ORIGINAL PAPER

GmPAP4, a novel purple acid phosphatase gene isolated from soybean (Glycine max), enhanced extracellular phytate utilization in Arabidopsis thaliana

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Abstract

Key message GmPAP4, a novel plant PAP gene in soybean, has phytase activity. Over-expressing GmPAP4 can enhance Arabidopsis growth when phytate is the sole P source in culture.

Abstract Phosphorus (P) is an important macronutrient for plant growth and development. However, most of the total P in soils is fixed into organic phosphate (Po). Purple acid phosphatase (PAP) can hydrolyze Po in the soil to liberate inorganic phosphate and enhance plant P utilization. We isolated a novel PAP gene, GmPAP4, from soybean (Glycine max). It had an open reading frame of 1,329 bp, encoding 442 amino acid residues. Sequence alignment and phylogenetics analysis indicated that GmPAP4 was similar to other plant PAPs with large molecular masses. Quantitative real-time PCR analysis showed that the induced expression of GmPAP4 was greater in P-efficient genotype Zhonghuang15 (ZH15) than in P-inefficient genotype Niumaohuang (NMH) during the periods of flowering (28–35 days post phytate stress; DPP) and pod formation (49–63 DPP). Moreover, peak expression, at 63 DPP, was about 3-fold higher in 'ZH15' than in 'NMH'. Sub-cellular localization showed that GmPAP4 might be on plasma membrane or in cytoplasm. Over-expressing GmPAP4 in Arabidopsis resulted in significant rises in P acquisition and utilization compared with the wild-type (WT). Under phytate condition, transgenic Arabidopsis plants showed increases of approximately 132.7 % in dry weight and 162.6 % in shoot P content compared with the WT. Furthermore, when phytate was added as the sole P source in cultures, the activity of acid phosphatase was significantly higher in transgenic plants. Therefore, GmPAP4 is a novel PAP gene that functions in plant's utilization of organic phosphate especially under phytate condition.

Abbreviations

APase	Acid phosphatase
DPP	Days post phytate stress
GFP	Green fluorescent protein
ZH15	'Zhonghuang15' soybean
NMH	'Niumaohuang' soybean
ORF	Open reading frame
P	Phosphorus
PAP	Purple acid phosphatase
Phytate	Inositol hexaphosphate
Pi	Inorganic phosphate
Po	Organic phosphate
qRT-PCR	Quantitative real-time PCR
RT-PCR	Reverse-transcription PCR

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Introduction

As an essential nutrient, phosphorus (P) is involved in many physiological and biochemical processes in plants, such as the biosynthesis of nucleic acids, ATP production, and membrane stability (Marschner 1995; Hammond et al. 2004). Inorganic phosphate (Pi) is absorbed from the soil by the roots for plant growth and development. Approximately 50–80 % of the total P in soils is fixed into organic phosphate (Po). Of that, however, 60–80 % is inositol hexaphosphate (phytate), a form mostly unavailable to plants (Hayes et al. 2000; George and Richardson 2008). Hence, to improve the P availability is of significance for enhancement of crop yield and quality.

Acid phosphatases (APases) are hydrolytic enzymes that hydrolyze phosphoric acid monoesters and diesters or anhydrides to release Pi with acidic pH optima (Duff et al. 1994; Schenk et al. 2000a). These enzymes are generally divided into two groups, non-specific and specific, based on their particular catalyzing substrate. Purple acid phosphatases (PAPs) are primarily non-specific APases, and their presences are generally defined by the purple color of protein solutions and their tartrate-insensitive activities. According to their molecular masses and protein structures, PAPs are designated as either small (about 35 kDa) or large (about 55 kDa). The PAP active center mainly consists of five conserved motifs and seven invariant metal-ligating amino acid residues (**D**XG/G**D**XX**Y**/G**N**H(D/E)/VXX**H**/G**H**X**H**) (Schenk et al. 1999). These motifs have important roles in coordinating bi-metal nuclear centers, i.e., Fe(III)–Zn(II) or Fe(III)–Mn(II) in plants and Fe(III)–Fe(II) in mammals (Schenk et al. 2000b; Olczak et al. 2003).

To tolerate the stress associated with a low supply of soil P, plants employ several sophisticated mechanisms to enhance P acquisition and utilization. Those steps include modifying root morphology and architecture, enhancing the expression of Pi transporter genes, and improving the activity of PAPs (Devaiah et al. 2007a, b; Zhou et al. 2008). This increase in the activities of internal and secreted PAPs under a P deficiency has been demonstrated in many species, including Arabidopsis (Hurley et al. 2010), white lupin (Gilbert et al. 1999), common bean (Liang et al. 2010), and rice (Zhang et al. 2011). Results from those investigations have suggested that PAPs play an important role in recycling the internal or external Po from old plant leaves and the rhizosphere.

Over-expressing AtPAP15 in soybean can enhance Po efficiency when phytate is supplied as the sole source of P in a culture medium (Wang et al. 2009). Arabidopsis plants transformed with MtPAP1 are better able to cope with Po (Xiao et al. 2006a). PvPAP3, a novel purple acid phosphatase, can enhance extracellular ATP utilization in common bean (Liang et al. 2010). Although 29 PAPs have been identified from Arabidopsis, only AtPAP7–AtPAP13 are induced by P starvation (Li et al. 2002). To date, although some other PAP genes were also studied, it was documented only in some model plants such as Arabidopsis and rice. While in soybean, which is a valuable crop because of its abundant protein and oil contents in the seed, only three PAP genes have been analyzed. Furthermore, there are no obvious evidences to testify that these PAPs can catalyze the phytate in rhizosphere to maintain the plant growth under low P condition. For instance, GmPAP3 is induced by NaCl stress but not by P starvation (Liao et al. 2003). Therefore, it is extremely important to search soybean PAP gene that can efficiently hydrolyze organic phosphate.

The availability of a P-efficient soybean line can help improve P acquisition and utilization (Fageria et al. 2008; Yan et al. 2001). Several soybean varieties show high P efficiency in response to low P soil (Liu 1997). Nutrient-efficient plants are generally defined as those that can produce relatively higher yields or maintain other desirable traits when nutrient levels are reduced (Wang et al. 2010). In present study, by use of two soybean genotypes, Zhonghuang15 (ZH15, P efficient) and Niumaohuang (NMH, P inefficient), with morphological and physiological differences under P-starvation condition (Li et al. 2010; Liu et al. 2012), a novel low P-induced PAP gene, GmPAP4, was isolated from the roots of the soybeans and the functional characteristics were verified of this gene.

Materials and methods

Plant materials and growth conditions

Two varieties 'ZH15' and 'NMH' previously identified as P efficient and P inefficient, respectively, were used from among hundreds of soybean varieties. These two genotypes were with morphological differences under P-starvation condition such as the relative values of shoot dry weight (0.85, 0.52), root dry weight (1.06, 0.80) and whole-plant dry weight (0.89, 0.57), as well as the physiological such as root architecture, P content, P-utilization efficiency and root APase activity (Li et al. 2010; Liu et al. 2012).

