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Research Article

Overexpression of *OsPAP10a*, A Root-Associated Acid Phosphatase, Increased Extracellular Organic Phosphorus Utilization in Rice

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Abstract

Phosphorus (P) deficiency is a major limitation for plant growth and development. Among the wide set of responses to cope with low soil P, plants increase their level of intracellular and secreted acid phosphatases (APases), which helps to catalyze inorganic phosphate (Pi) hydrolysis from organophosphates. In this study we characterized the rice (*Oryza sativa*) purple acid phosphatase 10a (*OsPAP10a*). *OsPAP10a* belongs to group Ia of purple acid phosphatases (PAPs), and clusters with the principal secreted PAPs in a variety of plant species including *Arabidopsis*. The transcript abundance of *OsPAP10a* is specifically induced by Pi deficiency and is controlled by *OsPHR2*, the central transcription factor controlling Pi homeostasis. In gel activity assays of root and shoot protein extracts, it was revealed that *OsPAP10a* is a major acid phosphatase isoform induced by Pi starvation. Constitutive overexpression of *OsPAP10a* results in a significant increase of phosphatase activity in both shoot and root protein extracts. *In vivo* root 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) assays and activity measurements on external media showed that *OsPAP10a* is a root-associated APase. Furthermore, overexpression of *OsPAP10a* significantly improved ATP hydrolysis and utilization compared with wild type plants. These results indicate that *OsPAP10a* can potentially be used for crop breeding to improve the efficiency of P use.

Keywords: Purple acid phosphatase; phosphate; rice; OsPAP10a; root-associated APase.

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Introduction

Phosphorous (P) is an important macronutrient required for plant growth, and plays a pivotal role in metabolism. Of the many different sources of P present in a given soil solution, plants can only use a small portion: inorganic phosphate (Pi) (Sánchez-Calderón et al. 2010). It has been estimated that over 30% of the world's arable land is P-limited, which in turn limits plant growth and yield (Vance et al. 2003). To cope with P deficiency, plants have evolved diverse adaptive response pathways, including enhancement of P uptake through root morphological modification, activation of Pi transporters, alteration of cellular P metabolism, and increased uptake of available P in soil through the secretion of organic acids, phosphatases, ribonucleases and phosphodiesterases into the rhizosphere (Ticconi and Abel 2004; Sánchez-Calderón et al. 2010;

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Chiou and Lin 2011). Acid phosphatases (APases; EC 3.1.3.2) catalyze the hydrolysis of attached phosphate groups from phosphate esters and anhydrides at an optimal pH below 7 (Duff et al. 1994). Purple acid phosphatases (PAPs) that display a purple or pink color are a distinct group of APases (Bozzo et al. 2004). They have a bimetal nuclear center coordinated with a conserved motif in their carboxyl end (Klabunde et al. 1995; Schenk et al. 1999). The purple or pink color of purified PAPs is caused by a charge transfer transition at ~560 nm from a tyrosine to the metal ligand Fe^{3+}. Biochemistry and molecular studies suggest that PAPs are multi-function proteins and exist in bacteria, plants, and animals (Schenk et al. 2000; Olczak et al. 2003). The induction of intracellular and secreted APases is a universal plant response to low Pi conditions. While intracellular APases are believed to remobilize and scavenge Pi from intracellular P monoesters and anhydrides, secreted APases hydrolyze Pi from organo-phosphates, the principal form of phosphorus found in soil, which cannot be utilized by plants.

There are 29 and 26 putative PAP genes in the *Arabidopsis* and rice genome, respectively, which are divided into seven groups (Li et al. 2002; Zhang et al. 2011). While some PAPs are induced by phosphate starvation and regulate phosphate homeostasis in plants, others have been shown to be involved in development or other stress responses (del Pozo et al. 1999; Liao et al. 2003; Zhu et al. 2005; Kuang et al. 2009; Zhang et al. 2011). Gel phosphatase activity assays from leaves, roots protein extracts, and root exudates have shown that there are several major PAP isoforms in *Arabidopsis*, which led to the identification of *AtPAP17*, *AtPAP12*, *AtPAP26*, *AtPAP10*, Phosphatase Under Producer 1 (PUP1) and other previously unknown PAPs (del Pozo et al. 1999; Tomscha et al. 2004; Hurley et al. 2010; Tran et al. 2010). While *AtPAP12* and *AtPAP26* are believed to be released into the rhizosphere, *AtPAP10a* or PUP1 is a cell wall bound PSI-secreted PAP associated with the root surface, which is also necessary for optimal adaptation to low Pi conditions in *Arabidopsis* and can utilize rhizosphere organophosphate (Tomscha et al. 2004; Wang et al. 2011; Wang and Liu 2012). In addition to these *Arabidopsis* PAPs, several Pi-starvation-induced PAP proteins were also purified in tomato, Lupinus and common bean (Bozzo et al. 2004; Bozzo et al. 2006; Liang et al. 2010), suggesting that their functions are important and conserved across plant species.

