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Agrobacterium rhizogenes transformed soybeans with *AtPAP18* gene show enhanced phosphorus uptake and biomass production

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ABSTRACT

Low-phosphorus stress is a challenging factor in limiting plant development. Soybean is cultivated in soils often low in phosphorus. However, on average 65% of total P is in the form of organic phosphates, which are unavailable to plants unless hydrolyzed to release inorganic phosphate. One approach for enhancing crop P acquisition from organic P sources is boosting the activity of acid phosphatases (APases). This study seeks to understand the role of an Arabidopsis (*Arabidopsis thaliana*) purple APase gene (*AtPAP18*) in soybean. Thus, the gene was isolated and a final vector (*AtPAP18/pK7GWG2D*) was built. Composite soybean plants were created using *Agrobacterium rhizogenes*-mediated transformation. *A. rhizogenes* K599 carrying the *AtPAP18/pK7GWG2D* vector with *egfp* as a reporter gene was used for soybean hairy root transformation. Analysis of *Egfp* expression detected fluorescence signals in transgenic roots, whereas there was no detectable fluorescence in control hairy roots. The enzyme assay showed that the APase activity increased by 2-fold in transgenic hairy roots. The transformed hairy roots displayed an increase in plant soluble P and total P contents, as compared with the control plants, leading to improved biomass production. RT-PCR analysis revealed high expression levels of *AtPAP18* in transformed hairy roots. It is noteworthy that these primers amplified no *PAP18* transcript in control hairy roots. Taken together, the findings demonstrated that overexpression of the *AtPAP18* gene offers an operative tactic to reduce the utilization of inorganic phosphorus (Pi) fertilizer through increased acquisition of soil Pi, especially improving the crop yield on soils low in available P.

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KEYWORDS

APase; GFP; soybean; hairy root; P acquisition

Introduction

The fundamental role of phosphorus (P) in maintaining the vitality of organisms is unquestionable [1]. The crucial role of P lies in its involvement in various metabolic and biosynthesis processes and also contributes to signalling cascades by functioning as mediators of signal transduction. On the other hand, phosphorus deficiency is a major challenge for the cultivation of crops in the world [1]. Various estimates suggest that more than 30% of the world's arable soils are deficient in phosphorus, which in turn restricts plant growth and yield [2]. *Glycine max* L. Merr. growing soils mainly suffer from a lack of phosphorus [3]. As a result, to keep the highest levels of soybean production, phosphorus fertilizers should be added to soils with phosphorous concentrations less than 10 mmol/L [4]. However, most of the annually added phosphorus fertilizers are fixed in the soil in

organic forms by adsorption, sedimentation and transformation, which is challenging because the additional dissolution of phosphates is potentially harmful to the environment [5]. These changes, along with other factors like P fixation by free Fe and Al oxides, lead to conversion of the major part of the soil phosphorus into organic phosphorus (50%–80% of the total soil P). Consequently, P becomes unavailable to plants unless hydrolyzed to release inorganic phosphate [6]. Therefore, despite the high level of phosphorus in the soil, it is not accessible. In addition, the worldwide P resources are non-renewable and scientists have estimated that its resources will be depleted in the future [1]. Consequently, understanding the different players that help to remobilize and recycle inorganic phosphorus (Pi) from its compounds as well as scavenge Pi from organic soil matter is a sustainable, economic and important approach in producing Pi

efficient soybean plants, which are capable of efficient utilization of organic phosphates and P added as fertilizers.

The role of PAPs (*Purple acid phosphatases*) genes in improved crops phosphorus efficiency is remarkable, since they are involved in P acquisition and recycling in plants [7,8]. On the other hand, some other studies have documented the positive relationship between the level of plant tolerance to P starvation and the amount of released PAPs from plant roots into the rhizosphere. These enzymes catalyze the breakdown of organic phosphates and added P fertilizers into Pi. PAPs are highly induced by P starvation and can be secreted or located in the cellular organelles to utilize externally available Pi in the soil or to recycle it from intracellular P sources [4]. PAP family enzymes are distributed across different plants, for example the genomes of *Arabidopsis thaliana*, *Oriza sativa* and *Glycine max* include 29, 26 and 35 PAP family members, respectively [7,9,10]. It has been proved that creating a transgenic plant overexpressing one of the APase-encoding genes would be a helpful method to reduce the consumption of chemical Pi fertilizers, since in the given species, the transgenic plants with improved P efficiency would obtain more Pi from the soil, minimizing the need for P fertilizers, in comparison with untransformed plants.

