Running Title: PvPAP3 Enhancing ATP Utilization

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Research area: Environmental Stress and Adaptation to Stress
Biochemical and Molecular Characterization of PvPAP3, a Novel Purple Acid Phosphatase Isolated from Common Bean Enhancing Extracellular ATP Utilization

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Footnotes

Financial source:

This work is supported in part by grants from the National Natural Science Foundation of China (Grant No. 30890131) and National Program on the Development of Basic Research of China (Grant No. 2005CB120902) to X.Y. and H.L.; and the Hong Kong RGC Earmarked Grant (CUHK4434/04M) and the Hong Kong UGC AoE Plant & Agricultural Biotechnology Project (AoE-B-07/09) to H.-M.L.

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ABSTRACT

Purple acid phosphatases (PAPs) play diverse physiological roles in plants. In this study, we purified a novel PAP, PvPAP3 from the roots of common bean (*Phaseolus vulgaris*) grown under phosphate (Pi) starvation. PvPAP3 was identified as a 34 kDa monomer acting on the specific substrate, ATP, with a broad pH range and a high heat stability. The activity of PvPAP3 was insensitive to tartrate, indicating that PvPAP3 is a PAP-like protein. Amino acid sequence alignment and phylogenetic analysis suggest that PvPAP3 belongs to the group of plant PAPs with low molecular mass. Transient expression of 35S:*PvPAP3-GFP* in onion epidermal cells verified that it might anchor on plasma membrane and be secreted into apoplast. Pi starvation led to induction of *PvPAP3* expression in both leaves and roots of common bean, and expression of *PvPAP3* was strictly dependent on P availability and duration of Pi starvation. Furthermore, induction of *PvPAP3* expression was more rapid and higher in P-efficient genotype, G19833, than that in P-inefficient genotype, DOR364, suggesting possible roles of PvPAP3 in P efficiency in beans. *In vivo* analysis using a transgenic hairy root system of common bean showed that both growth and P uptake of bean hairy roots from the *PvPAP3* overexpression transgenic lines were significantly enhanced when ATP was supplied as the sole external P source. Taken together, our results suggest that PvPAP3 is a novel PAP which might function in the adaptation of common bean to P deficiency, possibly through enhancing utilization of extracellular ATP as a P source.
INTRODUCTION

As one of the essential macronutrients, phosphorus (P) is directly or indirectly involved in many important physiological and biochemical processes in plants (Marschner, 1995). In soils, P is easily fixed by organic compounds, Fe or Al oxides into the forms that are unavailable to plants. Therefore, low P availability is one of the major factors limiting crop production (Raghothama, 1999; Vance et al., 2003). Plants have developed a group of adaptive strategies to enhance P acquisition and utilization, such as modifying root morphology and architecture (Liao et al., 2004; Devaiah et al., 2007a and b; Zhou et al., 2008), increasing acid phosphatase (APase) activity (del Pozo et al., 1999; Bozzo et al., 2002; Wang et al., 2009) and organic acid exudation (Ligaba et al., 2004), as well as enhancing expression of a diverse array of genes (Raghothama, 1999; Vance et al., 2003). Among them, APases are generally believed to be important for P acquisition and utilization (Bieleski, 1973; Duff et al., 1994).

Plant APases function to hydrolyze Pi from orthophosphoric monoesters and have a pH optimum below 7.0. They are traditionally divided into two groups according to their substrate specificity, including non-specific versus specific APases (Duff et al., 1994). Purple acid phosphatases (PAP) belong to a special group of APases that are characterized by the purple color of purified proteins in water solution and tartrate-insensitive metallo-phosphatase activity (Schenk et al., 1999; Schenk et al., 2000; Li et al., 2002). Based on their molecular mass and protein structure, PAPs can be further divided into two groups, including small PAPs with a molecular mass of about 35 kD, and large PAPs that are mostly homodimeric proteins with a subunit molecular mass of about 55 kD (Schenk et al., 1999; Schenk et al., 2000; Li et al., 2002; Olczak et al., 2003). Among all of the PAPs, seven invariant residues are highly conserved and are required for metal coordination (Schenk et al., 1999; Schenk et al., 2000; Li et al., 2002; Olczak et al., 2003).

Increased PAP activity by Pi starvation has been demonstrated in various plants, including Arabidopsis (Arabidopsis thaliana), tobacco (Nicotiana tabacum), potato (Solanum tuberosum) and tomato (Lycopersicon esculentum) (del Pozo et al., 1999; Bozzo et al., 2002; Kaida et al., 2003; Zimmermann et al., 2004), indicating that these Pi starvation responsive PAPs, like some other APases, might be involved in P acquisition.
and mobilization. Transgenic expression of MtPAP1 resulted in improved P acquisition and growth in Arabidopsis when phytate P was supplied as the sole external P source under sterile conditions (Xiao et al., 2006). Recent results showed that overexpressing AtPAP15 could enhance P efficiency in soybean (Wang et al., 2009). However, it has been difficult to describe the general physiological functions of all plant PAPs due to their diversity and ubiquity. In the Arabidopsis genome, a total of twenty-nine PAP-like genes have been identified. Among them, the transcription of only four PAP-like genes was induced by Pi starvation, including PAP11, PAP12, PAP17 and PAP26 (Li et al., 2002; Veljanovski et al., 2006). Furthermore, PAP17 showed both APase and peroxidase activities, indicating a multi-functional nature for the enzyme (del Pozo et al., 1999). In addition to P acquisition and utilization, other functions of plant PAPs, such as protection against oxidative stress and involvement in cell wall synthesis have been suggested in soybean (Glycine max) and tobacco (Klabunde et al., 1995; Kaida et al., 2003; Liao et al., 2003; Li et al., 2008).

