

Identification of soybean purple acid phosphatase genes and their expression responses to phosphorus availability and symbiosis

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• **Background and Aims** Purple acid phosphatases (PAPs) are members of the metallo-phosphoesterase family and have been known to play important roles in phosphorus (P) acquisition and recycling in plants. Low P availability is a major constraint to growth and production of soybean, *Glycine max*. Comparative studies on structure, transcription regulation and responses to phosphate (Pi) deprivation of the soybean *PAP* gene family should facilitate further insights into the potential physiological roles of *GmPAPs*.

• **Methods** BLAST searches were performed to identify soybean *PAP* genes at the phytozome website. Bioinformatic analyses were carried out to investigate their gene structure, conserve motifs and phylogenetic relationships. Hydroponics and sand-culture experiments were carried out to obtain the plant materials. Quantitative real-time PCR was employed to analyse the expression patterns of *PAP* genes in response to P deficiency and symbiosis.

• **Key Results** In total, 35 *PAP* genes were identified from soybean genomes, which can be classified into three distinct groups including six subgroups in the phylogenetic tree. The expression pattern analysis showed flowers possessed the largest number of tissue-specific *GmPAP* genes under normal P conditions. The expression of 23 *GmPAPs* was induced or enhanced by Pi starvation in different tissues. Among them, nine *GmPAP* genes were highly expressed in the Pi-deprived nodules, whereas only two *GmPAP* genes showed significantly increased expression in the arbuscular mycorrhizal roots under low-P conditions.

• **Conclusions** Most *GmPAP* genes are probably involved in P acquisition and recycling in plants. Also we provide the first evidence that some members of the *GmPAP* gene family are possibly involved in the response of plants to symbiosis with rhizobia or arbuscular mycorrhizal fungi under P-limited conditions.

Key words: Soybean, *Glycine max*, purple acid phosphatase, expression analysis, phylogenetic analysis, phosphorus nutrition, symbiosis.

INTRODUCTION

Purple acid phosphatases (PAPs) are members of the metallo-phosphoesterase family. They are characterized by the purple/pink colour as the result of a charge-transfer transition at 550–560 nm from the tyrosine residue to ‘chromophoric’ ferric ion (Oddie *et al.*, 2000). PAPs often carry seven invariant amino acid residues distributed in five blocks (**DXG/GDXXY/GNH(D/E)/VXXH/GHXH**; bold letters indicate metal-ligating residues), which are required for the co-ordination of the bimetal nuclear centre in the active site (Li *et al.*, 2002).

Plant PAPs are traditionally classified into two groups: high-molecular-weight large PAPs and low-molecular-weight small PAPs. Large PAPs belong to oligomeric proteins with a polypeptide of about 55 kDa, which generally possess two domains and are more homologous to the enzymes found in fungi and mycobacteria (Li *et al.*, 2002; Olczak *et al.*, 2003; Tran *et al.*, 2010a). Conversely, small PAPs have only one domain in their structure and are typically monomeric proteins with a molecular mass around 35 kDa and are closely related to mammalian PAPs (Li *et al.*, 2002; Olczak *et al.*, 2003).

The physiological functions of plant PAPs are diverse, including phosphorus (P) acquisition (Tomscha *et al.*, 2004; Lung *et al.*, 2008; Liang *et al.*, 2010), generation of reactive oxygen species (del Pozo *et al.*, 1999; Liao *et al.*, 2003; Zhang *et al.*, 2008), involvement in flower development (Zhu *et al.*, 2005) and cell wall biosynthesis (Kaida *et al.*, 2008, 2009, 2010). Plant PAPs have mostly been studied for their potential involvement in P nutrition, which can catalyse the hydrolysis of various phosphate esters and anhydrides in the pH range 4–7 (Oddie *et al.*, 2000; Li *et al.*, 2002). And the majority of PAPs are inducible by phosphate (Pi) deprivation. Phosphorous is one of the most important macronutrients involved in many physiological and biochemical processes in plants and is also a vital structural constituent of many biomolecules (Duff *et al.*, 1994; Tran *et al.*, 2010a). However, P concentration in soil solution is often low because of intensive erosion, weathering and strong P fixation by organic compounds, free Fe or Al oxides. Therefore, low P availability is a major constraint to plant growth and development. During long-term evolution, plants have developed a number of adaptive strategies to cope with low P, including enhancing secretory and intracellular PAP activity

to mobilize the sparingly soluble P from the growth medium and recycle P in plants (Olczak *et al.*, 2003; Xiao *et al.*, 2006; Wang *et al.*, 2009, 2010). Under low-P conditions, a variety of PAP activities are up-regulated, indicating that PAP activity plays a very important role in P acquisition (Li *et al.*, 2002; Bozzo *et al.*, 2006; Zhang *et al.*, 2011). Meanwhile, Nodulin PvNOD33, a putative phosphatase from common bean, was induced during nodule development in mature nodules, and was possibly involved in carbon metabolism (Roussis *et al.*, 2003). In common bean, the activities of phosphatases and phytases had also been increased in the nodules under low-P conditions, indicating that N₂-fixing legumes can enhance P utilization within the nodules to tolerate P deficiency (Araújo *et al.*, 2008). In addition, the up-regulation of PAP gene expression and the subsequent secretion of PAP by arbuscular mycorrhizal (AM) roots were also reported to accelerate mineralization of organic P in the rhizosphere through the extraradical hyphae as well as the roots (Ezawa *et al.*, 2005).

PAPs are commonly found in a wide range of plant species. In Arabidopsis, 29 different PAP genes were annotated, several of which are transcriptionally induced during Pi deprivation (del Pozo *et al.*, 1999; Li *et al.*, 2002; Hurley *et al.*, 2010; Tran *et al.*, 2010b). Zhang *et al.* (2011) identified 26 rice PAP genes from *O. japonica* genomes. Soybean (*Glycine max*) is one of the most important food and oil crops, and also serves as an important protein source for both human consumption and animal feed (Kereszt *et al.*, 2007). Until now, only four soybean PAPs have been reported (LeBansky *et al.*, 1992; Schenk *et al.*, 1999, 2000; Hegeman and Grabau, 2001; Liao *et al.*, 2003). In the present study, we identified 35 soybean PAP genes, and subsequently performed a more thorough phylogenetic analysis of the GmPAP family. A study on expression patterns of these PAPs in soybean was also conducted to investigate the potential functions of this gene family in plant P nutrition. Also we provide the first evidence that some members of the GmPAP gene family are possibly involved in the response of plants to symbiosis with rhizobia or arbuscular mycorrhizal fungi under P-limited conditions.