The accumulating body of data on the structure, function and expression pattern of PAP genes would be meaningful in cloning and transgenic overexpression of related genes to create P efficient crops. For example, *GmPAP4* is a PAP gene involved in the utilization of organic phosphate in plants especially under phytate conditions. Transgenic overexpression of this gene in *Arabidopsis* resulted in a significant rise in P acquisition and utilization in comparison with the wild-type [11]. The overexpression of another PAP gene (*AtPAP15*) in soybean hairy roots led to a 1.5-fold increase in the APase activity in the transgenic hairy roots. Also, transgenic plants exhibited enhanced P efficiency and better root and shoot growth rates versus the controls [6]. Overexpression of *AtPAP18* in *Nicotiana tabbaccum* led to significantly increased acid phosphatase activity, total P and Pi contents, resulting in improved biomass production in both Pi-deficient and Pi-sufficient conditions [12,13].

Based on some promising results from previous works on improving crops P efficiency, in this study, we isolated the *Arabidopsis thaliana* *PAP18* (*AtPAP18*) gene and subsequently introduced it into soybean hairy roots mediated by *Agrobacterium rhizogenes*. The transgenic soybeans overexpressing the gene exhibited remarkable increases in their APase activity, total P and free Pi contents, and root growth rates. This study could have

significant contribution for successful crop production on low-P soils, which is an agronomic limitation worldwide.

Materials and methods

Vector construction and *A. rhizogenes* strain

The complete coding sequence of *AtPAP18* were amplified by using polymerase chain reaction (PCR) from the cDNA of *Arabidopsis*, using primers specially designed for the gene of interest (forward primer: 5'-ATGGAAAAATGGGGGATTTTGC-3', reverse primer: 5'-TAAGGTTCCAAGAGCATTTTCC-3'). The PCR product with 3' A-overhangs, was subcloned into a pCRTM8/GW/TOPO[®] vector, following the vector transformed into competent *E.coli* cells. True colonies were selected and analysed; then positive transformants were chosen and the plasmid was isolated. To build the final vector, the LR recombination reaction proceeded between Gateway[®] destination vector pK7GWIG2D (II) [14] and donor vector. The final vector encompasses the following segments: (1) the selectable marker *nptII*, which codes for neomycin phosphotransferase and induces kanamycin resistance, (2) enhanced green fluorescent protein (*egfp*) driven by the 35S promoter (p35S), the (3) spectinomycin resistance gene (*Sm/Spr*) for plasmid selection and the gene of interest *AtPAP18* (Figure 1). We transformed this vector into the *A. rhizogenes* (strain K599), which was subsequently used to induce transformed hairy roots in

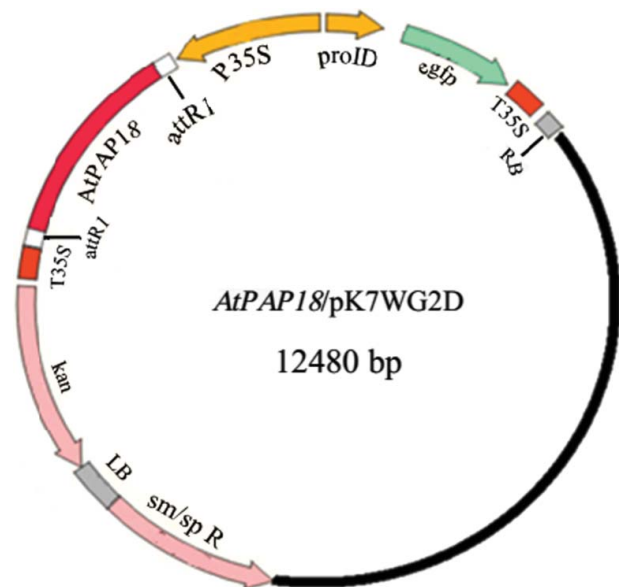


Figure 1. Map of the *AtPAP18*/pK7WG2D vector introduced into *A. rhizogenes* K599, as part of the hairy root transformation system.

soybean plants. *A. rhizogenes* K599, which was used for injection of *G. max*, was prepared by Prof. Peter M. Gresshoff, The University of Queensland.

