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Original Article

OsPAP10c, a novel secreted acid phosphatase in rice, plays an important role in the utilization of external organic phosphorus

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ABSTRACT

Under phosphate (P_i) starvation, plants increase the secretion of purple acid phosphatases (PAPs) into the rhizosphere to scavenge organic phosphorus (P) for plant use. To date, only a few members of the PAP family have been characterized in crops. In this study, we identified a novel secreted PAP in rice, *OsPAP10c*, and investigated its role in the utilization of external organic P. *OsPAP10c* belongs to a monocotyledon-specific subclass of Ia group PAPs and is specifically expressed in the epidermis/exodermis cell layers of roots. Both the transcript and protein levels of *OsPAP10c* are strongly induced by P_i starvation. *OsPAP10c* overexpression increased acid phosphatase (APase) activity by more than 10-fold in the culture media and almost fivefold in both roots and leaves under P_i-sufficient and P_i-deficient conditions. This increase in APase activity further improved the plant utilization efficiency of external organic P. Moreover, several APase isoforms corresponding to *OsPAP10c* were identified using in-gel activity assays. Under field conditions with three different P_i supply levels, *OsPAP10c*-overexpressing plants had significantly higher tiller numbers and shorter plant heights. This study indicates that *OsPAP10c* encodes a novel secreted APase that plays an important role in the utilization of external organic P in rice.

Key-words: *Oryza sativa* (rice); purple acid phosphatase (PAP).

INTRODUCTION

Phosphorus (P) is an essential macronutrient for plant growth and development. Plants take up P only in the form of inorganic phosphate (P_i) from the soil. Although P is abundant in the earth's crust, it is usually present in soil in the form of either organic P or fixed with calcium, iron and aluminium; plants are unable to take up P in these forms (Wu *et al.* 2013). In many countries, P is one of the most restricted nutrients in the soil during crop production. To obtain high crop yields, P fertilizer is widely used in farming; use of these fertilizers accelerates soil degradation and water eutrophication. It is estimated that quality rock P_i, the primary source of P fertilizer, will be depleted at the end of this century (Gilbert 2009). Therefore, the development of crop cultivars with high P_i uptake and utilization efficiency is essential for sustainable agriculture practices and the maintenance of global food supply (Fan *et al.* 2012; Veneklaas *et al.* 2012).

Purple acid phosphatases (PAPs) (EC 3.1.3.2) are a family of binuclear metalloenzymes that hydrolyse a wide range of phosphate esters and anhydrides under acidic conditions. PAPs are so named because the extracted enzymes in solutions appear purple or pink in colour. PAPs show acid phosphatase (APase) activity and differ from other APases in their insensitivity to tartrate inhibition (Olczak *et al.* 2003). In plants, many members of the *PAP* gene family have been identified; these members include 29 *PAPs* in *Arabidopsis*, 26 *PAPs* in rice, 35 *PAPs* in soybean and 33 *PAPs* in maize (Li *et al.* 2002, 2011; Zhang *et al.* 2011; Gonzalez-Munoz *et al.* 2015). These PAPs can be divided into three groups and eight subgroups based on the diversity of their amino sequences. Among these, some *PAP* transcripts are induced in response to P_i deficiency; other *PAPs* are regulated at the protein level (Tran *et al.* 2010b; Zhang *et al.* 2011). Therefore, it has been hypothesized that PAPs play a pivotal role in plant adaptation to P_i deficiency (Tran *et al.* 2010a; Plaxton & Tran 2011).

Over the past decade, several PAPs were found to participate in the utilization and mobilization of extracellular and intracellular organic P in *Arabidopsis*. AtPAP26 is a dominant APase isozyme that is induced post-transcriptionally by P_i starvation. It is dual targeted to both the vacuole and extracellular space in *Arabidopsis* (Veljanovski *et al.* 2006; Hurley *et al.* 2010). In the *atpap26* mutant, the APase activity was significantly decreased in both leaves and roots regardless of the P_i status (Hurley *et al.* 2010; Robinson *et al.* 2012b; Wang *et al.* 2014b). Under P_i deprivation conditions, the mutant showed stronger growth inhibition than the wild type (WT); this finding indicates a specific function of *AtPAP26* in phosphate utilization during P_i starvation (Hurley *et al.* 2010). *AtPAP12*, a close homolog of *AtPAP26*, is partially functionally redundant with *AtPAP26* (Robinson *et al.* 2012b). AtPAP12, AtPAP26 and AtPAP10 were found to be the major root-associated APase isozymes; these proteins were also secreted into the growth

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media (Wang *et al*. 2011; Robinson *et al*. 2012b). *AtPAP10* expression is induced by both local and systemic P_i starvation signals and is involved in utilization of organic P (Wang *et al*. 2011; Zhang *et al*. 2014). AtPAP25 was purified together with AtPAP12 and AtPAP26 from the cell walls of P_i-starved suspension cells (Del Vecchio *et al*. 2014). AtPAP25 appears to be a phosphoprotein phosphatase that regulates P_i starvation signalling rather than a non-specific scavenger of organic P, as demonstrated for AtPAP10, AtPAP12 and AtPAP26 (Del Vecchio *et al*. 2014; Wang *et al*. 2014b). AtPAP15 exhibits a high phytase activity and is involved in phytate metabolism (Zhang *et al*. 2008; Kuang *et al*. 2009). The overexpression of *AtPAP15* in soybean resulted in increased yields in low-P_i soils (Wang *et al*. 2009). Mutations of several other PAPs, including *AtPAP1*, *AtPAP2*, *AtPAP3*, *AtPAP4* and *AtPAP5*, resulted in reduced APase activity under P_i starvation conditions in the roots of *Arabidopsis* (Wang *et al*. 2014b). However, the physiological functions of these PAPs in P_i-limiting conditions are unknown.

Functional analyses of PAPs in crop species are more limited. Although a number of P_i starvation-induced PAPs have been identified by RT-PCR and microarray analyses in rice, soybean and maize, the functions of the majority of these PAPs remain unknown (Li *et al*. 2011; Zhang *et al*. 2011; Gonzalez-Munoz *et al*. 2015). Our previous study demonstrated that *OsPAP10a* expression in rice leaves and roots is greatly induced by P_i starvation and phosphate starvation response protein 2 (*OsPHR2*), the central transcription factor controlling P_i signalling. *OsPAP10a* overexpression significantly increases APase activity and growth performance when organic P was supplied (Tian *et al*. 2012). *PvPAP3* expression in the common bean is induced by P_i starvation. Purified PvPAP3 hydrolyses extracellular ATP (Liang *et al*. 2010). PAPs possessing phytase activity have been cloned in rice, maize, barley and wheat; these proteins are homologs of *AtPAP15* (Dionisio *et al*. 2011). These PAPs are predicted to play roles in phytate metabolism during grain germination (Dionisio *et al*. 2011; Madsen *et al*. 2013).

In *Arabidopsis*, the major extracellularly secreted, root-associated, cell-wall-bound and intracellularly secreted APases (SAPs) are all encoded by PAP genes of the Ia group. Genetic and physiological analyses of mutants and overexpression lines demonstrated the roles these PAPs play in the plant adaptation to P_i limitation (Hurley *et al*. 2010; Wang *et al*. 2011; Robinson *et al*. 2012b; Del Vecchio *et al*. 2014; Wang *et al*. 2014b). Studies in other species have also indicated the importance of the Ia group of PAP proteins in organic P_i utilization or the adaptation to P_i stress (Bozzo *et al*. 2004, 2006; Tian *et al*. 2012). In this study, we demonstrated that *OsPAP10c*, a close homolog of *OsPAP10a*, is a SAP exhibiting substantially higher APase activity than *OsPAP10a*. In contrast with previously described *PAPs*, *OsPAP10c* belongs to a monocotyledon-specific subclass of Ia group PAPs and is primarily expressed in the epidermis/exodermis cell layers of roots and root tips. Constitutive *OsPAP10c* overexpression significantly increased APase activity in leaves, roots, the root surface and culture media. *OsPAP10c* represents a novel secreted PAP in roots that scavenges organic P under P_i starvation. This enzyme has great potential for practical applications in the improvement of P use efficiency in crops.

MATERIALS AND METHODS

Plant material and growth conditions

The Nipponbare cultivar of *Oryza sativa* (japonica group) was used for all physiological experiments and rice transformations. Hydroponic experiments were conducted using a modified 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 $MgSO_4$, 0.25 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 ethylenediaminetetraacetic acid (EDTA)-Fe (II) (Wang *et al*. 2012). This nutrient solution was referred to as a sufficient P_i (+P) condition. For the P_i-deficient (−P) treatment, seedlings were transferred to a nutrient solution without P_i or as indicated in the text. For ATP degradation and utilization experiments, 0.1 mM ATP (equivalent to 0.3 mM P_i when completely hydrolysed) was added to the nutrient solution to replace the NaH_2PO_4. The pH of all nutrient solutions was adjusted to 5.5. Rice seedlings 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 at 60% relative humidity (RH).