For gene cloning experiments, soybean seeds were sown in quartz sand with half-strength Hoagland nutrient solution. Seven days after germination, uniform seedlings were transplanted into full-strength Hoagland solution supplemented with phytate (1 mmol L^{-1}). This nutrient solution was continuously aerated and replaced once a week. After 21 days of culturing under those conditions, roots were harvested and stored at −80 °C for RNA extraction, and

the other seedlings were still cultured to observe the growth differences of 'ZH15' and 'NMH' under phytate condition. For quantitative real-time PCR (qRT-PCR), seeds of each variety were germinated in vermiculite with double distilled water. After 7 days of growth, the seedlings were exposed to one of two phosphate forms (1 mmol L^{-1} each): KH_2PO_4 (+Pi, control) or phytate (+Po, treatment). Roots were sampled after 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 days, respectively.

cDNA and DNA cloning of GmPAP4

Total RNA was extracted from the roots, using TRIzol Total RNA Reagent (Tiangen, China) according to the manufacturer's instructions. The RNA was treated with DNaseI (Takara, Japan) to prevent contamination with genomic DNA (gDNA). First-strand cDNA was synthesized from 1-µg samples of total RNA with a Prime-Script™ 1st Strand cDNA Synthesis Kit (Takara).

According to the sequence of *AtPAP15* (GenBank Accession No. NM_111593), we performed sequence splicing via a Blast search of soybean ESTs. The putative open reading frame (ORF) of novel isolated gene, *GmPAP4*, was estimated from the NCBI database (http://www.ncbi.nlm.nih.gov/). Two gene-specific primers (F1, 5'-ATGGAACTCAAACAACAAAAACTC-3'; and R1, 5'-TTAGGGCGTCAAAAGTGTACTTCTG-3') were designed according to the *in silico* ORF sequence. To verify the putative sequence, we amplified cDNA and gDNA from both 'ZH15' and 'NMH' with primers F1/R1. Afterward, the target fragments were cloned into the pMD18-T vector (Takara), and 10 positive clones per sample were sequenced by Sangon (China).

Bioinformatics analysis of GmPAP4

The molecular weight and isoelectric point of GmPAP4 were predicted by DNAStar. Multiple sequences were aligned with DNAMAN and a phylogenetic tree was constructed by MEGA5.1. The trans-membrane region and signal peptide were predicted by TMHMM 2.0 and SignalP 4.1 (http://www.cbs.dtu.dk/services), respectively. Subcellular localization was estimated with TargetP1.1 (http://www.cbs.dtu.dk/services/) and http://www.predictprotein.org/. Exons, introns and copy numbers of *GmPAP4* were analyzed and predicted by the blast results through phytozome genomics program (http://www.phytozome.net).

Quantitative real-time PCR

First-strand cDNA was synthesized with a PrimeScript™ Reagent Kit and the gDNA Eraser (Takara). The qRT-PCR analysis was performed with SYBR Green Mix (Takara)

and the Roche LightCycler 2.0 (Roche, Germany). The following primers were used: F2, 5'-CGACCTCTTCCTC GTAAAACC-3' and R2, 5'-GTGCTTGTCTCCTGCCAA AG-3' for *GmPAP4*; and F3, 5'-ATCTTGACTGAGCGTG GTTATTCC-3' and R3, 5'-GCTGGTCCTGGCTGTCTC C-3' for the housekeeping gene *Actin11* (Wang et al. 2012). The qRT-PCR data, as the means of three replicates, for *GmPAP4* were evaluated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Expression and purification of GmPAP4 in *Escherichia coli*

A partial ORF of *GmPAP4*, lacking the predicted signal peptide (21 amino acids of the N terminus) was sub-cloned into pET-32a and transformed into *E. coli* strain Transetta (DE3). Specific primers for PCR amplification were the following: F4, 5'-<u>GGTACC</u>TCTTACCCTCAACAGGTA CACATTTC-3' (*Kpn*I restriction site) and R4, 5'-<u>CTCG AG</u>TTAGGGCGTCAAAAGTGTACTTCTG-3' (*Xho*I restriction site). The transformed *E. coli* cells were cultured in a Luria–Bertani medium containing 100 µg mL^{-1} of ampicillin and 50 µg mL^{-1} of chloromycetin until the density reached OD$_{600}$ = 0.5 (culture conditions: darkness, 37 °C, 200 rpm). Afterward, 0.8 mmol L^{-1} of isopropyl β-D-1-thiogalactopyranoside was added to induce gene expression. GmPAP4 was extracted and purified according to the instructions for the His-bind Purification Kit (CWBIO, China). Finally, the quality of the purified GmPAP4 was visualized by SDS-PAGE. The purified GmPAP4-His fusion protein was further verified by Western blotting. Briefly, the primary anti-His-tag monoclonal mouse antibody and the secondary goat anti-mouse IgG, AP conjugated antibody (CWBIO) were diluted at 1:3,000 and 1:5,000, respectively. Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Amresco, USA) was used as substrate.

Assay for APase and phytase activity in GmPAP4

The activity of APase was analyzed using ρ-nitrophenol phosphate (ρ-NPP) as standard substrate (Sigma, USA). The enzyme detection system entailed 20 µL of purified GmPAP4, which was activated at 37 °C for 30 min in 1,480 µL of NaOAc buffer (200 µmol L^{-1}; pH 5.0) that contained 1 mmol L^{-1} of ρ-NPP. The reaction was terminated by adding 0.5 mL of NaOH (0.5 mol L^{-1}) for a total volume of 2.0 mL. Afterwards, the reaction product ρ-nitrophenol (ρ-NP) was measured spectrophotometrically at 410 nm (Bradford 1976). Similarly, the pH optimum for GmPAP4 was assessed at pH values of 3.0–8.0, adding different buffers that included NaOAc for pH 3.0 through 6.0 and Tris–HCl for pH 7.0 through 8.0 (Ching et al. 1987; Cashikar et al. 1997). APase activity was

defined as ρ-NP released per hour per unit protein at the same condition, and the data were the means of three replicates.

Meanwhile, the activity of phytase in GmPAP4 was analyzed using phytate as substrate (sigma, USA). The molybdenum blue assay was used to measure the phytase activity (Murphy and Riley 1962; Liu et al. 2012, 2011), and the activity was defined as Pi released per hour per unit protein, and the data were the means of three replicates.

In addition, to determine substrate specificity, ρ-NPP and phytate were measured according to the methods described by Ching et al. (1987), and the final concentration of substrate (ρ-NPP and phytate) in 0.1 mol L^{-1} NaOAc reaction buffer (pH 5.0) was 1 mmol L^{-1}. The released Pi content was determined by the molybdenum blue method (Murphy and Riley 1962), and the data were the means of three replicates.