MATERIALS AND METHODS

Database search and bioinformatics analysis of PAP genes in the soybean genome

A BLAST search was performed with the known soybean (*Glycine max*) PAP AY151271 at the phytozome website (<http://www.phytozome.net>). The predicted amino acid sequences of 22 different PAPs with conserved sequence motifs (DXG/GDXXY/GNH(E/D)/VX2H/GHXXH) were identified in the soybean genome. Then the BLAST searches using the other known soybean PAPs, AAF60316, AAF19820 and AAK49438, were separately performed using the same website, resulting in 13 additional predicted soybean PAPs. Finally, the predicted protein sequences of 35 PAPs were each used as query sequences for another round of blastp searches on the phytozome website. No more predicted PAPs were found, suggesting that the total number of predicted PAPs in the soybean genome was likely to be 35. The 35

soybean PAPs, with the prefix 'GmPAP' for *Glycine max* PAP, were named according to their positions on the different chromosomes (Li *et al.*, 2002).

Genome sequences of the GmPAPs members were retrieved from the phytozome website. A phylogenetic tree of GmPAPs, AtPAPs and OsPAPs based on entire protein sequence alignments using ClustalX was constructed by the neighbor-joining method with 1000 bootstrap replicates in the MEGA 4.1 program. Complete deletion was used to deal with gaps or missing data in sequences. The distance between sequences was estimated after Poisson correction.

Numbers of exons and introns, first exon length and total exon length were extracted from the phytozome website. The molecular weight and isoelectric point of the protein were predicted by the ExPASy server (www.expasy.org). The subcellular location prediction was performed by Target P 1.1. The signal peptide of GmPAPs was predicted by the Signal P 3.0 server. The N-glycosylation site of the protein was predicted by the NetNGlyc 1.0 server.

Plant materials and treatments

The P-efficient soybean genotype HN66 was used as the plant material, which was also the start material for soybean transformation in our laboratory (Wang *et al.*, 2009). Soybean plants were cultured in solution in a glasshouse. For expression analysis of GmPAPs in the different tissues of soybean plants, 1 week after germination, the seedlings were transplanted into full-strength nutrient solution containing 250 μM KH₂PO₄, 3000 μM KNO₃, 2000 μM Ca(NO₃)₂, 250 μM MgSO₄, 25 μM MgCl₂, 12.5 μM H₃BO₃, 1 μM MnSO₄, 1 μM ZnSO₄, 0.25 μM CuSO₄, 0.25 μM (NH₄)₆Mo₇O₂₄ and 25 μM Fe-Na-EDTA. The seedlings were grown for 10 d till the first trifoliolate leaves were fully developed and then they were treated with different P concentrations (5 μM and 500 μM KH₂PO₄). At 18 d after treatment, roots, stems, leaves and flowers were harvested separately. At 29 d after treatment, young pods and seeds were harvested separately. All tissue samples were stored at -80 °C for RNA extraction. Shoots and roots were harvested at 29 d after treatments for total root length, P content and biomass determination.

For the rhizobium inoculation experiment, 1 week after germination, the soybean seedlings were inoculated with rhizobia, *Bradyrhizobium* sp. BXYD3 and transplanted into LN (100 μM N added) nutrient solution containing 5 μM or 500 μM KH₂PO₄. Fifty days after planting, the nodules were harvested for RNA extraction and quantitative real-time PCR (q-PCR) analysis.

For the AM fungi infection experiment, soybean seeds were germinated in sand culture with or without the inoculants containing the infected maize roots, *Glomus mosseae* spores and mycelium. One week after germination, the seedlings were transplanted into sand culture and irrigated by nutrient solution containing 5 μM KH₂PO₄ every day. Fifty days after planting, the roots were harvested for RNA extraction and q-PCR analysis. The sub-samples from lateral roots of soybean plants were harvested for AM assay according to Wang *et al.* (2011). All the experiments, unless specified otherwise, had four biological replicates.

Determination of root traits and P content

Plants were harvested after appropriate treatments as described above. Roots were scanned and the digital images were quantified with computer image analysis software (Win-Rhizo Pro, Régent Instruments, Québec, Canada) for measuring total root length (Zhao *et al.*, 2004). P contents of shoots and roots were determined using the phosphorus–molybdate blue colour reaction and by measuring absorbance at 700 nm (Murphy and Riley, 1962).

RNA extraction and quantitative real-time PCR

Total RNA was extracted from roots, stems, leaves, flowers, young pods and seeds of soybean plants using RNAiso™ Plus reagent (TaKaRa) according to the manufacturer's instructions. RNA samples were treated with RNase-free DNase I (Invitrogen) to avoid amplification from genomic DNA. The first cDNA strand was synthesized from total RNA using the MMLV-reverse transcriptase (Promega, USA) according to the protocol from the supplier. Quantitative real-time PCR was performed using SYBR® Premix EX Taq™ (TaKaRa). All the reactions were done on a Rotor-Gene 3000 (Corbett Research, Australia). The soybean housekeeping gene *EF-1a*

was used as an endogenous control to normalize the samples. The specific primer sequences for 35 predicted *GmPAPs* are listed in Supplementary Data Table S1 (available online). Reaction conditions for thermal cycling were: 95 °C for 1 min, 40 cycles of 95 °C for 15 s, 54–62 °C for 15 s and 72 °C for 30 s. The annealing temperature (54–62 °C) was adjusted to suit the amplification of the individual *PAP*. Fluorescence data were collected during the cycle at 72 °C.

Statistical analyses

All of the data were analysed using Microsoft Excel 2000 (Microsoft Company, USA) for calculating mean and standard error, and SAS 8.1 (SAS Institute Inc., Cary, NC, USA) for one-way ANOVA.