Seed germination

To sterilize soybean cv. Williams 82 seeds, they were transferred into a bottle containing a hydrogen peroxide/ethanol mix (10 mL of 30% (w/w) H₂O₂ to 75 mL of 96% (v/v) ethanol made up to a final volume of 100 mL with sterile distilled water). They were inverted gently for 2 min into the mix and then washed several times (5–6 times) with sterile distilled water [15]. After sterilization, they were positioned into wet vermiculite at a depth of 1–2 cm in pots. The pots were incubated for 5–6 d at 26 °C in a chamber with the following illumination conditions: 100 PAR, 12-h day, 12-h night. The seedlings were allowed to grow until the cotyledons reached

a folded stage, in which the seedlings height would be roughly 5 cm (Figure 2(a)).

Preparation of *A. rhizogenes* K599 and inoculation

Simultaneously with sowing the seeds, *A. rhizogenes* K599 containing the related vector was cultured in lysogeny broth (LB) medium (from glycerol stock). After 24 h, a loop of bacteria were transferred onto the LB plates containing 50 mg/L rifampicin and were incubated at 28 °C for 1 d. On the following day, a single colony was inoculated onto a new plate containing 50 mg/L spectinomycin and incubated at 28 °C again. Five-day old healthy plantlets with open green cotyledons were inoculated with *A. rhizogenes* by piercing at the cotyledonary node with a sterile syringe needle [15]. A drop of bacteria from the incubated plates was injected into the cotyledonary node site (Figure 2(b)). Control soybean plants

