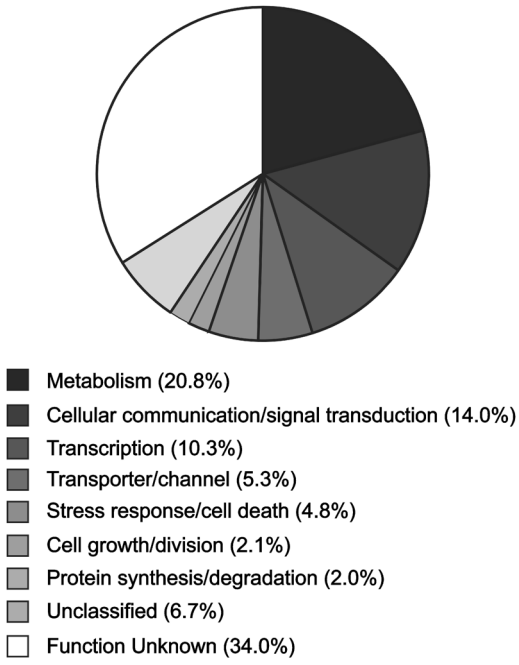

Figure 1. Gene ontology analysis of TvX-induced genes controlled by OsTPC1. Classification of genes whose expression differed by more than two-fold in wild-type and *Ostpc1* after TvX (30 μg ml^{-1}) treatment.

TAG TAT GC-3′. Relative mRNA abundances were calculated using the standard curve method and normalized to corresponding *OsActin1* mRNA levels.

We identified a total of 4,480 up-regulated probes (p-value log ratio <0.01; fold change >2) at each time point after elicitation by TvX in the wild type cells. Among them, 1,472 probes were suppressed less than twice at any time point in *Ostpc1* cells. Moreover, the expression of 1,140 out of 1,472 probes (77%) was restored more than 1.5-fold by introducing the wild type *OsTPC1* gene (Supplemental Table 1), suggesting possible involvement of OsTPC1 in the expression of these genes. Gene ontology analysis showed that OsTPC1 affects a variety of TvX-induced gene expression (Figure 1 and Supplemental Tables 1–3), such as a number of WRKY-type transcription factors including OsWRKY45, 53, and 71 that have been shown to be involved in defense responses against pathogens (Chujo et al. 2007; Liu et al. 2007; Shimono et al. 2007), was affected by the disruption of OsTPC1 (Table 1). This suggests that OsTPC1-mediated $[Ca^{2+}]_{cyt}$ rise may take part in the regulation of expression of transcription factors leading to the activation of various immune responses.

Hypersensitivity-related (*hsr*) gene, *Hsr203J*, that encodes a serine hydrolase with esterase activity, is postulated to regulate either the establishment or limitation of cell death in tobacco (Pontier et al. 1998). The expression of a rice *hsr203J* gene (Accession No. AK100934) was induced 0.5 h after the application of TvX in the wild-type cells, and the induced expression was severely suppressed in *Ostpc1* cells (Table 1). This result