Common bean (Phaseolus vulgaris) is a major food leguminous crop, mostly cultivated on P deficient soils in the tropics (Beebe et al., 2006). The origin of this crop from two major gene pools representing Middle America and Andean mountain areas has provided excellent genetic resources for characterization and improvement of bean P efficiency (the ability to maintain yield under low P conditions). Two common bean genotypes, G19833 (P-efficient) and DOR364 (P-inefficient), as well as their recombinant inbred lines, have been used by several groups to elucidate the morphological, physiological, and genetic mechanisms underlying P efficiency, including development of a shallower root system, greater exudation of organic acids and protons, and formation of more cortical aerenchyma in roots (Shen et al., 2002; Fan et al., 2003; Liao et al., 2004). Genes involved in the response network of common bean to P deficiency have been identified through the sequencing of 3,165 ESTs from a Pi-starved root cDNA library and via in silico approaches (Ramírez et al., 2005; Graham et al., 2006). More recently, works from our lab have suggested that enhanced expression of the P-responsive genes, PvPT1 and PvPS2, might contribute to efficient P acquisition and translocation in the P-efficient genotype, G19833, under P limiting conditions (Tian et al., 2007).
In an earlier study, one principal APase isoform in the leaves of the P-inefficient genotype, DOR364, was found to be entirely defective in the leaves of the P-efficient genotype, G19833, and its activity was not associated with P acquisition and utilization in common bean (Yan et al., 2001). The study of other APases, especially the APase isoforms induced by Pi starvation, could be more productive for understanding P efficiency in crops such as common bean. In the present study, one Pi starvation-induced APase isoform, PvPAP3, was purified from the roots of common bean. Subsequently its biochemical and molecular characteristics, as well as its roles in P utilization were demonstrated.

RESULTS

Effects of Pi starvation on APase activity

The total APase activity in common bean was significantly increased by Pi starvation (Fig. 1A). In leaves, the APase activity generally increased by more than four folds in the P-inefficient genotype, DOR364, but much less in the P-efficient genotype, G19833. However, no significant difference was observed in root APase activity between G19833 and DOR364. In the non-denaturing polyacrylamide gel (PAGE) actively stained, one APase isoform (named as PvPAP3) with the highest mobility was obviously induced by Pi starvation in both leaves and roots of G19833 and DOR364 (Fig. 1B). Furthermore, activity staining patterns of APase isoforms in leaves were clearly different from those in roots. In leaves, a total of three APase isoforms were detected in DOR364 grown at low P, including one major APase isoform which was fully defective in G19833. This might be the reason why the total APase activity in the leaves of G19833 was much lower than that of DOR364.

Purification and biochemical characterization of the PvPAP3 protein

PvPAP3 was purified from the roots of Pi starved G19833 using three consecutive purification steps: selective precipitation with ammonium sulphate (60±80%), non-denaturing PAGE, and Q-sepharose ion-exchange chromatography. The purified protein at different steps was analyzed using denaturing SDS-PAGE gels. A major protein band with a molecular mass of approximately 34 kD was clearly and consistently detected on
the gel (Fig. 2A). The molecular mass of the native PvPAP3 was estimated to be about 34 kD by gel filtration using a Sephadex G-100 column and four protein size standards (Fig. 2C). Immunoblot analysis showed that the purified PvPAP3 was recognized by a PvPAP3 antibody (Fig. 2B).

A range of pH optimum for PvPAP3 activity using ρ-NPP as substrate was found spanning from pH 6.0 to 8.0 (Fig. 2D). To study thermal stability, PvPAP3 was incubated at various temperatures for 15 or 30 min, and its activity against ρ-NPP was subsequently measured. PvPAP3 activity significantly decreased as the reaction temperature was increased from 30°C to 70°C. PvPAP3 activity decreased by 80% at 70°C compared with 30°C after 30 min incubation (Fig. 2E).

The substrate specificity of purified PvPAP3 was examined (Table I). Phosphatase activity using ρ-NPP as substrate was set as 100% for comparison with the activity using other substrates. Interestingly, PvPAP3 exhibited the highest activity with ATP (307%) and the distant second activity with α-napthyl phosphate as substrates (55%). Very limited activity was found when phytate, AMP, and GMP were used as the substrates (Table I).

Effects of various reagents on PvPAP3 activity were also studied. PvPAP3 activity was significantly inhibited by the application of CuSO₄, NaCl, KH₂PO₄, CaCl₂ and ZnSO₄, and stimulated by MgCl₂ (Table II). Furthermore, PvPAP3 activity was not affected by the addition of tartrate, verifying that PvPAP3 belongs to the PAP subgroup of APase.