Rice (*Oryza sativa*) is one of the most important crop plants, providing the major calorie source for more than half of the world's population (Khush 2005). Breeding rice varieties with enhanced Pi uptake and Pi use efficiency is important for sustainable agriculture (Zhang 2007). Therefore, understanding the different players that help to remobilize and recycle Pi from expendable P-monoesters and anhydrides as well as scavenge Pi from organic soil matter is an important step in producing Pi efficient rice plants. In the common bean (*Phaseolus vulgaris*), *PvPAP3* was purified from a P-efficient genotype, and enhanced extracellular ATP utilization in overexpressing plants (Liang et al. 2010). Thus, manipulation of PAP expression in P-starved plants may improve their growth in Pi-limited soils. Compared with the advanced progress in other species, the study of rice PAPs is rare. *OsPAPhy_b* (*OsPAP15*) is the only rice APase that has been characterized. It has been shown that *OsPAP15* functions as a phytase during rice seed germination (Dionisio et al. 2011).

The Ia group members in *Arabidopsis*, *AtPAP10*, *AtPAP12* and *AtPAP26*, have been shown to be the predominant PSI-secreted PAPs, and to play an important role in low Pi adaptation (Hurley et al. 2010; Tran et al. 2010; Wang et al. 2011). Being located in the same group in the phylogenetic tree as *Arabidopsis* APases (Zhang et al. 2011), the rice PAP10a to PAP10d genes should have functions as important as those of their *Arabidopsis* homologies. To date, a number of studies have shown a significant induction of *OsPAP10a* mRNA under Pi-deficient conditions (Zhou et al. 2008; Wang et al. 2009a). However, no direct evidence has been shown to prove the induction of these APases at the protein or enzyme activity level by Pi deficiency. In this study, we isolated and characterized *OsPAP10a*, providing evidence that it is a major APase isoform in both shoot and root protein extracts, and is specifically induced by Pi-deficient conditions. We also demonstrated that *OsPAP10a* can be secreted and that overexpression of *OsPAP10a* is associated with improved hydrolysis capacity of external organic P and thus better phosphate use efficiency.

Results

Phylogenetic and expression analysis of *OsPAP10a*

In plants, the PAP family has been previously classified into seven groups and *OsPAP10a* belongs to group Ia (Zhang et al. 2011). In rice, group Ia contains 5 members, namely *OsPAP10a* to *OsPAP10d*, and *OsPAP26*. To further clarify the phylogenic relationship of the proteins within group Ia in plants, a phylogenetic tree was made using the group Ia PAP proteins from rice, *Arabidopsis*, maize (*Zea mays*), Soybean (*Glycine max*) and tobacco (*Nicotiana tabacum*). The results showed that the group Ia PAP proteins could be further divided into four subgroups (**Figure 1**). Subgroup Ia-1 includes proteins from all the plants tested, including *AtPAP10*, *AtPAP12* and *OsPAP10a*, while subgroup Ia-2 contains only *Arabidopsis* PAP proteins and subgroup Ia-3 is monocotyledon-specific. Interestingly, subgroup Ia-4 consists only of PAP26 proteins.

Previously, we demonstrated that expression of *OsPAP10a* was induced by Pi deprivation (Zhang et al. 2011). To determine whether the transcription level of *OsPAP10a* is specifically regulated by Pi starvation, 2-week-old rice seedlings were transferred to different nutrient-deprived solutions for an

