Research Article

Functional Assessment of an Overexpressed Arabidopsis Purple Acid Phosphatase Gene (*AtPAP26*) in Tobacco Plants

Mohammad Sadegh Sabet [1,2], Katayoun Zamani [3], Tahmineh Lohrasebi [1], Mohammad Ali Malboobi [1*], Mostafa Valizadeh [2]

[1]Department of Plant Biotechnology, National Institute of Genetic Engineering and Biotechnology, 14965/161, Tehran, Iran
[2]Department of Agronomy and Plant Breeding, Faculty of Agriculture, Tabriz University, 5166616471, Tabriz, Iran
[3]Department of Genetic Engineering and Biosafety, Agricultural Biotechnology Research Institute of Iran, Agricultural Research, Education, and Extension Organization, Karaj, Tehran, Iran

Corresponding author: Mohammad Ali Malboobi, Department of Plant Biotechnology, National Institute of Genetic Engineering and Biotechnology, 14965/161, Tehran, Iran. Tel: +98 21 44787325, Fax: +98 21 44787350, E-mail: malboobi@nigeb.ac.ir

Background: Overexpression of known genes encoding key phosphate (Pi)-metabolizing enzymes, such as acid phosphatases (APases), is presumed to help plants with Pi availability and absorption as they are mostly exposed to suboptimal environmental conditions for this vital element.
Objectives: In this study, the overexpression effect of *AtPAP26*, one of the main contributors in retrieving Pi from intracellular and extracellular compounds, was evaluated from various viewes in tobacco plant.
Materials and Methods: As a heterologous expression system, the encoding cDNA sequence of AtPAP26 was transferred into tobacco plants.
Results: A high growth rate of the transgenic lines was observed which could be due to an increased APase activity, leading to the high total phosphorus as well as the free Pi content of the transgenic plants. Interestingly, a significant increased activity of the other APases was also noticed, indicating a networking among them. These were accompanied by less branched and short primary roots and a decreased lateral root numbers grown in Pi-starvation condition compared to the wild type seedlings. Besides, a delayed germination and dwarf phenotype indicates the possible reduction in gibberellic acid biosynthesis in the transgenic lines.
Conclusions: Such transgenic plants are of interest not only for increased yield but also for the reduced need for chemical fertilizers and removal of excessive Pi accumulation in soils as a consequence of fertilizers' or poultry wastes' over-usage.

Keywords: Arabidopsis, *AtPAP26,* Heterologous expression, Phosphate starvation, Purple acid phosphatase, Tobacco

1. Background

Phosphorus (P) is an essential nutrient for all living organisms being the second important element after nitrogen for plant growth (1-3). Also, as the most vital nutrient, it is a structural constituent of essential biomolecules, such as nucleic acids, phospholipids and phosphor-sugars; a central element in almost all metabolic reactions including photosynthesis, and respiration, as well as a key component of energy transfer in the form of ATP, NADPH, and so on (4).

Plants absorb P in its soluble inorganic form of $H_2PO_4^-$ or HPO_4^{2-} ions (Pi). Despite the existence of high amounts of P compounds in the soil (5), the concentration of Pi in many soil solution averages at about 1 μM and seldom exceeds 10 μM (6); far below the cellular Pi concentrations (5-20 mM) required for the optimal plant growth and development (7). Such a limitation in more than 30 percent of the world arable land often lead to a reduced productivity in the natural ecosystems as well as cropping systems, unless supplied as fertilizer (8). In fact, As estimated, the world resources of affordable Pi will be exhausted by 2050 (9, 10). Whilst, our limited global Pi reserves are non-renewable, Pi is being built

up to harmful level in many agricultural soils.

In order to secure agricultural sustainability through a reduction in the Pi fertilizer usage, one major approach is to bioengineer Pi efficient transgenic crops. As only near15–30% of the applied phosphate fertilizer is taken up by crops in the field, potentially large gains in efficiency can be made by improving phosphate acquisition (8). This necessitates our detailed understanding of transcriptional regulation response and the complex biochemical adaptations in the Pi deficient plants (11).

The vacuolar Pi concentration is estimated to range from µM to mM depending on the extracellular inorganic phosphate concentrations. Manipulation of tonoplast Pi transporters expression would indeed affect the relatively constant cytoplasmic Pi levels, which highlights the importance of the vacuole in maintaining cytoplasmic Pi homeostasis (12). After a prolonged deprivation, cytoplasmic Pi is maintained at a minimum level of homeostasis by mobilizing and recycling Pi from expendable molecules. This requires the cooperation of a series of enzymes including acid phosphatases (APases) that catalyze the hydrolysis of a wide range of phosphoric acid monoesters and diestersase well as anhydrides at pH in the range of 4 to 7 (13, 14). Now, it is well known that the expression and activity of many APases, both intracellular and extracellular, are commonly increased in the plant tissues and in the rhizosphere during Pi starvation in the higher plants (for a review see 7).

Also, post-transcriptional mechanisms have a major role in the regulation of Pi starvation inducible gene expression and enzymes activity (7). A family of intracellular and secreted APases known as purple acid phosphatases (PAPs) is characterized by a bimetallic active site that gives a pink or purple color in solution (15-18). Amongst these, *Arabidopsis thaliana* PAP26 (AtPAP26) has been identified with both intra and extracellular APase activity in *Arabidopsis thaliana* seedlings and suspension cell culture (18, 19). The basal level of *AtPAP26* transcripts is relatively abundant and invariant regardless of available Pi in all tissues (20). The loss of *AtPAP26* expression in the Arabidopsis mutant plants resulted in the abolition of the corresponding transcripts and the 55-kDa immunoreactive polypeptides, correlated with a 9- and 5-folds decrease in the extractable shoot and root APase activity, respectively, and a 40% reduction in the secreted APase activity as well. The impaired growth and development was accompanied by 35% and 50% reduction in the total P and free Pi levels, respectively, in the mutant shoots compared to the wild-type seedlings, while those were unaffected under Pi-sufficient conditions (21). The similar results were observed for *OsPAP26* gene, which was expressed in all *Oryza sativa* tissues during leaf senescence. Furthermore, phosphate deprivation and leaf senescence result in a large increase in the abundance of OsPAP26 protein (22).

2. Objectives

Previous results have suggested that AtPAP26 is targeted to both vacuoles and the culture medium (23) and makes a significant contribution to the recycling and scavenging of Pi through the hydrolysis of a wide range of substrates in Arabidopsis plants subjected to Pi starvation (21). In this study, the overexpression of AtPAP26-encoding gene in tobacco was evaluated from various points of view. Over-expression of *AtPAP26* led to an increased APase activity, Pi accumulation, biomass production, the total P and Pi contents in the transgenic plants. However, developmental traits such as inflorescence formation and seed germination were hindered in the tobacco plants.