**Cloning, expression analysis of the PvPAP3**

Using MALDI-TOF/TOF MS analysis, the following sequences of trypsin-digested peptides of PvPAP3 were obtained: EGYTSVLGNHDYR, KPKSKLHLLVVDGWGR and VLLVGDWGR. Comparison with sequences in databases revealed that one fragment was highly similar to a putative PAP in Arabidopsis (AAL49808), while the other two sequences had highest similarity to a PAP (AAP51881) in rice (*Oryza sativa*). The full length cDNA coding PvPAP3 was isolated from a cDNA library constructed from G19833 plants under P deficiency by PCR amplification using degenerate and gene specific primers (detailed in Materials and Methods).

The *PvPAP3* cDNA clone (FJ464333) contains a 993-bp open reading frame
encoding a polypeptide of 331 amino acid residues. A putative signal peptide containing 29 amino acid residues was found in the N terminus, suggesting that the molecular mass of the mature PvPAP3 protein is approximately 34 kD. Using PvPAP3 as a query sequence with the BLASTx algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that the conserved domain and metal-binding residues of deduced amino acid sequence of PvPAP3 was similar to the other PAPs from common bean, soybean, potato and Arabidopsis. The alignment of amino acid sequences of these proteins is illustrated in Fig. 3 with five distinct conserved motifs highlighted.

A phylogenetic tree was generated through analysis of the PvPAP3 and the protein sequence of other PAPs including four PAPs (CAA04644, BAD05166, AAF60317 and TC14892) in common bean (Schenk et al., 2000; Vogel et al., 2002; Yoneyama et al., 2004). The phylogenetic analysis showed that there were two distinct PAP groups in plants, denoted as PAPs with high molecular mass (Group I) and low molecular mass (Group II) (Fig. 4). Based on this analysis, PvPAP3 protein was grouped in Group II and had the high similarity to one PAP (NP_178297) from Arabidopsis (Fig. 4).

To test whether the expression of PvPAP3 is responsive to changes in plant nutrient status, G19833 (P-efficient genotype) plants were subjected to starvation of nitrogen (N), potassium (K), iron (Fe), or phosphate (Pi). Transcript accumulation of PvPAP3 revealed by qRT-PCR data was only induced in both leaves and roots under Pi starvation, but not under the other nutrient starvation conditions (Fig. 5).

A dose response experiment was then carried out to investigate the expression patterns of PvPAP3 in common bean in response to different P status conditions by qRT-PCR. Transcript accumulation of PvPAP3 in both leaves and roots was strictly dependent on P availability in the medium (Fig. 6A, B). In leaves and roots of G19833 and DOR364, a noticeable accumulation of PvPAP3 transcripts was observed for plants grown on nutrient solution containing 5, 50 and 100 µM Pi, respectively, and the transcript abundance decreased with increasing Pi concentration. Interestingly, the induction of PvPAP3 transcripts was stronger in both leaves and roots of G19833 than that of DOR364 at the same P level. Furthermore, when Pi concentration in the nutrient solution was raised from 5 to 50 µM, greatly reduced expression of PvPAP3 was only observed in the roots of DOR364, but not of G19833.
The time course for *PvPAP3* expression in response to the onset of P deficiency was also investigated. The induced *PvPAP3* expression in the leaves and roots of both genotypes increased with increasing duration of Pi starvation and reached highest level at 12 d after P deficiency treatment (Fig. 6C, D). However, the induction of *PvPAP3* was more rapid and intensive in G19833 compared to DOR364 in response to Pi starvation, as indicated by that the induction of *PvPAP3* in the leaves of G19833 was observed at 4 d after Pi starvation, but at 8 d in DOR364. And the relative expression of *PvPAP3* in the leaves of G19833 was significantly greater than that of DOR364 at the same day after P deficiency treatment. In roots, expression level of *PvPAP3* was about two fold higher in G19833 than that in DOR364 at 8 d after growing in P deficiency, but this difference was diminished at 12 d of Pi starvation.

**Subcellular localization of PvPAP3**

Bioinformatics analysis showed that 29 amino acid residues in the N terminal of PvPAP3 were predicted as signal sequences (http://www.expasy.ch/). 17 amino acids of the signal peptides were identified to be hydrophobic (http://www.enzim.hu/hmmtop/html/submit.html). These indicated that the PvPAP3 might anchor on the plasma membrane or be secreted outside of the cells.

To determine the subcellular localization, *PvPA3* was combined with *GFP* reporter gene and transiently expressed in onion (*Allium cepa*) epidermal cell. The *GFP* empty vector was used as a control. After the cells were plasmolyzed by adding 30% sucrose solution, laser confocal scanning microscopy was used to check whether PvPAP3 was located on plasma membrane or cell wall. The results clearly suggested that most the signals of PvPAP3-GFP were detected on the plasma membrane and a few signals were located in the apoplast region (Fig. 7). However, the signal of GFP of the empty vector control was detected throughout the intracellular areas.