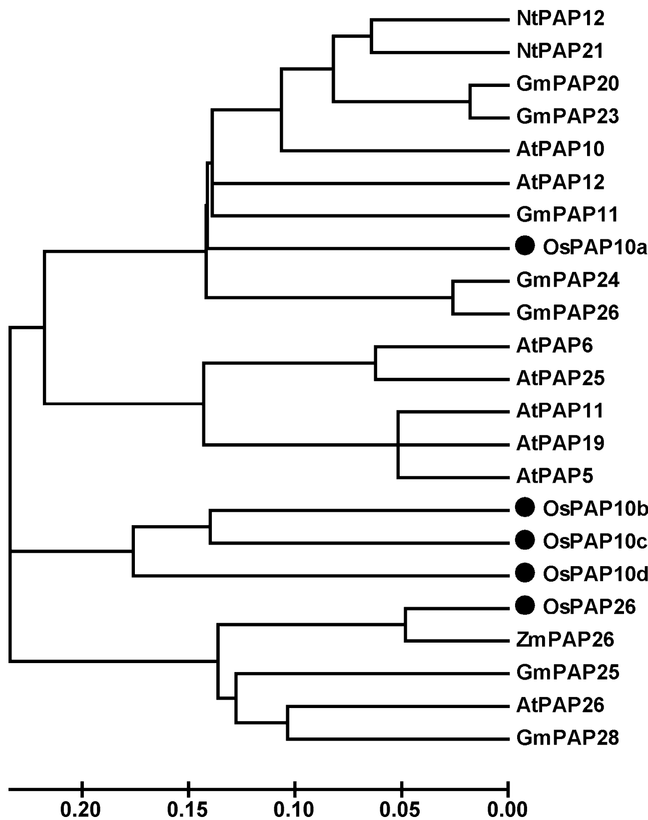

Figure 1. Phylogenetic trees of subgroup Ia PAP proteins in selected plants constructed using the Mega 4.1 program.

Only bootstrap values of ≥ 50% are indicated for major branch points. Species' names were abbreviated as follow: At, *Arabidopsis*; Os, *Oryza sativa*; Zm, *Zea mays*; Nt, *Nicotiana tabacum*; Gm, *Glycine max*.

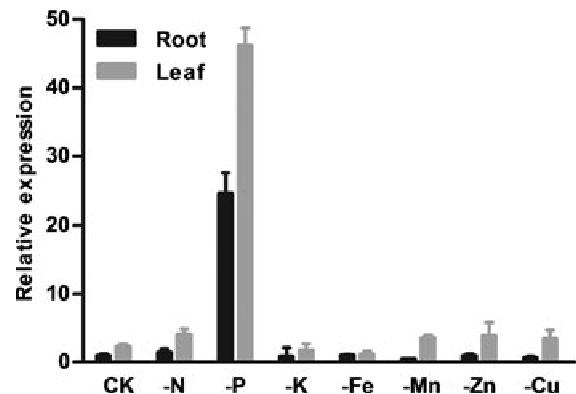

Figure 2. Expression analysis of *OsPAP10a* by qRT-PCR.

Two-week-old seedlings were transferred to a nitrogen, phosphate, potassium, iron, manganese, zinc or copper-deprived solution for 10 d. The leaves or roots of the plants were sampled and extracted for RNA. The samples were normalized to *OsACTIN* and calculated for fold change.

additional 10 days. Quantitative RT-PCR (qRT-PCR) showed that the expression of *OsPAP10a* was highly and specifically induced by Pi starvation in both roots and leaves (Figure 2). In contrast, other nutrient stresses had no or little influence on the expression of *OsPAP10a*.

Overexpression of *OsPAP10a* increased APase activity in transgenic rice

To further characterize *OsPAP10a*, we constitutively overexpressed *OsPAP10a* in rice. Full length cDNA of *OsPAP10a* was inserted into the binary vector *pCAMBIA1300-mod* under the double CaMV 35S promoter (Wang et al. 2009a). The *pCAMBIA1300-mod-OsPAP10a* vector was introduced into the *japonica* cultivar Nipponbare via *Agrobacterium*-mediated transformation. As expected, Pi starvation highly induced the level of expression of *OsPAP10a* in wild type (WT) plants. In *OsPAP10a*-overexpressing plants, *OsPAP10a* transcript abundance was higher in both Pi-sufficient and deficient conditions than it was in WT plants (Figure 3A). Interestingly, the transcript level of *OsPAP10a* in *OsPAP10a* overexpressing plants grown under Pi-replete conditions was even higher than that in WT plants grown under Pi-deficient conditions. The expression of *OsIPS1* was low under normal conditions but was greatly induced under Pi-starvation conditions in both WT and transgenic plants. These results suggest that the transgenic lines have both a higher and a more constitutive expression of *OsPAP10a* compared to WT plants.

APase activity was then measured in shoot and root protein extracts in both WT and transgenic plants. In WT and transgenic plants, APase activity was higher in root extracts compared to shoot extracts. Overexpression of *OsPAP10a* increased the total APase activity by ~1.5 and ~2 fold in roots and shoots, respectively (Figure 3B).

OsPAP10a is an APase induced by Pi deficiency and regulated by *OsPHR2*

To test if *OsPAP10a* is one of the main APases induced by Pi deficiency in rice, an in-gel APase isoform analysis was performed on both WT and *OsPAP10a*-overexpressing plants. Root and shoot protein extracts were run on a native PAGE gel and stained for APase activity. Remarkably, only the APase isoform displaying the slowest mobility with both shoot and root protein extracts, named E1, was induced by Pi deficiency (Figure 4A, B). A similar result was previously obtained in *Arabidopsis*, with the only Pi up-regulated isoform also displaying the slowest mobility (Tomscha et al. 2004). Furthermore, the E1 band was

