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RESEARCH PAPER

Identification of rice purple acid phosphatases related to posphate starvation signalling

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ABSTRACT

Purple acid phosphatases (PAPs) are a family of metallo-phosphoesterases involved in a variety of physiological functions, especially phosphate deficiency adaptations in plants. We identified 26 putative PAP genes by a genome-wide analysis of rice (*Oryza sativa*), 24 of which have isolated EST sequences in the dbEST database. Amino acid sequence analysis revealed that 25 of these genes possess sets of metal-ligating residues typical of known PAPs. Phylogenetic analysis classified the 26 rice and 29 *Arabidopsis* PAPs into three main groups and seven subgroups. We detected transcripts of 21 PAP genes in roots or leaves of rice seedlings. The expression levels of ten PAP genes were up-regulated by both phosphate deprivation and over-expression of the transcription factor *OsPHR2*. These PAP genes all contained one or two *OsPHR2* binding elements in their promoter regions, implying that they are directly regulated by *OsPHR2*. Both acid phosphatase (AP) and surface secretory acid phosphatase (SAP) activity assays showed that the up-regulation of PAPs by Pi starvation, *OsPHR2* over-expression, *PHO2* knockout or *OsSPX1* RNA interference led to an increase in AP and SAP activity in rice roots. This study reveals the potential for developing technologies for crop improvement in phosphorus use efficiency.

INTRODUCTION

Purple acid phosphatases (PAPs), a family of binuclear metal-containing acid hydrolases, have been identified in animals, plants and some bacterial species (Schenk *et al.* 2000a,b; Olczak *et al.* 2003). PAPs have a conserved motif in their carboxyl end, including seven invariant amino acid residues in five blocks (**D**XG/G**D**XX**Y**/G**N**H(D/E)/VXX**H**/G**H**X**H**; bold letters represent metal-ligating residues) (Li *et al.* 2002). These seven conserved residues are involved in coordinating the bimetal nuclear centre, which is Fe(III)–Fe(II) in mammalian PAPs and Fe(III)–Mn(II) or Fe(III)–Zn(II) in plant PAPs (Klabunde *et al.* 1995; Strater *et al.* 1995; Schenk *et al.* 1999). The purple colour in the purified PAPs is caused by a charge transfer transition at 560 nm from the metal-coordinating tyrosine residue to the metal ligand Fe(III).

The majority of plants PAPs catalyse the hydrolysis of phosphoric acid esters and anhydrides, displaying broad and non-specific acid phosphatase activity. Physiological and molecular investigations suggest that plant PAPs are multi-functional proteins. Under Pi-limited conditions, both the transcript abundance and the protein levels of plant PAPs accumulate with significantly increased intracellular and secretory acid phosphatase activity (del Pozo *et al.* 1999; Bozzo *et al.* 2002, 2006; Wu *et al.* 2003). The enhanced acid phosphatase activity plays an important role in phosphate (Pi) acquisition and recycling by plants (Ticconi & Abel 2004; Tomscha *et al.* 2004; Veljanovski *et al.* 2006). In addition to their function for Pi homeostasis, several PAPs have also been shown to have peroxidation activity, suggesting that they could be involved in the metabolism of reactive oxygen species (del Pozo *et al.* 1999; Bozzo *et al.* 2004; Veljanovski *et al.* 2006). Consistent with this hypothesis, the expression of *AtPAP17* (*AtACP5*) and *GmPAP3* is induced by salt and oxidative stress (del Pozo *et al.* 1999; Liao *et al.* 2003). *AtPAP15* was shown to have phytase activity and regulates foliar ascorbate synthesis, which alters plant resistance to salt and oxidative stress (Zhang *et al.* 2008a,b). In addition, PAPs also have a role in plant growth and development under normal growth conditions. Several *Arabidopsis* PAPs are predominantly expressed in flowers, suggesting their potential function in flower development (Zhu *et al.* 2005). Recently, a wall-bound PAP was shown to be involved in regulation of cell wall biosynthesis by activation of β-glucan synthases (Kaida *et al.* 2008, 2009).

Phosphorus (P) is one of the most important macronutrients for plant growth and development (Maathuis 2009). Plants have evolved a wide range of adaptive strategies for P deficiency, including remobilisation of Pi from organic P resources by acid phosphatases (Vance *et al.* 2003; Ticconi & Abel 2004). Several key regulators of Pi starvation signalling have been reported. For example, *Arabidopsis* phosphate response 1 (*AtPHR1*) and its homologue *OsPHR2* are transcription factors for the positive regulation of a subset of phosphate starvation-induced (PSI) genes, including the

best-studied PAP gene, *AtPAP17* (Rubio *et al.* 2001; Zhou *et al.* 2008). *PHO2*, an E2 ubiquitin-conjugating enzyme (*UBC24*) downstream of *PHR1* (phosphate starvation response 1), negatively regulates Pi remobilisation and uptake (Aung *et al.* 2006; Bari *et al.* 2006; Chiou *et al.* 2006). Recently, *OsSPX1* was identified as a suppressor of Pi starvation signalling, forming a feedback loop with *OsPHR2* (Duan *et al.* 2008; Wang *et al.* 2009a). Studies have shown that the expression of PAP genes increases during P starvation (Bozzo *et al.* 2006; Veljanovski *et al.* 2006; Tran & Plaxton 2008). However, induced PAP proteins or activities are not always correlated with an increase of PAP transcripts (Tomscha *et al.* 2004; Veljanovski *et al.* 2006). How the P starvation signal regulates the expression of the individual PAPs, and how many of these PAPs are regulated by the Pi starvation signal remain largely unknown.