**PvPAP3 enhances extracellular ATP utilization in transgenic bean hairy roots**

Functional analysis of *PvPAP3* was performed using transgenic hairy roots of common bean. The transgenic common been hairy roots overexpressing *PvPAP3* were generated, and the overexpression of *PvPAP3* was confirmed by qRT-PCR analysis using *PvPAP3* specific primers and Western blot analysis using a PvPAP3 antibody (Fig. 8A, B). The average APase activity against ATP and ρ-NPP in 35S:*PvPAP3* transgenic hairy...
roots were 84% and 39% higher than those in the empty vector control, respectively (Fig. 8C, D). Furthermore, the fresh weight and P content of 35S:PvPAP3 hairy roots was significantly higher than those of the control when 500 μM ATP was used as the sole external P source in the growth medium, as indicated by 61% and 35% increase of the average fresh weight and P content in 35S:PvPAP3 transgenic hairy roots, respectively (Fig. 9).

DISCUSSION

In this study, we purified and sequenced a novel specific Pi starvation-induced APase isoform (PvPAP3) from common bean roots. Subsequently, the full-length PvPAP3 cDNA was isolated. Blast analysis showed that six reported ESTs in common bean had high homology with PvPAP3 in the common bean gene index from DFCI computational biology and functional genomics laboratory (http://compbio.dfci.harvard.edu/tgi/). Among them, one 397-bp EST (FD795350) has 99% identities with PvPAP3, indicating the EST is a partial fragment of PvPAP3. Expression of all the EST as related to P availability has not been characterized except that one EST (TC7351) expression has been reported to be up-regulated by Pi starvation in roots of common bean (Hernández et al., 2007). However, no further information has been documented about their biochemical and molecular properties. The biochemical properties of PvPAP3, together with its predicted peptide sequence, strongly suggest that PvPAP3 is a bona fide PAP in common bean. Firstly, PvPAP3 activity was resistant to tartrate (Table II), a common property of PAPs (Vincent and Averill, 1990; Olczak et al., 1997; Nakazato et al., 1998; Bozzo et al., 2002), indicating that PvPAP3 belongs to the PAP subfamily of acid phosphatase. Secondly, the predicted protein of PvPAP3 contained five motifs that are highly conserved in other plant PAPs including seven invariant residues required for metal coordination (Fig. 3) (Li et al., 2002). Furthermore, PvPAP3 is a monomeric protein with a molecular mass around 34 kD determined by Sephadex G-100 chromatography (Fig. 2C), which agrees with the molecular mass of the mature protein predicted from the sequence of PvPAP3. This indicates that PvPAP3 belongs to the group of low molecular mass PAPs, which are generally monomeric protein with a molecular mass of about 35 kD (Li et al., 2002). On the contrary, except the PAP recently purified from tobacco
(Lung et al., 2008), most high molecular mass PAPs are homodimeric proteins with a single subunit of about 55 kD (Schenk et al., 1999; Schenk et al., 2000; Li et al., 2002; Olczak et al., 2003). Our further results from alignment and phylogenetic analysis of PvPAP3 with other PAPs also revealed that PvPAP3 has a high degree of similarity with other low molecular mass PAPs (Fig. 4). Taken together, these findings strongly suggest that PvPAP3 is a novel PAP with low molecular mass in common bean.

Another unique feature of PvPAP3 is that it was found to be most active with ATP as substrate, suggesting that the in vivo substrate for PvPAP3 might be ATP. It was previously speculated that ATP is the substrate for KBPAP purified from common bean seeds (Cashikar et al., 1997). However, despite extensive investigations made on the understanding of biochemical properties, primary structure, x-ray structure and subcellular localization of KBPAP, its physiological functions remained controversial (Cashikar et al., 1997; Grote et al., 1998). In the present study, part of the PvPAP3-GFP signals was detected in the apoplast of onion cells, while a significant fraction of the signals was found on the plasma membrane (Fig. 7). Consistent with this results, bioinformatics analysis shows that 17 amino acids of the signal peptides on the N terminal of PvPAP3 were predicted as hydrophobic. These results together indicate that the hydrophobic signal peptides at the N terminus of PvPAP3 might be a transmembrane domain and anchor the protein on the plasma membrane. However, due to the proteolytic removal, the association of PvPAP3 with membrane might be unsteady, which could be one of the reason for the detection of certain PvPAP3-GFP signals in the apoplast. Another possibility is that the protein could be directly released into the extracellular regions.

Given the high activity of PvPAP3 against ATP and the subcellular localization, we speculate that PvPAP3 could function in P nutrition by enhancing the extracellular ATP utilization. Our data showed that overexpressing PvPAP3 could significantly increase the ATP hydrolyzing activity of the transgenic hairy roots (Fig. 8). Subsequently, improved growth and P accumulation of these transgenic hairy roots were observed when ATP was supplied as the sole P source (Fig. 9). These indicate that enhanced expression of PvPAP3, as well as increased accumulation of PvPAP3, may help bean hairy roots to utilize ATP in the root apoplast/rhizosphere. Consistent with our findings, Thomas et al.
(1999) showed that overexpression of \textit{Apyase} resulted in augmentation of the accumulation of Pi derived from extracellular ATP in pea (\textit{Pisum sativum}). Even ATP content is generally low in soils, it has been suggested that ATP content could reach mM levels in certain special microenvironments, such as in the regions adjacent to cell injury or places where there is a high exocytotic activity in roots (Thomas et al., 1999; Jeter and Roux, 2006). Thus, it is possible that utilization of ATP could be important for plant adaptation to environments that are low in P availability, but rich in organic matter. In addition to mobilizing phosphate from ATP, PvPAP3 might also be involved in quenching extracellular ATP signals which can affect cytosolic Ca$^{2+}$ concentration, mitogen-activated protein kinase (MAPKs) activity, and production of NO and reactive oxygen species (Demidchik et al., 2003; Jeter et al., 2004; Song et al., 2006; Wu et al., 2008; Wu and Wu, 2008). Since expression of \textit{PvPAP3} is also strongly induced in leaves by Pi starvation, we can not exclude the possibility that PvPAP3 might be involved in the utilization or signaling of extracellular ATP released by plants.