Sub-cellular localization of GmPAP4-GFP

For sub-cellular location of GmPAP4, the ORF of *GmPAP4* (without TAA) was cloned into the pCamE-*GFP* expression vector using Primers F5 (5′-GTCGACATGGA ACTCAAACAACAAAAACTC-3′; *Sal*I restriction site) and R5 (5′-GGTACCGGGCGTCAAAAGTGTACTTC-3′; *Kpn*I restriction site). Subsequently, fusion vector pCamE-*GmPAP4-GFP* and controlled vector pCamE-*GFP* were transferred into the epidermal cells of onion (*Allium cepa*) by a helium-driven accelerator (PDS/1000; Bio-Rad) (Scott et al. 1999). The plasmid DNA and gold particles were prepared as described previously (Scott et al. 1999); bombardment conditions included 5 μL of gold particles, 1,100 psi pressure, and 6-cm bombardment distance. The transformed cells were incubated on MS medium at 28 °C, and sub-cellular localization of GmPAP4-GFP was observed with a fluorescence microscope after 18 h. For plasmolysis, the transformed cells were treated with 30 % sucrose solution.

Arabidopsis transformation and confirmation of transgenic plants

The ORF of *GmPAP4* was cloned into the over-expression vector pCamE after PCR amplification with Primers F6 (5′-GTCGACATGGAACTCAAACAACAAAAACTC-3′; *Sal*I restriction site) and R6 (5′-GGTACCTTAGGGCGTCAAAA GTGTACTTCTG-3′; *Kpn*I restriction site). Binary vector pCamE-*GmPAP4* was then transformed into *Agrobacterium tumefaciens* strain GV3101 by a freeze–thaw procedure, and was transferred into *Arabidopsis* through the floral dipping method (Clough and Bent 1998). The T_3 transgenic homozygous lines were screened by hygromycin selection, PCR amplification, and DNA sequencing. To verify that *GmPAP4*

was being expressed in the transgenics, RT-PCR was performed with cDNA from the transformed and *Arabidopsis* ecotype Columbia (wild-type, WT) plants.

Bioassays of transgenic *Arabidopsis* with *GmPAP4*

To investigate the functions of *GmPAP4*, transgenic *Arabidopsis* (GmPAP4) and WT plants were cultured in sand separately. Half-strength Hoagland solution, supplemented with either KH_2PO_4 or phytate at the same P concentration (1 mmol L^{-1}), was added to each pot in alternate weeks. One month after germination, the *Arabidopsis* plants were harvested for phenotypic observations, as well as physiological and biochemical determinations. To study the production of primary and lateral roots, we cultured transgenic and WT plants on MS medium containing KH_2PO_4 or phytate as the sole P source, and recorded lengths and numbers from 14-day-old seedlings. These experiments were repeated three times, with ten plants per replicate.

Total P contents in the shoots were analyzed as described by Ames (1966). Fresh tissues were dried at 80 °C for 24 h and flamed to ashes, which were then incubated in 100 μL of 30 % HCl and 10 % HNO_3. Afterwards, 20-μL dissolved samples were mixed with 1.48 mL of distilled deionized water, to which we added 500 μL of a Pi reaction buffer (5 % ammonium molybdate solution and 10 % ascorbic acid; 6:1, v:v). The reaction mixture was incubated at 37 °C for 1 h. Shoot P contents were determined at 820 nm with a spectrophotometer, and were calculated according to a premade standard curve. The experiments were repeated three times, with six plants per replicate.

To quantify chlorophyll, transgenic and WT plants were grown on +Pi or +Po media for 4 weeks. Fresh leaves were harvested and soaked for 1 day under darkness in absolute ethanol to achieve full decolorization. The eluents were evaluated at 649 and 665 nm. Chlorophyll concentrations were computed based on a previous method (Kooten and Snel 1990). Experiments were repeated three times, with six plants per replicate.

For measuring APase activity in the rhizosphere, 20-day-old seedlings of both transgenic and WT lines were transferred to 2-mL Eppendorf tubes containing 1.5 mL of a liquid medium supplemented with 1 mmol L^{-1} of ρ-NPP. After being held for 1 day at 24 °C, 500 μL of 0.5 mmol L^{-1} NaOH was added to terminate the reaction. Absorbance was measured at 410 nm, and the situ staining was also completed using ρ-NPP as substrate (Wang et al. 2009; Liu et al. 2012). APase activity was expressed as ρ-NP released per hour per plant. All experiments were repeated three times, with five plants per replicate.

To measure phytase activity in the rhizosphere, we transferred 20-day-old seedlings, both transgenic and WT,

to 2-mL Eppendorf tubes containing 1.5 mL of a liquid medium with 1 mmol L^{-1} phytate. After 2 days at 24 °C, the liquid medium was carbonized with 30 % HCl and 10 % HNO_3. We then incubated 1 mL of this carbonized medium plus 500 μL of P reaction buffer with 5 % ammonium molybdate solution and 10 % ascorbic acid (6:1, v:v) at 37 °C for 1 h. The P content in the liquid medium was determined at 820 nm with a spectrophotometer (Murphy and Riley [1962]). Phytase activity was defined as Pi released per hour per plant. All experiments were repeated three times, with five plants per replicate.

A Western blotting experiment was performed to detect the secreted GmPAP4-GFP in liquid medium. The seeds of over-expressing *GmPAP4-GFP* and WT *Arabidopsis* were sown. Then, 20-day-old seedlings were transferred into liquid medium. After 2 days, the liquid medium was collected, and 1/10 volume 100 % trichloroacetic acid was added, mixed and kept overnight at 4 °C. The mixture was centrifuged at 4 °C 12,000 revolutions per minute for 15 min. Then the supernatant was abandoned and the protein precipitation was washed twice with 1 mL cold acetone (acetone kept at −20 °C). The protein was dried and dissolved with double distilled water for Western blotting. The fusion GmPAP4-GFP protein was determined on SDS-PAGE. Subsequently, Western blotting was performed. Briefly, the primary anti GFP-tag mouse monoclonal antibody and the secondary goat anti-mouse IgG, AP conjugated antibody (CWBIO) were diluted at 1:2,000 and 1:4,000, respectively. Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Amresco) was used as substrate.

Data analysis

All data were analyzed by one-way ANOVA, using the LSD at $P < 0.05$ or $P < 0.01$ to identify the differences between the observations with the statistical program SPSS 17.0 (Kuang et al. [2009]).

Results

Molecular cloning and bioinformatics analysis of *GmPAP4*

By amplifying the cDNA and gDNA sequences of *GmPAP4*, an ORF of 1,329 bp (GenBank Accession No. HQ162477) was determined, encoding a polypeptide of 442 amino acid residues. Bioinformatics analysis suggested that the molecular mass was approximately 50.3 kDa, with an isoelectric point at 6.14.

Protein alignments indicated that GmPAP4 shared significant similarity with other plant PAPs in their conserved domain and metal-binding residues (Fig. 1). The protein contained five distinct conserved motifs and seven invariant metal-coordinating amino acid residues. Phylogenetics analysis of these PAPs revealed two distinct groups: high molecular mass (Group I) and low molecular mass (Group II). Here, GmPAP4 was classified into Group I, having high similarity with AtPAP23 (Fig. 2).