3. Materials and Methods

3.1. Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Col-0 plants were cultivated hydroponically following the procedure described previously (24). Twenty-eight days old *A. thaliana* plant roots were used for RNA extraction and *AtPAP26* cDNA cloning as described (25). *Nicotiana tabacum* cultivar Xanthi was used as a heterologous host for the transgene. Seeds were washed in 70% (*v/v*) ethanol-water, rinsed in distilled water and surface sterilized with 10% hydrogen peroxide and then germinated in MS medium culture (26) containing 1.2 mM KH_2PO_4 and supplemented with 1% sucrose in dark at 4 °C for 2 days to break dormancy and to produce a uniform germination. Seven days after germination, seedlings were transplanted into MS medium with none or 1.2 mM KH_2PO_4 and were grown under a 16 h light and 8 h dark regime at 24 °C. After 14 days of growth, shoots and roots were separately harvested and frozen in liquid N_2 prior to storage at -80 °C.

3.2. PCR Amplification and Gene Cloning

The full length, 1487-bp cDNA of *AtPAP26* (locus No. At5g34850) coding sequences was PCR amplified from the cDNA pool of Arabidopsis using gene specific oligonucleotide primers (forward primer: 5′-TCGAG**GGATCC**TGACGTGATGAATCATTTG-3′, and reverse primer: 5′-ACTT**GGATCC**AAAGCAT GAGGCAGACT-3′) in which the restriction sites for *Bam*HI were introduced at both 5′ and 3′ ends of the gene (bold residues). The PCR reaction was performed using Expand High Fidelity *Taq* DNA polymerase (Roche, Applied Sciences, Germany) using the following thermal profile: denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 66 °C for 1 min, and 72 °C for 1 min, plus a final 20 min terminal extension step at 72 °C. The amplification product was gel purified and cloned into pTZ57R/T vector (Fermets, Lithuania) to generate pTZ-*AtPAP26* construct. After confirming the

sequence accuracy, the insert was cloned downstream of the cauliflower mosaic virus 35S (CaMV-35S) promoter in a binary vector pARM1 (27) to yield the final construct pARM1-26 which was propagated in *Escherichia coli* strain DH5α as the bacterial host.

3.3. Genetic Transformation of Tobacco

Agrobacterium tumefaciens strain GV3101 was used for tobacco leaf disk transformation. The bacteria were cultured in LB medium culture composed of 10 g.L^{-1} tryptone, 10 g.L^{-1} NaCl, and 5 g.L^{-1} yeast extract. The construct was introduced into *A. tumefaciens* by freeze and thaw method (28). Transformation of the tobacco leaf disc was performed according to Hirschi (29). The transformed shoots were selected on MS media containing 100 mg.mL^{-1} kanamycin and were subcultured every 7 days. The occurrence of the transformation was further verified by PCR using gene-specific primers as above. The transgenic plants were regenerated on the same medium. After adequate rooting, seedlings were transferred to peat:perlite:vermiculite at a ratio of 1:1:1 (*v/v/v*) and were allowed to produce self-fertilized seeds.

Mature seeds were collected from the self-fertilized flowers and resistance of the seedlings (T$_1$ progeny) to kanamycin was tested as described above. For pot culture, the regenerated transgenic or wild type seedlings were transferred into pots and were grown to maturity in peat-based compost that was treated regularly with liquid fertilizer containing 1.2 mM KH$_2$PO$_4$.

3.4. cDNA Synthesis and Semi-quantitative RT-PCR

Total RNA was extracted from tobacco seedlings grown as above using a commercially prepared guanidine reagent, TRIzol (SinaClonBioScience Co., Tehran, Iran) according to the manufacturer instructions. To eliminate genomic DNA contamination, the RNA samples were further treated with the RNase A-free DNase (Roche Applied Sciences, Germany) according to the supplier instruction. RNA integrity was monitored on a 1.5% (*w/v*) agarose gel containing ethidium bromide. For cDNA synthesis, 20 μg of the RNA was mixed with 1 μL of 100 μM oligo-dT (12-20) primer, 1 μL of 10 mM dNTP mix, and sterile water up to a volume of 10 μL. The reaction was heated at 70 °C for 20 min and cooled on ice. 10 μL of the reaction mix containing 2 μL RT buffer, 0.1 M dithiothreitol, 50 mM MgCl$_2$, 40 units of RNase inhibitor (Roche, Applied Sciences, Germany) and 50 units of Superscript II reverse transcriptase (Fermente, Lithuania) was added to the mixture and incubated at 42 °C for 1.5 h prior to PCR reactions with the gene-specific primers (forward primer: 5′-TAGGCGATATGGGTCAGACATTC-3′ and the reverse primer: 5′-CAGCGTACCAAAGAGGACTGCTAC-3′). The amplified transcripts of α-tubulin with the

specific primers (forward primer: 5′-GCTTTCAAC AACTTCTTCAG-3′ and reverse primer: 5′-CATCG TACCACCTTCAGACAC-3′) was used as an internal control. The PCR reactions were conducted as above at annealing temperature of 56 °C. To quantify the expression level, the intensity values of the amplified *AtPAP26* gene were divided by that of the α-tubulin gene in two replicates, accordingly.

3.5. Genomic DNA Extraction and PCR Analysis

DNA was extracted from the mature leaves of the seedlings' tissue and Polymerase Chain Reaction (PCR) was carried out with the above gene specific primers with *Taq* DNA polymerase (SinaClon BioScience Co., Tehran, Iran) to verify transgenic lines. The following amplification program was used in the PCR amplification: 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and final extension for 10 min at 72 °C.

3.6. Measurement of APase Activities

The treated wild type or transgenic T$_1$ seedling samples were ground to a fine powder in the liquid nitrogen and were homogenized in the extraction buffer (10 mM sodium acetate, pH 5.6). The homogenates were centrifuged twice at 12,000 ×*g* for 30 min and the supernatants were used for enzyme assays. All extraction steps were carried out at 4 °C. Based on the previous studies (18, 21, 30, 31), *p*-nitrophenyl-phosphate (*p*NPP), phosphoenolpyruvate (PEP), phosphatidylcholine (PLP), phytate (IP6) and glucose-6-phosphate (G6P) were chosen as representative of the different substrate types. Enzymatic activities were assayed at 37 °C for 30 min using 100 mM sodium acetate buffer (pH 5.6) containing 5 mM of each substrate. To calculate activities, a standard curve was constructed for each set of assays using known concentrations of PO$_4$$^{3-}$. Each unit of activity was defined as 1 μmol of the released Pi per min. The total protein concentration was determined by Bradford (32) using bovine serum albumin as a standard. APase activity was expressed as units per mg of the soluble proteins.

3.7. Soluble Pi and Total P Assays

Pi released by APase activity and the total P were measured by a modified Ames (33) method as described below. For determination of the Pi level, about 50 mg fresh tissue samples were ground in the liquid nitrogen and were homogenized in one fold of the above extraction buffer (*v/v*). Following centrifugation at 12,000 ×*g* for 30 min, 50 μL of the supernatant was diluted with 250 μL water and 700 μL of the assay reagent composed of 10% ascorbic acid and 6 volumes of 0.42% ammonium molybdate in 1N H$_2$SO$_4$ and were incubated at 45 °C for 20 min. The

soluble Pi content was measured at A_{820} and was expressed as micromoles of the soluble $Pi.mg^{-1}$ of fresh weight using a standard calibration curve.