Reverse-transcription PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from the leaves and roots using a TRIzol reagent according to the manufacturer's instructions (Invitrogen, CA, USA). Reverse transcription and qRT-PCR were performed according to Wang *et al*. (2012). The rice actin gene (*OsACTIN*) was used as a reference gene. The sequences of the *OsPAP10c* primers are as follows: 5′-GTACAATCTAAAAGGGAGTCAC-3′ and 5′-CTCTGTTCTGGATAATCTCACTG-3′. The sequences of the *OsACTIN* primers are as follows: 5′-GGAACTGGTATGGTCAAGGC-3′ and 5′-AGTCTCATGGATACCCGCAG-3′.

Phylogenetic analysis

The amino acid sequence of OsPAP10a was used to search the National Center for Biotechnology Information (NCBI) non-redundant protein database via the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify other group Ia PAPs. The full-length amino acid sequences of the selected group Ia PAPs were aligned using ClustalX. The non-conserved regions of the amino acid sequences were deleted based on the results from the first round of multiple alignments; this step was performed to avoid misalignments derived from substantial sequence differences in PAPs from different species. The modified amino acid sequences were then used for a second round of multiple alignments building. Based on the multiple alignments from the second round of analysis, a

neighbour-joining tree with 1000 bootstrap replicates was constructed using the MEGA program with the identified PAPs from *Arabidopsis* and the following crop species: rice, maize (*Zea mays*), turnip rape (*Brassica rapa*), sorghum (*Sorghum bicolor*) and soybean (*Glycine max*). Proteins were named according to their *Arabidopsis* homolog. The names and NCBI accession numbers of these genes are as follows: *ZmPAP10a*, GRMZM2G093101; *ZmPAP10b*, GRMZM2G073860; *ZmPAP10c*, GRMZM2G077466; *ZmPAP26*, AC211394.4_FG004; *SbPAP10a*, Sb03g036210; *SbPAP10c*, Sb09g030100; *BrPAP10a*, LOC103860461; *BrPAP10b*, LOC103837767; *BrPAP10c*, LOC103860459; *BrPAP12a*, LOC103864734; *BrPAP12b*, LOC103864735; *BrPAP5a*, LOC103837841; *BrPAP5b*, LOC103828428; *BrPAP6*, LOC103862464; *BrPAP25*, LOC103834978; and *BrPAP26*, LOC103833746.

Vector construction and plant transformation

Vectors for *OsPAP10c* overexpression and an *OsPAP10c* promoter *β*-glucuronidase (*GUS*)–reporter ($P_{OsPAP10c}$-GUS) were constructed and introduced into the Nipponbare rice as described previously (Wang *et al.* 2012). In brief, full-length *OsPAP10c* was cloned and inserted into the pTF101-ubi vector after digestion with *Bam*HI and *Sma*I. The primer sequences for the amplification of *OsPAP10c* are as follows: 5′-CGGGATCCATGGGGATGCTGCGGTGG-3′ and 5′-CCCCCGGGTTATACATCGTCGTTGGTGGG-3′. For the $P_{OsPAP10c}$-GUS construct, the 2 kb fragment upstream of the start codon ATG was amplified from the Nipponbare genomic DNA. The sequences of the primer are as follows: 5′-ACCATGATTACGCCAGATAAAGACTCAAAGTAT-3′ and 5′-GATCTACCATGGTACGGTGACTGTGAGCTAGCC-3′. The purified PCR product was cloned into the binary vector pBIGUS-plus using a GBclonart Seamless Assembly Kit according to the manufacturer's instructions (GBI, Suzhou, China). Transgenic lines were produced with resulting plasmids via *Agrobacterium*-mediated rice transformation.

Protein extraction from plant tissues and culture media

Root and leaf tissues were ground separately into fine powders in liquid nitrogen and homogenized in ice-cold extraction buffer containing 0.1 M potassium acetate, 20 mM $CaCl_2$, 2 mM EDTA and 0.1 mM phenylmethylsulphonyl fluoride at pH 5.5. Samples were gently agitated on ice for 0.5 h and then centrifuged at 14 000 *g* at 4 °C for 20 min. The supernatant was transferred to a fresh tube before use. The protein content was quantified using Coomassie Plus protein assay reagent.

Rice calli with uniform size were suspended in a 100 mL flask containing 10 mL of R2S liquid media and gently shaken in a growth chamber (80 rpm, 28 °C). R2S liquid medium contains the following components: 39.57 mM KNO_3, 2.54 mM $(NH_4)_2SO_4$, 1.01 mM $MgCl_2$, 1.75 mM NaH_2PO_4, 1.02 mM $CaCl_2$, 0.02 mM $FeSO_4$, 0.02 mM Na_2EDTA, 49 *μ*M H_3BO_3, 7 *μ*M $MnSO_4$, 8 *μ*M $ZnSO_4$, 0.5 *μ*M $CuSO_4$, 0.52 *μ*M Na_2MoO_4, 555 *μ*M myo-inositol, 4 *μ*M nicotinic acid, 2 *μ*M pyridoxine HCl, 1.48 *μ*M thiamine HCl, 27 *μ*M glycine, 87.65 mM sucrose, and 9 *μ*M 2,4-D. Before treatment, the calli were transferred into normal or P_i-deficient R2S media. The media were refreshed every 5 d. After 7 d of treatment, the calli were removed using filter papers; the liquid culture media were collected. Proteins were concentrated and isolated using centrifugal filter devices with a 3 kDa cut-off at 4 °C according to the manufacturer's instructions (Merck KgaA, Darmstadt, Germany). The protein content was quantified using Coomassie Plus protein assay reagent.

OsPAP10c and OsACTIN antibodies

The open reading frame (ORF) of *OsPAP10c* was amplified and inserted into the expression vector pET-30a. OsPAP10c protein was purified from *Escherichia coli* cells that were transformed with pET-30a. An anti-OsPAP10c polyclonal rabbit antibody was raised and affinity purified against the immunogen sequence. The final concentration of affinity-purified antibodies used for the immunoblot analyses was 1:500 (v/v). An antibody against ACTIN was purchased from EarthOx LLC, Millbrae, CA, USA (Plant-ACTIN Rabbit Polyclonal Antibody).

Western blot analysis of OsPAP10c proteins

Total proteins were extracted as described earlier and separated on 10% sodium dodecyl sulphate (SDS)–polyacrylamide gels. Proteins were then transferred to a polyvinylidene difluoride membrane in transfer buffer (123.8 mM Tris, 77.26 mM glycine, 1.28 mM SDS and 20% methanol). The membrane was blocked with 5% milk in TBST buffer (0.01 M Tris-HCl, 0.5 M NaCl and 0.05% Tween-20) at room temperature for 1 h and then incubated with OsPAP10c antibodies (1:500) at room temperature for 1 h in TBST buffer containing 0.5% milk. The membrane was then incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody at a dilution of 1:10 000 for 1 h. After all reactions were completed, the membrane was washed three times with TBST (5 min each) and incubated for 5 min at room temperature in a mixture (1:1) of the two enhanced chemiluminescence solutions. The blotted membrane was detected using a storage phosphor imaging device (UVP, Upland, CA, USA).

5-Bromo-4-chloro-3-indolyl-phosphate staining and APase activities

In vivo root APase activity staining was performed as described (Tian *et al.* 2012). Seeds were geminated on +P and −P nutrient solutions and grown for 10 d. The roots of seedlings were excised and incubated on a 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)–agar solution containing 10 mM phosphate buffer (pH 5.2), 0.5% agar and 0.01% BCIP for 1 h at 25 °C. Cleavage of BCIP by APase produces blue precipitates, which were imaged.

To measure root surface-associated APase (RSAP) activity against the substrate *p*-nitrophenyl phosphate (pNPP), 2-week-old seedlings were transferred into nutrient solutions with different P supply levels (+P, −P and 0.1 mM ATP). After 10 d of treatment, seedlings were rinsed in distilled water and transferred into separate tubes containing 30 mL of culture solution with 10 mM pNPP. Plants were then placed in a growth chamber at 30 °C for 10 min. The reaction was stopped with 1 M NaOH. The pNPP substrate was measured at 410 nm using a Spectroquant NOVA60 spectrophotometer (Merck, Darmstadt, Germany). The pNPP content was calculated using a standard curve generated with varying concentrations of pNPP. Root-associated APase activity was expressed as micromoles of pNPP per hour per milligram of fresh weight. The measurements were repeated three times with three biological replicates for each sample.