The genome of higher plants generally includes numerous PAP genes. Multiple PAP genes were identified in *Arabidopsis*, canola, sweet potato, soybean and red kidney bean based on conserved structural features (Schenk *et al.* 2000a; Li *et al.* 2002; Lu *et al.* 2008). Rice is the most important cereal crop and is used to feed more than half of the world's population (Miyao *et al.* 2007). Compared with the extensive studies on *Arabidopsis* and tomato PAP genes (Li *et al.* 2002; Bozzo *et al.* 2006), little has been reported about the gene number, structure, expression or regulation of rice PAPs. Molecular dissection of the rice PAP gene family would help to unravel the phosphate limitation adaptation mechanism in rice. In this study, we identified 26 rice PAP genes from *O. japonica* genomes and performed bioinformatic analyses on their full-length cDNA support, phylogenetic relationships and protein motifs. The expression patterns of these PAPs in wild-type rice (Nipponbare), a *pho2* mutant, an *OsPHR2* over-expression line (*PHR2-O*) and an *OsSPX1* RNA interference line (*SPX1-Ri*) were analysed. Our results show that ten of the 26 *PAP* genes are responsive to Pi starvation. All of their promoters contain binding sites for *OsPHR2* and therefore are controlled by the *OsPHR2*-mediated Pi starvation signalling pathway.

MATERIALS AND METHODS

Plant material and growth conditions

The japonica rice variety Nipponbare was used for all experiments. Hydroponic experiments were performed using a normal rice culture solution containing 1.425 mM NH_4NO_3, 0.323 mM NaH_2PO_4, 0.513 mM K_2SO_4, 0.998 mM $CaCl_2$, 1.643 mM $MgSO_4$, 0.009 mM $MnCl_2$, 0.075 μM $(NH_4)_6Mo_7O_{24}$, 0.019 mM H_3BO_3, 0.155 μM $CuSO_4$, 0.036 mM $FeCl_3$, 0.070 mM citric acid and 0.152 μM $ZnSO_4$ (Yoshida *et al.* 1976). Rice plants were grown in growth chambers with a 12-h photoperiod (200 μmol photons $m^{-2}{\cdot}s^{-1}$) and a day/night temperature of 30/22 °C after germination, as previously reported (Wang *et al.* 2009b). For the no Pi (−P) treatment, NaH_2PO_4 was omitted.

The seeds of wild-type (WT) plants, an *OsPHO2* loss-of-function mutant (*pho2*) (Wang *et al.* 2009a), an *OsPHR2*-over-expression line (*PHR2-O*) (Zhou *et al.* 2008) and an *OsSPX1*-interference line (*SPX1-Ri*) (Wang *et al.* 2009a) were germinated and grown in a normal culture solution for 10 days. The 10-day-old seedlings were then transferred to a hydroponic medium with (+P) or without (−P) 0.323 mM phosphate for 7 days, after which RNA sampling and root surface secretory acid phosphatase (SAP) activity analyses were performed.

Identification of PAP genes in the rice genome and gene annotation

Blast searches using the amino acid sequences of *AtPAP10* from *Arabidopsis* were conducted at the TIGR rice genomic Blastp website (http://rice.plantbiology.msu.edu/) and the International Rice Genome Sequencing Project (IRGSP; http://rgp.dna.affrc.go.jp) using the default settings. Fifteen different PAPs were identified in the rice genome. A second search using these 15 rice PAPs was performed at the same web pages, which resulted in several additional predicted rice PAPs. The Blast search was based on two criteria: the sequences had to be at least 20% identical to the query sequence and contain the conserved sequence motif (**D***X*G/G**D***XX***Y**/G**N**H(E/D)/V*X*2**H**/G**H***X***H**) described previously for *Arabidopsis* PAPs (Li *et al.* 2002). Eleven additional putative rice PAPs were found in this step. These newly identified 11 rice PAPs were each used as the query sequences for the final Blastp search at the above websites, which resulted in no additional hits. The full-length cDNA sequences of the 26 predicted PAP genes and 1 kb DNA sequences up-stream of the start codon were obtained from the Knowledge-Based *Oryza* Molecular Biological Encyclopedia (KOME; http://cdna01.dna.affrc.go.jp/cDNA/) and GenBank (http://www.ncbi.nlm.nih.gov). Expressed sequence tag (EST) sequences of all rice PAPs were collected from the dbEST database (http://www.ncbi.nlm.nih.gov/dbEST/). Promoters were analysed using DNASTAR (DNASTAR, Inc., Madison, WI, USA) to search for the imperfect palindromic sequence (P1BS element: GNATATNC) that is the *AtPHR1*-binding *cis*-element (Rubio *et al.* 2001).

Phylogenetic analysis

The phylogenetic tree of PAPs was constructed using a previously described method (Flanagan & Huber 2007). The full-length amino acid sequences of rice and *Arabidopsis* PAPs were aligned using ClustalX (Thompson *et al.* 1997). We then modified the amino acid sequences by splicing the conserved regions, according to results from the first round of multiple alignments, to avoid misalignments derived from substantial sequence differences between rice and *Arabidopsis* PAPs. The modified amino acid sequences were then used for a second round of multiple alignments. Based on the results from the second round multiple alignments, a neighbour-joining tree with 1000 bootstrap replicates was constructed and visualised using TREEVIEW (Zhang *et al.* 2008a,b).

Semi-quantitative reverse transcription PCR and quantitative real-time PCR

Total RNA was extracted from the shoots and roots of rice plants using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was synthesised from 5 μg of DNase I-treated total RNA using SuperScript II reverse transcriptase (Invitrogen). Semi-quantitative reverse transcription PCR (sqRT-PCR) was performed using gene-specific primers designed using

OligoPerfectTM (http://www.invitrogen.com/oligos). All primers for the RT-PCR are listed in Table S1. The sqRT-PCR conditions were as follows: initial denaturing at 94 °C for 4 min followed by 28–36 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 25 s, followed by a final extension period for 2 min at 72 °C. Quantitative real-time PCR (qRT-PCR) was performed using the FastStart DNA Master SYBR Green I kit (Takara Biotechniques, Dalian, China) on a LightCycler480 machine (Roche Diagnostics, Basel, Switzerland) following the manufacturer's instructions. The amplification programme for SYBR Green I consisted of 94 °C for 10 s, 58 °C for 10 s, and 72 °C for 10 s. Triplicate quantitative assays were performed on each cDNA sample. The amplification of actin cDNA was used as an endogenous control to normalise the samples.