Unlike most studies regarding the expression patterns of plant \textit{PAP}s in response to environmental stresses, in this research we started by purifying the target protein and then obtained the full-length \textit{PvPAP3} cDNA from a cDNA library of the P-efficient bean genotype, G19833. Subsequently, the expression pattern of \textit{PvPAP3} was investigated under different nutrient conditions. The expression of \textit{PvPAP3} was strongly induced by Pi starvation in both leaves and roots but not by other nutrient deficiencies, including N, K and Fe (Fig. 5), indicating that \textit{PvPAP3} was specifically regulated by P availability at the transcriptional level. Induction of \textit{PAP} expression by Pi starvation has been well documented in other plant species such as Arabidopsis and potato (de Pozo et al., 1999; Li et al., 2002; Zimmermann et al., 2004). It has been suggested that APase activity in plants may contribute to improve P uptake from organic P in soils (Schachtman et al., 1998; Rahothama, 1999; Richardson et al., 2001). Enhanced expression of \textit{PAP} genes in response to Pi starvation suggests that PAPs, like the other APases, might play an important role in P remobilization and acquisition in plants.

It has been well documented that low P availability increases APase exudation or enhanced internal APase activity in plants. It has also been found that there is a positive relation between root APase activity and P uptake from inositol hexaphosphate in bean.
(Helal, 1990) and barley (Asmar et al., 1995), showing that APase activity might be closely related to plant P efficiency. However, Yan et al. (2001) demonstrated that a major APase isoform in leaves was missing in the P-efficient genotype, and thus could not confer P efficiency in common bean. In our present study, there was no difference in total APase activity in roots between the P-efficient genotype, G19833 and P-inefficient genotype, DOR364, confirming that at least in common bean, overall APase activity can not be considered a critical index for P efficiency. This could be due to the heterogeneity of APases in plants. In the case of common bean, there are two major APase isoforms that contribute to APase activity in roots under low P conditions. The APase studied here, PvPAP3, is Pi starvation induced, but the other isoform is not (Fig.1), suggesting that it is important to separate different APase isoforms in order to study their functions as related to P efficiency. Interestingly, the speed and intensity of transcription induction of PvPAP3 were much faster and greater in the P-efficient genotype, G19833, compared to the P-inefficient genotype, DOR364 (Fig. 6), strongly suggesting the contribution of PvPAP3 to effective P efficiency in common bean. It has been verified that the effective P efficiency of G19833 is possibly contributed by shallower root system, exudation of more organic acids and protons, formation of more aerenchyma in roots, as well as higher expression of Pi transporter gene (PvPT1) (Shen et al. 2002; Fan et al. 2003; Liao et al. 2004; Tian et al., 2007). This suggests that highly induced expression of PvPAP3, along with changes of root morphological and physiological traits, might cause the superior P efficiency in G19833.

In conclusion, our results demonstrate that PvPAP3 is a novel plant PAP which might function in the adaptation of common bean to Pi starvation, possibly through its involvement in ATP hydrolysis and the use of extracellular ATP as a P source from the environment.

MATERIALS AND METHODS

Plant materials and growth conditions

Two common bean genotypes, G19833 and DOR364, were employed. Among them, G19833 was characterized as a P-efficient genotype with a better adaptation to low P conditions (Yan et al., 1995a, b).
For protein purification, seeds of G19833 were surface sterilized for 1 min in 10% (v/v) H$_2$O$_2$, then germinated in dark on germination papers soaked with 1/2 strength modified nutrient solution as described by Yan et al. (2001). The full strength modified nutrient solution without P was composed of (in µM) 3000 KNO$_3$, 2000 Ca(NO$_3$)$_2$, 250 MgSO$_4$, 25 MgCl$_2$, 12.5 H$_3$BO$_3$, 1 MnSO$_4$, 1 ZnSO$_4$, 0.25 CuSO$_4$, 0.25 (NH$_4$)$_6$Mo$_7$O$_24$, and 25 Fe-Na-EDTA. Seven days after germination, uniform seedlings were transplanted into nutrient solution without P addition. Nutrient solution was continuously aerated and replaced once a week. Roots were harvested for protein purification at 10 d after Pi starvation. For analysis of APase activity and isoforms in leaves and roots, uniform seedlings were transplanted into the nutrient solution with or without P addition (500 µM KH$_2$PO$_4$). Leaves and roots were separately harvested at 10 d after treatment.