To measure specific APase activity, 1 μg (roots and media) or 10 μg (leaves) of proteins was added to 600 μL of pre-warmed 10 mM pNPP in 50 mM sodium acetate at pH 5.5. The reactions were allowed to proceed at 25 °C for 10 min for root and leaf proteins or 20 min for media proteins. The reactions were stopped by the addition of 1.2 mL of 1 M NaOH. The absorbance was determined at 410 nm using a Spectroquant NOVA60 spectrophotometer (Merck). APase activity was expressed as micromoles of pNPP per minute per milligram of protein.

In-gel APase profiling

The proteins were separated on a 10% non-reducing polyacrylamide gel electrophoresis (PAGE) gel at 4 °C. The gels were gently washed in cold distilled water (10 min per wash for a total of six washes) to remove salt. The gels were washed twice (15 min each) by gentle shaking in a buffer containing 50 mM sodium acetate (pH 4.9) and 10 mM $MgCl_2$. After equilibration in buffer, the gels were stained with 0.5 mg mL^{-1} Fast Garnet GBC (Sigma, St Louis, MO, USA) and 0.5 mg mL^{-1} alpha-naphthyl phosphate (Sigma) dissolved in sodium acetate buffer. Gels were then washed in distilled water and imaged.

GUS histochemical analysis

The T1 generation of $P_{OsPAP10c}$-GUS transgenic plants was grown in +P and −P rice nutrient solutions. Different tissues were sampled for GUS staining as described previously (Wang *et al.* 2014a). In brief, plant tissues from the GUS transgenic lines were submerged in GUS staining solution and vacuum infiltrated for 10 min; this process was followed by incubation at 37 °C for 2 h. After staining, the tissues were washed with 70% ethanol to remove chlorophyll and observed under a stereoscope. For the sectioning, the roots and leaves were embedded in 3% agar and cut into 30 µm sections using a VT1000S vibratome (Leica, Nußloch, Germany) and observed/imaged.

Measurement of P_i concentration and total P content

The leaves and roots of the WT and transgenic seedlings from different treatments were sampled separately. The P_i concentration was measured using the procedure described previously (Wang *et al.* 2012). Briefly, 50 mg of fresh tissue was homogenized with 50 μL of 5 M H_2SO_4 and 3 mL of H_2O. The homogenate was transferred to 1.5 mL tubes and centrifuged at 10 000 *g* for 10 min at 4 °C. The supernatant was collected and diluted to an appropriate concentration. The diluted supernatant was mixed with a malachite green reagent (19.4 mM H_3BO_3, 27.64 mM $(NH_4)_6MO_7O_{24}$, 2.38 M H_2SO_4, 627.5 μM malachite green and 0.1% polyvinyl alcohol) at a 3:1 ratio and analysed after 20 min. The absorption values for the solution at 650 nm were determined using a Spectroquant NOVA60 spectrophotometer (Merck). The P_i content was quantified based on a standard curve generated using various concentrations of KH_2PO_4. Free P_i released from ATP in the nutrient solutions was directly measured using the method described earlier. If the added ATP is completely cleaved into adenosine and P_i by APase, 1 M of ATP will release 3 M of P_i into the solution. Therefore, the ratio of P_i released from ATP can be calculated as follows: $[P_i]/[ATP]_{mol} \times 3 \times 31$. Here, $[P_i]$ is the P_i concentration (mg mL^{-1}) in solution. $[ATP]_{mol}$ is the molar concentration of ATP (mol L^{-1}) added to the solution, which is 0.1 mM in this study. The molecular weight of P is 31.

For the total P measurement, the plant tissues were dried under 80 °C to a constant weight. A total of 30 μg of dried samples were pre-digested in glass tubes with H_2SO_4 for 2 h. The tubes were then heated to 180 °C, and 50 µL of H_2O_2 was added every 10 min until the solution turned colourless. The digestion was continued for an additional 30 min. The digested solution was cooled to room temperature, and the pH was adjusted to 5.0 with 30% NaOH. The solution was diluted to an appropriate concentration, and the P_i content was analysed as described earlier.

Statistical analysis

Data were statistically analysed in the Data Processing System (DPS Version 7.05, Zhejiang, China). Student's *t*-test was used to determine the significance of differences between the WT and transgenic lines in each treatment. A least significant difference (LSD) test was used to conduct the pairwise comparison of samples.

RESULTS

Phylogenetic analysis of Ia group PAPs in plants

Genomic searches for PAP proteins identified Ia group PAP proteins in *Arabidopsis* (8), turnip rapeseed (*B. rapa*) (10), soybean (*G. max*) (7), rice (5), maize (*Z. mays*) (4) and sorghum (*S bicolor*) (2). A phylogenetic tree was constructed with these 36 PAP proteins. These proteins were divided into four subfamilies; this division is in contrast with a previous

analysis using only *Arabidopsis* PAPs, which identified two subfamilies (Supporting Information Fig. S1, Li *et al.* 2002). Among these four subfamilies, the Ia-1 subfamily is the most divergent group and includes members from all six species. The Ia-2 and Ia-3 subfamilies exclusively contain PAPs from dicotyledons and monocotyledons, respectively (Supporting Information Fig. S1). Ia-4 subfamily members are homologs of AtPAP26 and are dual targeted to the extracellular space and the vacuole (Hurley *et al.* 2010).

While OsPAP10a is classified as an Ia-1, this subgroup contains AtPAP10. The remaining rice PAP10s, including OsPAP10b, OsPAP10c and OsPAP10d, were classified as Ia-3 proteins. It is noteworthy that these three PAP genes cluster together within a 10 kb region of the genome (Supporting Information Fig. S2a). Moreover, *LOC_Os12g44030*, *LOC_Os12g44040* and *LOC_Os12g44050* are mis-annotated as three ORFs. Indeed, *LOC_Os12g44030* and *LOC_Os12g44050* encode the N-terminal and C-terminal regions of the OsPAP10d protein, which is disrupted by the putative transposon gene *LOC_Os12g44040* (Supporting Information Fig. S2a,b).

OsPAP10c expression is specifically induced by P_i starvation in roots

All five PAP genes in the Ia group are expressed in rice, as isolated expressed sequence tag (EST) sequences have been found in dbEST (Supporting Information Table S1). The expression of these PAP genes in different tissues was analysed using the data collected from the microarray database (Zimmermann *et al.* 2005). According to the microarray data, *OsPAP26* showed high expression levels in all tissues; *OsPAP10a* had moderate expression levels across different tissues (Fig. S3). By contrast, *OsPAP10c* was predominantly expressed in roots compared with other parts of the plant; the expressions of *OsPAP10b* and *OsPAP10d* were lower in all tissues (Supporting Information Fig. S3).

Previously, we demonstrated that *OsPAP10a* was specifically induced by P_i starvation in both roots and leaves (Tian *et al.* 2012). Expression analysis of *OsPAP10c* under different P_i-deficient conditions showed that the induced expression of *OsPAP10c* by P_i starvation occurred only in the roots. Induction occurs at P_i concentrations in media lower than 3.23 μM. The abundance of *OsPAP10c* transcripts in the leaves was not affected by the P_i level (Fig. 1b). Immunoblotting for OsPAP10c via Western blot using an affinity-purified rabbit anti-OsPAP10c-IgG showed that a prominent 57 kDa peptide band was greatly induced in the protein extracts of P_i-starved root tissues (lanes 3 and 4 in Fig. 1c). However, the band could not be detected by the OsPAP10c antibody in leaf tissues (data not shown).