Measurement of soluble Pi concentrations in plants

Leaves and roots of the WT and transgenic seedlings from either Pi-sufficient or Pi-deficient treatments were sampled separately. The soluble Pi concentration was measured using the procedure described previously (Wang *et al.* 2009b). Briefly, 50 μg fresh samples were homogenised with 50 μl 5 M H_2SO_4 and 3 ml H_2O. The homogenate was transferred to 1.5-ml tubes and centrifuged at 10000*g* for 10 min at 4 °C. The supernatant was collected and diluted to an appropriate concentration. The diluted supernatant was mixed with malachite green reagent in a 3:1 ratio and analysed 30 min later. The absorption values for the solution at 650 nm were determined using a Spectroquant NOVA60 spectrophotometer (Merck, Darmstadt, Germany). Pi concentration was calculated from a standard curve generated with varying concentrations of KH_2PO_4.

Protein extraction and APase activity assays

Leaf samples were ground to a powder in liquid N_2, and homogenised in ice-cold extraction buffer (100 mM potassium acetate, pH 5.5, 20 mM $CaCl_2$, 2 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulphonyl fluoride, 1.5% (w/v) polyvinylpolypyrrolidone) with a small scoop of acid-washed sand. Samples were centrifuged at 14,000 *g* and 4 °C for 20 min, the supernatant was then removed to a fresh tube. Protein content was quantified using Coomassie Plus protein assay reagent and stored at −80 °C.

To measure specific APase activity, 1 μg protein was added to 600 μl prewarmed 10 mM *p*-nitrophenyl phosphate (pNPP) in 50 mM sodium acetate, pH 5.5. Reactions proceeded for 10 min at 25 °C, were stopped with 1.2 ml 1 N NaOH. and pNP accumulation was read at A_{412} and converted to nanomoles by plotting values against a pNP standard curve generated with assay reagents. Phosphatase activity was expressed as nmol substrate hydrolysed per μg of soluble protein per min (Tomscha *et al.* 2004).

Root surface secretory acid phosphatase (SAP) activity staining

Root surface SAP activity was detected as described by Bozzo *et al.* (2006). The 10-day-old seedlings grown hydroponically were transferred to a culture solution in the presence or absence of Pi. After a 7-day treatment, roots of the seedlings were excised and incubated with a 5-bromo-4-chloro-3--indolyl-phosphate (BCIP)–agar overlay solution containing 50 mM Na-acetate, pH 5.3, 10 mM $MgCl_2$, 0.6% (w/v) agar and 0.1 $mg \cdot ml^{-1}$ BCIP for 2 h at 25 °C for visualisation of root SAP activity. The BCIP solution is a colourless acid phosphatase substrate, and the root SAP activity is visualised as a blue colour, resulting from the hydrolysis of BCIP.

RESULTS

Identification of PAP genes in the rice genome and motif analysis

A total of 26 putative PAP genes were identified in the rice genome (Table 1). Twenty-four of these were found in the EST database, except *OsPAP27c* and *OsPAP21c*, indicating that most of the PAP genes are expressed proteins (Table 1). An additional search of the KOME database revealed that 22 of the PAP genes had full-length cDNA (Table 1).

Sequence analysis indicated that 25 of the rice PAPs contained all five blocks of amino acid sequences and the seven invariant residues involved in ligation of the dimetal nuclear centre characteristic of known PAPs (Table 2). The remaining PAP protein (OsPAP21c) lacks the fourth block, VXX**H**, and its conserved residues in the fifth block, G**H**X**H**, were replaced by residue I (Table 2, shaded gene). The gene was still considered a putative PAP gene because its overall amino acid sequence displayed significant levels of homology to known PAPs. SignalP 3.0 predicted that 24 rice PAP proteins contain a signal peptide, and TargetP 1.1 predicted 23 as secretory proteins. TMpred and NetNGlyc 1.0 predictions suggested that 24 PAP proteins had a strong N-terminal trans-membrane helix and several N-glycosylation sites (Table 2).

Phylogenetic analysis and nomenclature of rice PAP genes

A neighbour-joining phylogenetic tree was constructed based on multiple alignments of the conserved amino acid sequences of both rice and *Arabidopsis* PAPs (Fig. 1). Similar to the previous analysis in *Arabidopsis* (Li *et al.* 2002), the 55 PAP proteins were divided into three major groups (group I, II and III), which were further classified into seven subgroups (subgroup Ia, Ib, Ic, IIa, IIb, IIIa and IIIb), each with more than 95% bootstrap support (Fig. 1). Twenty-six rice PAPs were divided into six subgroups (except subgroup IIIa) and were mixed with the *Arabidopsis* PAPs, suggesting a close association between PAPs from different species. The rice PAP genes were named after their closest *Arabidopsis* homologues. The 26 rice PAPs were classified into 12 sets: OsPAP1, OsPAP3, OsPAP7, OsPAP9, OsPAP10, OsPAP15, OsPAP18, OsPAP20, OsPAP21, OsPAP23, OsPAP26 and OsPAP27. The individual gene within each set was named by adding additional letters (a, b, c or d) at the end of the set name (Table 1; Fig. 1).