To measure the total P contents, a volume of up to 1.5 mL of 10% magnesium nitrate in 95% ethanol was added to about 50 mg fresh tissue sample in a Pyrex tube. The biomaterial was dried and burned to ash by shaking the tube over a strong flame until the brown fume was disappeared, then the tube was allowed to cool down before adding 500 µL of the concentrated perchloric acid. The capped tube was heated in a boiling water bath for 60 min to hydrolyze any pyrophosphate formed in the ash and the final volume was adjusted to 5 mL by water. 300 µL of this sample was assayed as above.

3.8. Measurement of Dry and Fresh Weight

Roots and shoots of 14 days transgenic and wild-type plants grown in MS medium culture with none or 1.2 mM Pi were harvested and assayed separately. Twenty plants per line (*n*=20) were detached and dried at 70 °C for 72 h to measure dry weight.

3.9. Statistical Analysis

The segregation ratios of the non/transgenic plant progenies were examined by *Chi*-square test at $p < 0.05$ using SPSS (Statistical Package for the Social Science) software V.16. Data collected for assays and measurements were statistically analyzed as factorial experiments in a completely randomized design with at least three replications. Walter-Duncan K ratio or *t*-test were used to determine the significance difference between means at $p < 0.05$ using SPSS software V.16. In order to discount the positional effects of the gene insertion and genotype variations, the mean of the two selected transgenic lines was evaluated.

4. Results

4.1. Generation of Tobacco AtPAP26 Transgenic Lines

The full length of 1487-bp cDNA sequence of *AtPAP26* encoding a purple APase from *A. thaliana* was isolated via a PCR-cloning strategy. *AtPAP26* cDNA encodes a protein of 55 kDa with 475 amino acid residues that exhibits high sequence identity with the other plants PAPs (data not shown) including gall seven known motifs as shown before (17, 34). The cDNA sequence was integrated downstream of CaMV-35S promoter and introduced into tobacco plants using *A. tumefaciens*-mediated transformation method. A total of 30 T_0 kanamycin-resistant plants were regenerated and transferred to the greenhouse where grown in soil and tested for the presence of the *AtPAP26* gene by PCR analysis. The transgenic T_0 plants were allowed to self-fertilize and produce T_1 seeds. Several transgenic lines

showing segregation ratios close to 3:1 for 50 to 80 examined seeds (kanamycin resistant:sensitive seedlings) were selected as the single locus insertion lines for further analysis.

4.2. Heterologous Expression of AtPAP26 Gene in Tobacco

As a screening step, crude leaf proteins were extracted from transgenic seedlings and were assayed using *p*NPP as the substrate for selecting those with high APase activity (data not shown). Subsequently, semi-quantitative RT-PCR was applied using gene-specific primers for selecting lines with a higher APase activity. Quantification of the RT-PCR products revealed variations in the *AtPAP26* transcripts levels being unrelated to the APase activity in the transgenic lines. The specific primers amplified no endogenous *PAP26* transcript in the wild type tobacco plants. Among the fifteen single-locus transgenic plants, two lines: 26-15 and 26-17 with the highest transcription levels were selected for the subsequent comparative experiments (**Fig. 1**).
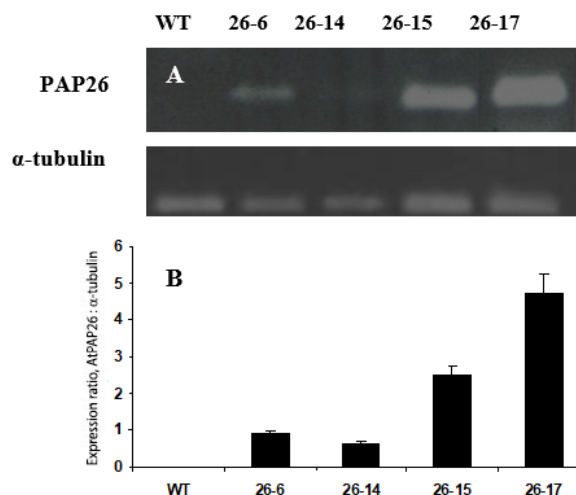

Figure 1. The expression analysis of the *AtPAP26* gene in the transgenic tobacco plants by RT-PCR. (**A**) Relative RT-PCR of *AtPAP26* expression level (top) in the wild type and the overexpressed lines. Alpha-tubulin (bottom) was used as the loading control. The *AtPAP26* signal was first normalized against alpha-tubulin signal and then compared to the wild type. (**B**) Means values of the relative expression of the wild type (WT) and overexpressed lines (numbered). Each value is the mean of the two biological replicates. Data was collected for at least 30 seedlings of each line of non/transgenic plants.

4.3. Comparison of Total P and Free Pi Contents

As the indicators for the rate of Pi accumulation, we measured the levels of total P and free Pi in the *AtPAP26* overexpressed lines and wild type plants grown for 14 days in the culture medium with or without Pi. The levels
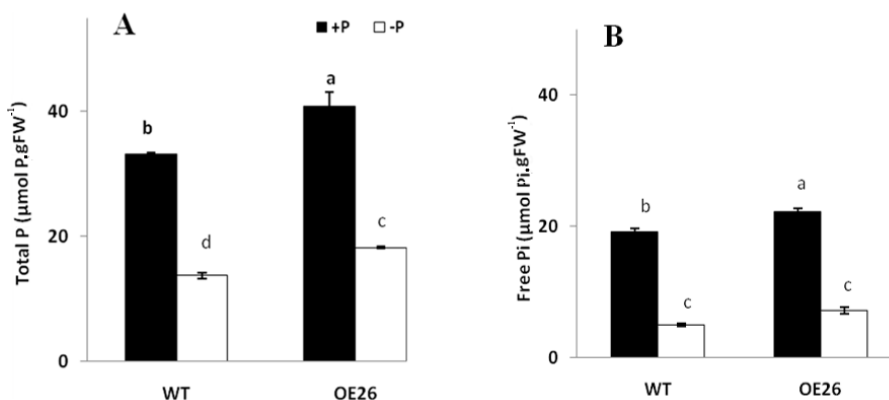
Figure 2. Alterations in the P accumulation in transgenic and the wild type tobacco plants. Total P (**A**) and free Pi (**B**) contents of the wild type (WT) or overexpressed lines (OE26) seedlings grown for 14 days on solid medium containing none (-P) or 1.2 mM Pi (+P). All values represent means (± SE) of four replicates. Mean values with the same letter have no significant difference as compared by Duncan's mean comparison method at $p < 0.05$.

of both indicators were quite low in the P-starved wild type plants relative to the Pi-fed seedlings (**Fig. 2**). In comparison, the total P and Pi contents were increased in the transgenic plants grown in both Pi-starved and Pi-fed concentrations. Quantitatively, the levels of total P and free Pi were increased for 1.3 and 1.2-folds in transgenic plants compared to the wild type ones when grown in Pi sufficiency, while in Pi-starved plants they were 1.3 and 1.5-folds, respectively.

4.4. Alterations in APase Activity Profiles

The effect of production of AtPAP26, as a heterologous protein in the crude protein extracts, was examined on total APase activities against a series of the substrates including *p*NPP as a general organic substrate, PEP, as an enolic-Pi substrate, PLP as a phospholipid Pi substrate, IP6 as a phytate substrate, and G6P as a sugar phosphate substrate, respectively. While the total APase activity was remarkably increased in the Pi-starved seedlings compared to the well-fed ones in all cases (**Fig. 3**), variations were not significantly different between overexpressing lines and wild type plants. In contrast, the total APase activities were significantly higher in the well-fed overexpressed lines than in the wild type plants for all examined substrates. Although the highest phosphatase activity level was observed for G6P, the highest difference was found for PLP and PEP substrates which were approximately 1.8 and 1.6 folds higher in overexpressed lines than wild type plants, respectively.