OsPAP10c is predominantly expressed in the root epidermis

To precisely analyse the expressions of *OsPAP10c* in different tissues, 17 independent transgenic events resulting in the expression of an *OsPAP10c* promoter::GUS construct were generated. Fifteen of these transgenic lines showed similar GUS expression patterns. Staining of the transgenic plants

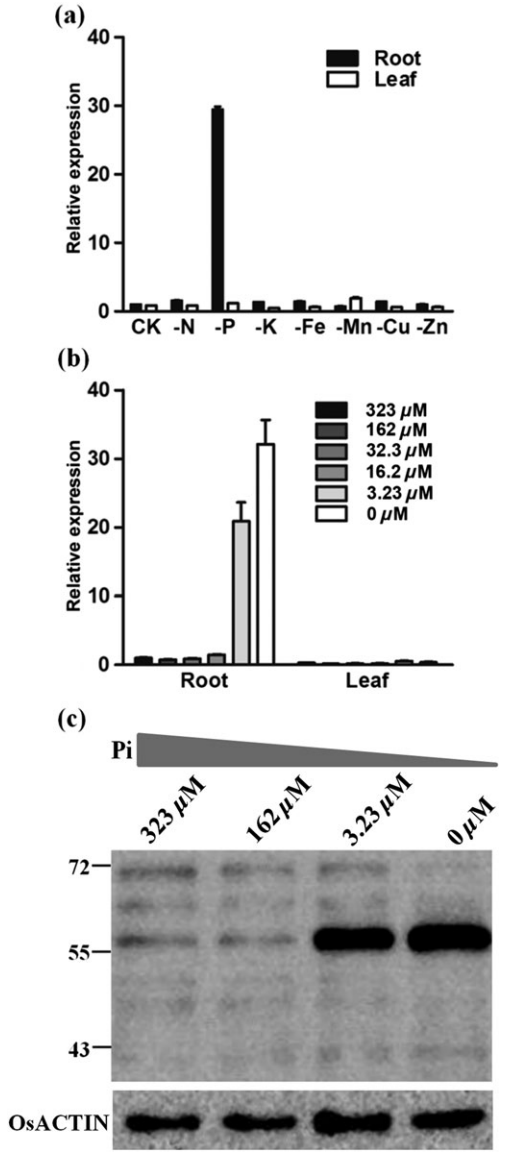

Figure 1. (a) Expression analysis of *OsPAP10c* under different nutrient deficiency conditions. Two-week-old seedlings were transferred to normal nutrient solution (CK) or solutions deprived of nitrogen (−N), phosphate (−P), potassium (−K), iron (−Fe), manganese (−Mn), zinc (−Zn) or copper (−Cu). Leaves and roots were sampled after 10 d of treatment, and RNA was extracted for quantitative RT-PCR (qRT-PCR). *OsPAP10c* expression was normalized to that of *OsACTIN*. (b) Expression analysis of *OsPAP10c* under different P_i-deficient conditions. Two-week-old seedlings were transferred to nutrient solutions with different P_i concentrations. Leaves and roots were sampled after 10 d of treatment, and RNA was extracted for qRT-PCR. *OsPAP10c* expression was normalized to *OsACTIN*. (c) Detection of OsPAP10c in a Western blot of the leaf protein extracts from WT plants grown under different P_i supply levels. Two-week-old seedlings were transferred to nutrient solution with different P_i concentrations. Leaves or roots were sampled after 10 d of treatment, and proteins were extracted. Six micrograms of total protein was loaded in each lane.

revealed that the *OsPAP10c* is basally expressed in all of the examined tissues, including leaves, flowers, roots and pollen (Figs 2 & S4). In accordance with the qRT-PCR results, *OsPAP10c* expression was induced by P_i starvation in roots but not leaves; a very high expression was observed in root tips (Fig. 2a,b). Cross sections of the different regions of the roots showed that *OsPAP10c* was predominantly expressed in epidermis and exodermis cells (Fig. 2c,d,e,f).

OsPAP10c overexpression increased APase activity in both roots and leaves

To generate transgenic rice plants with enhanced APase activity, the full-length cDNA of *OsPAP10c* was expressed in Nipponbare rice; transgenic expression was driven by the maize ubiquitin promoter. Three independent transgenic lines that highly expressed *OsPAP10c* were selected for further physiological analysis (Fig. 3a). Immunoblotting revealed that the OsPAP10c protein was highly expressed in the leaves of the *OsPAP10c* overexpression lines (Fig. 3b). The OsPAP10c antibody was not able to detect any bands in the leaf protein extracts of WT or *OsPAP10a*-overexpressing plants (Fig. 3b). The data also verified that the OsPAP10c antibody specifically recognizes the protein and does not cross react with its homolog OsPAP10a.

5-Bromo-4-chloro-3-indolyl-phosphate staining is a simple and effective way to measure APase activity on the root surface. Both P_i starvation and *OsPAP10a* overexpression significantly increased the level of BCIP staining (Supporting Information Fig. S5); these findings are in agreement with the previously reported results (Tian *et al.* 2012). Compared with *OsPAP10a*-overexpressing plants, *OsPAP10c* overexpression lines showed a more pronounced staining (Supporting Information Fig. S5). To quantify the RSAP, the *OsPAP10a*-overexpressing and *OsPAP10c*-overexpressing plants were grown under different P_i supply conditions for 10 d. Plants were transferred to a culture solution containing 10 mM pNPP, the phosphatase substrate. Under P_i-sufficient conditions, RSAP increased 9-fold to 11-fold in *OsPAP10c*-overexpressing plants, which are significantly higher than that in the WT. RSAP in *OsPAP10a*-overexpressing plants was twofold higher than that in WT plants (Fig. 4a). P_i starvation significantly increased RSAP in the WT plants and *OsPAP10a*-overexpressing plants (approximately fivefold). In contrast, the RSAP in *OsPAP10c*-overexpressing plants remained at a similar level as that in P_i-sufficient conditions. *OsPAP10c*-overexpressing plants remained nearly twofold greater than that in WT plants (Fig. 4a).

The intracellular APase activities (IAPs) in the leaves and roots of *OsPAP10c*-overexpressing plants were significantly greater than those in WT plants or *OsPAP10a*-overexpressing plants. Under both P_i-sufficient and P_i-deficient conditions, the IAPs in the leaves and roots of *OsPAP10a*-overexpressing plants were only 1.5-fold to 2-fold higher than those in WT plants (Fig. 4b,c). In contrast, IAPs in the leaves and roots of *OsPAP10c*-overexpressing plants were approximately five times greater than those in WT plants (Fig. 4b,c). While P starvation did not significantly change the leaf and root IAPs in the WT and *OsPAP10a*-overexpressing plants, the leaf

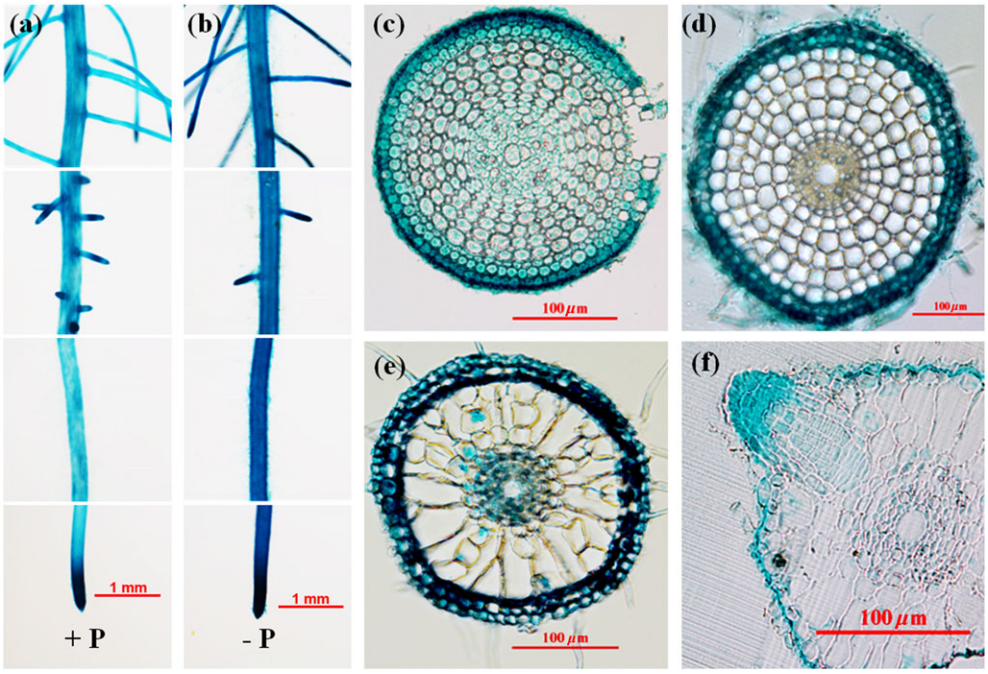

Figure 2. β-Glucuronidase (GUS) reporter gene expression in transgenic plants expressing the *OsPAP10c* promoter–GUS constructs. Seeds were geminated on +P and −P solutions and grown for 10 d. The roots of the seedlings were excised and stained with GUS solution. Bar = 100 μm. (a, b) GUS staining of the roots of $P_{OsPAP10c}$-GUS transgenic plants grown in P_i-sufficient (a) or P_i-deficient (b) conditions. (c, d, e, f) Transverse section of different regions of GUS-stained roots of $P_{OsPAP10c}$-GUS transgenic plants grown under P_i-deficient conditions: (c) 3 mm from root tip, (d) 1 cm from root tip, (e) 3 cm from root tip; (f) the region of lateral root emergence.