Responses of rice PAP genes to phosphate deprivation

To determine the transcriptional responses of the rice PAPs to Pi starvation, 10-day-old seedlings were transferred into culture solution lacking Pi for 7 days. Seven days of Pi starvation resulted in a significantly decreased Pi

Table 1. General information about the 26 *OsPAP* genes and the corresponding deduced OsPAP proteins in rice.

gene loci	name	BAC/PAC clone	accession No. of cDNA	protein accession No.	total EST	ORF (aa)	predicted Size (kDa)
Os03g11530	OsPAP1a	AC135158	AK101976	NP_001049368	80	615	68.1
Os12g38770	OsPAP1b	AP008218	–	ABA99053	7	564	63.7
Os12g38760	OsPAP1c	AP008218	AK073512	NP_001067110	45	611	68.9
Os12g38750	OsPAP1d	AP008218	AK121432	NP_001067109	8	607	68.4
Os11g34710	OsPAP3a	AC151599	AK069660	ABA94167	4	294	32.8
Os10g02750	OsPAP3b	AC145127	AK110804	NP_001064054	18	335	37.4
Os03g13540	OsPAP3c	AC118981	AK070684	NP_001049500	130	339	38.3
Os11g34720	OsPAP7	AC151599	AK106004	ABA94168	49	330	36.2
Os07g02090	OsPAP9a	AP003746	AK071643	NP_001058738	80	653	73.7
Os01g58640	OsPAP9b	AP003344	AK100372	NP_001044534	25	630	68.2
Os01g56880	OsPAP10a	AP003293	AK066643	NP_001044416	86	465	53.0
Os12g44010	OsPAP10b	AP008218	AK119972	EEE53671	69	462	52.0
Os12g44020	OsPAP10c	AP008218	AK058368	NP_001067369	187	463	52.1
Os12g44030	OsPAP10d	AP008218	AK241534	ABA99980	10	337	38.7
Os03g63074	OsPAP15	AC099324	AK065415	ABF99890	5	1025	119.4
Os03g37130	OsPAP18	AC084319	AK060200	NP_001050513	35	470	52.5
Os11g05410	OsPAP20a	AC116949	–	AAX94838	15	439	21.1
Os12g05540	OsPAP20b	AP008218	AK107301	NP_001066169	19	445	48.4
Os04g33530	OsPAP21a	AP008210	AK121649	NP_001052730	10	452	49.8
Os11g05400	OsPAP21b	AC116949	AK073222	AAX94837	22	549	61.9
Os12g05510	OsPAP21c	AP008218	–	NP_001066168	0	390	42.5
Os08g17784	OsPAP23	AP004556	AK072150	NP_001061440	5	335	68.2
Os06g43640	OsPAP26	AP003568	AK061635	NP_001058182	78	476	55.1
Os09g32840	OsPAP27a	AC108759	AK102346	NP_001063606	48	630	69.3
Os08g41880	OsPAP27b	AP004015	AK072408	NP_001062330	92	623	69.9
Os09g32830	OsPAP27c	AC108759	–	EAZ09673	0	651	69.9

Table 2. Domain organisation of the 26 OsPAPs.

name	motif					signal peptide (length, cleave site)	number of N-glycosylation site	[b]subcellular location	N-terminal transmembrane helix (length, location)
OsPAPla	GDMG	GDICY	GNHE	FLAH	GHVH	23, VAA–AG	2	S	21, Y5–Y25
OsPAPlb	GDMG	GDLSY	GNHE	FLAH	GHIH	28, ARG–DE	2	S	24, Y3–Y26
OsPAPlc	GDMG	GDICY	GNHE	FLAH	GHVH	17, AAA–AV	4	S	18, Y2–Y19
OsPAPld	GDMG	GDICY	GNHE	FLAH	GHVH	16, CAA–AH	5	S	18, Y2–Y19
OsPAP3a	GDWG	GDNFY	GNHD	VVGH	GHDH	22, AAA–EM	1	S	23, Y3–Y25
OsPAP3b	GDWG	GDNFL	GNHD	AVGH	GHDH	23, ATA–EL	1	S	17, Y1–Y17
OsPAP3c	GDWG	GDNFY	GNHD	AVGH	GHDH	28, ATA–SG	1	S	24, Y9–Y32
OsPAP7	GDWG	GDNFY	GNHD	VVGH	GHDH	29, VAG–EL	6	S	19, Y6–Y24
OsPAP9a	GDMG	GDISY	GNHE	FQGH	GHVH	19, AAA–AT	4	S	19, Y1–Y19
OsPAP9b	GDMG	GDISY	GNHE	FIGH	GHVH	19, AAA–AS	3	S	17, Y4–Y20
OsPAPl0a	GDLG	GDLCY	GNHE	VLLH	GHVH	25, CLA–GE	5	S	21, Y5–Y25
OsPAPl0b	GDLG	GDLSY	GNHE	VCVH	GHVH	25, ARG–GV	4	S	19, Y5–Y23
OsPAPl0c	GDLG	GDLSY	GNHE	VLMH	GHVH	24, CAG–AG	3	S	18, Y10–Y27
OsPAPl0d	GDLG	GDLSY	GNHE	MASH	GHVH	No	3	M	No
OsPAP15	GDLG	GDVSY	GNHE	AGWH	GHVH	21, AEA–AP	9	S	19, Y5–Y23
OsPAP18	GDLG	GDLSY	GNHE	VLLH	GHVH	34, AAA–AS	1	S	24, Y13–Y36
OsPAP20a	GDLG	GDLSY	GNHE	ALVH	GHVH	30, SLA–VT	1	S	22, Y3–Y24
OsPAP20b	GDLG	GDLSY	GNHE	ALVH	GHVH	28, AAS–LA	3	S	22, Y6–Y27
OsPAP2la	GDLG	GDLSY	GNHE	VLLH	GHVH	29, VAA–TE	3	S	24, Y5–Y28
OsPAP2lb	GDLG	GDLSY	GNHE	ALVH	GHVH	45, VLA–LI	1	S	24, Y24–Y47
[a]OsPAP2lc	GDLG	GDLSY	GNHE	–	GIMI	No	0	Unknown	No
OsPAP23	GDLG	GDMTY	GNHE	AAWH	GHVH	24, VVG–SR	5	S	19, Y10–Y28
OsPAP26	GDLG	GDLSY	GNHE	VLMH	GHVH	22, VSC–GR	2	S	19, Y5–Y23
OsPAP27a	GDMG	GDITY	GNHE	FIAH	GHVH	21, AAA–GG	6	S	20, Y1–Y20
OsPAP27b	GDMG	GDIPY	GNHE	FAAH	GHVH	25, ASA–SR	5	M	20, Y7–Y26
OsPAP27c	GDMG	GDLSY	GNHE	FAAH	GHVH	29, AAA–AA	3	S	23, Y10–Y32

[a]The spectrum of potential metal-ligating residues in the shaded PAP differs to some extent from that of typical PAPs.
[b]S and M = secretory and mitochondrial, respectively.