Bioinformatics analysis showed that the 21 amino acids of the N terminus in GmPAP4 were considered as a signal peptide sequence, which indicated that the protein might be on the plasma membrane or be secreted outside of the cells. Meanwhile, the results by blasting against the sequence of soybean genome showed that *GmPAP4* contained five exons and four introns, which was identical to the structure of *AtPAP15*. And there were two copies of *GmPAP4* in soybean genome, chromosomes 2 and 14 with one copy each. In addition, the sequence of *GmPAP4* in 'ZH15' and 'NMH' shared 100 % identical via the detections of PCR and DNA sequencing.

Expression patterns for *GmPAP4* in soybean

We monitored the temporal expression of *GmPAP4* in roots from 'ZH15' and 'NMH' soybeans under two types of P availability. When phytate was the sole source, the pattern of expression differed between varieties (Fig. 3). For 'ZH15', expression was much higher at 28–35 and 49–63 days post phytate stress (DPP), with the peak occurring at 63 DPP. By contrast, expression in 'NMH' was relatively consistent over time, with peak levels being only 1.4-fold higher than what was recorded on Day 0 of phytate treatment. When relative expression was compared between 'ZH15' and 'NMH', levels of induced transcripts were higher in the former from 28 to 35 DPP and from 49 to 63 DPP, with peak transcription being approximately threefold higher in 'ZH15' than in 'NMH' at 63 DPP.

Prokaryotic expression and GmPAP4 activity

A specific electrophoretic band on the SDS-PAGE showed a molecular mass of about 61.2 kDa (Fig. 4a), which was further confirmed by Western blotting (Fig. 4b). The purified GmPAP4-His fusion protein was nearly consistent with the predicted size.

Enzymatic assays demonstrated that the pure GmPAP4 protein had significant APase activity when compared with the control products from pET-32a ($P < 0.05$; Fig. 4c). Maximum APase activity was noted at pH 5.0 (Fig. 4d). Furthermore, when phytate was used as substrate, GmPAP4 also had a significant phytase activity compared with pET-32a control ($P < 0.05$; Fig. 4e). The substrate specificity of purified GmPAP4 was examined. APase activity using ρ-NPP as substrate was set as 100 % for comparison with the phytase activity using phytate.

Fig. 1 Amino acid sequence alignment of GmPAP4 (HQ162477) with GmPAP1 (AF19820), GmPhy (AF19820), OsPAP (AAM00197), SpPAP (BAA92365), MtPAP1 (AY804257), and AtPAP15 (NP_187369). Conserved sequence motifs were *boxed*; metal-binding residues were indicated by *asterisks*

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GmPAP1   RQFWFVTPPEIGPDVPYTFGLI GDLG QSFDSNKTLSHYELNPRKGQTVLFV GDLSYA DNY  198
GmPhy    IYYFRTMPISGSKSYPGKVAVV GDLGL TYNTTTTIGHLTSNEP..DLLLLI GDVTYA NLY  236
OsPAP    REFWFETPPAIDPDASYTFGLI GDLG QTFNSLSTLQHYEKSEG..QTVLFV GDLSYA DRY  196
SpPAP    RLFWFKTPPEVGPDVPYTFGLI GDLG QTFDSNVTLTHYESNPG.GQAVLYV GDLSYA DVY  195
MtPAP1   RQFWFTTPPEIGPDVPYTFGLI GDLG QSYDSNKTLSHYELNPTKGQTVLFV GDLSYA DNY  218
AtPAP15  IHHFRTMPVSSPSSYPGRIAVV GDLGL TYNTTDTISHLIHNSP..DLILLI GDVSYA NLY  228
GmPAP4   FELKTPP.....AQFPITFAVA GDLG QTGWTKSTLAHIDQCKY..DVYLLP GDLSYA DCM  187
                                *                          *   *

GmPAP1   PNH....................DNIRWDSWGRFTERSVAYQPWIWTA GNHE NHFAPEIGE  239
GmPhy    LTNGTGSDCYSCSFPLTPIHETYQPRWDYWGRFMQNLVSNVPIMVVE GNHE IEK...QAE  293
OsPAP    QHN...................DGVRWDSWGRLVERSTAYQPWIWSA GNHE IEYRPDLGE  237
SpPAP    PDH...................DNVRWDTWGRFVERSTAYQPWIWTT GNHE IDYAPEIGE  236
MtPAP1   PNH...................DNVRWDTWGRFAERSVAYQPWIWTV GNHE LDFAPEIGE  259
AtPAP15  LTNGTSSDCYSCSFPETPIHETYQPRWDYWGRFMENLTSKVPLMVIE GNHE IEL...QAE  285
GmPAP4   QH....................LWDNFGKLVEPFASTRPWMVTE GNHE EEN..ILLL  222
                                                        *

GmPAP1   TVPFKPYTHRYHVPYKASQSTSPFWYSIKRASAHIIVLASYSAYGKYTPQYKWLEKELPK  299
GmPhy    NRTFVAYSSRFAFPSQESGSSSTFYYSFNAGGIHFIMLGAYINYDKTAEQYKWLERDLEN  353
OsPAP    TSTFKPYLHRCHTPYLASKSSSPMWYAVRRASAHIIVLSSYSPFVKYTPQWTWLKYELKH  297
SpPAP    YVPFKPFTHRYHVPHKSSGSGSPFWYSIKRASAYIIVLASYSAFGKYTPQSEWLEQEFPK  296
MtPAP1   TKPFKPYSHRYRTPYKASQSTSPFWYSIKRASAHIIVLASYSAYGKYTPQYKWLEQELPK  319
AtPAP15  NKTFEAYSSRFAFPFNESGSSSTLYYSFNAGGIHFVMLGAYIAYDKSAEQYEWLKDLAK  345
GmPAP4   TDEFVSYNSRWKMPFEESGSTSNLYYSFEVAGVHVIMLGSYADYDVYSEQYRWLKEDLSK  282

GmPAP1   VNRTETPWLI VLMH SPWYNSYNYHYMEGETMRVMYEPWFVQYKVDDVVFA GHVHAY ERSER  359
GmPhy    VDRSITPWLVVTWHPPWYSSYEAHYREAECMRVEMEDLLYAYGVDIIFNGHVHAYERSNR  413
OsPAP    VDREKTPWLI VLMH SPMYNSNEAHYMEGESMRAAFEKWFVKYKVDLVFA GHVHAY ERSYR  357
SpPAP    VNRSETPWLI VLMH SPLYNSYNYHYMEGETMRVMYEPLFVTYKVDVIFA GHVHAY ERSYR  356
MtPAP1   VNRTETPWLI VLMH SPWYNSYNYHYMEGESMRVMYEPWFVKYKVDVVYA GHVHAY ERSER  379
AtPAP15  VDRSVTPWLVAS WH PPWYSSYTAHYREAECMKEAMEELLYSYGTDIVFNGHVHAYERSNR  405
GmPAP4   VDRKRTPWLL VLFH VPWYNSNKAHQGAGDDMMAAMEPLLYAASVDLVI GHVHAY ERSKR  342
                    *                                        *  *
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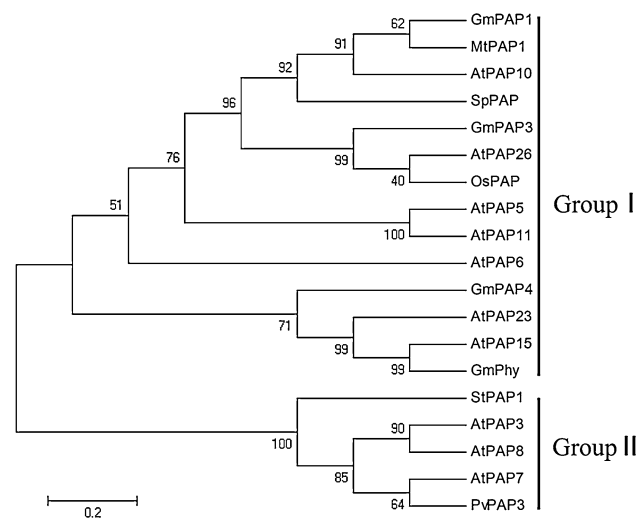