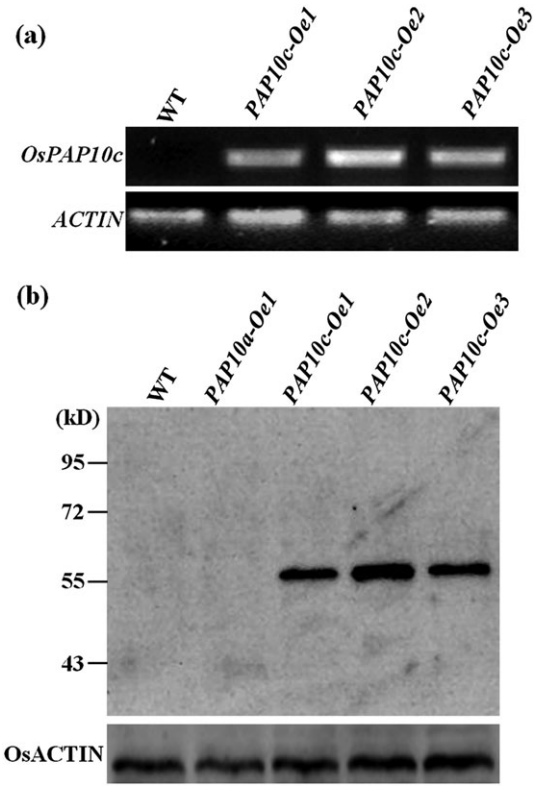

Figure 3. Expression analysis (a) and Western blot detection (b) of *OsPAP10c* in wild-type (WT) and transgenic plants under P_i-sufficient conditions. RNA and protein were extracted from the leaves of WT and transgenic plants. Six micrograms of total protein was loaded in the gels used for Western blotting. *PAP10a-Oe1*, overexpression of *OsPAP10a* line 1; *PAP10c-Oe1*, *PAP10c-Oe2* and *PAP10c-Oe3*, overexpression of *OsPAP10c* line 1, line 2 and line 3, respectively.

IAP, but not the root IAP, in *OsPAP10c*-overexpressing plants was significantly induced by P_i deprivation (Fig. 4b,c).

Overexpression of *OsPAP10a* and *OsPAP10c* generates different APase isoforms

In-gel activity assays were conducted to compare the APase isoforms present in both the roots and leaves of *OsPAP10a*-overexpressing and *OsPAP10c*-overexpressing plants. As previously reported, P_i starvation significantly induced the accumulation of a major APase isoform (A2) in both the leaves and roots of WT plants. Under P_i-sufficient conditions, the A2 isoform also accumulated to a high level in the leaves and roots of *OsPAP10a*-overexpressing plants (Fig. 5a,b, Tian *et al.* 2012). *OsPAP10c* overexpression increased the accumulation of several other APase isoforms, including A1, A3, A4 and A5, but not A2 (Fig. 5a,b). A1, A3, A4 and A5 APase isoforms were not observed in either the leaves or roots of WT and *OsPAP10a*-overexpressing plants (Fig. 5a,b). To verify the composition of these isoforms, immunoblotting of non-denaturing gels was performed using the OsPAP10c antibody. As shown in Fig. 6a,b, the OsPAP10c antibody detected high levels of the A4 and A5 isoforms in both leaf and root protein

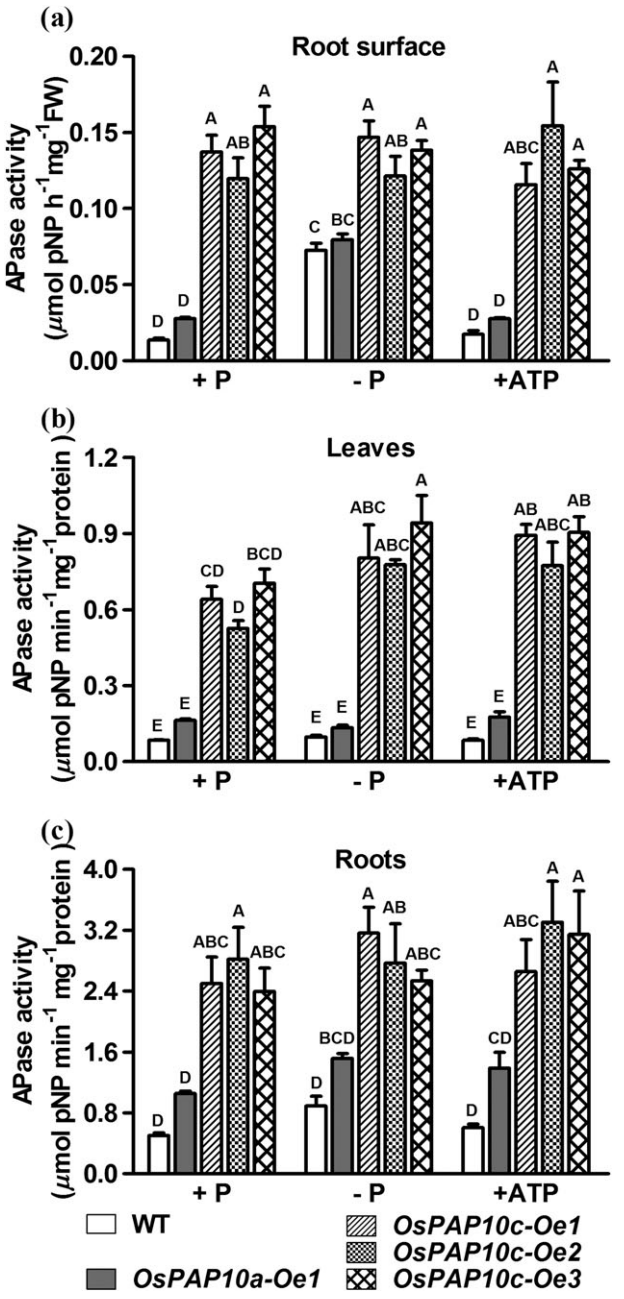

Figure 4. Acid phosphatase (APase) activities in the root surface (a), leaves (b) and roots (c) of wild-type (WT) and transgenic plants under different P_i supply levels. Germinated seeds were grown in normal nutrient solution for 2 weeks and transferred to nutrient solutions containing 0.323 mM P_i (+P), without P_i (−P) or containing 0.1 mM ATP (+ATP) for 10 d. Seedlings were washed and transferred into media containing 10 mM *p*-nitrophenyl phosphate (pNPP) for 10 min. The amount of released pNPP was quantified to calculate the APase activity at the root surface. To determine APase activity in the leaves and roots, leaf and root tissues were separately sampled for protein extraction. The hydrolysis of pNPP was used to measure the APase activity. Data are represented as the means of three replicates with standard error of the mean (SEM). Least significant difference (LSD) test was used to perform the pairwise comparison of different samples. Means with different capital letters indicate significantly different at the $P < 0.01$ level.