4.5. Phenotyping of the Overexpressed Lines

The germination of the T_1 progenies transgenic lines were delayed compared to the wild type. Considering the expression level of the transgene, a significant correlation was evident ($p < 0.001$) between *AtPAP26* transcript levels

and germination delay (**Fig. 4**).

No abnormal phenotypes were found in the transgenic lines except for being shorter and having larger leaves than wild-type plants grown in soil (**Fig. 5A**). There were obvious differences in the root architecture of the 21-day old seedlings between Pi-starved or fed transgenic lines and the wild type plants. The lateral roots of transgenic seedlings were abundant in Pi sufficiency, while it was noticeably reduced in the Pi deficiency (**Figs. 5B and F**).

We also compared the fresh and dry weight of the pooled roots and shoots of the seedlings after 14 days of growth in 1.2 mM or no Pi conditions (**Figs. 5C and D**). In general, the fresh weights of the shoots were reduced significantly whiles there was no change for the roots in the Pi-starved versus Pi-fed wild type seedling. Interestingly, both roots and shoots fresh or dry weights of transgenic lines were higher than those of the wild type plants when grown on either Pi-deficient or Pi-sufficient conditions. There were 15 and 40 percent increase in the root's fresh weight or 10 and 28 percent increase in the shoot fresh weight of the Pi-fed and P-starved transgenic lines versus the wild type seedlings, respectively. As a result, the whole transgenic seedlings had on average 8 and 31 percent higher fresh weight in the Pi sufficient and starvation conditions, respectively, relative to the wild type ones. Whereas dry weights of the wild type Pi-starved roots were significantly higher than those grown in the Pi-fed condition, the transgenic lines showed a non-significant alteration in the root dry weight in these conditions.

The increased lateral root number and elongation of the primary root are the morphological hallmarks for the Pi starvation response in plants (for a review see 35). Therefore, we examined the effect of *AtPAP26* overexpression on rooting of the tobacco plants grown in the media with different Pi concentrations (**Figs. 5E and F**). In no Pi
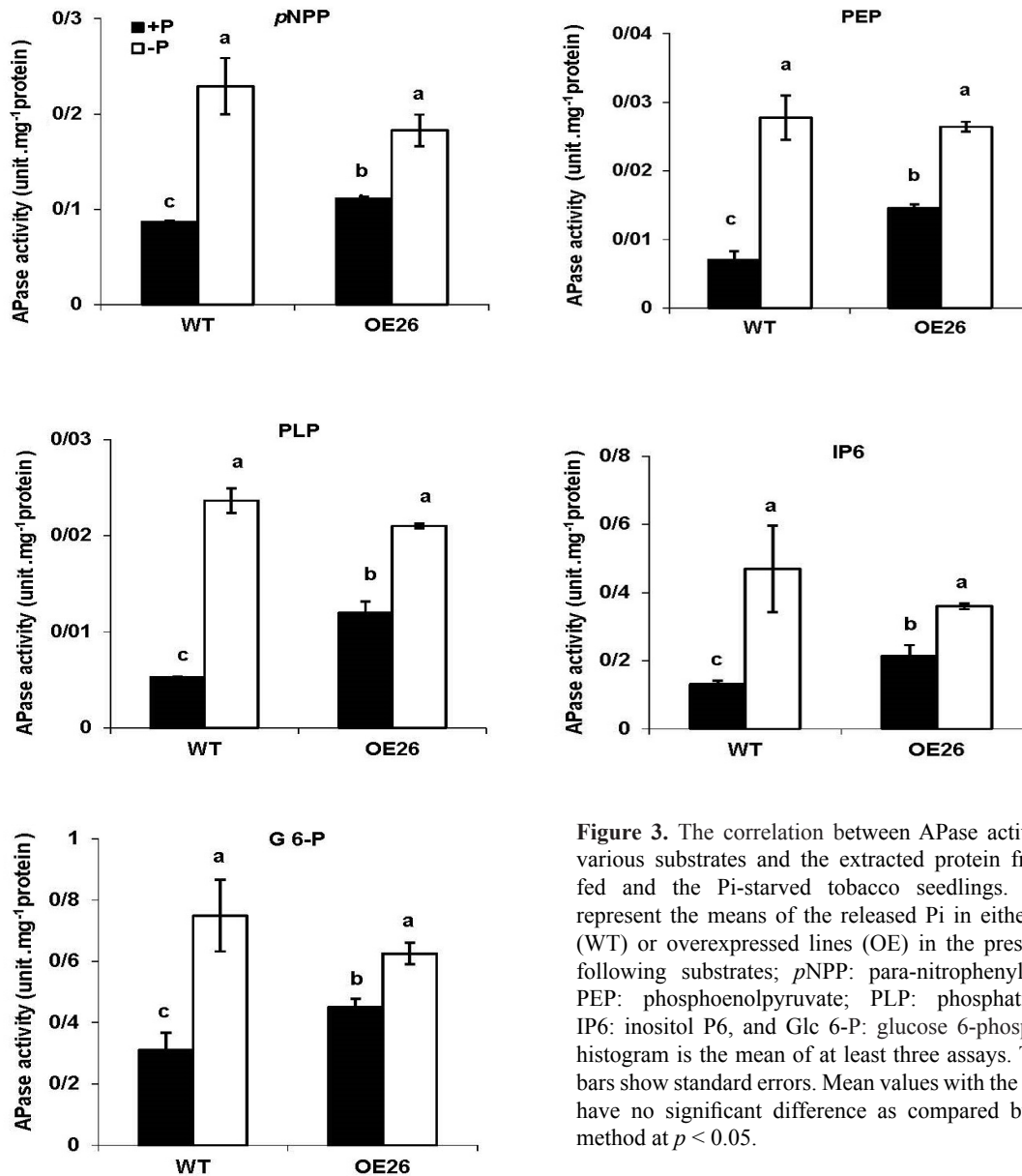
Figure 3. The correlation between APase activity against various substrates and the extracted protein from the Pi-fed and the Pi-starved tobacco seedlings. Histograms represent the means of the released Pi in either wild type (WT) or overexpressed lines (OE) in the presence of the following substrates; *p*NPP: para-nitrophenyl-phosphate; PEP: phosphoenolpyruvate; PLP: phosphatidylcholine; IP6: inositol P6, and Glc 6-P: glucose 6-phosphate. Each histogram is the mean of at least three assays. The vertical bars show standard errors. Mean values with the same letters have no significant difference as compared by Duncan's method at $p < 0.05$.
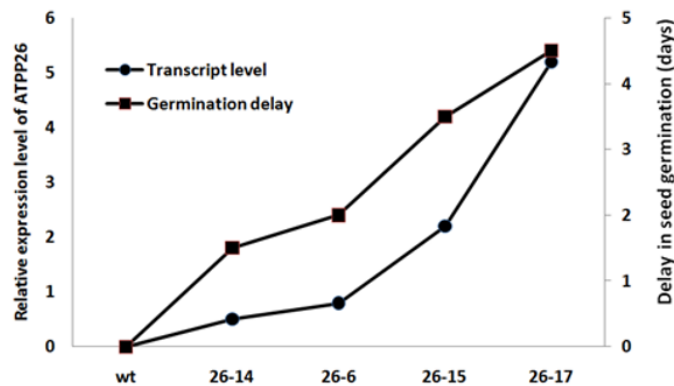