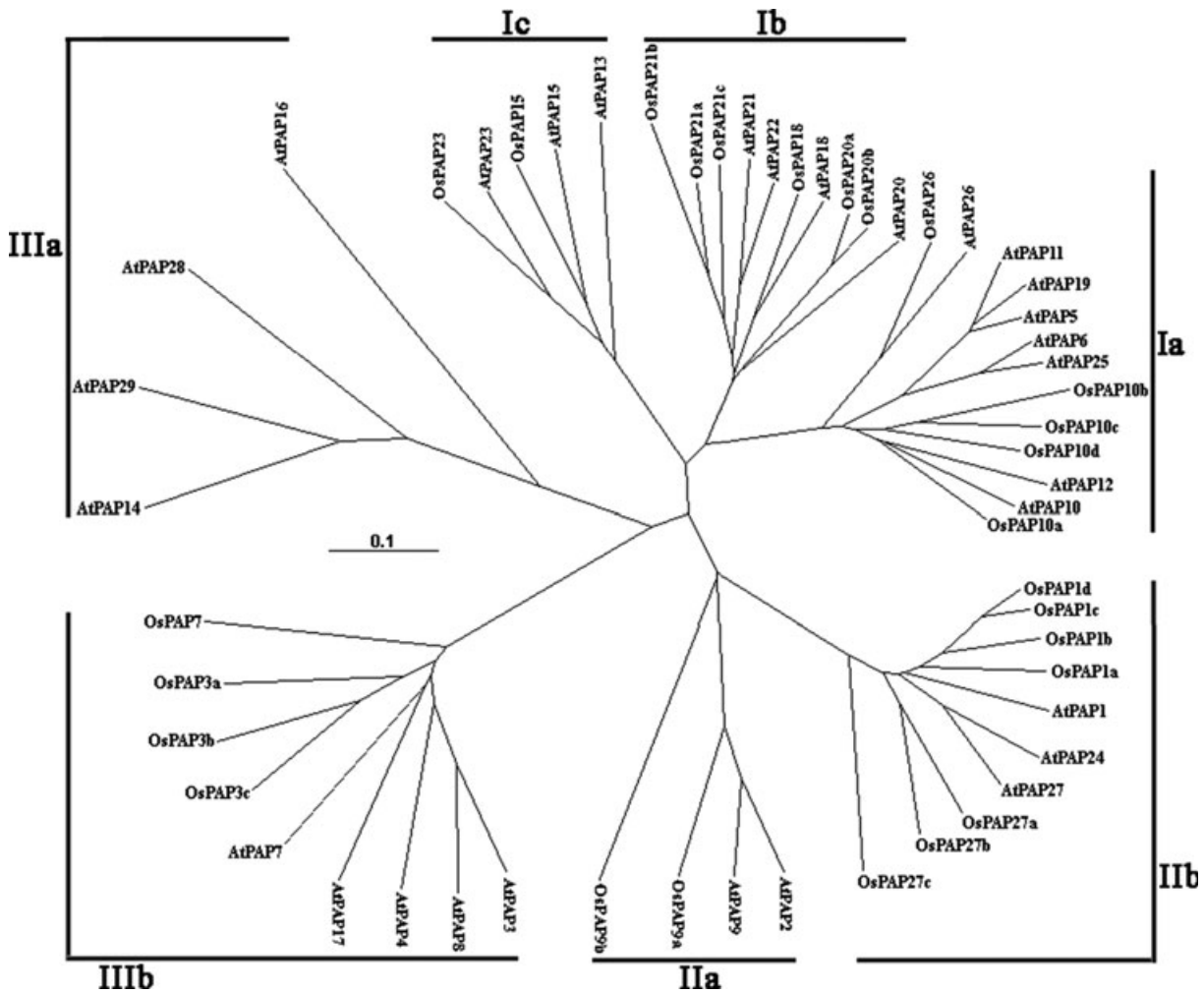

Fig. 1 Neighbour-joining phylogenetic trees of *OsPAP* and *AtPAP* genes constructed using ClustalX.

concentration and increased root/shoot ratio of fresh seedlings (Fig. 2A,B). Semi-quantitative reverse transcription PCR (sqRT-PCR) analysis showed that expression of ten PAP genes was induced by Pi starvation (Fig. 3). The expression of *OsPAP1a*, *OsPAP10c*, *OsPAP20b* and *OsPAP27a* was only induced in roots, whereas that of *OsPAP1d, OsPAP3b*, *OsPAP9b*, *OsPAP10a, OsPAP21b* and *OsPAP23* was induced in both roots and shoots (Fig. 3; Table 3). Nine PAPs were constitutively expressed regardless of P supply (Fig. 3). Interestingly, the expression of *OsPAP15* and *OsPAP21a* was inhibited by Pi starvation in leaves and roots, respectively. Transcripts of *OsPAP3a, OsPAP27c*, *OsPAP20a*, *OsPAP21c* and *OsPAP10b* were not detected in the RT-PCR analysis. It is possible that these genes were expressed in tissues other than roots and leaves, similar to other *Arabidopsis* PAP genes (Zhu *et al.* 2005).