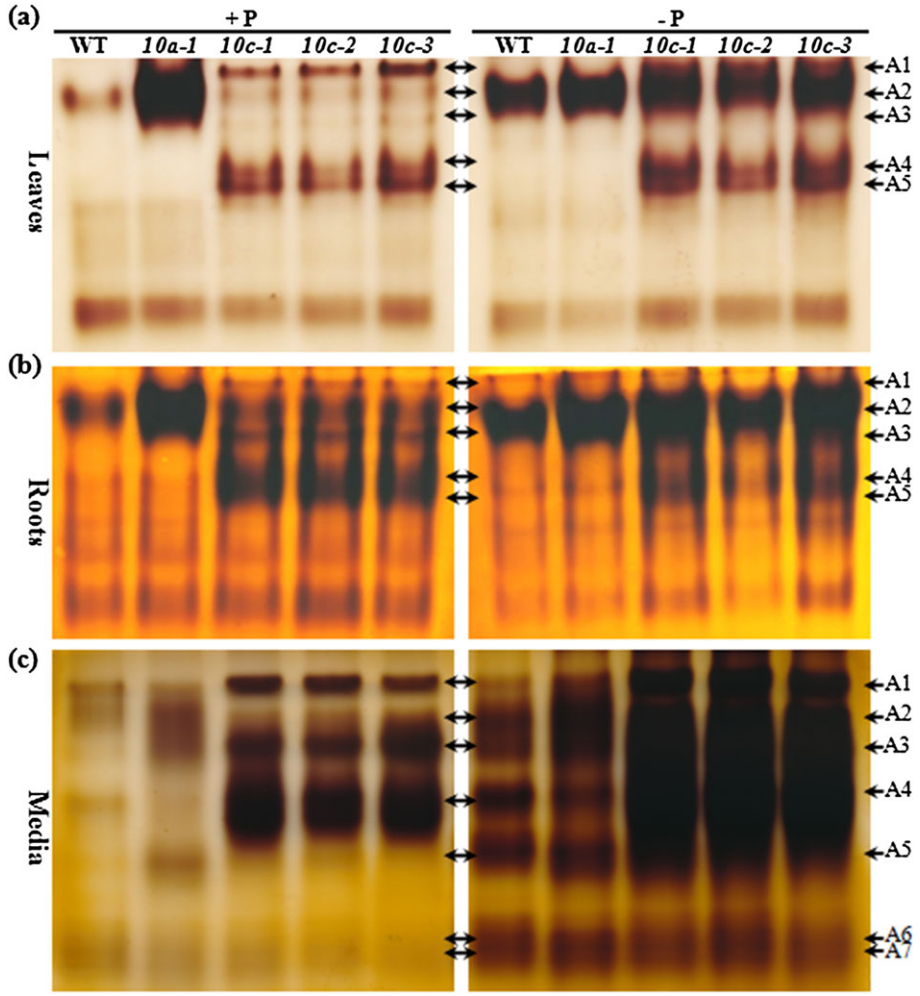

Figure 5. In-gel acid phosphatase (APase) activity assay of leaves (a), roots (b) and culture media (c) of the wild-type (WT) and transgenic plants grown under P_i-sufficient and P_i-deficient conditions. Proteins were extracted from leaves, roots or the culture media as described in the Materials and Methods section. The proteins were electrophoresed in native discontinuous polyacrylamide gel electrophoresis (PAGE) and stained for APase activity using Fast Garnet GBC and alpha-naphthyl phosphate. Different APase isoforms are indicated by arrows.

extracts of *OsPAP10c*-overexpressing plants. These bands were not detectable in samples from WT and *OsPAP10a*-overexpressing plants (Fig. 6a,b).

OsPAP10c overexpression significantly increases APase secretion

To determine whether P_i deficiency induced OsPAP10c secretion into the media, suspension cultures derived from the calli generated from WT plants and *OsPAP10a* and *OsPAP10c* overexpression lines were established. Proteins in the culture media were harvested and concentrated. The APase activity in these protein extracts represents the SAP activity of the calli grown in the media. While P_i starvation and *OsPAP10a* overexpression increased SAP activity by approximately twofold, *OsPAP10c* overexpression resulted in a 10-fold increase in SAP (Fig. 7a). Furthermore, SAPs in *OsPAP10c* overexpression lines were significantly elevated by P_i deprivation (Fig. 7a).

Western blotting using these medium protein extracts confirmed that OsPAP10c was indeed secreted into the media in all measured samples (Fig. 7b). The amounts of secreted *OsPAP10c* protein were induced by P_i starvation in the WT, *OsPAP10a*-overexpressing and the *OsPAP10c*-overexpressing calli (Fig. 7b). To further verify these findings, in-gel activity assays were performed to measure the presence of different APase isoforms present in the culture media. In leaf or root protein extracts, P_i starvation induced only the accumulation of the A2 isoform in WT and *OsPAP10a*-overexpressing plants. In the growth medium protein extracts, at least six APase isoforms were significantly induced; these isoforms were A2, A3, A4, A5, A6 and A7 (Fig. 5c). Under P_i-sufficient conditions, *OsPAP10c* overexpression induced the secretion of A1, A3 and A4 APase isoforms into the culture media. The SAP activities of these APase isoforms were further induced by P_i starvation in suspended *OsPAP10c*-overexpressing calli (Fig. 5c). A Western blot of the non-denatured proteins showed that OsPAP10c consisted of several different isoforms, including A1, A3, A4 and A5 (Fig. 6c).

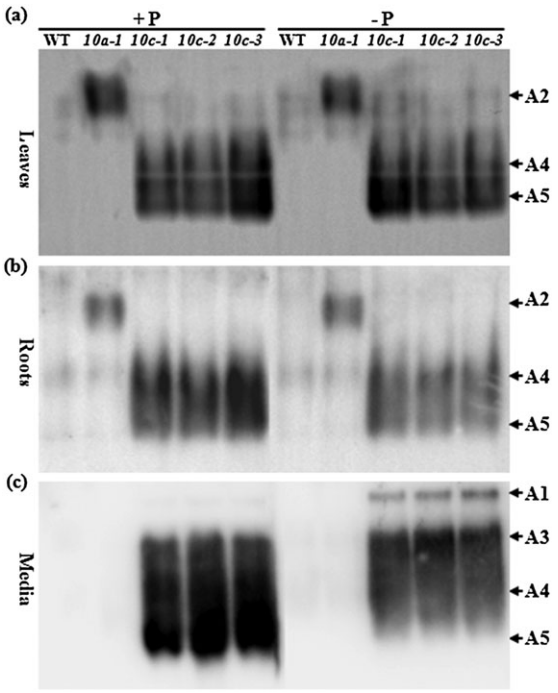

Figure 6. Western blotting with the native proteins of leaves (a), roots (b) and culture media (c) of the wild-type (WT) and transgenic plants grown under P_i-sufficient and P_i-deficient conditions. An OsPAP10c antibody was used to detect the target protein. Proteins were extracted from leaves, roots or the culture media as described in the Materials and Methods section. Six micrograms of total protein was loaded in the gels used for Western blotting. Acid phosphatase isoforms are indicated by arrows.

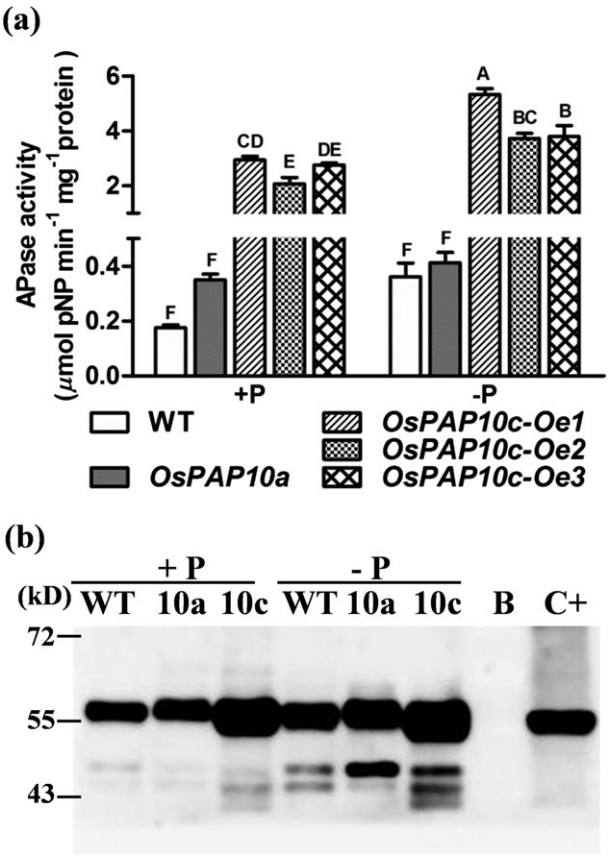

Figure 7. Extracellular acid phosphatase (APase) activity (a) and Western blot detection (b) of proteins extracted from the culture media of wild-type (WT) and transgenic calli grown in P_i-sufficient and P_i-deficient conditions. Calli were suspended in P_i-sufficient and P_i-deficient media for 1 week. Proteins were extracted from the culture media. (a) *p*-Nitrophenyl phosphate (pNPP) hydrolysis was used to measure APase activity. The data are the means of three replicates with standard error of the mean (SEM). Least significant difference (LSD) test was used to perform the pairwise comparison of different samples. Means with different capital letters indicate significant difference at the $P < 0.01$ level. (b) Western blot detection of proteins using the OsPAP10c antibody. Six micrograms of secreted proteins was loaded. 10a, *OsPAP10a* overexpression line; 10c, *OsPAP10c* overexpression; B, blank control; C+, proteins extracted from the leaves of *OsPAP10c*-overexpressing plants were used as a positive control.