Fig. 2 Phylogenetic tree constructed with MEGA 5.1 for sequences of GmPAP4, GmPAP1 (AF19820), MtPAP1 (AAX20028), AtPAP10 (NP_179235), SpPAP (BAA92365), GmPAP3 (NP_001236677), AtPAP26 (NP_198334), OsPAP (AAM00197), AtPAP5 (NP_564619), AtPAP11 (NP_179405), AtPAP6 (NP_176033), At-PAP23 (NP_193106), AtPAP15 (NP_187369), GmPhy (AAK49438), StPAP1 (AAT37529), AtPAP3 (NP_172923), AtPAP8 (NP_178298), AtPAP7 (NP_178297), and PvPAP3 (ACO25293). PAP groups were marked I and II. Bootstrap values indicated major branches as percentages

Fig. 3 Expression of *GmPAP4* in roots from 'ZH15' and 'NMH' soybean. Seven-day-old plants were treated with either phytate as P deficiency or KH_2PO_4 as control for 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, or 70 days. Total RNA isolated from roots was used for qRT-PCR. Data were evaluated by $2^{-\Delta\Delta CT}$ method and were the means of three replicates. *Error bars* represented SE for all samples tested. *Asterisks* indicated that values were significantly different between two varieties at $P < 0.05$

Interestingly, GmPAP4 exhibited the relatively high activity with phytate (45 %) (Supplementary Table 1).

Sub-cellular localization of the GmPAP4 protein

Results from the bioinformatics analysis indicated that the 21 amino acid residues in the N terminus of the GmPAP4 could be considered as signal peptide sequences. We had predicted that 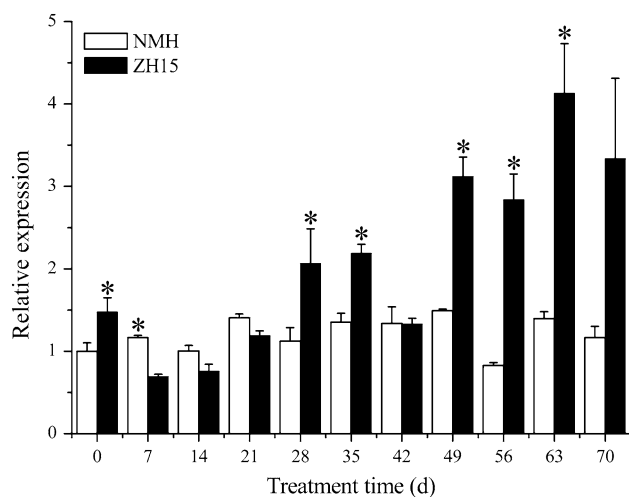GmPAP4 was either attached to the plasma membrane or secreted outside of the cell. To test that prediction, we constructed the pCamE-*GmPAP4*-GFP vector for transient expression in onion epidermal cells. Plasmolysis experiment was used to check whether GmPAP4 was located on plasma membrane or cell wall. The results clearly showed most of pCamE-GmPAP4-GFP fluorescence signals were observed on the plasma membrane or in cytoplasm, while the signals of controlled vector (pCamE-GFP) were detected throughout the intracellular cells (Fig. 5). In addition, we also observed the fluorescence of pCamE-GmPAP4-GFP moved from internal cell to plasma membrane (Supplementary Fig. 1).
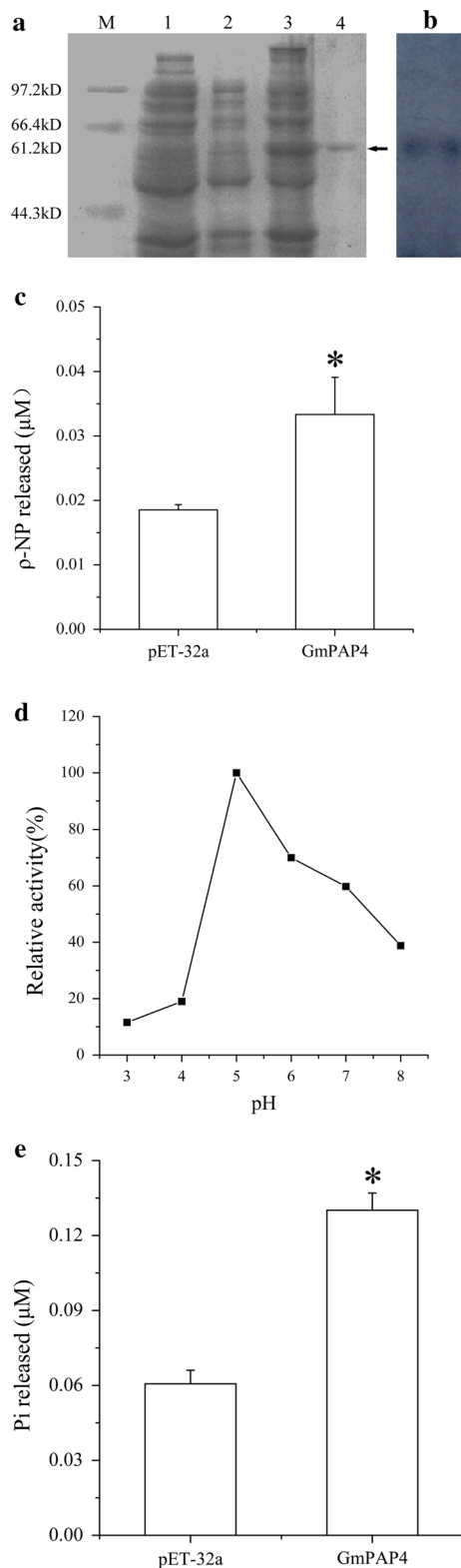
Characterization of transgenic *Arabidopsis* under organic P conditions

Over-expression of GmPAP4 promotes Arabidopsis growth under phytate condition

The T_3 transgenic homozygous *Arabidopsis* plants were confirmed by PCR, RT-PCR (Fig. 6a) and DNA sequencing. When grown with KH_2PO_4, morphology was very similar between transgenic and WT plants. Under phytate condition, however, the transgenic plants were more robust and had longer petioles (Supplementary Fig. 2) when compared with the WT (Fig. 6b). When plants were grown on +Pi or +Po media, primary root lengths were similar between the transgenic and WT (Fig. 6c). However, the transgenic plants had 35 % more lateral roots, which was a significant increase ($P < 0.05$) over that of the WT (Fig. 6d). The chlorophyll concentration was 10.8 % lower in the transgenic plants under phytate conditions (Fig. 7a).