Figure 4. Correlation between *AtPAP26* transcript levels (circles) of the wild type (WT) and the transgenic lines (numbered) as well as seed germination delay (square). The delayed time corresponds to when the majority of seeds germinated after transferring from 4 °C to room temperature. Data were collected for at least 150 seeds of each line of non/transgenic plants.
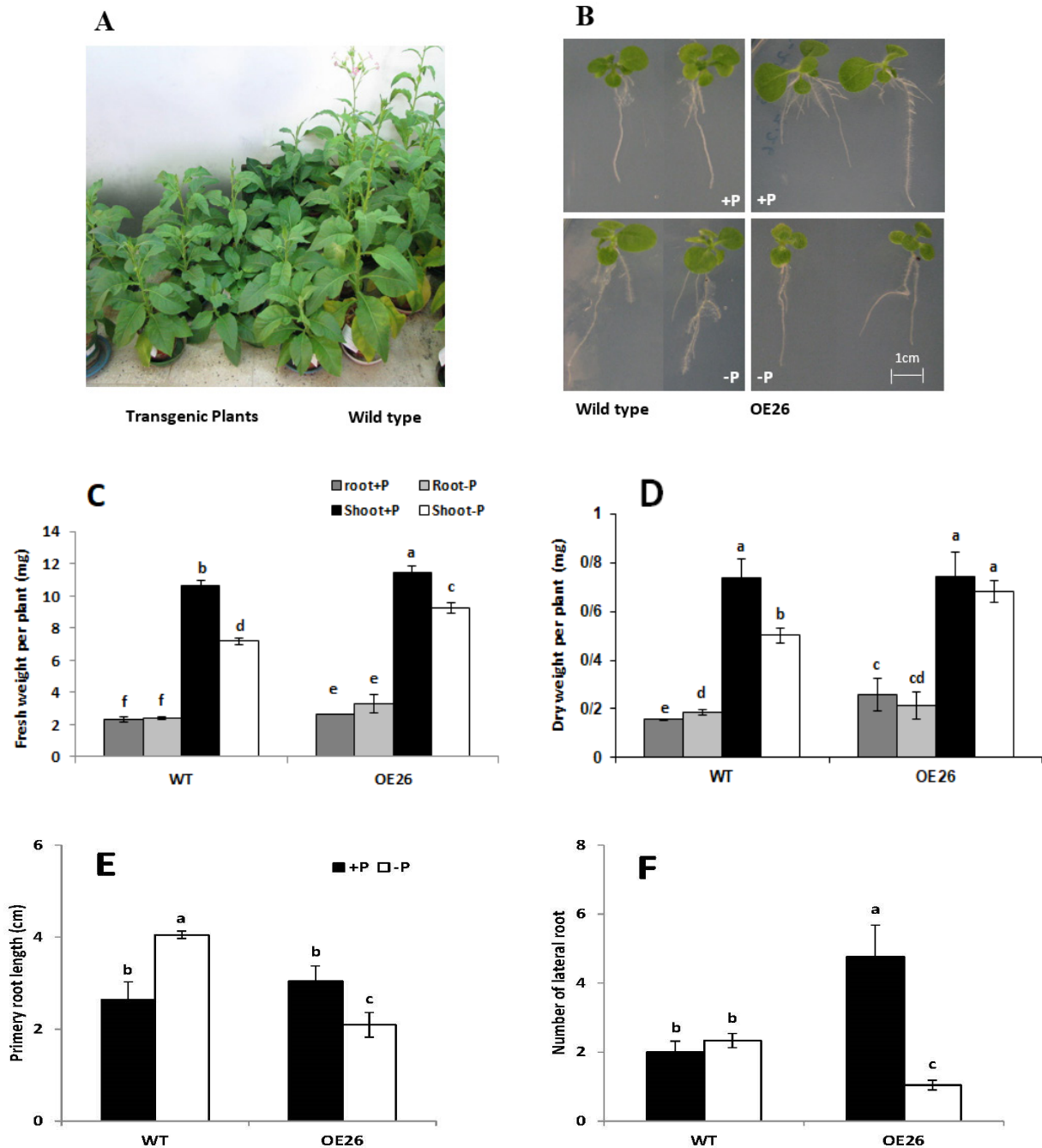
Figure 5. The phenotype of *AtPAP26* over-expressing tobacco plants. The comparisons of the height differences in pot-grown plants (A), and the morphology of the roots (B), fresh weight (C), dry weight (D), primary root length (E), and lateral root numbers (F) of the several wild type and transgenic seedlings plated on medium containing 1.2 mM Pi (+P) or no Pi (-P) for 14 days are shown. All values represent means ± SE of n = 20 in two independent experiments. Mean values with the same letters have no significant difference as compared by Duncan's method at $p < 0.05$.

condition, transgenic plants showed a significant decrease in the primary root lengths compared to the wild type plants. This was coincident with a decrease in the number of lateral roots for 2.2 folds. However, the number of lateral roots of *AtPAP26* overexpressed lines was remarkably increased when seedlings were grown in 1.2 mM Pi in compared to those grown in no Pi medium as well as wild type plants.

5. Discussion

Veljanovski and colleagues (2006) have clearly shown that a pronounced decrease in the intracellular free Pi levels was correlated with the accumulation of the vacuolar AtPAP26 polypeptides and concomitant with a marked increase in the intracellular APase activity of

the Pi-starved Arabidopsis suspension cells or seedlings. Recent molecular and phenotypic analysis of an *atpap*26 T-DNA inserted mutant lacking the relevant transcripts and immunoreactive polypeptides confirmed that the corresponding gene is the main contributor to the intracellular APase activity and, consequently, to the Pi metabolism in the Pi-starved Arabidopsis (21). Also, the RT-PCR analysis and *AtPAP26*:GUS plants showed a widespread expression and GUS activity in all tissues irrespective of the plant's age or P status (36). Similarly, *OsPAP26*, an orthologous of *AtPAP26* in rice genome, is constitutively expressed in all tissues (22).

It is postulated that *AtPAP26* is mainly regulated at translational and/or post-translational levels. There are two additional glycoforms of AtPAP26 secreted in the Pi-starved suspension cell and seedling culture of Arabidopsis (7, 21). Under Pi starvation conditions, the OsPAP26 protein was also markedly increased in rice leaves and roots. According to the results, the *OsPAP26* expression is regulated post-transcriptionally based on phosphate supply (22). These results collectively indicate that AtPAP26 is a predominant PAP existed both in vacuolar and secreted forms. The broad substrate selectivity and pH activity profiles of AtPAP26 are consistent with the hypothesis that its combined activities help Pi-starved Arabidopsis to efficiently scavenge Pi from a wide range of intracellular and extracellular Pi-esters.