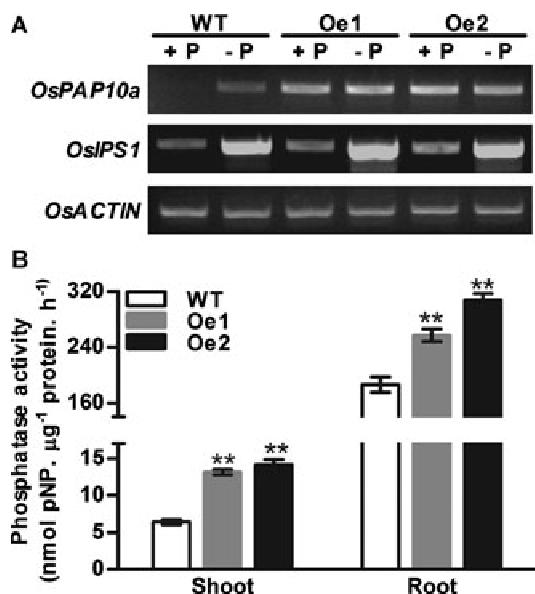

Figure 3. Gene expression and protein activity analysis of *OsPAP10a*-overexpressing plants.

(A) RT-PCR analysis of *OsPAP10a* in WT and transgenic plants. The *OsIPS1* gene was used as a Pi-starvation-induced marker. The samples were normalized by comparing *OsACTIN*.

(B) APase activity using pNPP as the substrate. The hydrolysis of pNPP at 25 °C over 15 min was measured for 1 μg protein. Data are the means of 3 replicates with *SD*. WT, wild type; Oe3 and Oe7, overexpression lines 3 and 7. Values marked by an asterisk are significantly different from WT (**, $P < 0.01$). 2-week-old seedlings were transferred to phosphate-deprived solutions for 10 d. The root and shoot tissues were separately sampled for analysis.

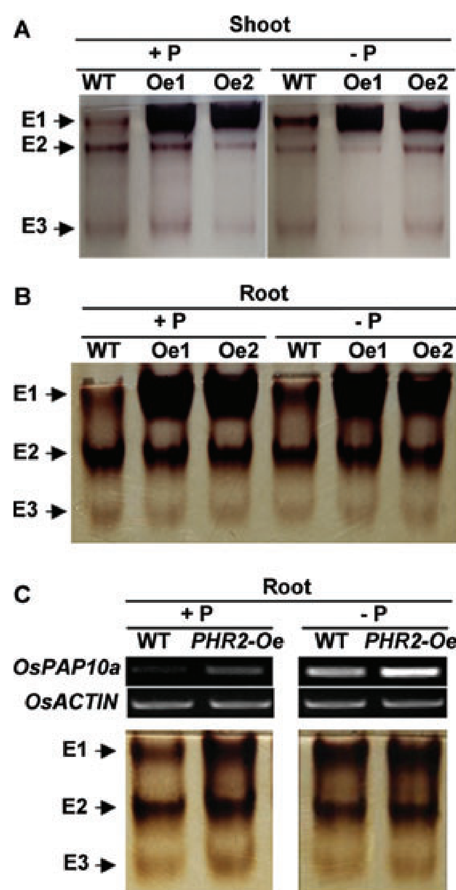

Figure 4. In-gel APase activity and isoform analysis in WT and transgenic plants under Pi-sufficient and deficient conditions.

Two-week-old seedlings were transferred to phosphate-deprived solutions for 10 d.

Shoot **(A)** and root **(B)** proteins were extracted for electrophoresis under native discontinuous PAGE and stained for APase activity using Fast Garnet GBC and alpha-naphthyl phosphate. The arrow indicates the slowest-running APase, identified as *OsPAP10a*.

(C) The expression of *OsPAP10a* and in-gel APase activity analysis in WT and *OsPHR2*-overexpressing plants under Pi-sufficient and deficient conditions. 2-week-old seedlings were transferred to phosphate-deprived solutions for 10 d. Roots were sampled for the analysis as described above.

the only band which increased dramatically in *OsPAP10a* overexpressing plants compared to WT plants in both shoot and root protein extracts under Pi-replete and deficient conditions (**Figure 4A, B**), which indicates that the E1 band contains *OsPAP10a*.

Previous studies have shown that *OsPHR2* is the central transcription factor regulating Pi-starvation responses in rice (Zhou et al. 2008; Wang et al. 2009a). Overexpression of *OsPHR2* (*PHR2-Oe*) was shown to activate the Pi starvation signaling pathway and cause Pi overaccumulation in the plants (Zhou et al. 2008). To assess if *OsPAP10a* could be controlled by *OsPHR2*, in-gel APase isoform analyses were performed on protein extracts from WT and *PHR2*-overexpressing plants. Analysis of transcript abundance in *PHR2-Oe* plants showed an increased transcript level for *OsPAP10a* under both Pi-replete and deficient conditions. This increase in transcript abundance was also associated with an increase in APase activity (**Figure 4C**), suggesting that *OsPAP10a* is regulated by *OsPHR2*.