OsPAP10c overexpression increased extracellular ATP degradation and utilization

To determine whether *OsPAP10c* overexpression enhances the release and utilization of organic P_i, the degradation kinetics of 0.1 mM ATP in WT, *OsPAP10a*-overexpressing and *OsPAP10c*-overexpressing plants were compared. The ATP in the nutrient solution in which WT rice plants were grown gradually degraded from days 1 to 4; 41% of free P_i was released from ATP at day 5 (Fig. 8a). Compared with WT plants, *OsPAP10c* overexpression significantly increased the release of P_i from ATP at day 2; nearly 90% of free P_i was released at day 5 in *OsPAP10c*-overexpressing plants (Fig. 8a). *OsPAP10a*-overexpressing plants served as a control; 77% of free P_i was released from ATP at day 5. These results were in agreement with the APase activities determined in the WT plants and in *OsPAP10a* and *OsPAP10c* overexpression lines under ATP growth conditions (Fig. 4). P_i concentrations in the leaves of cultured WT plants and *OsPAP10a* and *OsPAP10c* overexpression lines grown in solution using 0.1 mM ATP as the sole P source were measured daily. At the beginning of the treatment, both *OsPAP10a* and *OsPAP10c* overexpression lines showed significantly higher P_i concentrations compared with the leaves of WT plants (Fig. 8b). When grown in solution containing ATP, the P_i concentration in the leaves of WT plants gradually decreased (Fig. 8b). The P_i concentration declined by almost half at day 5, suggesting that WT plants have a limited ability to break down ATP. In contrast, *OsPAP10a* and *OsPAP10c* overexpression lines had more stable leaf P_i concentrations than the WT. After 5 d of treatment, the P_i concentration decreased by 25% in the *OsPAP10a* overexpression line, while a 8–24% decline in P_i concentrations was observed in different *OsPAP10c* overexpression lines (Fig. 8b).

Analyses of agronomic performance in the field under different P levels revealed that the tiller numbers in *OsPAP10c*-overexpressing plants significantly increased and the height of *OsPAP10c*-overexpressing plants decreased under all P_i conditions (Fig. 9a,b,c). However, the total P content in *OsPAP10c*-overexpressing plants was only significantly increased under a low P_i supply (Fig. 9d).

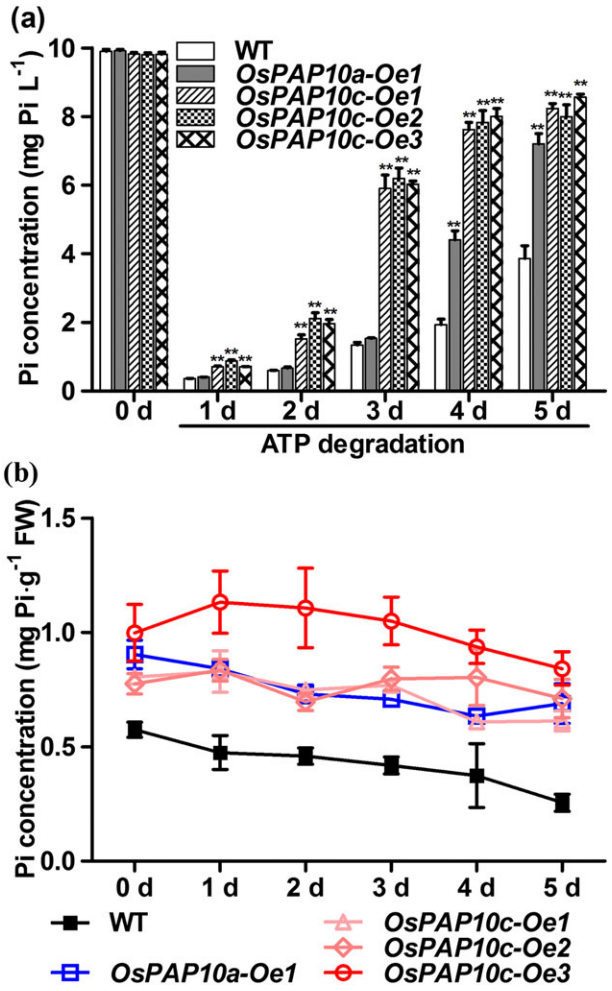

Figure 8. Degradation (a) and utilization (b) of ATP in the nutrient solution by wild-type (WT) and transgenic plants. Germinated seeds were grown in normal nutrient solution (10 mg L^{-1} P_i) for 2 weeks and transferred to nutrient solution containing 0.1 mM ATP as the only source of P. (a) Free P_i concentrations in the solution were measured daily. Before the ATP treatment was commenced was defined as day 0. Student's *t*-test was used to determine the significance of differences between the WT and transgenic lines in each treatment. *P* values < 0.01 are indicated with two asterisks. (b) P_i concentrations of WT and transgenic plants grown in nutrient solution containing 0.1 mM ATP as the only source of phosphorus. The P_i concentrations of the second leaves from different plants were measured daily during the treatment. Before the ATP treatment was commenced was defined as day 0. The data are the means of three replicates with standard error of the mean (SEM).

DISCUSSION

One of the most important functions of plant PAPs is to hydrolyse extracellular organic P sources into an inorganic form. Previously, we showed that *OsPAP10a* overexpression increased APase activity by 1.5-fold to 2-fold in rice. The APase activity and organic P utilization capability of OsPAP10c, a monocotyledon-specific PAP, were compared with those of OsPAP10a in the present study. *OsPAP10c* overexpression conferred approximately 2.5-fold to 5-fold greater IAP activity compared with that of *OsPAP10a* overexpression (Fig. 4a,b). Furthermore, the SAP activity from suspension cells of the *OsPAP10c* overexpression lines was approximately eightfold greater than that of the *OsPAP10a* overexpression lines (Fig. 4a). The significantly increased APase activities of *OsPAP10c* overexpression plants resulted in a more efficient use of ATP as an organic P source (Fig. 8a,b). Thus, *OsPAP10c* has great potential for use in the generation of crops with enhanced organic P utilization.

P_i starvation significantly induces several intracellular and extracellular APases in plants, the majority of which belong to the Ia group of PAPs (Bozzo *et al.* 2006; Hurley *et al.* 2010; Tian *et al.* 2012). The functions of *AtPAP10*, *AtPAP12*, *AtPAP25* and *AtPAP26*, which are from the Ia family in *Arabidopsis*, have been well elucidated in the P_i starvation response and in signalling regulation (Wang *et al.* 2011; Robinson *et al.* 2012a, 2012b; Del Vecchio *et al.* 2014; Wang *et al.* 2014b). PAPs of the Ia family were previously divided into two subfamilies based on *Arabidopsis* PAPs (Li *et al.* 2002). A more comprehensive collection of homologs of AtPAP12 and AtPAP26 from different plant species showed that the defined Ia-2 family of proteins actually belong to two major groups (Tran *et al.* 2010b). However, these analyses only included PAPs from a single species or selected PAPs from a few species. In this study, we performed a phylogenetic analysis on the Ia subfamily of PAPs from six plant species. Our result divided the Ia group of PAPs into four subfamilies. The Ia-3 subfamily is monocotyledon specific and contains *OsPAP10b*, *OsPAP10c* and *OsPAP10d* from rice. It appears that these PAPs evolved via gene duplication; they are adjacent to each other within a 10 kb region (Supporting Information Fig. S2). Digital expression data showed that the expression of *OsPAP10b* and *OsPAP10d* is very low (Supporting Information Fig. S3), suggesting that these two genes may be pseudogenes. Unlike that of *OsPAP10a* and the other Ia-1 subfamily members, the expression of which is induced by P_i starvation in leaves and roots, the induction of *OsPAP10c* occurred only in roots (Fig. 1). This finding implies that *OsPAP10c* has a different function and regulatory mechanism.

A 57 kDa band was detected in the protein extracts of *OsPAP10c*-overexpressing plants using a polyclonal antibody against OsPAP10c (Fig. 3). The size identified was 5 kDa greater than the predicted molecular mass (Supporting Information Table S1). The 5 kDa discrepancy may be attributed to the addition of glycan groups at the three predicted NXS/T glycosylation sites in OsPAP10c (Supporting Information Fig. S2b). *OsPAP10c* overexpression induced the accumulation of several APase isoforms (A1, A3, A4 and A5) in leaves and roots that did not exist in the WT or *OsPAP10a*-overexpressing plants (Fig. 5a,b,c). Western blotting of native PAGE gels confirmed that, at minimum, isoforms A4 and A5 were substantially accumulated in the leaves, roots and media protein extracts of *OsPAP10c* overexpression lines (Fig. 6a,b,c). In addition, the APase isoforms A1 and A3 were detected in the media protein extracts by Western blot (Fig. 6c). Taken together, we conclude that *OsPAP10c* overexpression resulted in the accumulation of several different APase isoforms, including A1, A3, A4 and A5.