RESULTS

Comparative analysis and nomenclature of soybean PAPs

In total, 35 putative *GmPAP* genes were identified according to repeated blastp searches of the protein database of soybean in phytozome website (Table 1). The amino acid sequences were available for all *GmPAPs* in the Phytozome website

TABLE 1. General information for the 35 *GmPAPs*

Gene loci	Proposed name	No. of exons/ introns	First/total exon length (bp)	Predicted size (kDa)	Signal peptide (length, cleave site)	Subcellular location*
Glyma02g13070	<i>GmPAP1</i>	11/10	76/1353	50.3	No	Unknown
Glyma02g45950	<i>GmPAP2</i>	5/4	162/1365	51.6	22, ATA-TP	S
Glyma03g35190	<i>GmPAP3</i>	5/4	141/1311	49.4	22, LLS-QE	S
Glyma03g35200	<i>GmPAP4</i>	4/3	265/1110	42.9	No	M
Glyma04g37180	<i>GmPAP5</i>	10/9	90/1281	48.9	No	M
Glyma05g03320	<i>GmPAP6</i>	12/11	204/1836	68.7	29, VYG-FA	S
Glyma05g26920	<i>GmPAP7</i>	9/8	77/930	35.2	No	Unknown
Glyma05g26930	<i>GmPAP8</i>	7/6	198/1008	37.9	30, SIA-EL	S
Glyma05g33630	<i>GmPAP9</i>	7/6	192/996	37.6	28, ASA-EL	S
Glyma05g33640	<i>GmPAP10</i>	7/6	177/990	37.1	23, VSG-LL	S
Glyma06g03100	<i>GmPAP11</i>	20/19	108/3012	114.6	No	Unknown
Glyma06g17860	<i>GmPAP12</i>	12/11	198/1872	70.5	27, AFA-QS	S
Glyma08g06090	<i>GmPAP13</i>	7/6	183/987	37.4	25, ASA-EL	S
Glyma08g09880	<i>GmPAP14</i>	7/6	171/969	36.5	20, VSS-AV	S
Glyma08g09900	<i>GmPAP15</i>	7/6	195/999	37.3	29, SKA-KL	S
Glyma08g09910	<i>GmPAP16</i>	7/6	198/1008	37.9	30, SIA-EL	S
Glyma08g40210	<i>GmPAP17</i>	12/13	183/1851	69.1	28, VVS-DE	S
Glyma08g42670	<i>GmPAP18</i>	5/4	243/1410	53.1	No	Unknown
Glyma08g46550	<i>GmPAP19</i>	6/5	277/1584	59.7	29, GHC-HI	S
Glyma09g36360	<i>GmPAP20</i>	8/7	168/1395	53.2	26, GSS-SP	S
Glyma10g08300	<i>GmPAP21</i>	5/4	262/1137	58.1	No	Unknown
Glyma11g36510	<i>GmPAP22</i>	5/4	106/1018	48.9	No	Unknown
Glyma12g01000	<i>GmPAP23</i>	8/7	168/1395	53.1	23, CNG-GT	S
Glyma12g34960	<i>GmPAP24</i>	8/7	57/1275	49.4	No	Unknown
Glyma13g23090	<i>GmPAP25</i>	10/9	18/1428	54.5	29, GSA-GI	S
Glyma13g35610	<i>GmPAP26</i>	8/7	195/1407	54.1	32, CHG-GK	S
Glyma14g02790	<i>GmPAP27</i>	5/4	216/1383	52.2	45, ATP-QY	S
Glyma17g11790	<i>GmPAP28</i>	10/9	50/1539	59.5	47, VLA-SF	M
Glyma18g00410	<i>GmPAP29</i>	8/7	229/1653	62.2	No	Unknown
Glyma18g17580	<i>GmPAP30</i>	13/12	57/1839	69.1	No	Unknown
Glyma19g03220	<i>GmPAP31</i>	7/6	208/1532	57.2	No	Unknown
Glyma19g37850	<i>GmPAP32</i>	4/3	262/1140	43.0	No	Unknown
Glyma19g37860	<i>GmPAP33</i>	5/4	150/1290	48.6	27, VYG-YV	S
Glyma20g01470	<i>GmPAP34</i>	7/6	183/984	37.5	25, AFA-EL	S
Glyma20g03260	<i>GmPAP35</i>	2/1	705/1989	74.2	24, AES-KP	S

* S and M = secretory and mitochondrial, respectively.

(Supplementary Data Text File). Among them, 32 soybean PAPs had the conserved seven metal-binding residues (D, D, Y, N, H, H, H) of PAP metalloesterase in five consensus blocks (Supplementary Data Table S2). However, the three remaining soybean PAPs (GmPAP1, GmPAP5 and GmPAP7) differed in those conserved domains of PAPs. GmPAP1 lost two (fourth and fifth) motifs. In GmPAP5, one conserved residue in the first block was changed. In GmPAP7, three conserved residues in the first, second and fifth blocks, respectively, were changed (Supplementary Data Table S2). However, GmPAP1, GmPAP5 and GmPAP7 were still considered as potential PAPs because their overall amino acid sequences showed the highest homology with the known plant PAPs when BLASTing in NCBI.

Based on the prediction by Signal P 3.0 and Target P 1.1, 21 soybean PAPs contained a 20- to 45-amino acid N-terminal secretory pathway signal peptide, and three PAPs (GmPAP4, GmPAP5 and GmPAP28) had a mitochondrial-targeting transit peptide (Table 1). All soybean PAPs except GmPAP7 appeared to be heavily glycosylated, containing one to ten potential N-linked glycosylation sites as predicted by NetNGlyc 1.0 (Supplementary Data Table S2).

Thirty-five soybean PAP genes were unevenly distributed on all chromosomes except for chromosomes 1, 7, 15 and 16 (Table 1). Among them, chromosome 8 had the highest number of PAP genes with seven, followed by chromosome 5 with five (Table 1). The nomenclature of soybean PAPs followed the system for naming members of multigene families in arabidopsis, which was based on gene chromosome locations (Table 1; Li et al., 2002). This nomenclature would be of benefit to future studies on individual PAP genes in soybean.