OsPAP10a is a root-associated acid phosphatase in roots

The homolog of *OsPAP10a* in *Arabidopsis*, *AtPAP10*, has been shown to be a root-associated acid phosphatase (Wang et al. 2011). To confirm whether *OsPAP10a* is a root-associated secreted APase, its activity was analyzed in rice plants. While Pi starvation induced the root-associated APase activity in the roots of both WT and transgenic plants, the transgenic

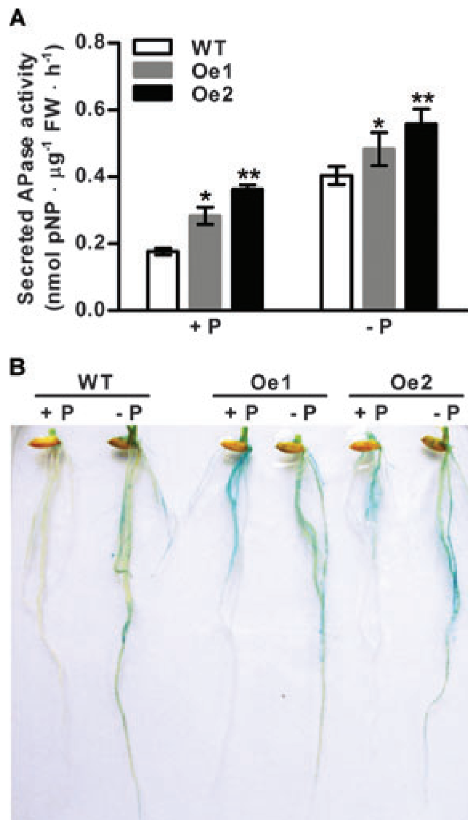

Figure 5. Root surface secretory APase activity in WT and transgenic plants under Pi-sufficient and deficient conditions.

(A) Fourteen-day-old seedlings were grown in the presence or absence of Pi, before being transferred to a fresh medium containing 10 mM pNPP for 1 h. pNP was then quantified, enabling the measurement of SAP activity.

(B) Germinated seeds were cultured in Pi-sufficient and deficient solutions for 10 d. Roots of the plants were then sampled for BCIP staining. WT, wild type; Oe3 and Oe7, overexpression lines 3 and 7.

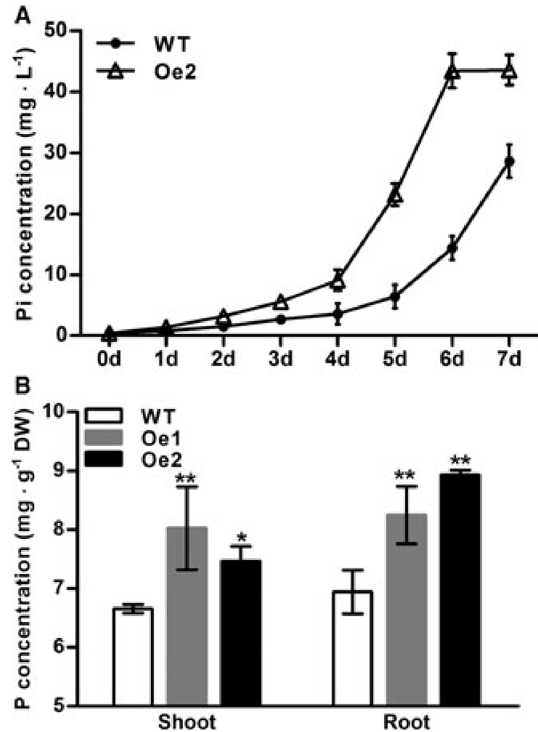

Figure 6. Enhanced release and uptake of Pi from P source ATP in *OsPAP10a*-overexpressing plants.

(A) Time course measurements of free Pi release by WT and *OsPAP10a*-overexpressing plants in culture solution supplied with ATP. Two-week-old plants were transferred to culture solution with 0.5 mM ATP as the only source of P. Pi content in the solution was recorded every day.

(B) Total P of shoots and roots of WT and *OsPAP10a*-overexpressing plants grown for 14 d with 0.5 mM ATP as the sole source of P. Values marked by an asterisk are significantly different from WT (*, $P < 0.05$; **, $P < 0.01$).

lines showed an increase in activity compared to that of WT under both Pi-sufficient and deficient conditions (**Figure 5A**). To validate this result, another approach was performed using an *in vivo* root activity staining. As shown in **Figure 5B**, while Pi starvation treatment induced root-associated secretary acid phosphatase activity in WT plants, the *OsPAP10a*-overexpressing plants showed enhanced root surface secreted acid phosphatase activity under Pi-sufficient conditions (**Figure 5B**).