Biomass and P content are increased in GmPAP4 over-expressing Arabidopsis

Transgenic *Arabidopsis* plants had markedly enhanced dry weights under phytate conditions, being approximately 132.7 % higher ($P < 0.01$) than in the WT (Fig. 7b). Furthermore, the transgenics had significantly greater P contents ($P < 0.01$) that were 162.6 % higher than in the WT (Fig. 7c). However, under +Pi conditions, those traits did not differ between the transgenics and WT.

Fig. 5 Sub-cellular localization of pCamE-GmPAP4-GFP in onion epidermal cells. **a–c** The fluorescence signals of vector control pCamE-GFP after plasmolyzing. **d–f** The fluorescence signals of pCamE-GmPAP4-GFP after plasmolyzing
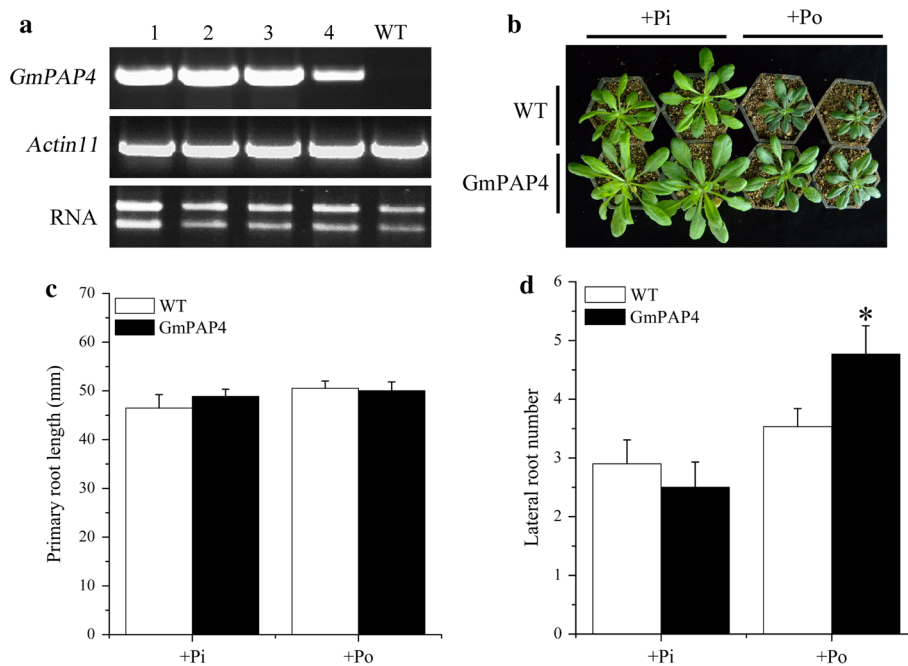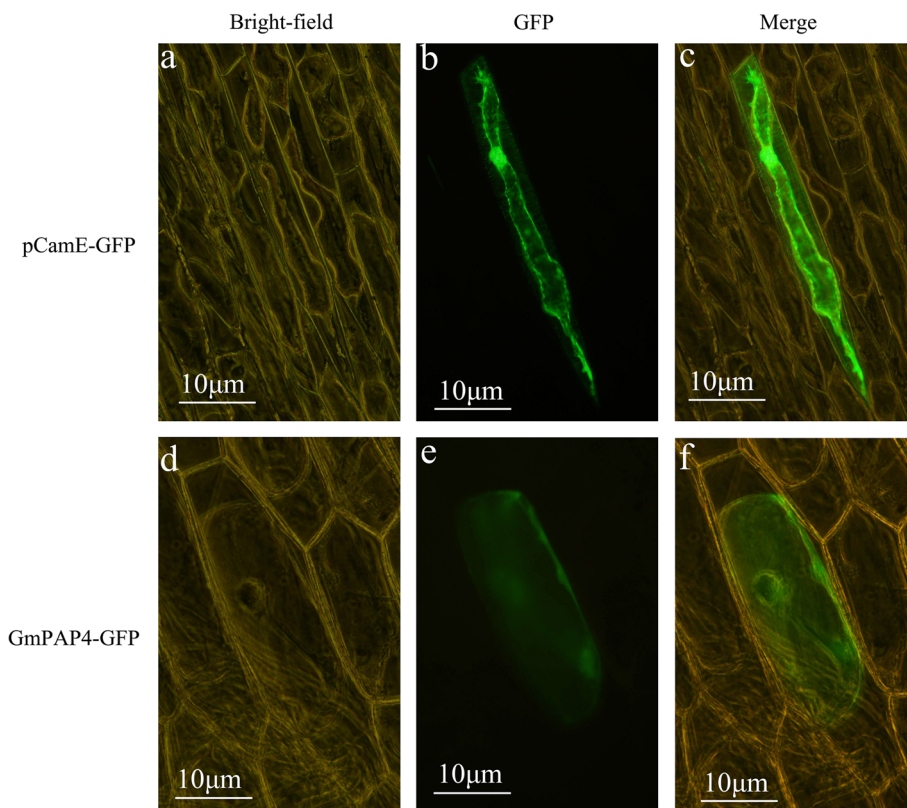