Similar to several other PAPs, the purified Arabidopsis AtPAP26 hydrolyzes Pi from a wide range of Pi compounds with the highest activity against PEP, phenyl-P, and *p*NPP (18). When *AtPAP26* was overexpressed in the transgenic tobacco plants, APase activity was increased significantly in the Pi-fed plants (**Fig. 3**). For 35S:*AtPAP12* and 35S:*AtPAP26* Arabidopsis lines, in contrast, the total intracellular APase activity was 150%–300% greater than in the wild type plants under both Pi-fed and no Pi conditions (37). Overexpression or RNA-silencingof *OsPAP26* gene in the rice transgenic plants was found to increase or reduce the APase activity significantly in the leaves, roots, and growth medium, respectively (22). The increase in the enzyme activity was parallel to the increase in the biomass production as well as total P and Pi contents in the transgenic plants versus wild type plants. Similar results were reported for *AtPAP18* in tobacco plants (38).

As reported by several researchers (for a recent review see Ref. 7), we observed a significant increase in the APase activity in the Pi starvation conditions in general. In such a condition, APases play an important role in optimizing remobilization, reallocation, recycling, and utilization of the Pi. A negative correlation between the levels of Pi and APase activity has been reported for many plants (16, 18, 23, 39-41). Due to induction of several endogenous APase-encoding genes, the effect of *AtPAP26* overexpression was

not distinguishable, such that, no significant difference in the APase activity was observed between wild type and transgenic lines in the Pi-starved condition. However, the non-significant lower APase activity of the transgenic plant in Pi-starved condition is probably the consequence of alterations in the gene expression profile of other APases influenced by the overrepresentation of *AtPAP26*. Whereas, in a sufficient Pi condition, due to the low expression of the Pi inducible APase genes, the overexpression of *AtPAP26* controlled by the CaMV-35S promoter in transgenic plants yielded a significant increase in the enzyme activity against all the examined substrates. As shown in **Figure 3**, the overexpression of *AtPAP26* in tobacco as a heterologous expression system has increased APase activity against PEP as the preferred substrate (18) as well as other substrates in the Pi-fed transgenic seedlings. These results demonstrate that presence of ectopic *AtPAP26* influences the expression of several other APase genes at the expression or activity levels by disturbing the interactive network of the numerous APases in plants (Lohrasebi and Malboobi, unpublished data). For instance, according to Veljanovski *et al.* (18), the purified AtPAP26 did not hydrolyze IP6, while we observed 65 percent increase in the IP6 activity in the crude protein extracted from transgenic plants. This is also an advantage for improving plant Pi acquisition, because phytates compose up to 60% of soil organic P and are poorly utilized by plants (42-44).

At phenotypic level, the transgenic seeds showed a delayed germination compared to the wild type ones which were highly correlated with the *AtPAP26* gene expression. Many roles have been postulated for the plant APases during seed germination and seedling growth (45). The current proposition is that such enzymes contribute to the mobilization of Pi from macromolecular organic Pi. An increase in the APase activity both in the dormant state and during seed germination was demonstrated in maize scutellum (46). APases are highly expressed and their activities are increased in *Glycine max* seeds during germination to release the stored materials for the growing embryo (47). Also, studies using microarrays have shown the abundance of *AtPAP26* transcripts in the dry seed of Arabidopsis (http://bar.utoronto.ca/). Altogether, these data suggest that *AtPAP26* is naturally up-regulated during seed development and germination and must not be responsible for the observed delayed germination.

The literature has shown that mature seeds of *atpap26* mutant plants exhibit a significant less total P relative to the wild type seeds. This demonstrates the importance of the AtPAP26 in Pi remobilization from senescing leaves as the liberated nutrients are often allocated to the developing seeds (8, 48). As the cultivars with a higher seed Pi contents establish seedlings with the higher, vigor, and ultimately produce a higher yield, the Pi content of the seed is very

important (18, 49, 50). For this reason, farmers try to increase available Pi around the germinating seeds by providing early-season Pi fertilizer (50). A clear result of decreased P reserves could be a noticeable delay in the germination rate and efficiency relative to the WT seeds that are displayed in *atpap26* seeds mutant plants (51).

Besides, other phenotypic alterations such as dwarfism in pot-grown transgenic lines imply the possible inhibition in gibberellic acid (GA) biosynthesis. GA regulates several aspects of plant growth such that longer seed dormancy or delayed germination events as well as dwarfism and late flowering; typical symptoms for GA deficiency (52). As the plants phytohormones, GAs stimulates seed germination in response to changes in light, temperature, and moisture. Inhibition of alpha-amylase during seed germination is one of the main causes of dwarfism, infertility, and failure of GA sensitive mutants in the rice and barley to mobilize the stored reserves. Inhibition of the Arabidopsis seed germination by the far-red light and stimulation of germination by red light or cold imbibition are associated with the decreased and increased GA accumulation, respectively (for a review see Ref. 53). Also, GA plays an important role in the regulation of the responses to phosphate stress. There is a cross-talk between Pi homeostasis and GA biosynthesis. The induction of MYB62 is a specific response in the Arabidopsis leaves during Pi deprivation. The responses to Pi starvation and GA biosynthesis are regulated by the MYB62 transcription factor. The increased expression of MYB62 suppresses the expression of the early GA biosynthetic genes and results in a GA-deficient phenotype and significant delay in the germination (54).

Since dwarfism was commonly observed in the overexpressed lines, we may propose that *AtPAP26* overexpression leads to a reduced GA biosynthesis. As one of the best described functions of AtPAP26 is known to be the hydrolysis of PEP to pyruvate; the main precursor for GA synthesis, such dwarfism in the overexpressed events were not expected. Although several others phosphorylated precursors in this pathway could also be substrates for the AtPAP26 or other APases with an altered expression pattern, the possible interruptions in one or more steps in the GA biosynthesis could also be hypothesized.

In conclusion, our results have clearly demonstrated that the overexpression of the plant APase-encoding gene(s) could be considered as an effective approach for improving P acquisition, and thus, offering a prospect of improving biomass accumulation and productivity of the soils with the limited free Pi. Considering that the excessive Pi runoff in these areas has caused severe environmental concerns of polluting surface or underground water, this approach could be very useful in removing excessive organic P from certain land areas where Pi fertilizers are over-used or too much of poultry litter are dumped.