Overexpression of *OsPAP10a* increased extracellular ATP utilization in solution culture and growth performance in soil

To assess the ability of *OsPAP10a*-overexpressing plants in scavenging and absorbing organic phosphate, 14-day-old seedlings were transferred to a liquid nutrient solution containing 0.5 mM ATP as the only source of P for 1 to 7 d (**Figure 6A**), with their media inorganic phosphate content measured every day before the transfer. As a result, inorganic phosphate concentration in the solution increased both for WT plants and transgenic plants in a time-dependent manner, indicating that both WT and *OsPAP10a*-overexpressing roots were able to hydrolyze the ATP into inorganic Pi (**Figure 6A**). However, the hydrolysis of ATP by the *OsPAP10a* over-expressing lines was much more rapid than that of the WT plants. After 4 d of incubation with ATP-containing media, Pi concentration in the solution of *OsPAP10a*-Oe2 plants reached 9.11 mg L^{-1}, which was significantly higher than that observed in the WT plants media (3.59 mg L^{-1}) (**Figure 6A**). The differences in Pi concentration in the solution between WT and transgenic plants

were most pronounced at 5 to 6 d. After 6 d, the ATP in the solution was almost completely hydrolyzed by transgenic plants (**Figure 6A**).

To confirm this result, 14-day-old WT Oe1 and Oe2 seedlings were grown in a nutrient solution with 0.5 mM ATP as the only source of P, and the solution culture was changed every 5 d. After 2 w, the shoot Pi concentration was measured. Results showed that Pi concentrations in the both shoots and roots of the transgenic plants were significantly higher than those of the WT plants (**Figure 6B**). Therefore, overexpression of *OsPAP10a* greatly enhanced extracellular ATP hydrolysis and uptake.

We further evaluated the growth performance of the transgenic plants in a soil pot experiment. Our results showed that *OsPAP10a*-overexpressing plants grow better and exhibit earlier heading than WT plants (**Figure 7A, B**). Moreover, the overexpressing lines have more tiller numbers (**Figure 7B**), which is an important trait for high-efficiency Pi uptake and utilization rice variety.

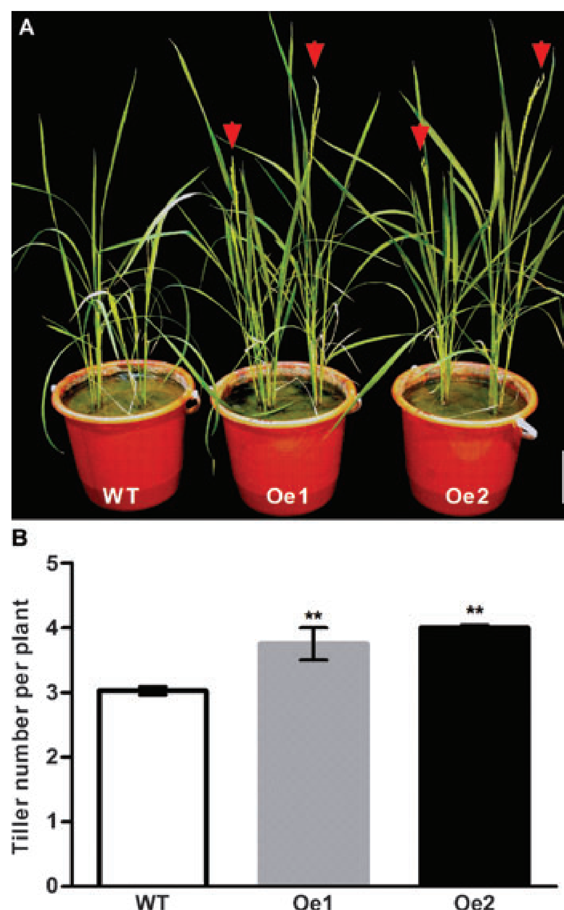

Figure 7. Growth of WT and transgenic plants cultured in soil pots.

(A) Growth performance of WT and transgenic plants cultured in soil pots. Earlier inflorescence of the transgenic plants is indicated by arrows.

(B) Tiller numbers of WT and transgenic plants. Data are the means of 4 plants. Values marked by an asterisk are significantly different from WT (*, $P < 0.05$, **, $P < 0.01$).

Discussion

Identifying the molecular components that allow plants to remobilize and recycle Pi from expendable P-monoesters and anhydrides as well as to scavenge and use the phosphate which is bound to organic matter is an importance approach to improving crop growth and yield under low-Pi conditions (Sánchez-Calderón et al. 2010; Chiou and Lin 2011). In this work, we demonstrated that *OsPAP10a* is one of the key compounds involved in the utilization of organic P.