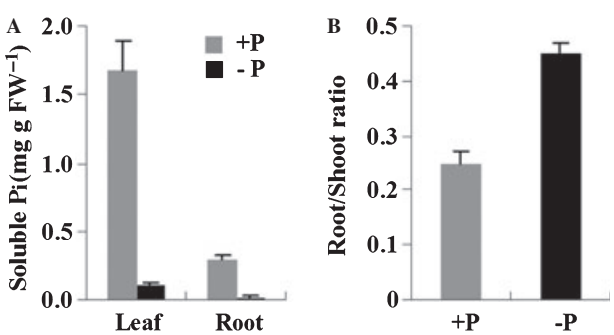

Fig. 2 Pi concentration (A) and root/shoot ratio of the fresh weigh (B) of wild-type seedlings grown under +P and −P conditions. Ten-day-old seedlings were transferred to +P and −P conditions for 7 days before measurement. All values represent means ± SD, n = 4.

Pi starvation-induced PAP genes are regulated by *OsPHR2*

AtPHR1/OsPHR2 is the central regulator in Pi starvation signalling. The transcription factor binds to an imperfect palindromic sequence (PHR1-binding element, P1BS), GNATATNC, found in the promoter regions of its downstream genes (Schachtman & Shin 2007; Zhou *et al.* 2008). Analysis of the 1-kb sequence upstream of the start codons of the rice PAP genes revealed 16 P1BS elements distributed in 12 *OsPAPs* (Table 3). The frequency of the P1BS element in the promoters of *OsPAP* genes is 0.615/kb, a value much higher than that in the entire rice genome (0.258/kb). Ten PAP genes were induced by Pi starvation. All of these PAPs contain P1BS sequences in their promoter regions (Table 3). To elucidate the

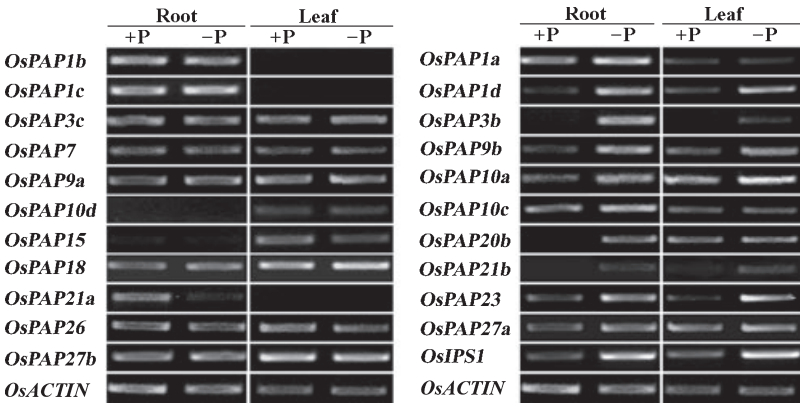

Fig. 3 Expression levels of *OsPAP* genes analysed by sqRT-PCR under both +P and −P conditions. Ten-day-old seedlings were transferred to normal and Pi-deprived solutions for 7 days. Total RNA was extracted from roots and leaves of the seedlings. The transcript levels of *OsACTIN* and *OsIPS1* were used as housekeeping and −P genes, respectively.

Table 3. Analysis of *OsPHR2* binding elements in the promoter regions of *OsPAPs*.

gene	PIBS element[a] number	PIBS element[a] location	root response to −P (qRT data)[b]
OsPAP1a	1	−280	+ (2.3 ± 0.2)
OsPAP1b	0		0
OsPAP1c	0		0
OsPAP1d	1	−227	+ (12.7 ± 0.5)
OsPAP3a	0		0
OsPAP3b	2	−426; −120	+ (49.1 ± 1.8)
OsPAP3c	0		0
OsPAP7	0		0
OsPAP9a	1	−103	0 (0.7 ± 0.1)
OsPAP9b	1	−225	+ (2.7 ± 0.1)
OsPAP10a	2	−990; −373	+ (9.9 ± 0.4)
OsPAP10b	0		0
OsPAP10c	2	−523; −146	+ (7.2 ± 0.2)
OsPAP10d	0		0
OsPAP15	2	−989; −511	0 (1.3 ± 0.1)
OsPAP18	0		0
OsPAP20a	0		0
OsPAP20b	1	−580	+ (8.8 ± 1.7)
OsPAP21a	0		–
OsPAP21b	1	−115	+ (17.5 ± 3.0)
OsPAP21c	0		0
OsPAP23	1	−147	+ (20.9 ± 2.3)
OsPAP26	0		0
OsPAP27a	1	−229	+ (61.5 ± 2.9)
OsPAP27b	0		0
OsPAP27c	0		0

[a]The average occurrence of the P1BS element in the rice genome is 0.258/kb.

[b]'+' = positive response to Pi starvation; '–' = negative response to Pi starvation; '0' = no response to Pi starvation; values in parentheses are calculated from qRT-PCR.

relationship between the PAP genes and *OsPHR2*, expression levels of the 12 PAP genes having the PIBS element in their promoter were measured by qRT-PCR in roots of wild-type and *OsPHR2* over-expressed seedlings grown under +P and −P conditions (Fig. 4; Fig. S1). During expression, the ten Pi starvation-induced PAP genes accumulated more transcripts in *OsPHR2* over-expressed plants compared with the wild type under both +P and −P conditions (Fig. 4, Table 3). It is clear that induction of rice PAP genes by Pi starvation is mediated through the *OsPHR2*-mediated P signalling pathway.