Phylogenetic analysis and classification of soybean PAPs

A phylogenetic tree of the amino acid sequences of the soybean, arabidopsis and rice PAPs is shown in Fig. 1. According to previous analysis in arabidopsis (Li et al., 2002), the 35 GmPAPs, 29 AtPAPs and 26 OsPAPs had been classified into three distinct groups (I, II and III) including eight subgroups (Ia-1, Ia-2, Ib-1, Ib-2, IIa, IIb, IIIa and IIIb; Fig. 1). Thirty-five GmPAPs covered six subgroups in the phylogenetic tree. Among eight subgroups, Subgroup Ia-1 only contained AtPAPs. Subgroup Ia-2 mainly consisted of GmPAPs 11, 20, 23 (previously

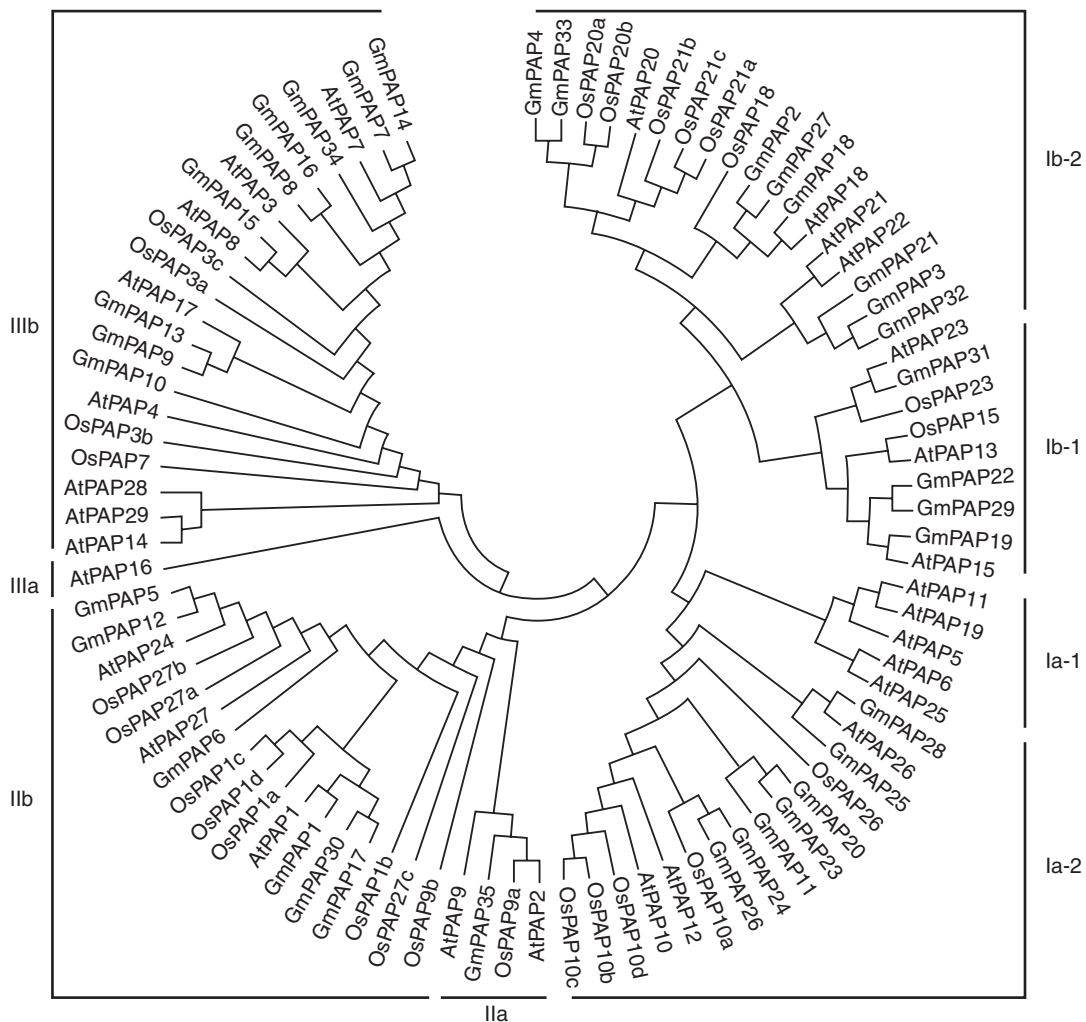


FIG. 1. Phylogenetic analysis of GmPAPs, AtPAPs and OsPAPs. Phylogenetic tree of 35 GmPAPs, 29 AtPAPs and 26 OsPAPs was constructed by the neighbor-joining method with 1000 bootstrap replicates in the MEGA 4.1 program.

named GmPAP1), 24, 25, 26 and 28 (previously named GmPAP3), AtPAPs 10, 12 and 26 and five OsPAPs. GmPAP29, which was previously named GmPhy and had high phytase activity in the cotyledons of germinated soybeans (Hegeman and Grabau, 2001), clustered with GmPAPs 19, 22 and 31, AtPAPs 13, 15 and 23 and OsPAPs 15 and 23 to form subgroup Ib-1. GmPAPs 2, 3, 4, 18, 21, 27, 32 and 33, which had high identity to AtPAPs 18, 20, 21, 22 and six OsPAPs, were in subgroup Ib-2. GmPAP35, AtPAP2, AtPAP9 and OsPAP9a were in subgroup IIa, which had a higher molecular weight. GmPAPs 1 and 5, of which the conserve metal-binding residues were changed, clustered with GmPAPs 6, 12, 17 and 30, AtPAPs 1, 24 and 27, and 8 OsPAPs to form the subgroup IIb. Group III consisted of subgroups IIIa and IIIb. Subgroup IIIa had only AtPAP16. Subgroup IIIb contained nine GmPAPs, eight AtPAPs and four OsPAPs. There was a close correlation between the classification of the groups and predicted molecular weight of the PAPs. Groups I and II comprised the high molecular weight PAPs. In contrast, group III consisted of the low molecular weight mammalian-like PAPs (Fig. 1).

Growth responses of soybean plants to Pi deprivation

Growth and P uptake of soybean plants were significantly affected by P availability (Fig. 2). High P significantly increased plant dry weight and P content by about 5- and 24-fold, respectively. Furthermore, sufficient P supply also promoted plant root growth as indicated by a 150% increase in total root length at the high P level compared with that at the low P level (Fig. 2).

Tissue-specific expression of soybean PAP genes and their responses to Pi deprivation

To identify the tissue-specific expression of *GmPAP* genes in soybean, the expression patterns of 35 *GmPAP* genes in the six tissues were investigated and analysed according to their tissue specificity. The results showed flowers possessed the largest number of tissue-specific *GmPAP* genes, followed by seeds and roots (six, four and three genes, respectively; Fig. 3 and Supplementary Data Table S3). This is similar to the results of the soybean gene expression atlas, which found the larger numbers of tissue-specific genes in flowers and seeds (Libault *et al.*, 2010; Severin *et al.*, 2010).