References

1. Kavanova M, Grimoldi AA, Lattanzi FA, Schnyder H. Phosphorus nutrition and mycorrhiza effects on grass leaf growth. P status- and size-mediated effects on growth zone kinematics. *Plant Cell Environ.* 2006;**29**(4):511-520. doi: 10.1111/j.1365-3040.2005.01428.x

2. Lambers H, Shane MW, Cramer MD, Pearse SJ, Veneklaas EJ. Root structure and functioning for efficient acquisition of phosphorus: matching morphological and physiological traits. *Ann Bot.* 2006;**98**(4):693-713. doi: 10.1093/aob/mcl114

3. Guo L, Zhao Y, Zhang S, Zhang H, Xiao K. Improvement of organic phosphate acquisition in transgenic tobacco plants by overexpression of a soybean phytase gene *Sphy1. Front Agric China.* 2009;**3**(3):259-265. doi: 10.1007/s11703-009-0021-2

4. Alexova R, Millar AH. Proteomics of phosphate use and deprivation in plants. *Proteomics.* 2013;**13**(3-4):609-623. doi: 10.1002/pmic.201200266

5. Rodríguez H, Fraga R. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotech Adv.* 1999;**17**(4):319-339. doi: 10.1016/S0734-9750(99)00014-2

6. Bieleski RL. Phosphate pools, phosphate transport and phosphate availability. *Annu Rev Plant Physiol.* 1973;**24**:225-252. doi: 10.1146/annurev.pp.24.060173.001301

7. Tran HT, Hurley BA, Plaxton WC. Feeding hungry plants: the role of purple acid phosphatases in phosphate nutrition. *Plant Sci.* 2010;**179**(1):14-27. doi: 10.1016/j.plantsci.2010.04.005

8. Veneklaas EJ, Lambers H, Bragg J, Finnegan PM, Lovelock CE, Plaxton WC, *et al.* Opportunities for improving phosphorus-use efficiency in crop plants. *New Phytol.* 2012;**195**(2):306-320. doi: 10.1111/j.1469-8137.2012.04190.x

9. Runge-Metzger A. Closing the cycle: Obstacles to efficient P management for improved global food security. In: H Thiessen (ed) *Phosphorus in the global environment: Transfers, cycles and management,* John Wiley & Sons, New York. 1995;**54**:27-42. doi: 10.1002/(SICI)1096-9837(199705)22:5<515::AID-ESP716>3.0.CO;2-Y

10. Vance CP, Uhde-Stone C, Allan DL. Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. *New Phytol.* 2003;**157**(3):423-447. doi: 10.1046/j.1469-8137.2003.00695.x

11. Pratt J, Boisson AM, Gout E, Bligny R, Douce R, Aubert S. Phosphate (Pi) starvation effect on the cytosolic Pi concentration and Pi exchanges across the tonoplast in plant cells: an *in vivo* 31P-nuclear magnetic resonance study using methylphosphonate as a Pi analog. *Plant Physiol.* 2009;**151**(3):1646-1657. doi:10.1104/pp.109.144626

12. Yang S-Y, Huang T-K, Kuo H-F, Chiou T-J. Role of vacuoles in phosphorus storage and remobilization. *Journal of experimental botany.* 2017. doi: 10.1093/jxb/erw481

13. Duff SMG, Sarath G, Plaxton WC. The role of acid phosphatases in plant phosphorus metabolism. *Physiol Plantarum.* 1994;**90**:791-800. doi: 10.1111/j.1399-3054.1994.tb02539.x

14. Klabunde T, Sträter N, Fröhlich R, Witzel H, Krebs B. Mechanism of Fe (III)–Zn (II) purple acid phosphatase based on crystal structures. *J Mol Biol.* 1996;**259**(4):737-748. doi: 10.1006/jmbi.1996.0354

15. Bozzo GG, Dunn EL, Plaxton WC. Differential synthesis of phosphate-starvation inducible purple acid phosphatase isozymes in tomato (*Lycopersicon esculentum*) suspension cells and seedlings. *Plant Cell Environ*. 2006;**29**(2):303-313. doi: 10.1111/j.1365-3040.2005.01422.x

16. del Pozo JC, Allona I, Rubio V, Leyva A, de la Pena A, Aragoncillo C, *et al*. A type 5 acid phosphatase gene from Arabidopsis thaliana is induced by phosphate starvation and by some other types of phosphate mobilising/oxidative stress conditions. *Plant J*. 1999;**19**(5):579-589. doi: 10.1046/j.1365-313X.1999.00562.x

17. Li D, Zhu H, Liu K, Liu X, Leggewie G, Udvardi M, *et al*. Purple acid phosphatases of Arabidopsis thaliana. Comparative analysis and differential regulation by phosphate deprivation. *J Biol Chem*. 2002;**277**(31):27772-27781.doi 10.1074/jbc. M204183200

18. Veljanovski V, Vanderbeld B, Knowles VL, Snedden WA, Plaxton WC. Biochemical and molecular characterization of AtPAP26, a vacuolar purple acid phosphatase up-regulated in phosphate-deprived Arabidopsis suspension cells and seedlings. *Plant Physiol*. 2006;**142**(3):1282-1293. doi: 10.1104/pp.106.087171

19. Tran HT, Plaxton WC. Proteomic analysis of alterations in the secretome of Arabidopsis thaliana suspension cells subjected to nutritional phosphate deficiency. *Proteomics*. 2008;**8**(20):4317-4326. doi: 10.1002/pmic.200800292s

20. Zhu H, Qian W, Lu X, Li D, Liu X, Liu K, *et al*. Expression patterns of purple acid phosphatase genes in Arabidopsis organs and functional analysis of *AtPAP23* predominantly transcribed in flower. *Plant Mol Biol*. 2005;**59**(4):581-594. doi: 10.1007/s11103-005-0183-0

21. Hurley BA, Tran HT, Marty NJ, Park J, Snedden WA, Mullen RT, *et al*. The dual-targeted purple acid phosphatase isozyme AtPAP26 is essential for efficient acclimation of Arabidopsis to nutritional phosphate deprivation. *Plant Physiol*. 2010;**153**(3):1112-1122. doi: 10.1104/pp.110.153270

22. Gao W, Lu L, Qiu W, Wang C, Shou H. OsPAP26 Encodes a Major Purple Acid Phosphatase and Regulates Phosphate Remobilization in Rice. *Plant Cell Physiol*. 2017;**58**(5):885-892. doi: 10.1093/pcp/pcx041

23. Haran S, Logendra S, Seskar M, Bratanova M, Raskin I. Characterization of Arabidopsis acid phosphatase promoter and regulation of acid phosphatase expression. *Plant Physiol*. 2000;**124**(2): 615-26. doi: 10.1104/pp.124.2.615

24. Malboobi MA, Lefebvre DD. A phosphate-starvation inducible β-glucosidase gene (psr3. 2) isolated from Arabidopsis thaliana is a member of a distinct subfamily of the BGA family. *Plant Mol Biol*. 1997;**34**(1):57-68. doi: 10.1023/A:1005865406382

25. Lohrasebi T, Malboobi MA, Samaeian A, Sanei V. Differential expression of *Arabidopsis thaliana* acid phosphatases in response to abiotic stresses. *Iran J Biotechnol* (IJB).2007;**5**(3).

26. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plantarum*. 1962;**15**(3):473-497. doi: 10.1111/j.1399-3054.1962.tb08052.x

27. Zamani K, Malboobi MA, Lohrasebi T, Esfahani K. Plant expression vectors for production and purification of recombinant proteins. *IR Iran Patent*. 2010;**No. 3058**.