For P dose responses experiment, G19833 and DOR364 seedlings were grown for 10 d in the nutrient solution supplied with 5, 50, 100 and 250 µM KH$_2$PO$_4$. For time course expression experiment, seedlings were grown in the full strength nutrient solution supplied with 250 µM KH$_2$PO$_4$ for 7 d, and then were transplanted to the Pi starvation solution. At 4, 8, 12 d after treatment, leaves and roots were separately harvested. In order to study the response specificity to nutrients, G19833 seedlings were grown in the nutrient solution lacking of K, N, Fe or P, respectively, and leaves and roots were harvested 10 d after treatment. All experiments, unless otherwise mentioned, had four biological replicates.

**Purification of PvPAP3**

Common bean roots were harvested and frozen at -80°C. About 80 g frozen tissues were homogenized with a Polytron at 4°C in 240 mL of extraction buffer containing 100 mM Tris-HCl (pH 8.0), 0.02 mM EDTA, 25 mM ascorbic acid, 10% (v/v) glycerol, and 0.0001 % (w/v) PMSF. After filtrated through four layers of cheesecloth and centrifuged, the supernatant was fractionated on ice with ammonium sulfate. The fraction precipitates at 60% and 80% were collected after 2 h incubation on ice by centrifugation. The pellets were resuspended in 20 mL of 20 mM Tris-HCl at pH 8.0, and then centrifuged to remove the insoluble materials. The supernatant was desalted by ultra-filtration in the same extraction buffer.
The retrieving of target protein from non-denaturing PAGE gel followed the procedures developed by Miller et al. (2001). Briefly, proteins in the supernatant were separated in non-denaturing 8% (w/v) polyacrylamide gels at constant voltage of 90 V for 1.5 h. A small section of the gel was cut away for activity staining as described by Yan et al. (2001). The activity-stained section was realigned with the unstained portion of each gel, and the gel area corresponding to the activity was excised. The gel slices were homogenized in 20 mM Tris-HCl at pH 8.0. After centrifuged at 12,000 g for 10 min, the supernatant was collected and concentrated by ultra-filtration.

About 0.5 mL supernatant was loaded to Q-Sepharose FF column, which had been connected to an ÄKTA PURIFIER system and pre-equilibrated with Tris-HCl buffer (20 mM, pH 7.5). The column was washed with Tris-HCl (20 mM, pH 7.5) buffer until the absorbance at 280 nm decreased to a baseline. Then the column was eluted with a linear gradient buffer (0-0.75 M, NaCl in 20 mM, Tris-HCl buffer, pH 7.5). The single activity peak was collected and concentrated to about 250 μL using an Amicon Ultra-15.

**Enzyme activity assays, protein determination and gel electrophoresis**

APase activity was determined by measuring the release of nitrophenol from p-nitrophenylphosphosphate (p-NPP) in 45 mM Na-acetate buffer (pH 5.0) as described by Yan et al. (2001). Extracts of plant tissues were incubated at 35°C for 15 min in 2 mL of 45 mM Na-acetate buffer containing 1 mM p-NPP (pH 5.0). The reaction was stopped by adding 1 mL of 1N NaOH and the absorbance was measured at 405 nm. Protein concentration was determined by the Coomassie blue method by using bovine serum albumin as a standard (Bradford, 1976).

The activities towards ATP and other substrates were measured according to the methods described by Ching et al. (1987). Briefly, enzyme extracts or purified PvPAP3 were incubated with 2 mL of 20 mM Tris-HCl buffer containing 4 mM ATP or alternative substrates at 35°C for 15 min. The released P content was determined by the photocolorimetric method (Murphy and Riley, 1963).

To study the thermal stability of PvPAP3, the purified PvPAP3 was separately incubated at 25, 30, 40, 50, 60 and 70°C for 15 and 30 min, and then its activity was measured as before. Enzyme activity at each temperature was expressed as the percentage of the enzyme activity incubated at 25°C. SDS-PAGE was carried out as described by...
Cellatly et al. (1994) and stained with Coomassie blue. APase isoforms were detected as described by Lefebvre et al. (1990). Totally, 50μg protein of extract from roots and leaves was separated in native gel for APase isoform detection. Since protein concentration in root extract was low, it was concentrated using ultra-filtration before being loaded into the native gels.

**Estimation of native molecular mass with Sephadex G-100 column**

About 1.0 mL solution from Q-Sepharose column, concentrated with an Amicon Ultra-15, was applied to the Sephadex G-100 (2×60 cm) column. The column was eluted with two column volumes of Tris-HCl (10 mM, pH 8.0) buffer at the rate of 0.5 mL.min⁻¹. Solution in the tubes was measured at 280 nm, native molecular mass (MM) was calculated from a plot of Ve (elution volume)/Vo (Void volume) against log MM, using the following proteins as standards: Albumin (67.8 kD), Ovalbinium (44.6 kD), Chymotrypsinogenb A (19.2 kD) and Ribonuclease A (14.3 kD).

**Tryptic in-gel digestion**

Target protein band of interest were manually excised from the SDS-PAGE gels. Gel chips were destained with 50% (v/v) methanol cotaining 25mM ammonium bicarbonate for 10 min twice, followed by soaking in 100% acetonitrile (ACN) until the gels turn opaque. Vacuum-dried gel chips were rehydrated with 10 μg.mL⁻¹ of Trypsin (Promega Corporation, Madison, Wisconsin, USA) in 25 mM ammonium bicarbonate (pH 8.0) and digested for 16 to 18 h at 30°C. After digestion, the liquid containing the tryptic peptides was extracted from gel chips and transferred into a new tube.