The transcriptional responses of 35 *GmPAP* genes to Pi deprivation in different tissues were also investigated using q-PCR with gene-specific primers. The results showed that the expression of 23 *GmPAPs* was induced or increased by Pi-deprivation treatment (Table 2 and Supplementary Data Table S4). Among them, roots and leaves possessed the largest number of P-deficient inducible *GmPAP* genes, followed by stems, indicating that GmPAPs play very important roles in P acquisition from the growth medium and P recycling in plants. The expressions of *GmPAP17* and *GmPAP34* were very highly induced in roots and leaves, respectively. Those of *GmPAP28* and *GmPAP35* were induced in stems and roots, respectively. The expressions of the other 19 *GmPAP* genes were induced by Pi deprivation in more than two

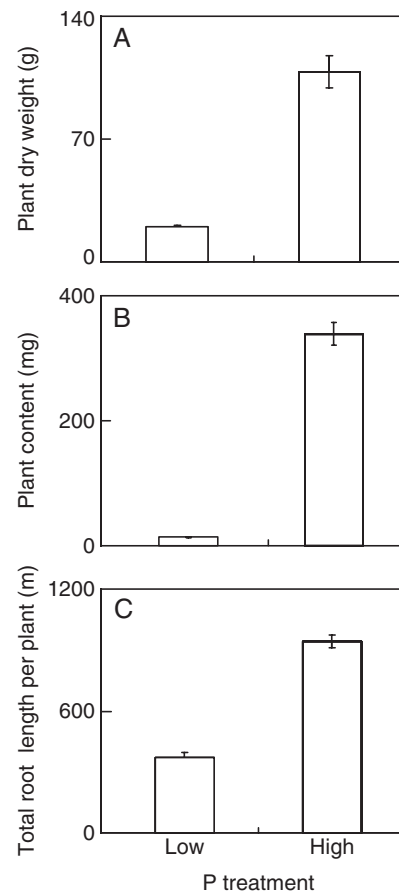


FIG. 2. Effects of different P treatments on individual plant dry weight, P content and total root length of soybean. Ten-day-old seedlings were transferred to solutions containing either a low P concentration ($5 \mu\text{M KH}_2\text{PO}_4$) or a high P concentration ($500 \mu\text{M KH}_2\text{PO}_4$) for 29 d before measurement. (A) Plant dry weight; (B) P content; (C) total root length. Data in the figure are the mean of four replicates \pm s.e. per plant.

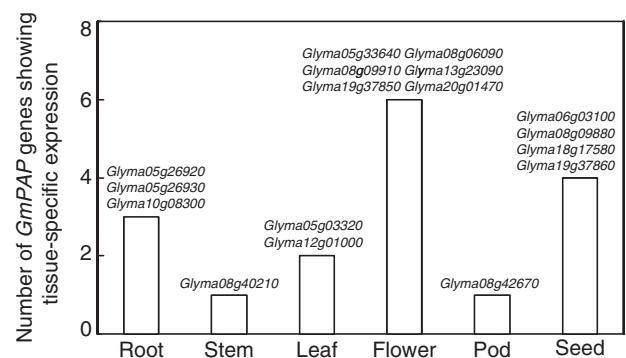


FIG. 3. Number and identity of *GmPAP* genes showing tissue-specific expression across six different tissues under normal P treatments. The fold-change between the expression levels of the most highly expressed and second most highly expressed *GmPAP* genes was calculated (Libault *et al.*, 2010). A threshold of 2-fold was used to determine the tissue specificity of *GmPAP* genes.

tissues. In contrast, *GmPAP2*, *GmPAP5*, *GmPAP6* and *GmPAP19* were constitutively expressed regardless of P supply (Table 2 and Supplementary Data Table S4).

TABLE 2. Transcriptional responses of 35 *GmPAP* genes to Pi deprivation in six tissues

Gene name	Root		Stem		Leaf		Flower		Pod		Seed	
	LP/HP	HP/LP	LP/HP	HP/LP	LP/HP	HP/LP	LP/HP	HP/LP	LP/HP	HP/LP	LP/HP	HP/LP
<i>GmPAP1</i>	+	.	+	.	+
<i>GmPAP2</i>
<i>GmPAP3</i>	++ +	.	.	.	+	.	+
<i>GmPAP4</i>	++	.	++	.	++	.	+	.	+	.	.	.
<i>GmPAP5</i>
<i>GmPAP6</i>
<i>GmPAP7</i>	+	+
<i>GmPAP8</i>	+	.	+	.	++
<i>GmPAP9</i>	++	.	++ +	.	++ +	.	++	.	++	.	.	.
<i>GmPAP10</i>	++	.	.	.	++	.	+	.	+	.	+	.
<i>GmPAP11</i>	.	.	++	.	++	---
<i>GmPAP12</i>	+	.	++	.	+
<i>GmPAP13</i>	++	.	++	.	++ +	.	+	.	++	.	.	.
<i>GmPAP14</i>	++	---
<i>GmPAP15</i>	++ +	.	.	.	+	--
<i>GmPAP16</i>	++	.	.	.	+	.	+	.	+	.	.	.
<i>GmPAP17</i>	++ +
<i>GmPAP18</i>	+	++ +	.
<i>GmPAP19</i>
<i>GmPAP20</i>	++	.	++	.	+
<i>GmPAP21</i>	++	.	+	.	+	.	+	.	+	.	.	.
<i>GmPAP22</i>	-	.	.	.	-	.	.
<i>GmPAP23</i>	++	.	++	.	+	.	+
<i>GmPAP24</i>	.	---	+	.	.	-	++ +	.
<i>GmPAP25</i>	++	.	++	.	++
<i>GmPAP26</i>	.	-
<i>GmPAP27</i>	.	.	+	++ +	.
<i>GmPAP28</i>	.	.	+
<i>GmPAP29</i>	-
<i>GmPAP30</i>	-
<i>GmPAP31</i>	++ +	.	+	.	.	.	+	.	.	.	++	.
<i>GmPAP32</i>	++	.	++	.	++	.	+	.	+	.	.	.
<i>GmPAP33</i>	++	.	++	.	++ +	.	+	.	+	.	.	.
<i>GmPAP34</i>	++ +
<i>GmPAP35</i>	+

The fold-changes of the expression of *GmPAP* genes between low P (LP) and high P (HP) were calculated and the expression differences were then classified into four groups, according to their response to Pi deprivation, as follows: +/-, induced/inhibited by Pi deprivation (≥ 3 - and < 10 -fold changes between the expression levels of LP and HP); ++ /-- highly induced/inhibited (≥ 10 - and < 100 -fold changes); ++ + /---, very highly induced/inhibited (≥ 100 -fold changes). A minus sign represents higher expression of *GmPAP* genes in HP than in LP.