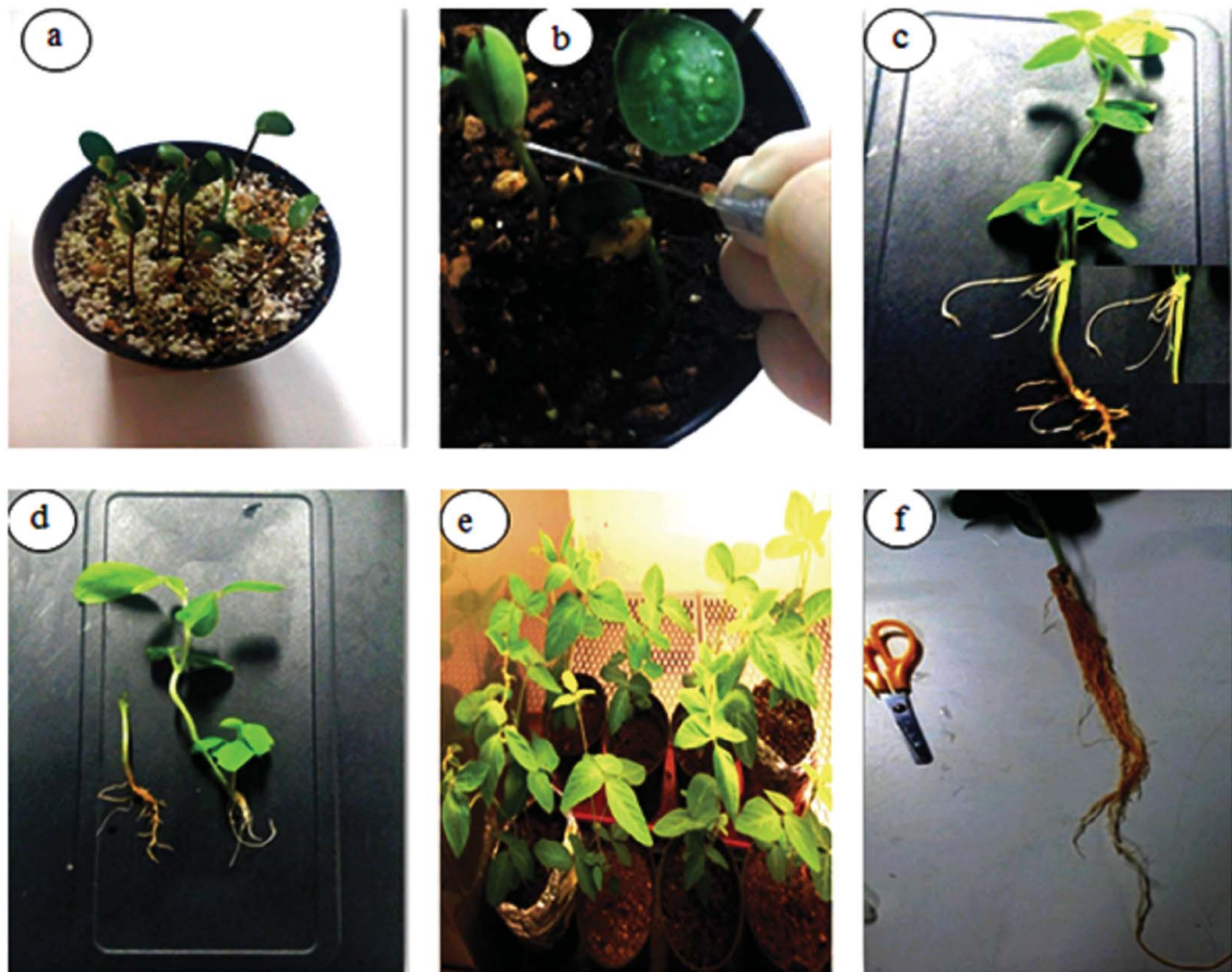


Figure 2. Different steps of *A. rhizogenes* mediated transformation of soybean hairy roots. (a) 5-day-old germinated soybean plants with open cotyledons. (b) Piercing of the hypocotyl close to the cotyledonary node site to inject bacteria. (c) Soybean plant 13 days after inoculation with *A. rhizogenes*. (d) Composite soybean plants after detaching the primary root. (e) Composite soybean plants after transferring to new pots. (f) Soybean hairy roots ready to be cut for performing different assays.

were injected with an *A. rhizogenes* without construct. The inoculated seedlings were sheltered with a transparent plastic and kept in a humid growth chamber with the following conditions: L/D: 16/8 h, T: 26/26 °C, RH: 66%, and they were watered with Broughton and Dilworth (BD) solution every day [15].

After 13 days of inoculation, induced hairy roots emerged at the injection site (Figure 2(c)). When the induced hairy roots reached a height of up to 5–10 cm, the primary roots were cut 1 cm under the cotyledonary node and detached from the plant (Figure 2(d)). Plants with induced hairy roots, which at that point are called composite plants, were moved into pots. The composite plants were watered with BD solution supplemented with 2 mmol/L KNO₃ as a nitrogen source. The composite plants were covered with a transparent plastic bag and kept in a growth chamber (L/D: 16/8 h, T: 26/26 °C, RH: 66%) for 20 d. After a few days, their plastic bags were removed to help gradual acclimation of composite plants to the growth chamber conditions (Figure 2(e)). Finally, the transformed and control hairy roots were detached to implement the required assays (Figure 2(f)).

Screening transgenic hairy roots

GFP screening

To examine the hairy root transformation, we used *Egfp* in the vector as a reporter gene. Approximately after the first week of transferring the composite plants into the new pots, green fluorescent protein (GFP) screening was used to identify the transgenic hairy roots. We used the e Fluorescence Stereo Microscope (Leica FLIII) with a Leica camera, to detect GFP expression in hairy roots. A 470-nm excitation filter was used. For GFP visualization, plants were removed from the pots and visualized on the stereomicroscope.

RNA isolation and RT-PCR

Total RNA was isolated from the transformed hairy roots of plants using a modified phenol/chloroform /isoamylalcohol mediated extraction protocol [16], and cDNA was synthesized from 2 mg RNA according to the kit's instructions (Applied Biosystems). PCR on cDNA was performed using specially designed primers for the gene of interest and 5'-ATCTTGACTGAGCGTGGTTATTCC-3' and R3, 5'-GCTGGTCTGGCTGTCTC C-3' for the *Actin* gene. The amplified fragments were 1336 bp long.

APase activity assay

Control or transgenic hairy root samples were powdered in liquid nitrogen and transferred to the extraction buffer (10 mmol/L sodium acetate, pH 5.6). Subsequently, the homogenized samples were centrifuged twice at

12,000 × *g*, 4 °C for 30 min and the supernatants were used to measure the APase enzymatic activity. We carried out all extraction steps on ice. The enzymatic activity measurements were performed at 37 °C for 30 min in 100 mmol/L sodium acetate buffer (pH 5.6) containing 5 mmol/L of p-nitrophenyl phosphate (pNPP). To determine the enzymatic activities, we constructed a standard curve using identified concentrations of PO₄³⁻. We defined each unit of APase activity as 1 μmol of released Pi per min [13].