**MALDI-TOF/TOF mass spectrometry**

Mass spectrometric analysis was carried out using a MALDI-TOF/TOF tandem mass spectrometer ABI 4700 proteomics analyzer (Applied Biosystems, Foster City, USA). For acquisition of mass spectra, 0.5 μL tryptic-digested samples were spotted onto a MALDI plate, followed by 0.5 μL matrix solution (4 mg.mL⁻¹ α-cyano-4- hydroxycinnamic acid in 35 % (v/v) ACN and 1% (w/v) TFA). The MS peaks (MH⁺) were detected on minimum S/N ratio ≥20 and cluster area S/N threshold ≥25 without smoothing and raw spectrum filtering. Fragmentation of precursor ions was performed using MS-MS 1 kV positive mode with CID on and argon as the collision gas. MS/MS spectra were accumulated from 3000 laser shots using default calibration with Glu-Fibrinopeptide B from 4700
Calibration Mixture (Applied Biosystems, USA). The MS/MS peaks were detected on minimum S/N ratio ≥3 and cluster area S/N threshold ≥15 with smoothing.

**MS database searching**

The MS and MS/MS data were loaded into the GPS Explorer™ software v3.5 (Applied Biosystems, Foster City, USA) and searched against NCBInr database by Mascot search engine version 1.9.05 (Matrix science, London, UK) using combined MS (peptide-mass-fingerprint approach) with MS/MS (DeNovo sequencing approach) analysis for protein identification. Known contaminant ions corresponding to keratin and trypsin were excluded from the peak lists before database searching. Top ten hits for each protein search were reported. Also, DeNovo Explorer™ software v3.5 was used to deduce the peptide sequence (sequence taq) of the selected peptide from MS scanning and the top ten deduced peptide sequences will be submitted for protein identification using BlastP.

**Isolation of full length *PvPAP3* cDNA**

With a pair of degenerate primers (forward: 5’-GTIRTIGGIGAYTGGGGIMG-3’ and reverse: 5’-CYRTARTCRTGRTTICCIAG-3’), a fragment was first amplified by PCR from the cDNA prepared from G19833 plants subjected to Pi starvation, then cloned into pGEM-T vector (Promega Inc., USA) and sequenced. Two gene-specific primers (PR1: 5’-CAACAAATGAACTGTAGAATGC-3’ and PF1: 5’-CAATCTTTTGTAGCCGATGCGATCA G-3’) were designed according to the sequence of the fragment determined. Pairs with PR1 and T3, PF1 and T7 primers were separately used to amplify the 5’ and 3’ terminals of *PvPAP3* from the same cDNA library. These three fragments were analyzed and combined together by the MEGA 4.1 program to generate a full length cDNA. Multiple sequence alignments and phylogenetic trees were constructed with Cluster X and MEGA 4.1 program, respectively.

**Quantitative real-time (qRT) PCR**

Total RNA was extracted using Trizol reagent according to the manual (Invitrogen Inc., USA), and treated with DNase I. Two μg total RNA of each sample was used for the first-strand cDNA synthesis in 20 μL reverse transcript reaction. Reverse transcription was performed according to the protocol (Promega Inc., USA). The first-strand cDNA was either used for SYBR Green monitored qRT-PCR (Toyobo Inc., Japan). The qRT-PCR analysis was performed by Rotor-Gene 3000 (Corbett Research, Australia). The
primer pairs were used for qRT-PCR analysis of \textit{PvPAP3} (5’-GAACGCTTGGTATTCCGTGT-3’ and 5’-TGTCTTCCCCAATCTCCAAC-3’), and housekeeping gene EF-1α (PvTC3216 from DFCI Computational Biology and Functional Genomics Laboratory) (5’-TGAACCACCTGGTCAGATT-3’ and 5’-TCCAGCATCACCATTCTTCA-3’).

**Expression of \textit{PvPAP3} in \textit{E. coli} and antiserum production**

The coding region of \textit{PvPAP3} was cloned into the pET28a expression vector and transferred to \textit{E. coli} (BL21) cells. To express \textit{PvPAP3}, cells were grown at 37°C till their density reached 6.0, then 1 mM IPTG was added. Extraction and purification of \textit{PvPAP3} was conducted after 2 h incubation at 28°C following the instruction of the His-bind purification kit (Novagen, Germany). The purified \textit{PvPAP3} from \textit{E. coli} was used to produce \textit{PvPAP3} antibody, which was provided by GL Biochem Company (China).

**Protein immunoblot analysis**

Western blot was carried out as described by Miller et al. (2001) with some modifications. Briefly, the purified \textit{PvPAP3} from plant roots and the extracted proteins from hairy roots were resolved on SDS-PAGE, and then electrophoretically transferred to a PVDF membrane (Bio-Rad, USA). Prior to exposure to \textit{PvPAP3} antiserum, the membrane was incubated overnight in 0.01 M Tris buffer (pH 8.0) containing 5% (w/v) non-fat dry milk (Bio-Rad, USA) and 0.15 M NaCl, followed by three washes with washing buffer containing 0.01 M Tris (pH 8.0) and 0.1% (v/v) Tween 20. The blot was then incubated 1 h with the \textit{PvPAP3} antibody (1:1000 dilution). After three washes with the same washing buffer, alkaline-phosphatase-tagged secondary antibody was added, and the bolt was developed.