Interestingly, the expressions of *GmPAP26*, *GmPAP29* and *GmPAP30* were inhibited by Pi deprivation in roots, leaves and seeds, respectively. Pi deprivation also inhibited the expression of *GmPAP22* in both leaves and pods (Table 2 and Supplementary Data Table S4). In addition, the expressions of *GmPAP11*, *GmPAP14* and *GmPAP15* were up-regulated in stems and leaves, leaves, roots and leaves, respectively, whereas, they were down-regulated in seeds by Pi deprivation. Conversely, the expression of *GmPAP24* was down-regulated in roots and pods, and up-regulated in flowers and seeds by Pi deprivation (Table 2 and Supplementary Data Table S4).

Expression patterns of soybean PAP genes in nodules and AM roots

Soybean genome annotation showed about 100 soybean genes played very important roles in nodulation (Schmutz *et al.*, 2010). RNA-Seq atlas of soybean found that the

nodule was one of the organs which was strongly enriched in highly tissue-specific genes (Libault *et al.*, 2010; Severin *et al.*, 2010). To reveal the responses of *GmPAP* genes in the nodules to Pi deprivation, q-PCR analyses were performed using total RNA from the nodules of soybean under both Pi-sufficient and Pi-deficient conditions (Fig. 4). The results showed that the expressions of nine *GmPAP* genes were induced by Pi deprivation and highly expressed in the nodules under low-P conditions. Eight *GmPAP* genes showed predominant expression in the Pi-sufficient nodules. The other members of *GmPAPs* were expressed in the nodules regardless of P supply (Fig. 4).

In addition, in order to investigate the effects of AM infection on the expression of *GmPAPs*, the Pi-deprived roots of soybean with and without AM inoculation were harvested for RNA extraction and q-PCR analysis (Fig. 5). The colonization percentage of the soybean roots was above 70 % when inoculated with AM fungi (data not shown). The results showed that only the expression levels of *GmPAP4*

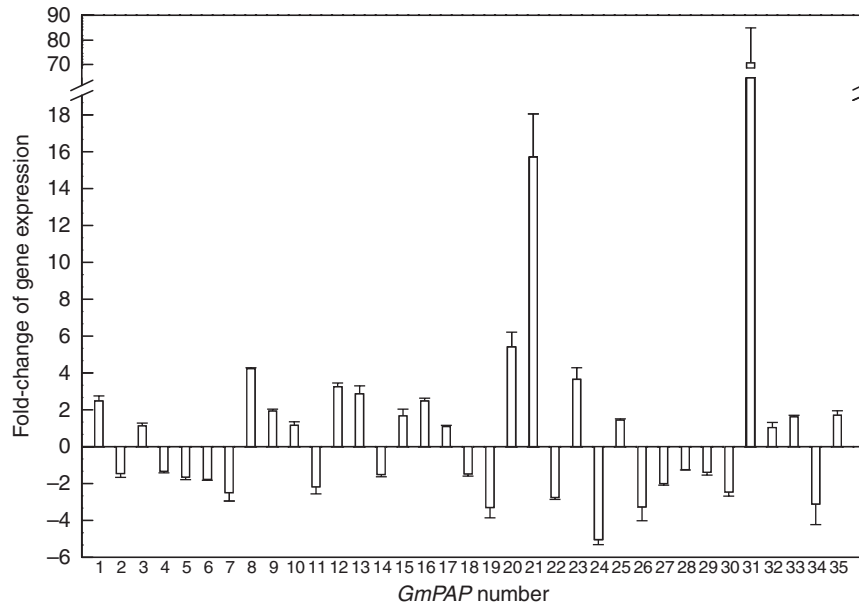


FIG. 4. The fold-change of the expression of *GmPAP* genes between low-P and high-P treatments in the nodules. Seven-day-old seedlings were inoculated with rhizobia, *Bradyrhizobium* sp. BXYD3 and transferred to solutions containing either a low P concentration ($5 \mu\text{M}$ KH_2PO_4) or a high P concentration ($500 \mu\text{M}$ KH_2PO_4) for 50 d before nodule harvest. The fold-change of the expression of *GmPAP* genes between low and high treatments in the nodules was calculated (y-axis). Negative values represent higher expression of *GmPAP* genes in high than in low P. A threshold of 2-fold was used to determine the responsiveness of *GmPAP* genes in the nodules to P treatments. Data in the figure are the mean of four replicates \pm s.e.

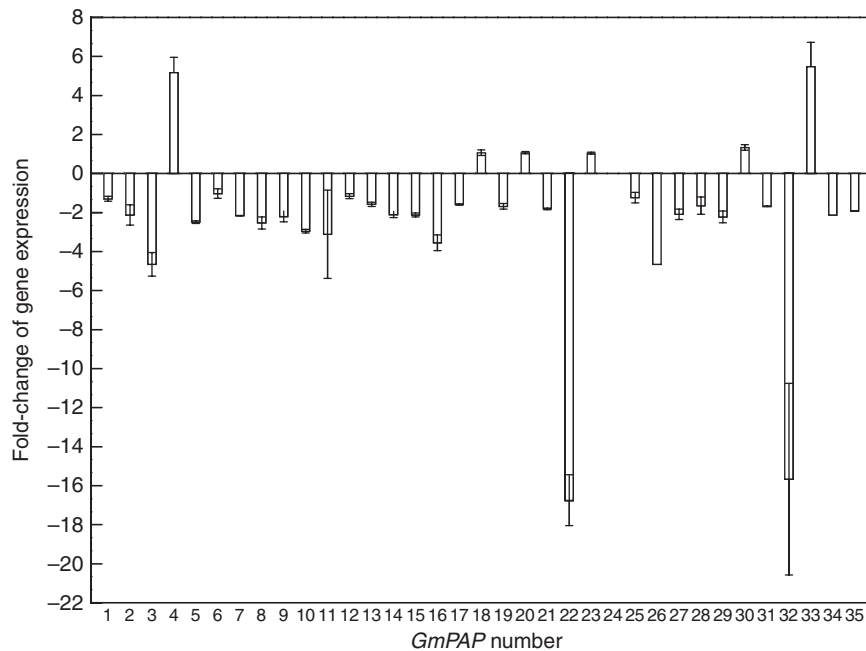


FIG. 5. The fold-change of the expression of *GmPAP* genes between roots with arbuscular mycorrhiza (+AM) and roots without arbuscular mycorrhiza (-AM) under low-P treatments. Soybean seeds were germinated in sand culture with or without the AM inoculants. Seven-day-old seedlings were transferred to solutions with a low concentration of P ($5 \mu\text{M}$ KH_2PO_4) sand culture for 50 d before harvest. The fold-change of the expression of *GmPAP* genes between +AM and -AM roots was calculated (y-axis). Negative values represent higher expression of *GmPAP* genes in -AM roots than that of +AM roots. A threshold of 2-fold was used to determine the responsiveness of *GmPAP* genes in the roots to AM formation under low-P conditions. Data in the figure are the mean of four replicates \pm s.e.

and *GmPAP33* were significantly increased in the roots inoculated with AM fungi compared with those not inoculated. Seventeen *GmPAP* genes showed predominant

expression under no-inoculation conditions. The rest of the *GmPAP* genes were expressed in the Pi-deprived roots regardless of AM inoculation (Fig. 5).