PHR2-O, *pho2* and *SPX1-Ri* seedlings show increased root AP activity

Under Pi sufficient conditions, *OsPHR2* over-expression lines (*PHR2-O*), *PHO2* knockout lines (*pho2)* and *OsSPX1* RNA interference lines (*SPX1-Ri*) enhance Pi acquisition and accumulation, resulting in Pi toxicity (Zhou *et al.* 2008; Wang *et al.* 2009a). Under both Pi sufficiency and deficiency conditions, *PHR2-O, pho2* and *SPX1-Ri* seedlings contained significantly more Pi compared to the wild type (Fig. 5A). To determine whether the alteration of PAPs expression in these transgenic or mutant plants contributed to the increase in Pi accumulation, the root acid phosphatase and secretory acid phosphatase activities of *PHR2-O, pho2* and *SPX1-Ri* were measured. Under +Pi conditions, root AP activity in the *pho2* mutant was similar to that of wild-type plants, while root AP activity in both *OsPHR2-O* and *OsSPX1-Ri* plants were significantly higher (Fig. 5B). Under −Pi conditions, root AP activities increased in all plants except *OsSPX1-Ri* (Fig. 5B). Moreover, the root AP activities of *PHR2-O* and *pho2* were higher than that of wild-type roots (Fig. 5B). To confirm this result, root secretory acid phosphatase (SAP) activity was determined colorimetrically using BCIP as substrate. Because the resultant indolyl derivative from hydrolysis of BCIP is a stable and insoluble blue compound, SAP activities of the samples can be compared according to the intensity of blue colour in the roots. As shown in Fig. 5C, while Pi deficiency induced SAP activity in all samples, the highest level of induction was observed in the *pho2* mutant (Fig. 5C).

DISCUSSION

In this study, 26 rice PAP genes were identified through genome-wide analysis, and were named based on their degree of similarity to *Arabidopsis* PAP genes. Protein sequence analyses predicted that 24 of the rice PAPs (except *OsPAP10d* and *OsPAP21c*) contain a signal peptide, various numbers of N-glycosylation sites and an N-terminal transmembrane helix (Table 2), which are motifs proposed to control secretion of PAPs (Olczak *et al.* 2003; Lu *et al.* 2008). Twenty-three *OsPAPs* are predicated to be secretory proteins (here, the cell

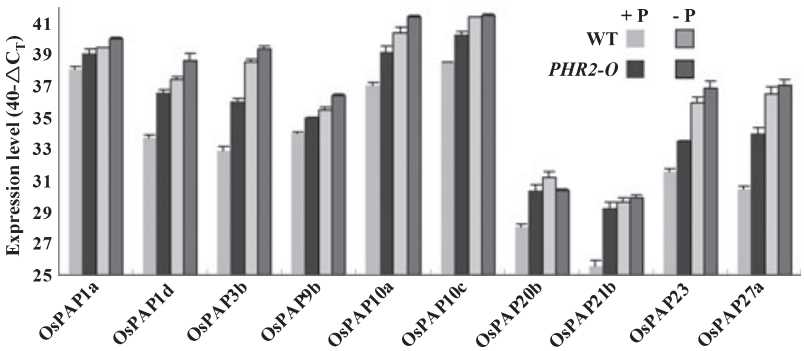

Fig. 4 Expression levels of *OsPAP* genes analysed by qRT-PCR in wild-type and *OsPHR2-O* plants. Ten-day-old seedlings were transferred to +P and −P conditions for 7 days. Total RNA was extracted from roots of the seedlings. Expression levels are given on a log scale, expressed as 40-ΔC_T, where ΔC_T is the difference in qRT-PCR threshold cycle number between the respective gene and the reference gene *OsACTIN*; 40 therefore equals the expression level of *OsACTIN*. The fold difference in expression in $\Delta\Delta^{C_T}$ when PCR efficiency is 2 (*e.g.* an ordinate value of 36 represents an eightfold lower expression than a value of 39).

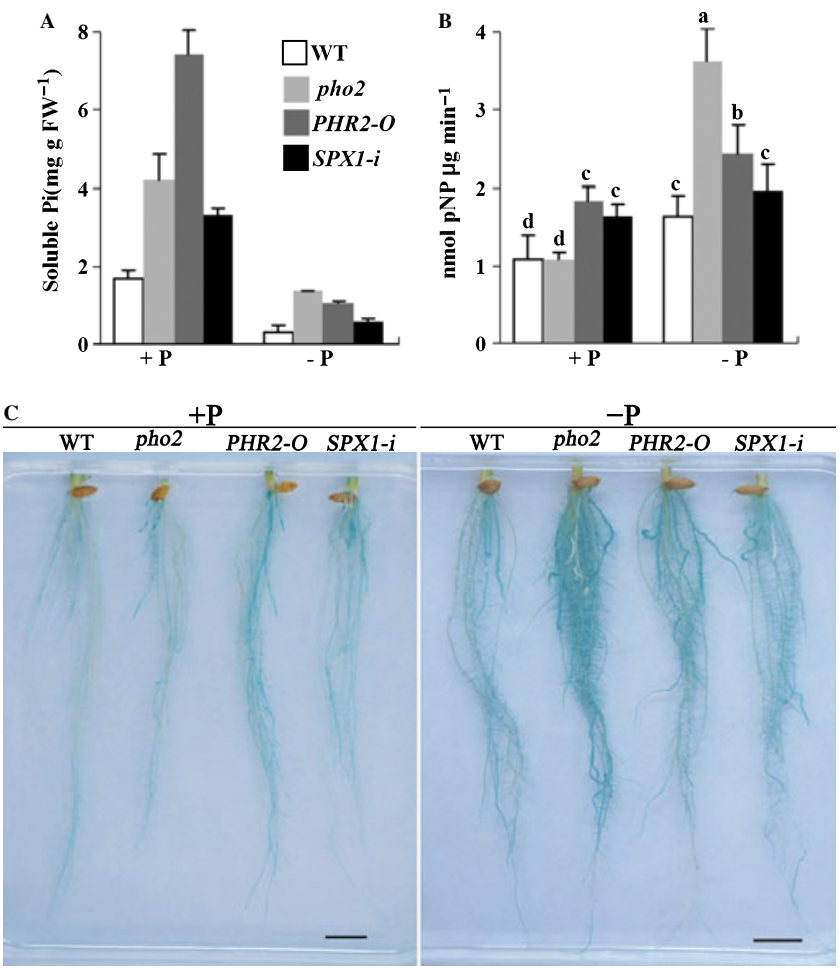

Fig. 5 Pi concentration (A), APase activity (B) and SAP activity (C) of *pho2*, *OsPHR2-O* and *OsSPX1-Ri* plants. Ten-day-old seedlings were transferred to +P and −P conditions for 7 days. WT, wild type; *pho2*, *OsPHO2* mutant; *PHR2-O*, *OsPHR2* over-expression line; *SPX1-Ri*, *OsSPX1* RNA-interference line. All values represent means ± SD, n = 4. Points with different letters (B) are significantly different at $P < 0.05$ (LSD test, $P < 0.05$).

vacuole is considered to be part of the 'secretory' pathway in addition to a secretome), in support of the initial hypothesis (Table 1).