Soluble pi and total p assays

To determine the phosphorous content, about 30 mg dry tissue samples were powdered in liquid nitrogen following extraction of tissue samples in 12.5% (w/v) TCA/ 25 mmol/L MgCl₂, overnight at 4 °C. Next, the extracts were centrifuged at 12,000 × *g* for 2 min. To measure the total P contents, 1 mL H₂SO₄ 1 N and 600 μL H₂O₂ were added to about 30 mg dry tissue samples in a Pyrex tube. The biomaterial was incubated on a 100 °C hot-plate for 24 h until the brown vapours vanished and then allowed the tube to cool down. Finally, to determine colourimetrically the soluble Pi and total P contents at A₆₅₀, 20 μL of the supernatant of each sample was diluted with 180 μL assay reagent (2 volumes of H₂O, 1 volume of 10% ascorbic acid, 1 volume of 2.5% ammonium molybdate and 1 volume of 1 N H₂SO₄) and incubated at 40 °C for 2 h. We constructed a standard curve and defined each unit of phosphorus content as micromoles of soluble Pi per milligram of dry weight [17].

Measurement of shoots and roots dry weight

The roots and shoots of the plants overexpressing *AtPAP18* and control ones grown in pots were harvested and assayed. Ten composite plants and three control plants were analyzed to measure the shoots and roots dry weights.

Data analysis

The results are presented as mean values from three and 10 samples, obtained from control and transformed plants, respectively, with their related standard deviations (±SD). Mean comparison was performed by using the Student's t-test and the SAS software version 9.2 was used to carry out statistical analysis.

Results and discussion

Hairy root induction and GFP assay

In this study, the hairy root transformation system was implemented to evaluate the effects of overexpressing

the *AtPAP18* gene in soybean on the plant P status. After injection of the *A. rhizogenes* harbouring the vector into the cotyledonary node, a small callus shaped at the concerned spot. From the injected spots, hairy root primordia and developing roots could be observed within 2 weeks after injection. By using this protocol, ~100% of *A. rhizogenes*-injected soybean plants successfully established roughly 10 hairy roots per injected plant on average. Monitoring of the transgenic hairy roots by fluorescence stereo microscope, revealed high levels of GFP expression (Figure 3(a)), whereas in the control hairy roots, there was no detectable GFP (Figure 3(b)).

The *A. rhizogenes*-mediated transformation method is a high efficient approach to obtain composite plants with transgenic hairy roots [18]. Many researchers have used this method to study the genes associated with morphological and physiological characteristics of roots through gene overexpression or silencing [18–[21]. Some APase genes, such as the *AtPAP10* gene, encode a root surface-bound enzyme which has a critical role in plant tolerance and adaptation to Pi deficiency [22]. In addition, another study about the effects of overexpressing the *AtPAP18* gene in *Arabidopsis* on the P status, revealed the great potential of this gene in reducing the consumption of chemical Pi fertilizers, since the plants overexpressing the gene could uptake more Pi from the soil and had the ability to remobilize the Pi from the internal resources [12]. Some APase genes have been cloned and overexpressed in model plant species [10,13,23,24] but only a few of these genes have been described in soybean and other important crops [6,9,11,16]. Therefore, in the present study, a hairy root transformation scheme was used to assay the influences of overexpressing an *Arabidopsis* purple APase gene in soybean on the phytate utilization in the rhizosphere.

Thus, full-length *AtPAP18* cDNA was isolated from *Arabidopsis* and cloned in the Gateway system. PSORT analysis and the results from ExPASy indicated that *AtPAP18* seems to have a cleavable signal peptide and is more likely localized extracellularly, including the cell wall. Similar results were observed when *AtPAP18::GFP* fusion construct was expressed in tobacco [13]. Further, the results from the bioinformatics analysis [25,26] suggested that the protein encoded by the *AtPAP18* gene can be considered an enzyme that is secreted outside the cells. Similarly, other PAP genes, including *AtPAP15*, encode a secretory PAP protein from hairy roots with phytase activity [6].