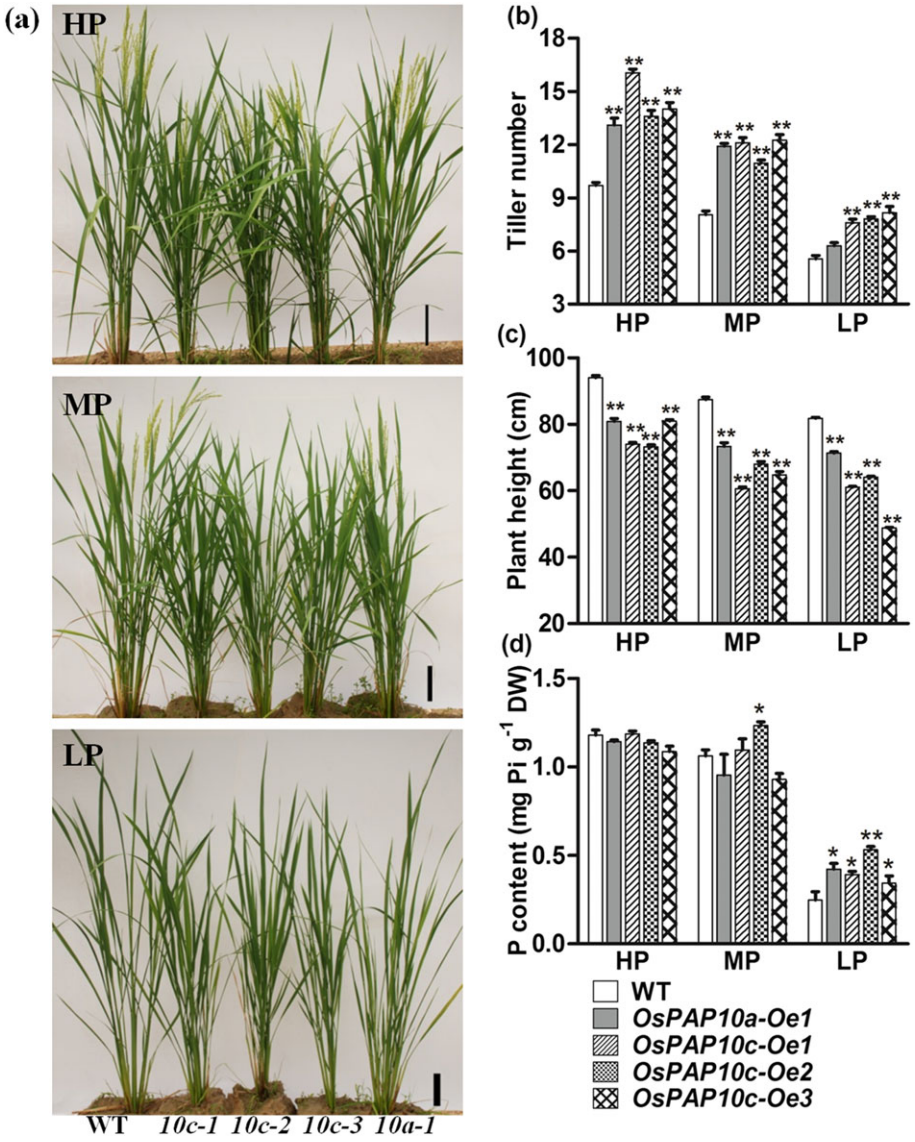

Figure 9. Agronomic performance of wild-type (WT) and transgenic plants grown in the field under different P_i supply levels. (a) Seventy-day-old WT and transgenic plants grown under high-P_i (HP), medium-P_i (MP) and low-P_i (LP) conditions. (b) Tiller numbers of the WT and transgenic plants. (c) Plant heights of the WT and transgenic plants. (d) P_i concentration of the WT and transgenic plants. The nutrient gradients in the field were controlled by applying different levels of P fertilizers over a period of years. The P_i concentrations in the HP, MP and LP fields were 10.5, 7.1 and 3.5 mg kg^{-1}, respectively. Student's *t*-test was used to determine the significance of differences between the WT and transgenic lines in each treatment. A *P* value ≤ 0.05 or < 0.01 is indicated with a single asterisk or two asterisks, respectively.

Conversely, P_i starvation only induced the accumulation of the A2 isoform in leaf and root tissues (Fig. 5a,b). These findings suggest that the majority of the P_i starvation-induced OsPAP10c in roots may be secreted to the extracellular matrix from root tissues. Several lines of evidence in this study support the hypothesis that OsPAP10c is a SAP in rice. Firstly, the SAP activity increased more than 10-fold in the culture media of *OsPAP10c*-overexpressing suspension cells (Fig. 7a). Secondly, significant amounts of OsPAP10c protein were detected in Western blots of medium extracts from the WT, *OsPAP10a* and *OsPAP10c* overexpression samples grown in both P_i-sufficient and P_i-deficient conditions (Fig. 7b). Furthermore, in-gel activity assays using protein extracts from the medium demonstrated that *OsPAP10c* overexpression significantly increased the accumulation of A1, A3 and A4 APase isoforms under both P_i-sufficient and P_i-deficient conditions. In the leaf and root protein extracts, only the A2 isoform was significantly induced by P_i starvation (Fig. 5a,b). Finally, *OsPAP10c* expression is induced predominantly in the epidermis and exodermis cell layers of the root tips (Fig. 2); this expression would facilitate the secretion of the protein into the rhizosphere. In a previous study, the SAP fraction in rice calli was purified (Fukuda *et al.* 2001). Two APase proteins, including OsPAP10c and an unknown APase, were found in the fraction. Indeed, OsPAP10c contains a conserved secreted N-terminal signal identified in AtPAP12 and AtPAP26 (Supporting Information

Fig. S2b) (Tran *et al*. 2010b). These findings suggest that there may be a highly conserved endoprotease ortholog throughout the plant kingdom that cleaves the transit peptides of PAPs targeted for the secretory pathway.

ATP degradation analysis showed that *OsPAP10c* overexpression increased the hydrolysis of scavenged organic P_i (Fig. 8). The released P_i from ATP maintained P_i levels in transgenic plants when grown in solution over time. To study the effect of *OsPAP10c* on P_i use efficiency, the agronomic performance of *OsPAP10c* overexpression lines was evaluated in the field under three P_i supply levels. As expected, the tiller number significantly decreased with the reduction in P_i supply (Fig. 9b). Compared with the WT, *OsPAP10c* overexpression significantly increased tiller numbers under all three P_i conditions (Fig. 9). However, the plant heights of both *OsPAP10a*-overexpressing and *OsPAP10c*-overexpressing plants were significantly decreased under all P_i conditions (Fig. 9). This finding may result from the constitutively increased APase activities in non-targeted tissues and the resulting detrimental effect on the growth of transgenic plants. Using native promoters to drive the *PAP* genes may be a better choice for the generation of P-efficient crops.

While *OsPAP10c* overexpression markedly enhanced SAP activity under P_i-sufficient condition, P_i starvation further significantly increased the leaf (Fig. 4b) and SAP activities in the *OsPAP10c*-overexpressing plants (Fig. 7a). These findings suggest that *OsPAP10c* may be regulated by P_i starvation at both transcriptional and post-transcriptional levels, as is the case for its *Arabidopsis* ortholog *AtPAP10* (Wang *et al*. 2011). The overexpression of *AtPAP10* or *AtPAP12* increased the accumulation of more than one APase isoform in *Arabidopsis* (Wang *et al*. 2014b). Similarly, *OsPAP10c* overexpression increased the accumulation of four APase isoforms in transgenic plants (Fig. 5). These results suggest that *OsPAP10c* may form different homomeric or heteromeric protein complexes. Further studies are needed to determine the molecular regulation of *OsPAP10c* expression.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Gene information for the Ia group of PAPs in rice. The data were downloaded from http://rice.plantbiology.msu.edu/index.shtml

Figure S1. Phylogenetic trees consisting of Ia group PAPs from *Oryza sativa* (Os), *Zea mays* (Zm), *Sorghum bicolor* (Sb), *Arabidopsis thaliana* (At), *Brassica rapa* (Br) and *Glycine max* (Gm). The conserved domains of these PAPs were selected, and an NJ tree was constructed using the MEGA program. PAPs from rice are marked with a dot.

Figure S2. (a) Structures of *OsPAP10b*, *OsPAP10c*, *OsPAP10d-N* and *OsPAP10d-C* in the genome of Nipponbare. Magnified gene structures of each locus are indicated. (b) Alignment of AtPAP10, OsPAP10a, OsPAP10b, OsPAP10c, OsPAP10d-N and OsPAP10d-C protein sequences. The red arrow indicates the conserved secreted signal site. The predicted 'NXS/T' glycosylation motifs are marked in red.

Figure S3. Expression levels of *OsPAP10a*, *OsPAP10b*, *OsPAP10c*, *OsPAP10d* and *OsPAP26* in different tissues based on microarray data retrieved from GENEVESTIGATOR (https://genevestigator.com/gv/).

Figure S4. GUS reporter gene expression in the leaves (a), flower (b, c) and pollen (d) of transgenic plants expressing the *OsPAP10c* promoter–GUS constructs.

Figure S5. BCIP staining of the wild-type and transgenic plants under P_i-sufficient and P_i-deficient conditions.