28. Holsters M, Waele D, Depicker A, Messens E, Montagu M, Schell J. Transfection and transformation of Agrobacterium tumefaciens. *Mol Gen Genet*. 1978;**163**(2):181-187. doi: 10.1007/BF00267408

29. Hirschi KD. Expression of Arabidopsis *CAX1* in tobacco: altered calcium homeostasis and increased stress sensitivity. *Plant Cell*. 1999;**11**(11):2113-2122. doi: 0.1105/tpc.11.11.2113

30. Coello P. Purification and characterization of secreted acid phosphatase in phosphorus-deficient Arabidopsis thaliana. *Physiol Plantarum*. 2002;**116**(3):293-298. doi: 10.1034/j.1399-3054.2002.1160303.x

31. Kuang R, Chan KH, Yeung E, Lim BL. Molecular and biochemical characterization of AtPAP15, a purple acid phosphatase with phytase activity, in Arabidopsis. *Plant Physiol*. 2009;**151**(1):199-209. doi:10.1104/pp.109.143180

32. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;**72**:248-254. doi: 10.1016/0003-2697(76)90527-3

33. Ames BN. Assay of inorganic phosphate, total phosphate and phosphatases. In: Elizabeth F. Neufeld VG, editor. Methods in Enzymology. Volume 8: Academic Press; 1966;**p**:115-118. doi: 10.1016/0076-6879(66)08014-5

34. Schenk G, Ge Y, Carrington LE, Wynne CJ, Searle IR, Carroll BJ, *et al*. Binuclear metal centers in plant purple acid phosphatases: Fe–Mn in sweet potato and Fe–Zn in soybean. *Arch Biochem Biophys*. 1999;**370**(2):183-189. doi: 10.1006/abbi.1999.1407

35. Chevalier F, Pata M, Nacry P, Doumas P, Rossignol M. Effects of phosphate availability on the root system architecture: Large-scale analysis of the natural variation between Arabidopsis accessions. *Plant Cell Environ*. 2003;**26**(11):1839-1850. doi: 10.1046/j.1365-3040.2003.01100.x

36. Robinson WD, Park J, Tran HT, Del Vecchio HA, Ying S, Zins JL, *et al*. The secreted purple acid phosphatase isozymes AtPAP12 and AtPAP26 play a pivotal role in extracellular phosphate-scavenging by Arabidopsis thaliana. *J Exp Bot*. 2012;**63**(18):6531-6542. doi: 10.1093/jxb/ers309

37. Wang L, Lu S, Zhang Y, Li Z, Du X, Liu D. Comparative genetic analysis of Arabidopsis purple acid phosphatases AtPAP10, AtPAP12, and AtPAP26 provides new insights into their roles in plant adaptation to phosphate deprivation. *J Integr Plant Biol*. 2014;**56**(3):299-314. doi: 10.1111/jipb.12184

38. Zamani K, Sabet M, Lohrasebi T, Mousavi A, Malboobi M. Improved phosphate metabolism and biomass production by overexpression of AtPAP18 in tobacco. *Biologia*. 2012;**67**(4):713-720. doi:10.2478/s11756-012-0072-3

39. Liu J, Samac DA, Bucciarelli B, Allan DL, Vance CP. Signaling of phosphorus deficiency induced gene expression in white lupin requires sugar and phloem transport. *Plant J*. 2004;**41**(2):257-268. doi:10.1111/j.1365-313X.2004.02289.x

40. Morcuende R, Bari R, Gibon Y, Zheng W, Pant BD, Blasing O, *et al*. Genome-wide reprogramming of metabolism and regulatory networks of Arabidopsis in response to phosphorus. *Plant Cell Environ*. 2007;**30**(1):85-112. doi: 10.1111/j.1365-3040.2006.01608.x

41. Müller R, Morant M, Jarmer H, Nilsson L, Nielsen TH. Genome-wide analysis of the Arabidopsis leaf transcriptome reveals interaction of phosphate and sugar metabolism. *Plant Physiol*. 2007;**143**(1):156-171. doi: 10.1104/pp.106.090167

42. Mudge SR, Smith FW, Richardson AE. Root-specific and phosphate-regulated expression of phytase under the control of a phosphate transporter promoter enables Arabidopsis to grow on phytate as a sole P source. *Plant Sci*. 2003;**165**(4):871-878. doi: 10.1016/S0168-9452(03)00286-3

43. Richardson AE, Hadobas PA, Hayes JE. Extracellular secretion of Aspergillus phytase from Arabidopsis roots enables plants to

obtain phosphorus from phytate. *Plant J.* 2001;**25**(6):641-649. doi: 10.1046/j.1365-313x.2001.00998.x

44. Zimmermann P, Zardi G, Lehmann M, Zeder C, Amrhein N, Frossard E, *et al*. Engineering the root–soil interface via targeted expression of a synthetic phytase gene in trichoblasts. *Plant Biotechnol J.* 2003;**1**(5):353-360. doi: 10.1046/j.1467-7652.2003.00033.x

45. Senna R, Simonin V, Silva-Neto M, Fialho E. Induction of acid phosphatase activity during germination of maize (Zea mays) seeds. *Plant Physiol Bioch.* 2006;**44**(7):467-473. doi: 10.1016/j.plaphy.2006.03.008

46. Teno AM, Palma MS, Rossi A. Acid phosphatase from maize scutellum: properties as a function of seed germination. *Phytochem*. 1987;**26**(1):55-58. doi: 10.1016/S0031-9422(00)81480-6

47. dos Prazeres JN, Ferreira CV, Aoyama H. Acid phosphatase activities during the germination of Glycine max seeds. *Plant Physiol Biochem.* 2004;**42**(1):15-20. doi: 10.1016/j.plaphy.2003.10.009

48. Buchanan☐Wollaston V, Earl S, Harrison E, Mathas E, Navabpour S, Page T, *et al*. The molecular analysis of leaf senescence–a genomics approach. *Plant Biotech J*. 2003;**1**(1):3-22. doi: 10.1046/j.1467-7652.2003.00004.x

49. Ros C, Bell RW, White PF. Effect of seed phosphorus and soil phosphorus applications on early growth of rice (*Oryza sativa* L.) cv. IR66. *Soil Sci Plant Nutr.* 1997;**43**(3):499-509. doi: 10.1080/00380768.1997.10414777

50. White PJ, Veneklaas EJ. Nature and nurture: the importance of seed phosphorus content. *Plant Soil*. 2012;**357**(1-2):1-8. doi: 10.1007/s11104-012-1128-4

51. Robinson WD, Carson I, Ying S, Ellis K, Plaxton WC. Eliminating the purple acid phosphatase AtPAP26 in *Arabidopsis thaliana* delays leaf senescence and impairs phosphorus remobilization. *New Phytol*. 2012;**196**(4):1024-1029. doi: 10.1111/nph.12006

52. Reyes D, Rodríguez D, González-García MP, Lorenzo O, Nicolás G, García-Martínez JL, *et al*. Overexpression of a protein phosphatase 2C from beech seeds in Arabidopsis shows phenotypes related to abscisic acid responses and gibberellin biosynthesis. *Plant Physiol*. 2006;**141**(4):1414-1424. doi: 10.1104/pp.106.084681

53. Hauvermale AL, Ariizumi T, Steber CM. Gibberellin signaling: a theme and variations on DELLA repression. *Plant Physiol.* 2012;**160**(1):83-92. doi: 10.1104/pp.112.200956

54. Devaiah BN, Madhuvanthi R, Karthikeyan AS, Raghothama KG. Phosphate starvation responses and gibberellic acid biosynthesis are regulated by the MYB62 transcription factor in Arabidopsis. *Mol Plant.* 2009;**2**(1):43-58. doi: 10.1093/mp/ssn081