RT-PCR assay

To verify the *AtPAP18* expression in the transgenic hairy roots, RT-PCR assay was implemented with the cDNA from the transgenic/control hairy roots. The results from this assay showed a high expression level of *AtPAP18* in the hairy roots of plants, indicating overexpression of the gene, whereas the assay did not amplify a detectable *PAP18* transcript in the control plants (Figure 4).

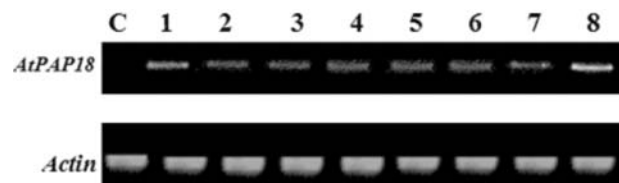


Figure 4. RT-PCR analysis of *AtPAP18* transcripts in soybean hairy roots. Lane C, control hairy root; Lane 1–8, transformed hairy roots. Note: The product fragments were 1040 bp long. The actin transcripts levels were used as internal controls (lowest panel).

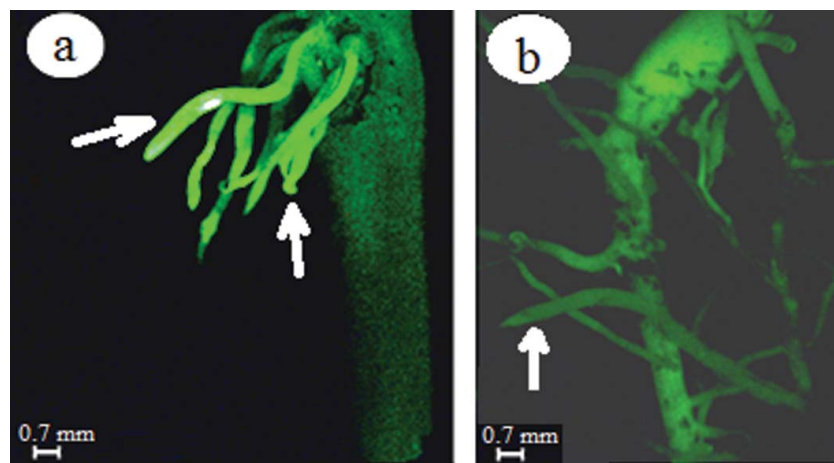


Figure 3. GFP detection of transformed soybean hairy roots. (a) Transgenic hairy roots with GFP expression. (b) Control plant hairy root without GFP expression. Note: bar = 0.7 mm.

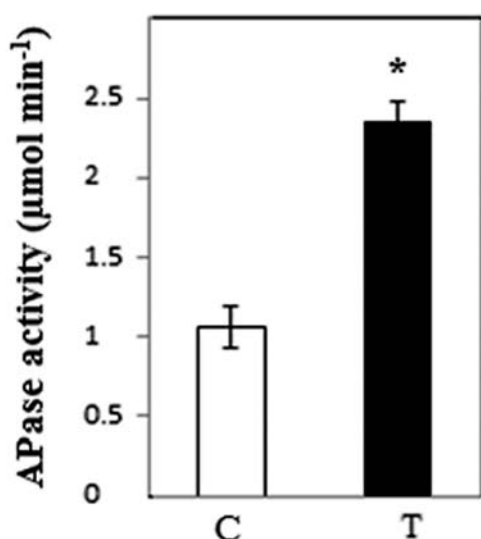


Figure 5. APase activity in transformed hairy roots and controls. Note: Data are mean values from three replicates for controls and 10 plants with transformed hairy roots. Asterisks indicate significantly different differences at $P \leq 0.05$.

APase activity in transformed hairy roots

To further characterize *AtPAP18* and its effect on APase activity, we constitutively overexpressed *AtPAP18* in soybean hairy roots. Subsequently, for additional analyses, 10 transformed hairy roots were selected taking into account their high level of *AtPAP18* transcript. Enzymatic assays revealed that the protein encoded by the *AtPAP18* may be a key contributor for APase activity, since its activity in the transgenic hairy roots was significantly higher than in the controls. The APase activity in the transformed hairy roots showed a 2-fold increase approximately as compared to the controls ($P \leq 0.05$; Figure 5).