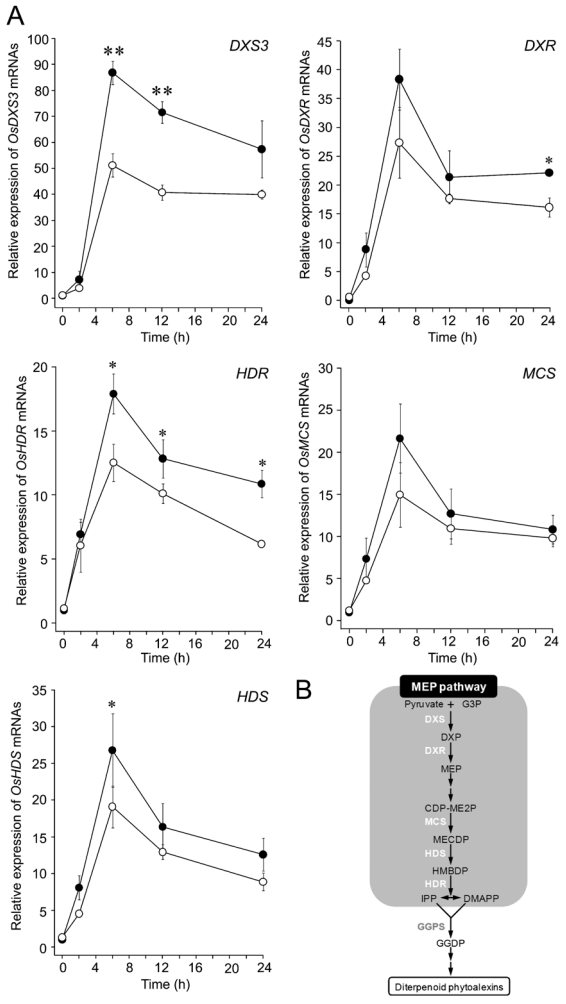

Figure 2. Expression levels of MEP pathway genes after TvX treatment in wild-type and *Ostpc1* cells. (A) Relative mRNA levels of *OsDXS3*, *OsDXR*, *OsMCS*, *OsHDS*, and *OsHDR* in the wild-type (solid circle) and *Ostpc1* (open circle) cells 0, 2, 6, 12, and 24 h after TvX treatment (30 μg ml^{-1}). mRNA levels were determined using real-time quantitative PCR. Average values and standard errors of three experiments for each line are shown. * $p<0.05$; ** $p<0.005$, significantly different from wild-type. (B) The MEP pathway genes induced by TvX-induced and affected by OsTPC1. G3P, glyceraldehydes-3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; CDP-ME2P, 2-phospho-4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol; MECDP, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate; HMBDP, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGDP, geranylgeranyl diphosphate; DXS, DXP synthase; DXR, DXP reductoisomerase; MCS, MECDP synthase; HDS, HMBDP synthase; HDR, HMBDP reductase; GGPS, GGDP synthase.

is consistent with a previous report and confirms that OsTPC1 plays a role in the regulation of TvX-induced hypersensitive cell death (Kurusu et al. 2005).

Plant defense reactions against pathogen infection include the synthesis and accumulation of low-molecular antimicrobial substances, known as phytoalexins (Ahuja et al. 2012; Okada et al. 2009; Umemura et al. 2002). 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 major phytoalexins, momilactones and phytocassanes (Okada et al. 2009). Induced expression of momilactone biosynthetic genes (*OsKSL4*, *CYP99A2* and *OsMAS*) and some phytocassane biosynthetic genes (*OsCPS4* and *OsKSL7*) were partially suppressed in *Ostpc1* cells (Table 1). These results are consistent with the previous report and confirm that OsTPC1 plays a role in the regulation of TvX-induced phytoalexin biosynthesis (Hamada et al. 2012).

The methylerythritol phosphate (MEP) pathway is required for the biosynthesis of geranylgeranyl diphosphate (GGDP), which is an early precursor of diterpenoid phytoalexins such as momilactones and phytocassanes in rice (Okada et al. 2007; Okada 2011). TvX-induced expression of genes for a GGDP synthase (*GGPS*) as well as for the MEP pathway, such as *OsDXS3* (*DXP synthase3*) was significantly suppressed in *Ostpc1* in comparison with the wild type (Table 1). The induction of other MEP pathway genes such as *OsHDS* (*HMBDP synthase*), *OsDXR* (*DXP reductoisomerase*), *OsMCS* (*MECDP synthase*) and *OsHDR* (*HMBDP reductase*) was also partially reduced in *Ostpc1* relative to that in the wild type. In particular, OsDXS3 and OsDXR have been suggested to be functional enzymes that catalyze the first two steps of the MEP pathway, and the application of ketoclomazone and fosmidomycin, inhibitors of DXS and DXR, respectively, represses the accumulation of diterpene-type phytoalexins induced by elicitors in rice cells (Okada et al. 2007). Real-time RT-PCR analysis revealed that the expression of these genes was induced by TvX, which was significantly suppressed in *Ostpc1* cells (Figure 2A). Moreover, partial reduction of other TvX-induced MEP pathway genes was also confirmed by real-time RT-PCR analysis (Figure 2A). These results suggest that OsTPC1 may be involved in signaling to activate the MEP pathway (Figure 2B) by supplying the precursor of diterpenoid phytoalexins in defense responses.

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