Fig. 6 Phenotypes of transgenic (GmPAP4) and wild-type (WT) *Arabidopsis* under different P conditions. **a** Transcription analysis of *GmPAP4* in T_3 transgenic lines 1 through 4. *Actin11* served as internal control. **b** The growth of plants after 30 days in sand culture containing either KH_2PO_4 (+Pi) or phytate (+Po); P concentration was 1 mmol L^{-1}. **c** Lengths of primary roots from transgenic and WT plants under +Pi or +Po. **d** Numbers of lateral roots from transgenic (GmPAP4) and WT plants under +Pi or +Po. Data were the means of three replicates (ten plants each). *Error bars* represented SE of all samples tested. *Asterisks* indicated that values were significantly different between transgenic plants and WT at $P < 0.05$
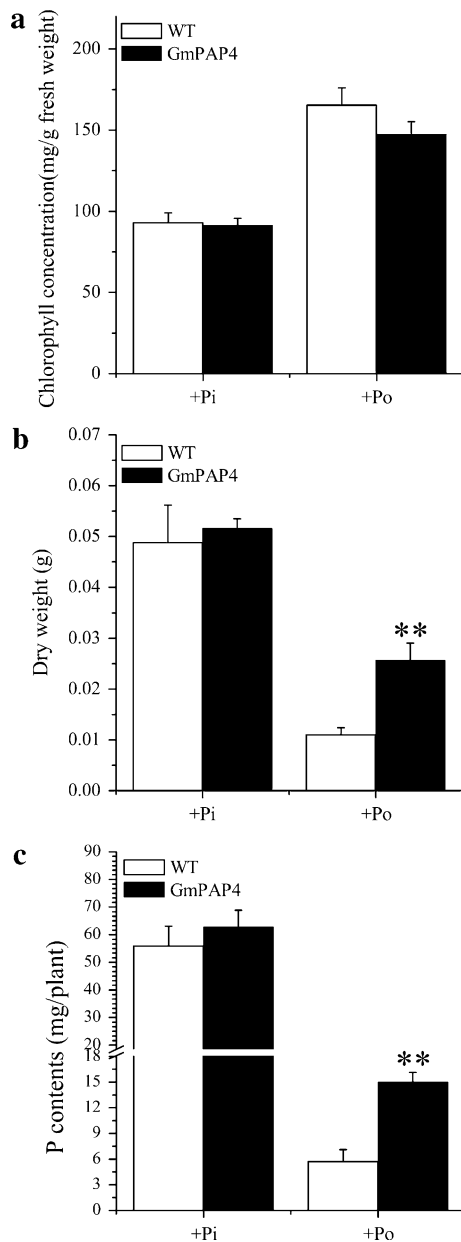
Fig. 7 Biochemical measurements of transgenic (GmPAP4) and wild-type (WT) *Arabidopsis* under different P conditions. **a** Chlorophyll concentrations in transgenic (GmPAP4) and wild-type (WT) *Arabidopsis* grown in sand for 30 days under +Pi or +Po. **b** Dry weights from WT and GmPAP4 plants. **c** P contents in WT and GmPAP4 plants. For **a–c**, data were the means of three replicates (six plants each). *Error bars* represented SE of all samples tested. *Asterisks* indicated that values were significantly different between transgenic plants and WT at $P < 0.01$

Over-expressing GmPAP4 improves Arabidopsis root APase and phytase activity

Compared with the WT, secreted APase activity was 22.3 % higher in transgenic plants that over-expressed

GmPAP4 (Fig. 8a). Moreover, the culture solution for the transgenics showed more intense yellow coloring (Fig. 8b). Meanwhile, the secreted phytase activity increased by 232.1 % in the transgenic plants when phytate was added to the solution as the sole P source (Fig. 8c). In addition, in transgenic *Arabidopsis*, GmPAP4-GFP fusion protein secreted into the liquid medium was verified by Western blotting analysis with GFP-tag antibody (Fig. 8d, e). This demonstrated that *GmPAP4*, a novel purple acid phosphatase gene, functioned in hydrolyzing extracellular phytate and improved the utilization of organic phosphate by *Arabidopsis* plants.

Discussion

Although some APase genes have been cloned and shown to be functional in some model plant species (Gilbert et al. 1999; Hegeman and Grabau 2001; Xiao et al. 2006b; Hurley et al. 2010; Liang et al. 2010; Zhang et al. 2011), only a few such genes have been characterized in soybean. More importantly, there were no direct evidences to testify that these PAPs could catalyze the phytate in culture medium to maintain the plant normal growth and development under low P condition. For example, *GmPAP1* and *GmPAP2* were only confirmed to be induced by low P stress, and *GmPAP3* was approved to associate with the adaptation of soybean to NaCl stress, rather than to low P starvation (Liao et al. 2003). Thus, to obtain a soybean PAP gene related to phytate utilization in rhizosphere, we performed RT-PCR to clone and sequence *GmPAP4* from soybean roots. The Blast search indicated that GmPAP4 was a purple acid phosphatase. Multiple alignment and phylogenetics analysis revealed high similarities between GmPAP4 and other PAPs in the high molecular mass Group I. Its conserved domain had five distinct conserved motifs and seven invariant metal-coordinating residues that were typical of PAPs. Furthermore, many obviously experimental evidences testified that *GmPAP4* enhanced extracellular phytate utilization in *Arabidopsis* and soybean subsequently.

As species have evolved, plants have developed a series of adaptive strategies to improve P acquisition and utilization from the soil, such as altering root morphology and architecture or enhancing APase activity (Chiou and Lin 2011). The diverse mechanisms that operate in response to low phosphorus are controlled by a coordinated array of genes. The polyploidy soybean has a large and complex genome, and most of its genes have multiple copies, thereby leading to different patterns of expression (Schmutz et al. 2010). The exon–intron structure of its genes also influences transcription via alternative splicing (Fedorova and Fedorov 2003; He et al. 2007, 2009). We
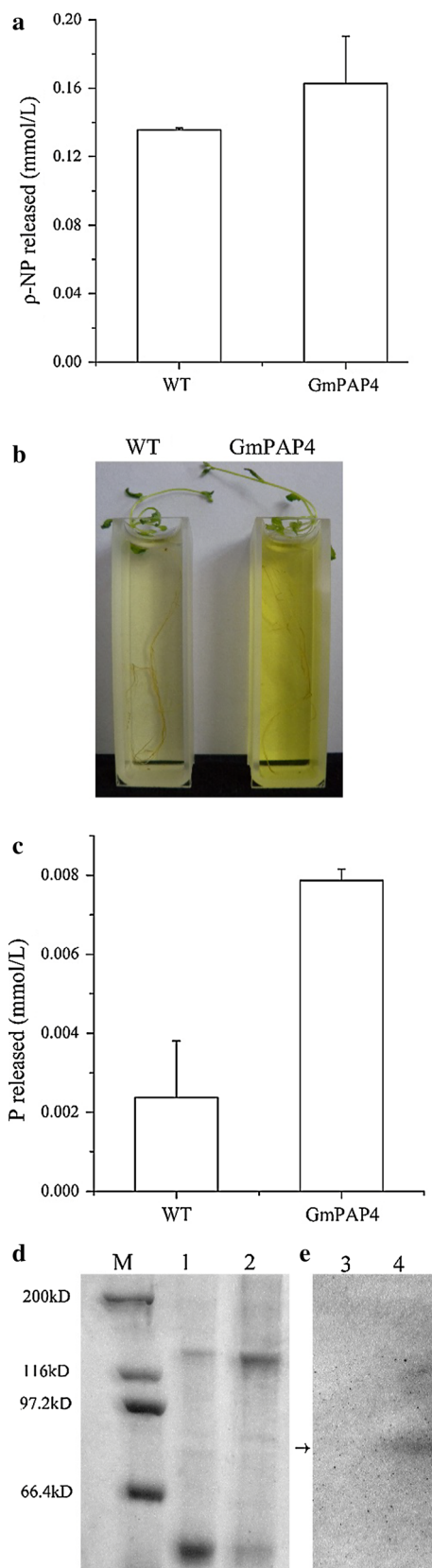
◀ **Fig. 8** Assays of enzyme activity in transgenic *Arabidopsis*. **a** APase activity in wild-type (WT) and transgenic *Arabidopsis* (GmPAP4), expressed as ρ-NP released per hour per plant. **b** Secretion of APase from roots of WT and GmPAP4, indicated by *yellow color*. **c** Phytase activity in WT and GmPAP4, expressed as amount of Pi released per hour per plant. Data were the means of three replicates (five plants each). *Error bars* represented SE of all samples tested. **d** Detection of total protein in root exudates by SDS-PAGE analysis. *M* protein marker, *1* wild-type *Arabidopsis* (WT), *2* transgenic *Arabidopsis* (GmPAP4). **e** Detection of GmPAP4-GFP in root exudates by Western blotting. *3* wild-type *Arabidopsis* (WT), *4* transgenic *Arabidopsis* (GmPAP4) (color figure online)

found that the gDNA sequence of *GmPAP4* had five exons and four introns, which was consistent with our bioinformatics predictions. Because two varieties did not differ in either their cDNA or gDNA sequences, it may be concluded that *GmPAP4* was conserved, similar to that noted with the deduced sequences for the whole genome of 'Williams 82' soybean (Supplementary Fig. 3; Schmutz et al. 2010).