Although PAP protein sequences display high levels of similarity, their functions and modes of regulation vary considerably. Using in-gel activity assays, we were able to show that the induction of APase activity by Pi deficiency is predominantly attributed to the increased expression of *OsPAP10a,* which is under the control of *OsPHR2*, the central transcription factor controlling Pi homeostasis in rice. Overexpression of *OsPAP10a* in rice increased shoot and root-associated APase activities in both Pi-replete and Pi-deficient conditions. As a consequence, the transgenic plants were able to hydrolyze extracellular organic P sources, such as ATP, into an inorganic form more efficiently than WT plants. However, substrate specificity still needs to be investigated.

Phylogenetically, *OsPAP10a* and *OsPAP10c* belong to subfamily Ia of the plant PAP family (Li et al. 2011; Zhang et al. 2011). Among group Ia, subgroups 1 and 4 are the only clusters in which all the plant species tested in this study have at least one member, suggesting that the function of these protein is important and conserved between mono- and di-cotyledons. Indeed, *Arabidopsis AtPAP10*, *AtPAP12* and *AtPAP26* have been shown to be the three predominant PAP isozymes secreted during Pi-starvation conditions and involved in low-Pi stress adaptation (Veljanovski et al. 2006; Hurley et al. 2010; Tran et al. 2010; Wang et al. 2011). Homologues of *AtPAP10*, *AtPAP12* and *AtPAP26* have been cloned and characterized in a wide range of plant species, suggesting that the functions of these proteins are important and conserved between mono- and di-cotyledons (Bozzo et al. 2002, 2004; Tomscha et al. 2004; Kaida et al. 2010; Tran et al. 2010). *AtPAP10* is mainly associated with the root surface, and was highly active on the substrates of organic phosphates ATP, ADP, pyrophosphate and polyphosphate (Wang et al. 2011). In our study, we found that overexpression of *OsPAP10a* significantly increased the root extracts and surface-associated APase activity. Consistent with the results, overexpression of

OsPAP10a in rice increased the ability of transgenic rice to hydrolyze ATP to release the inorganic form of phosphorus, which facilitated acquisition of Pi by the plants. Moreover, soil growth experimentation showed that overexpression of *OsPAP10a* could improve the P efficiency of plants (**Figure 7**). These results suggest that *OsPAP10a* can potentially be used for crop improvement of P use efficiency similar to *AtPAP15* and *PvPAP3*, which were shown to improve P use efficiency when overexpressed in soybean (Wang et al. 2009b; Liang et al. 2010).

Materials and Methods

Plant materials and growth condition

Oryza sativa japonica var Nipponbare was used for all physiological experiments and rice transformation. Hydroponic experiments were conducted using a modified rice culture solution (Yoshida et al. 1976) 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 $MgSO_4$, 0.25 mM $NaSiO_3$, 0.009 mM $MnCl_2$, 0.075 μM $(NH_4)_6Mo_7O_{24}$, 0.019 μM H_3BO_3, 0.155 μM $CuSO_4$ and 0.152 μM $ZnSO_4$ with 0.125 mM EDTA-Fe. The pH of the solution was adjusted to 5.5. Rice plants were grown in growth chambers with a 12 h photoperiod (200 μmol photons m^{-2} s^{-1}) and a day/night temperature of 30/22 °C after germination. Humidity was controlled to approximately 60%. In the P-deficient treatment, seedlings were transferred to a nutrient solution without a supply of P. In the treatment with added ATP, 0.5 mM ATP was added to the nutrient solution to replace the medium component of 0.323 mM NaH_2PO_4.

Phylogenetic analysis

A protein sequence of *OsPAP10a* was used to search the National Center for Biotechnology Information (NCBI) non-redundant protein database using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Group Ia PAP proteins were selected for alignment with ClustalX (Version 1.81). A neighbour-joining tree with 1000 bootstrap replicates was generated using the Mega 4.1 program.

Reverse transcript PCR (RT-PCR)

Total RNA was extracted from the leaves and roots using a Trizol reagent according to the manfacturer's instructions (Invitrogen, CA, USA). First-strand cDNAs were synthesized from total RNA using SuperScript II reverse transcriptase and this cDNA was used to amplify target genes using RT-PCR. The amount of cDNA template in each sample was normalized based on that of *OsACTIN*. The following primers were used for specific PCR: for *OsPAP10a* (*LOC_01g56880*), (5′-GAGATAGATTTTGCCCCAGAACT-3′ and 5′-AGCTTCAAGCCACTTGTACTGAG-3′); for *OsACTIN* (*LOC_Os03g50885*), (5′-CAACACCCCTGCTATGTACG-3′ and 5′-CATCACCAGAGTCCAACACAA-3′).