**Subcellular localization of \textit{PvPAP3}-GFP fusion protein**

The plasmids of the \textit{PvPAP3}-GFP fusion protein constructs and 35S:GFP empty vector were transformed into the onion (\textit{Allium cepa}) epidermal cell by particle bombardment using the helium-driven accelerator (PDS/1000, Bio-Rad). Bombardment parameters were shown as follows: 1100 p.s.i. bombardment pressure, 1.0-mm gold particles, a distance of 9 cm from macrocarrier to the samples, and a decompression vacuum of 88,000 Pa. The transformed epidermal cells were cultured on MS medium for 16 h before observed by a confocal scanning microscope system (TCS SP2, Leica).
plasmolysis, the transformed cells were treated with 30% sucrose solution. To distinguish the cell wall, the transformed cells were stained by propidium iodide (PI).

**Overexpression constructs of **\textit{PvPAP3}

For overexpression of \textit{PvPAP3} driven by the 35S promoter, the coding region of \textit{PvPAP3} was amplified with primers, \textit{PvPAP3F}: 5’-TCAGAAGCTTGGCATGGCGTTATC-3’ and \textit{PvPAP3R}: 5’-CTGACGCGTGATTCTTTACCGGTCTTT-3’. The amplified fragment was digested with \textit{Hind}III and \textit{MluI} and cloned into the binary vector obtained from South China Agricultural University (Fig. S1).

**Development of transgenic common bean hairy roots**

Common bean hairy roots were produced as described by Cho et al. (2000) with minor modifications. Briefly, the seeds of DOR364 were surface-sterilized by incubating overnight in Cl₂ gas produced by the mixture of 100 mL NaClO and 4.2 mL HCl. Sterilized seeds were germinated on 1/2 MS medium in dark for 35 to 48 h and the cotyledons were harvested. The abaxial of cotyledons were wounded with a scalpel previously dipped into the overnight cultures of the \textit{A. rhizogenes} strain (K599) containing 35S:\textit{PvPAP3} or empty binary vector. The wounded cotyledons were incubated with abaxial side up on filter paper pre-moistened in sterilized water for 3 d. Cotyledons were then transferred to a solid MS medium containing 500 µg.mL⁻¹ carbenicillin disodium and 20 µg.mL⁻¹ hygromycin. Hairy root tips (1 to 2 cm in length) of different lines were separately transferred to the fresh MS medium with the same antibiotics as mentioned above. The established hairy roots were used for assays of qRT-PCR analysis, phosphatase activity and Western blot analysis. Four independent transgenic lines, including two overexpressing lines and two empty controls, were separately selected and regenerated for further analysis. In the ATP utilization experiment, 500 µM ATP was used as the sole external P source, fresh weight of the hairy roots from control and the overexpressor with \textit{PvPAP3} was analyzed at 7 d after growth. The hairy roots were then kept at 75°C until completely dried. Phosphorus content was analyzed colorimetrically after ash digestion (Murphy and Riley, 1963). Each treatment for different lines had five biological replicates. Data were analyzed using the SAS program (Statistical Analysis Systems Institute, version 6.12).

**ACKNOWLEDGEMENTS**
We are grateful to Drs. Leon Kochian from Cornell University, Yongchao Liang from Chinese Academy of Agricultural Sciences, Lixing Yuan from China Agricultural University, and Hong Mei from Columbia University for critical comments, Dr. Yaoguang Liu for providing the over-expression vector and Dr. Peter M Gresshoff for Agrobacterium rhizogenes strain K599, Ruibin Kuang and Sau-Na Tsai for technical help and Drs. Xiurong Wang, Yingxiang Wang, Jing Zhao and Wenbing Guo for valuable discussions.

LITERATURE CITED


Wu SJ, Wu YJ (2008) Extracellular ATP-induced NO production and its dependence on


**Figure legends**

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**Figure 9.** Fresh weight and P content of hairy roots over expressing *PvPAP3* (OX) and control hairy roots (CK) grown on the medium supplied without ATP (open bar) and with
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Supported information

Supplemental Figure 1. The diagram of binary construct for expression of PvPAP3 in common bean hairy roots. P35S, the CaMV35S promoter. Nos3’, 3’ terminator from nopaline synthase gene.

Table I. Substrate specificity of PvPAP3

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
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<tr>
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<tr>
<td>Substrate</td>
<td>Activity (%)</td>
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<tr>
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<tr>
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<td>Phytate</td>
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Note: PvPAP3 activity was determined with 4.0 mM of each substrate and expressed as the percentage of the rate of Pi hydrolysis from ρ-NPP.
<table>
<thead>
<tr>
<th>Regents</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
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<tr>
<td>MgCl$_2$</td>
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<td>Tartrate</td>
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</tr>
</tbody>
</table>

$^a$ PvPAP3 activity against $\rho$-NPP was determined with 5 mM of each reagent applied in the reaction buffer and expressed as the percentage of PvPAP3 activity without application of these regents.
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