The results from different investigations have suggested that the enzymatic activity of APases would be

higher in roots than in shoots [6], which is why we analysed extracts both from transformed and from non-transformed hairy roots to measure the APase activity. Studies with other crop species have indicated that increasing APase and phytase activity, following their secretion out of the cell or into the rhizosphere would be a major contributor to the plants' gaining organic P from soils [3]. Our results clearly showed a significant increase in the APase activity of the transformed hairy roots overexpressing the *AtPAP18* gene as compared with the control ones.

Overexpression of *AtPAP18* increases soybean p contents and dry weight

To study the effects of *AtPAP18* overexpression on Pi accumulation, the levels of total P and soluble P contents (or free P) were measured in the leaves of plants overexpressing the gene and in control ones. The total P and soluble P contents were high in the plants overexpressing *AtPAP18* compared to the control plants. The leaves of the plants with transformed hairy roots had a significantly higher (roughly 10% higher than controls) soluble P content ($P \leq 0.05$) in comparison with the control plants (Figure 6(a)). Furthermore, the shoots of plants with transformed hairy roots exhibited significantly enhanced total P content ($P \leq 0.05$; Figure 6(b)).

To understand the contribution of the *AtPAP18* overexpression to traits associated with growth and dry matter production, we compared the plants overexpressing *AtPAP18* with the control ones. There was an obvious difference in the shoots/roots production between the plants overexpressing *AtPAP18* and the control ones (Figure 7(a)). As shown in Figure 7(a), the plants overexpressing *AtPAP18* exhibited higher root and shoot dry weight than the controls (Figure 7(b)).

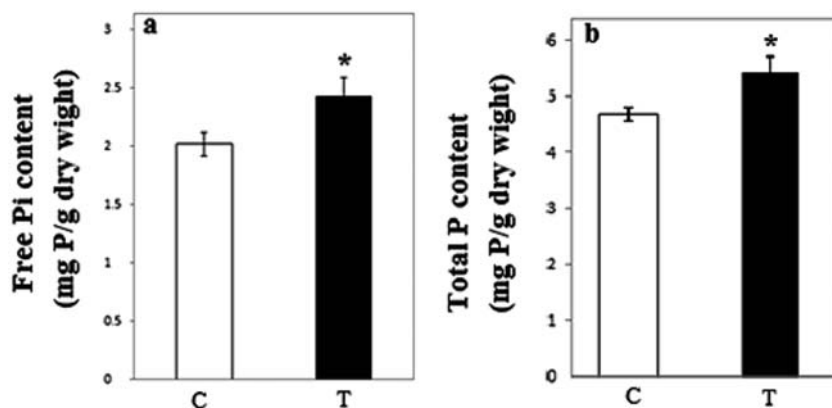


Figure 6. Free (a) and total (b) P content in leaves of transformed and control soybean. Note: Data are mean values from three replicates for controls and 10 plants with transformed hairy roots. Asterisks indicate significantly different differences at $P \leq 0.05$.