Analysis of temporal expression by *GmPAP4* showed that, after phytate treatment, the induced accumulation of transcripts was significantly greater at 28–35 DPP and 49–63 DPP in 'ZH15', with levels always being higher than in 'NMH', where expression remained low and relatively stable over time. The two spans parallel the stages of flowering (28–35 DPP) and pod formation (49–63 DPP) are critical times in soybean growth and development when more phosphorus is required. Thus, it can be inferred from these qRT-PCR data that *GmPAP4* had a vital function in hydrolyzing and utilizing soil-available organic phosphate by soybean plants. Our laboratory has previously confirmed that 'ZH15' and 'NMH' differed significantly in their phenotypic and biochemical characteristics under P-deficient conditions (Supplementary Fig. 4; Li et al. 2010; Liu et al. 2012). Those differences might be explained *GmPAP4* with different patterns of expression in these two varieties.

Arabidopsis genome-wide transcriptional analysis has shown that phosphate starvation-induced genes are directly regulated by transcription factors such as PHR1 (Schachtman and Shin 2007; Chiou and Lin 2011). Bustos et al. (2010) have reported that PHR1 is a central integrator of transcription regulation, based on observations from mutants in which transcriptional activation and repression are influenced in response to Pi starvation. In rice, one or two *OsPHR2* binding elements have been found in the promoter region of 10 PAP genes, implying that those genes are directly regulated by *Os-PHR2* (Zhou et al. 2008). Wang et al. (2011) have demonstrated that the promoter of *AtPAP10* is affected

by Pi deficiency, but not by K or N deficiencies. Using dose–response experiments with common bean, Liang et al. (2010) have shown that, at the same level of P, induction of *PvPAP3* transcripts is stronger in the leaves and roots of 'G19833' than those of 'DOR364'. All of these reports support our findings that differences exist in the transcriptional levels of *GmPAP4* from 'ZH15' and 'NMH' soybeans. Thus, the above results provided new focus in the regulation of transcription factors or cis-acting elements to *GmPAP4*.

PAP genes play an important downstream role in the signaling network for plant P acquisition and utilization (Schachtman and Shin 2007; Chiou and Lin 2011). Various researchers have investigated whether PAP can release Pi from organic phosphate and recycle internal phosphorus under a P deficiency. Testing the theory that PAPs in plants generally function under acidic conditions, Kuang et al. (2009) have proposed that AtPAP15 shows maximum APase activity at pH 4.5, and also found that AtPAP15 has a phytase activity, which is about 4 % relative to its APase activity. In addition, recombinant AtPAP10 protein has 29 % phytase activity compared with its APase activity (Wang et al. 2011). The result of in vitro enzyme assay showed that purified GmPAP4 possessed the features of APase, with maximum activity at pH 5.0. More importantly, GmPAP4 also significantly possessed a relative higher phytase activity in vitro assay (45 %) compared with its APase activity, which indicated that GmPAP4 in eukaryotic cells might have a relative higher capability to catalyze phytate in plant rhizosphere.

To verify whether GmPAP4 in plant has a relative higher activity of APase or phytase, the *GmPAP4* was over-expressed in *Arabidopsis*. The activity of APase in transgenic *Arabidopsis* showed that over-express GmPAP4 had relatively higher APase activity compared with the WT. The results from in situ staining using ρ-NPP as substrate for the activity of APase in the whole-plant roots also provided direct evidence that GmPAP4 could hydrolyze the organic P in the growth media and release Pi for plant growth. Meanwhile, bioinformatics analysis showed that GmPAP4 might be on the plasma membrane or secreted outside of the cell, and the subsequent sub-cellular localization of GmPAP4-GFP in onion epidermal cells showed that part of GmPAP4-GFP signals was detected on the plasma membrane. Furthermore, the Western blotting results of GmPAP4-GFP fusion protein extracted from *GmPAP4* over-expression plants liquid culture medium demonstrated that GmPAP4 had the ability to secrete outside of plant cells. Taken together, the novel GmPAP4 could secrete outside of plant cells, and had a higher APase activity to catalyze Po in growth medium.

Worldwide, P is the most important nutrient that can limit crop production (Marschner 1995). In soils, only the inorganic form is readily available for uptake by roots. However, abundant total P is fixed and exists as Po, with approximately 60–80 % being phytate. A few PAPs play roles in hydrolyzing phytate. For example, Xiao et al. (2006a) have found that transgenic *Arabidopsis* plants grow better on a medium when phytate is added as the sole P source, and Wang et al. (2011) have used enzyme assays to show that AtPAP10 has moderate phytase activity. Wang et al. (2009) have reported that dry weights are significantly increased in soybean plants over-expressed *AtPAP15*, and that their P contents are 18.2–90.1 % higher compared to the WT while both are grown on sand cultures containing phytate. In soybean, as we know, there are no clear evidences that the existing PAPs can promote plant growth under phytate condition to date (Liao et al. 2003). Here, *GmPAP4* was a phytate utilization-related PAP gene confirmed by some accurately experimental evidences. On media supplemented with phytate, *GmPAP4*-over-expressing *Arabidopsis* plants were more robust, and had longer petioles compared with the WT. The reduced concentration of chlorophyll in transgenic leaves implied that those plants had greater photosynthetic efficiency in a P-deficient environment. The significant increases in dry weight and total P content in transgenic shoots also demonstrated that *GmPAP4* improved the P efficiency of *Arabidopsis* when phytate was added as the sole source of phosphorus.

An interesting phenomenon recorded in this work was the plant root architecture altered. It had been generally known that increased number and length of lateral roots could help plants maintain Pi level under Pi starvation (Williamson et al. 2001). One reference documented that when transgenic plants over-expressing *AtPAP10* were grown under different phosphorus level conditions (0, 10, 50, 150 μM as low P level; normal P as control), the number and length of lateral roots at 150 μM P level were higher than the other P levels (Wang et al. 2011). It seemed to infer that lateral roots could grow more and better at the moderate low P stress level than the serious Pi deficiency or Pi sufficiency. In our study, transgenic plants over-expressing *GmPAP4* on phytate medium had significantly more and longer lateral roots than WT plants, which indicated that *GmPAP4* over-expressing made *Arabidopsis* hydrolyze phytate to supply a certain amount of Pi. However, whether *GmPAP4* can directly or indirectly affect lateral root growth needs to be further investigated.

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