Construction of the *OsPAP10a* overexpression vector and plant transformation

The binary vector pCAMBIA1300-mod was used to construct the overexpression vector of *OsPAP10a* as described by Wang et al. (2009a). Full-length cDNA of *OsPAP10a*, from the 5′ UTR to the 3′ UTR, was amplified from rice root cDNA. The purified PCR product was digested with *Bam*HI and *Spe*I, then inserted into pCAMBIA1300-mod under the double CaMV 35S promoter (Wang et al. 2009a). The resulting vector was verified by restriction analysis and DNA sequencing. The above construct was transformed into callus that were derived from mature seeds of WT plants via *Agrobacterium*-mediated transformation, as described by Chen et al. (2003).

Protein extraction and APase activity assay

Approximately 250 mg of root or shoot tissue samples were ground into powder in liquid nitrogen, and homogenized in ice-cold extraction buffer containing 100 mM Potassium acetate, pH 5.5, 20 mM CaCl2, 2 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and 1.5% (w/v) polyvinylpolypyrrolidone with a small scoop of acid-washed sand. Samples were centrifuged at 14 000 g at 4 °C for 20 min, and the supernatant was then removed and placed in 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 of pre-warmed 10 mM pNPP in 50 mM sodium acetate, at pH 5.5. Reactions proceeded for 15 min (root) or 30 min (shoot) at 25 °C, and were then stopped with 1.2 mL of 1 M NaOH. pNP accumulation was read at 412 nm wavelength using a spectrophotometer. The above values were converted to nanomoles by plotting values against a pNP standard curve generated with assay reagents. Phosphatase activity was expressed as nmol substrate hydrolyzed per μg of soluble protein per h (Tomscha et al. 2004).

In-Gel APase activity staining

Two micrograms of proteins were separated on a 4% stacking/10% resolving (w/v) native polyacrylamide gel. After electrophoresis, the gel was washed in ddH_2O and equilibrated with sodium acetate buffer (50 mM Sodium acetate, 10 mM $MgCl_2$, pH 4.9). The gel was stained with 0.5 mg/mL Fast Garnet GBC and 0.5 mg/mL alpha-naphthyl phosphate dissolved in sodium acetate buffer at 37 °C for 0.5 h, washed in distilled water and photographed.

Root-associated acid phosphatase activity assay

In vivo root activity staining of acid phosphatase was detected as described by Bozzo et al. (2006). Briefly, seeds were geminated on Pi-sufficient and deficient solutions and grown for 10 d. The 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, 10 mM $MgCl_2$, 0.6% agar, and 0.1 mg mL^{-1} BCIP at pH 5.3 for 2 h at 25 °C for the visualization of root SAP activity. The BCIP solution is a colorless acid phosphatase substrate, and the root SAP activity is visualized as a blue color, which results from the hydrolysis of BCIP.

To assay the secretary PAP activity in roots, 15-day-old seedlings were transferred into a solution culture with or without a supply of 323 mM Pi. After a 15-d treatment, individual plants were transferred into separate centrifuge tubes containing culture solution and 10 mM pNPP. Plants were then placed in a growth chamber at 30 °C for 1 h. Reactions were terminated using 1 mL 0.25 M NaOH, and the solution was then diluted with 20 mM NaOH. p-Nitrophenol (pNP) accumulation was measured at a 412 nm wavelength using a spectrophotometer. The absorbance values were converted to micromoles by plotting values against a pNP standard curve generated with assay reagents. Phosphatase activity was calculated as micromoles pNP per fresh weight. The experiment was conducted with three replications.

Measure of the total P concentration

The growth of leaves and roots of WT plants and transgenic seedlings grown in ATP nutrient solution were sampled separately and dried at 80 °C to a constant weight. Dried samples (30 mg) were pre-digested in glass tubes with H_2SO_4 for 2 h. The tubes were then heated to 180 °C and 50 μL H_2O_2 was added every 10 min until the solution turned colorless. The digestion was continued for another 30 min. The digested solution was cooled to room temperature and pH was adjusted to 5.0 with 30% (w/v) NaOH. The solution was diluted to an appropriate concentration and mixed with a malachite green reagent in a 3:1 ratio and analyzed 30 min afterwards. The absorption values for the solution at 650 nm were determined using a Spectroquant NOVA60 spectrophotometer (Merck, http://www.merck.com.cn). The Pi concentration was calculated from a standard curve generated with varying concentrations of KH_2PO_4.

Plant growth conditions

Two-week-old seedlings were transferred to pots loaded with 10 kg of natural soil. The soil contained 443 mg/kg total phosphorus and 56 mg/kg of inorganic phosphate. Each pot was planted with two seedlings. After 40 d, the phenotypes were evaluated. Four biological replicates were calculated.

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