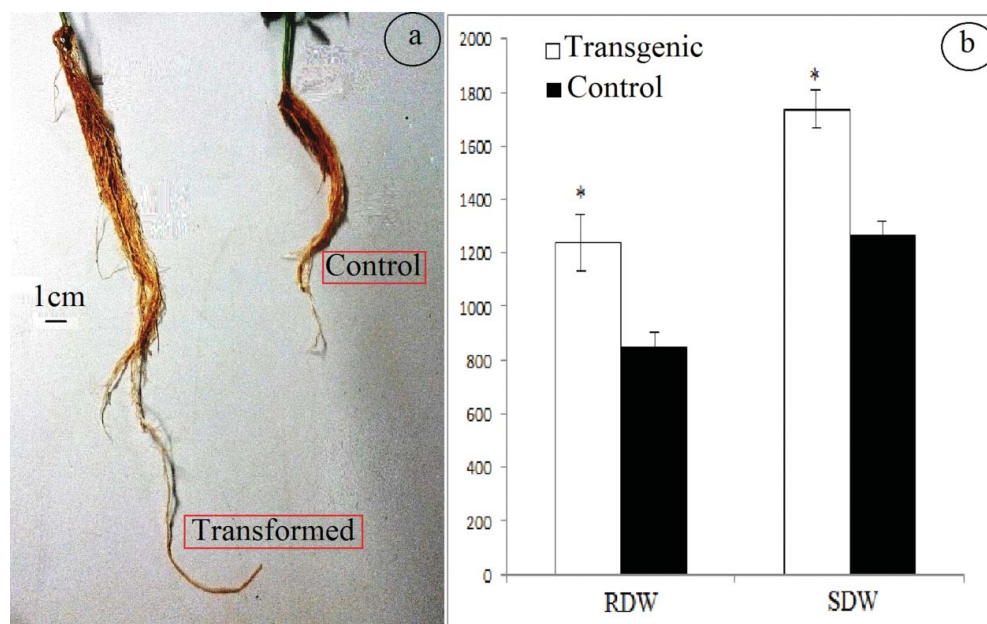


Figure 7. Roots (a) of an *AtPAP18* overexpressing plant compared to a control plant. Root and shoot dry weight (b) for transformed and control plants.

Note: RDW, root dry weight; SDW, shoot dry weight. Data are mean values from three replicates for controls and 10 plants with transformed hairy roots. Asterisks indicate significantly different differences at $P \leq 0.05$.

Subsequently, two concurrent phenomena, i.e. the increase in the APase activity and the total/soluble phosphorous, seem to be the reason for the higher growth and biomass in the plants overexpressing the *AtPAP18* gene (Figure 7(a)). Enhanced APase activity would result in upgraded P status in the plants, e.g. soybean crops overexpressing one of the relevant *PAP* genes would show improvement through substantial increases in their biomass, yield [6,13] and tiller numbers [27]. As reported [4,6,9,11,27], APases act as a key factor in the mineralization of organic P, remobilization, reallocation and utilization of Pi. Similar results have been reported for overexpression of *AtPAP18* in tobacco [13], *AtPAP15* in soybean [6], *AtPAP10* in *Arabidopsis* [22] and *OsPAP10c* in rice [27], in which the biomass and the P status showed substantial increasing, particularly in Pi deficient conditions [13]. In a study on overexpression of *OsPAP10a*, the APase activity in the shoots/roots of transgenic rice plants was significantly higher in comparison with controls, in both Pi abundance and Pi deficiency. Subsequently, rice plants overexpressing *OsPAP10a* showed enhanced ability to breakdown extracellular organic phosphate, such as ATP, into Pi in comparison to the wild type ones [2].

Our results are in agreement with similar studies that have also demonstrated the effects of *PAP* genes (including *AtPAP10* and *NtPAP12*) in increasing the plant biomass (root and shoot dry weight) under low

P conditions [22,28]. By overexpressing the *OsPAP21b* and *AtPAP18* genes, a noticeable increase in plant biomass has been expressed in rice and tobacco [13,29]. These morphological improvements, particularly the increase in root biomass production, are considered to increase the root surface area for soil Pi assimilation.

Identifying and boosting the enzymatic components that allow plants to recycle Pi from various P sources, particularly the phosphate contained in organic matter, is a critical approach to maintaining optimum levels of crop production in cultivation under conditions with a lack of phosphorous [30]. Our results indicated that *AtPAP18* may act as a key factor in the utilization of P. It is believed that, under Pi-deficient conditions, *PAPs* are able to increase the Pi availability through breakdown of organic compounds around the rhizosphere. The secreted APase may attach onto the root surface, making plants more capable of utilizing rhizosphere Pi across the area engaged by the roots [22].

This work provided evidence that overexpression of the *AtPAP18* gene increased the APase activity, which would result in improved plant growth and production under Pi-deficient conditions, where there is abundant organic phosphorus. Such conditions are prevalent in soybean cultivation lands despite the presence of adequate organic P in these soils, resulting in the cultivated area suffering from lack of phosphorous availability.

Conclusions

The results from this study confirmed that overexpression of an APase-encoding gene in plants would be an efficient strategy to improve Pi assimilation. Therefore, plants with improved P status would accumulate more biomass and would give higher productivity in soils with low free Pi. Taking into account the high costs of Pi fertilizers and increasing environmental concerns about high Pi contamination of soils and water resources (surface and underground water), this strategy would be very beneficial in reducing Pi fertilizer consumptions for soybean production. On the other hand, plants overexpressing *PAP* genes could probably eliminate the excessive Pi compounds from regions where Pi fertilizers are overused.

Acknowledgments

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Disclosure statement

No potential conflict of interest was reported by the authors.

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