DISCUSSION

Soybean PAP gene family

Although PAPs are found in a wide variety of organisms, only four *PAP* genes have previously been reported in soybean (LeBansky *et al.*, 1992; Schenk *et al.*, 1999, 2000; Hegeman and Grabau, 2001; Liao *et al.*, 2003). In the present study, more *PAP* genes were found in soybean than in arabidopsis and rice (Li *et al.*, 2002; Zhang *et al.*, 2011). In total, 35 GmPAPs were identified by blastp searches on the phytozome website (Table 1 and Supplementary Data Text File), 32 of which possessed the seven metal-binding residues characteristic of PAP metalloesterase (Supplementary Data Table S2). GmPAPs 1, 5 and 7 missed some of the conserved metal-binding residues, but showed the highest homology with the known AtPAPs 1, 24 and 7, respectively (Fig. 1). A search in Gbrowse on the phytozome site indicated that gene structures of GmPAPs 1, 5 and 7 were well supported by Blast and EST alignments, but the alignment evidence was sparse in some areas within these genes. Therefore, future work with PCR, ESTs or RNAseq might be needed to confirm the results of sequencing and assembly.

Previous studies have shown that the soybean genome has undergone at least two rounds of large-scale duplication events, approx. 59 and 13 million years ago, which resulted in more homeologous gene pairs in soybean, such as *GmPAP9* and *GmPAP13*, *GmPAP7* and *GmPAP14*, *GmPAP17* and *GmPAP30* and *GmPAP4* and *GmPAP33* (Schmutz *et al.*, 2010; Libault *et al.*, 2010). The two duplication events also resulted in more complex transcriptional controls in soybean. Many single-gene activities in arabidopsis were encoded by multigene families in soybean (Schmutz *et al.*, 2010). These might explain why there are more *PAP* genes in soybean than in arabidopsis and rice.

Structural and phylogenetic analysis of GmPAPs

Some studies have shown that most of the known PAPs are secreted and are soluble proteins (Olczak *et al.*, 2003). In this study, protein sequence analyses predicted that 24 out of the 35 GmPAPs contained an N-terminal secretory pathway signal peptide, indicating either vacuolar localization or secretion (Table 1). Three GmPAPs were located in the mitochondria, which was the source of reactive oxygen species, particularly in non-photosynthetic tissues (Table 1; Dat *et al.*, 2000). This implied these three GmPAPs had a unique physiological role distinguishing them from the other GmPAPs (Liao *et al.*, 2003). Glycosylation is an important post-translational modification, which influences protein folding, localization and trafficking, biological activity and half-life (Olczak *et al.*, 2003; Tran *et al.*, 2010a). All GmPAPs (except GmPAP7) were predicted to contain various numbers of N-glycosylation sites, implying that they are all initially targeted to the Golgi apparatus where glycosylation occurs, and control secretion of PAPs (Supplementary Data Table S2; Olczak *et al.*, 2003; Tran *et al.*, 2010a).

Gene expression could be closely related to gene size in soybean (Severin *et al.*, 2010). The soybean genes with large coding regions were often transcriptionally active. Our results found that all the 35 *GmPAP* genes had a greater

number of exons and a longer total exon length compared with the genes identified as neither highly confident nor transcriptionally active in previous studies, and belonged to highly confident genes and were transcriptionally active (Table 1; Severin *et al.*, 2010). However, the number of exons and first exon length varied greatly among the 35 *GmPAP* genes and did not show the same trends as previous reports, which found the genes with preferential expression for underground tissues had a longer first exon than seed and aerial tissues. Conversely, aerial tissues had a greater number of exons than seed and underground tissues (Table 1, Fig. 3 and Supplementary Data Table S3; Severin *et al.*, 2010).

In arabidopsis, phylogenetic analysis showed that there was a close correlation between the classification of the groups and the molecular weight of the predicted AtPAPs (Li *et al.*, 2002). Our studies also found the 35 GmPAPs, 29 AtPAPs and 26 OsPAPs can be classified into three distinct groups (I, II and III) with different molecular weights (Fig. 1 and Table 1). This indicates that this classification may reflect the structure and biochemical differences among the different types of PAPs (Li *et al.*, 2002). At the subgroup level, GmPAPs grouped with AtPAPs and OsPAPs together, suggesting a close relationship between PAPs from different species. In addition, PAPs clustered in the same group show similar biochemical properties (Li *et al.*, 2002). For instance, GmPAP29 (previously named GmPhy) grouped with AtPAP15 in subgroups Ib-1. Both of them had high phytase activity (Hegeman and Grabau, 2001; Zhang *et al.*, 2008; Kuang *et al.*, 2009; Wang *et al.*, 2009).

Tissue-specific expression of GmPAP genes and their involvement in P nutrition

The soybean genome annotation showed 69 145 putative genes were predicted in palaeopolyploid soybean, 46 430 genes of which were identified as highly confident genes (Schmutz *et al.*, 2010). All 35 *GmPAP* genes have already been annotated in the soybean genome, and could be found in the transcriptionally active genes identified by Libault *et al.* (2010) and Severin *et al.* (2010). Libault *et al.* (2010) reported that soybean nodule, flower and pod exhibited the highest degree of tissue-specific gene expression. Severin *et al.* (2010) found dramatic tissue-specific gene expression in seed development and nodule tissues. In agreement with these, our studies found that soybean flowers and seeds possessed the largest numbers of tissue-specific *GmPAP* genes (Fig. 3). In addition, some *GmPAP* genes showed similar tissue-specific expression patterns to the results reported by Libault *et al.* (2010) and Severin *et al.* (2010). For example, *GmPAP21* genes were very specifically expressed in the roots under normal P treatments in the present study (Fig. 3 and Supplementary Data Table S3), which was consistent with the results from Libault *et al.* (2010) and Severin *et al.* (2010). However, in the present study, *GmPAP14* genes were expressed very specifically in the seeds (Fig. 3 and Supplementary Data Table S3) but Libault *et al.* (2010) and Severin *et al.* (2010) found that they exhibited the highest expression in the roots. Such differences might be caused by the different sampling times, plant materials and *Rhizobium* inoculation in these three independent experiments.