Of the 26 PAP genes, 21 were expressed in rice young seedlings under either +P or −P conditions (Fig. 3). Expression of the remaining five genes, *OsPAP3a*, *OsPAP10b*, *OsPAP20a*, *OsPAP21c* and *OsPAP27c*, was not detectable with RT-PCR under our conditions. *OsPAP3a*, *OsPAP10b* and *OsPAP20a* have ESTs in the dbEST database (Table 1) and are expressed in a developmental or tissue-specific manner. For example, *AtPAP20* is specifically expressed in flowers and siliques in *Arabidopsis* (Zhu *et al.* 2005), so the rice homologues of *AtPAP20* and *OsPAP20a* may also be expressed in reproductive organs. *OsPAP21c* and *OsPAP27c* have no ESTs so far, and *OsPAP21c* does not contain the typical seven invariant metal-ligating residues found in other PAPs or any of the three elements at the N-terminal region (Table 2). Therefore, it is possible that *OsPAP21c* and *OsPAP27c* are pseudogenes.

The activities of intracellular acid phosphatase (IAP) and secreted acid phosphatase (SAP) generally increase in Pi deprivation conditions (Bozzo *et al.* 2002, 2006). This increased activity is regulated both transcriptionally and post-transcriptionally (del Pozo *et al.* 1999; Tomscha *et al.* 2004; Veljanovski *et al.* 2006). In this study, we show that Pi starvation induced the expression of ten rice PAP genes, suggesting that these play important roles in the acclimation of rice to low Pi conditions. Promoter analysis showed that all of these Pi starvation-induced PAP genes contain an *OsPHR2* binding element, P1BS, in their promoter region and their expressions can also be up-regulated by over-expression of *OsPHR2*. Therefore, induction of the ten PAP genes by Pi starvation was likely mediated by *OsPHR2*. A recent microarray analysis revealed that *OsPAP1d*, *OsPAP10c*, *OsPAP23* and *OsPAP27a* were induced by Pi starvation (Zheng *et al.* 2009). It was proposed that the −P-treated 10-day-old rice seedlings will carry over some nutrient from grains and only suffer moderate Pi starvation (Zheng *et al.* 2009). Therefore, the four genes detected by the microarray may represent early Pi starvation response PAP genes in rice. In our conditions, which used prolonged Pi starvation, while some other PAP genes were also induced, the expression of *OsPAP1d*, *OsPAP10c*, *OsPAP23* and *OsPAP27a* was induced to a relatively higher level (Table 3). Thus, these genes are good candidates for Pi starvation-responsive makers.

OsPHR2 negatively regulates expression of *OsPHO2* through *OsmiR399* and positively regulates *OsSPX1* (Panigrahy *et al.* 2009). Although over-expression of *OsPHR2*, knock-out of *OsPHO2* and RNA interference of *OsSPX1* all led to over-accumulation of Pi (Zhou *et al.* 2008; Wang *et al.* 2009a), expression of *OsPAPs* had a different pattern in these lines. As described above, the ten −Pi-induced rice PAP genes were directly regulated by *OsPHR2* (Fig. 4). Expression of these genes also increased in *OsSPX1-Ri* plants, although the levels were significantly less than those in *OsPHR2* plants (Fig. S2). Previously, we reported that *OsSPX1* suppressed the expression of many *OsPHR2*-induced genes (Wang *et al.* 2009a). Combined with the expression data of PAP genes, we propose that *OsPHR2* and *OsSPX1* form a feedback loop regulation system in Pi starvation signalling.

Although increased AP and SAP activity was observed in *OsPHR2-O*, *pho2* and *OsSPX1-Ri* plants under +P or −P conditions, the extent of induction differed among these lines. Under P sufficiency, *OsPHR2-O* and *OsSPX1-Ri* plants have higher AP and SAP activity than the wild type, which is correlated with induction of PAP gene expression (Figs 4, 5B,C and S2). A similar correlation between the expression of PAP genes and AP or SAP activity can be detected in wild-type seedlings under both P sufficiency and deficiency conditions (Figs 4 and 5B,C). It is clear that AP and SAP activity could be regulated at the transcriptional level; however, AP and SAP activity was not always correlated with PAP gene expression. The expression of Pi starvation responsive PAP genes was similar in *pho2* and *OsSPX1-Ri* plants (Fig. S2). Therefore, the different AP and SAP activity of *pho2* and *OsSPX1-Ri* seedlings may be due to different post-transcriptional regulation of PAP genes (Veljanovski *et al.* 2006). Further studies are needed to clarify where, when and how the individual PAP genes are expressed and regulated. A detailed understanding of these PAP genes and the corresponding PAP proteins may provide opportunities for enhancing technologies available for crop improvement for efficient P use.

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article:

Figure S1. Expression levels of *OsPAP9a* and *OsPAP15* genes analysed by qRT-PCR in wild-type and *OsPHR2-O* plants. The samples were used as described in Fig. 2.

Figure S2. Expression levels of *OsPAP* genes analysed by qRT-PCR in wild-type, *pho2* and *OsSPX1* RNA-interference plants. The samples were used as described in Fig. 2.

Table S1. Primer sequences used in this research.

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