A number of plant PAPs were induced by P deficiency and were suggested to play crucial roles in P acquisition and assimilation (Li et al., 2002; Lung et al., 2008; Tran et al., 2010b). Previous studies have demonstrated that two *GmPAP* genes (*GmPAP15* and *GmPAP23*) were induced in roots and leaves under Pi-starvation conditions to synthesize more PAP enzymes to cope with P deficiency (Liao et al., 2003). Our results showed the transcript level of 66% *GmPAP* genes were induced or increased by Pi-deprivation treatment (Table 2 and Supplementary Data Table S4), which was higher than those of *OsPAP* (48%) (Zhang et al., 2011) and *AtPAP* (14%) genes (Li et al., 2002; Tran et al., 2010a), indicating different evolution history in different plant species. The up-regulation of more *GmPAP* genes in Pi-deprived plants also implied that they could be involved in plant P nutrition. In contrast, four *GmPAP* genes were constitutively expressed regardless of P supply, and four *GmPAP* genes were highly expressed under high-P conditions, indicating that PAPs may also perform other biochemical functions in plants as well as being involved in P acquisition (Table 2 and Supplementary Data Table S4). Expression analysis also found that some *GmPAP* genes were expressed in specific tissues under Pi starvation conditions (Table 2 and Supplementary Data Table S4). For instance, two *GmPAP* genes were expressed specifically in the root, implying that their potential functions are in hydrolysing and mobilizing Pi from organic P in the soil for extracellular P acquisition. In contrast, several *GmPAP* genes were predominantly expressed in leaves or seeds, suggesting their involvement in the intracellular utilization of Pi reserves or other Pi-containing compounds in plants. It is interesting to note that the Pi starvation-induced *GmPAP*s often clustered together in the phylogenetic tree. However, PAPs with phytase activity in subgroup Ib-1 showed low responsive to P deficiency (Fig. 1, Table 2 and Supplementary Data Table S4).

The involvement of GmPAPs in plant symbiosis

The root nodule is formed by the interaction of the root systems of leguminous plants with rhizobia (Mylona et al., 1995). Generally, higher levels of phosphorus are required by nodulated legumes for optimal symbiotic performance (Deng et al., 1998). The biological N₂ fixation in P-deficient plants is usually impaired by a low P supply (Almeida et al., 2000). Some studies have revealed that acid phosphatase existed in the symbiosome membrane of soybean root nodules (Sarath et al., 1993). The RNA-Seq atlas showed that *GmPAP16* and *GmPAP30* had highest tissue-specific expression in nodules (Libault et al., 2010; Severin et al., 2010). Bean plants in Pi-deficient conditions showed increased activity of phosphatases and phytases in nodules (Araújo et al., 2008). Our results showed that nine out of the 35 *GmPAP*s were highly expressed in the nodules under low-P conditions (Fig. 4) and were possibly involved in Pi homeostasis in the nodules (Pereira and Bliss, 1987), Pi absorption directly by the nodules and bacteroids (Al-Niemi et al., 1998), or increased N₂ fixation ability to compensate for reduced nodulation (Almeida et al., 2000). In addition, it is interesting to note that all *GmPAP* genes expressed in Pi-deprived root nodules showed predominant expression in soybean plants

under P-limited conditions. In contrast, some *GmPAP* genes (not *GmPAP7* and *GmPAP34*) which were highly expressed in Pi-sufficient root nodules showed a low response to P deficiency in soybean plants (Fig. 4, Table 2 and Supplementary Data Table S4).

AM fungi play an important role in plant growth. Generally, AM formation improves P nutrition and results in decreased *PAP* gene expression. The expression of the *PAP* genes was reduced by AM formation in *Medicago* (Liu et al., 1998). In our studies, most of the *GmPAP* genes showed reduced expression levels in AM roots under low-P conditions (Fig. 5). However, it has also been found that in some plants, such as wheat and onion (Dodd et al., 1987), maize (Fries et al., 1998) and marigold (Ezawa and Yoshida, 1994; Ezawa et al., 2005), the increased acid phosphatase activity of the roots was in response to AM formation. Ezawa et al. (2005) found one *PAP* gene (*TpPAP1*) from marigold had higher transcript levels in response to AM formation under Pi-deficient conditions. Our studies also found that the expression levels of two *GmPAP* genes, *GmPAP4* and *GmPAP33*, were significantly increased by AM infection under P-limited conditions (Fig. 5), suggesting that AM fungi might activate part of the plant systems which respond to low-P, subsequently promoting hydrolysis of organic P in the rhizosphere, and salvaging P esters leaked from the root cell for P recycling (Barrett-Lennard et al., 1993; Ezawa et al., 2005). In addition, it is interesting to note that *GmPAP4* and *GmPAP33* were grouped into one small group in the phylogenetic tree, and also showed predominant expression in P-limited soybean plants (Fig. 5, Table 2 and Supplementary Data Table S4).

In summary, the present study has characterized and compared the molecular features of the *GmPAP* gene family, and conducted the expression pattern analysis of *GmPAP*s in soybean. The results suggested that flowers possessed the largest number of tissue-specific *GmPAP* genes under normal P conditions, and most *GmPAP* genes were possibly involved in P acquisition and recycling in plants. Also we provide the first evidence that some members of the *GmPAP* gene family are possibly involved in the response of plants to symbiosis with rhizobia or arbuscular mycorrhizal fungi under P-limited conditions. Further investigations are required to reveal their biological functions as well as the biochemical mechanisms of their actions by combined application of genetic, biochemical and immunological methods.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Text File: amino acid sequences of the 35 *GmPAP*s. Table S1: list of quantitative real-time PCR primers. Table S2: domain organization of the 35 *GmPAP*s. Table S3: expression specificity of *GmPAP* genes across six different tissues under normal P treatments. Table S4: quantitative real-time PCR analysis of expression levels of the *GmPAP* genes in different soybean tissues under different P treatments.

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