

RESEARCH PAPER

The secreted purple acid phosphatase isozymes AtPAP12 and AtPAP26 play a pivotal role in extracellular phosphate-scavenging by *Arabidopsis thaliana*

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Abstract

Orthophosphate (P_i) is an essential but limiting macronutrient for plant growth. Extensive soil P reserves exist in the form of organic P (P_o), which is unavailable for root uptake until hydrolysed by secretory acid phosphatases (APases). The predominant purple APase (PAP) isozymes secreted by roots of P_i -deficient ($-P_i$) *Arabidopsis thaliana* were recently identified as AtPAP12 (At2g27190) and AtPAP26 (At5g34850). The present study demonstrated that exogenous P_o compounds such as glycerol-3-phosphate or herring sperm DNA: (i) effectively substituted for P_i in supporting the P nutrition of *Arabidopsis* seedlings, and (ii) caused upregulation and secretion of AtPAP12 and AtPAP26 into the growth medium. When cultivated under $-P_i$ conditions or supplied with P_o as its sole source of P nutrition, an *atpap26/atpap12* T-DNA double insertion mutant exhibited impaired growth coupled with >60 and >30% decreases in root secretory APase activity and rosette total P_i concentration, respectively. Development of the *atpap12/atpap26* mutant was unaffected during growth on P_i -replete medium but was completely arrested when 7-day-old P_i -sufficient seedlings were transplanted into a $-P_i$, P_o -containing soil mix. Both PAPs were also strongly upregulated on root surfaces and in shoot cell-wall extracts of $-P_i$ seedlings. It is hypothesized that secreted AtPAP12 and AtPAP26 facilitate the acclimation of *Arabidopsis* to nutritional Pi deficiency by: (i) functioning in the rhizosphere to scavenge P_i from the soil's accessible P_o pool, while (ii) recycling P_i from endogenous phosphomonoesters that have been leaked into cell walls from the cytoplasm. Thus, AtPAP12 and AtPAP26 are promising targets for improving crop P-use efficiency.

Key words: *Arabidopsis thaliana*, cell walls, extracellular phosphate scavenging, functional genomics, phosphate nutrition, purple acid phosphatase, secreted hydrolases.

Introduction

Acid phosphatases (APases, EC 3.1.3.2) catalyse the hydrolysis of orthophosphate (P_i) from a broad range of phosphomonoesters and anhydrides with an acidic pH optimum. They function in the production, transport, and recycling of P_i , a critical macronutrient

Abbreviations: APase, acid phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; ELF, enzyme-labelled fluorescent; G3P, glycerol-3-phosphate; Glc-6-P, glucose-6-phosphate; GUS, β -glucuronidase; MS, Murashige and Skoog; PAP, purple acid phosphatase; PEP, phosphoenolpyruvate; PEPC, PEP carboxylase; $+P_i$, P_i sufficient; $-P_i$, P_i deficient; *p*NPP, *para*-nitrophenol phosphate; P_o , organic P; PSI, P_i starvation inducible.

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for cellular metabolism and bioenergetics. The induction of extra- and intracellular APases is a ubiquitous plant response to nutritional P_i deprivation, a common abiotic stress that frequently limits plant growth in natural ecosystems (Plaxton and Tran, 2011). Extracellular APases belong to a group of P_i -starvation-inducible (PSI) phosphohydrolases secreted by roots of P_i -deficient ($-P_i$) plants to hydrolyse P_i from external phosphomonoesters and phosphodiesteres derived from decomposing biomaterial, referred to as organic P (P_o). For example, the combined activities of secreted nucleases, phosphodiesterases, and APases allows $-P_i$ plants to efficiently scavenge extracellular nucleic acids as their sole source of P nutrition (Abel *et al.*, 2000; Chen *et al.*, 2000). P_o generally accounts for around 50% of the soil's total P content (Richardson *et al.*, 2009). Owing to microbial activity, extended periods of P-fertilizer application increase the proportion of applied P that accumulates in agricultural soils as labile P_o (George *et al.*, 2007). Given the abundance of P_o in most soils and its steady accumulation under various P_i fertilizer regimes, soil P_o makes an important contribution to plant P nutrition and overall efficiency of crop P_i uptake from applied fertilizers (Richardson *et al.*, 2009, 2011).

Soils have demonstrable APase activity, and substantial increases in APase activity have been documented in the rhizosphere of $-P_i$ plants, with several studies showing this to be associated with soil P_o depletion (Tarafdar and Claassen, 1988; Miller *et al.*, 2001; Nuruzzaman *et al.*, 2006; Richardson *et al.*, 2009). However, which soil P_o pools are accessible to roots remains unclear, and most plants have a limited capacity to obtain P_i from phytate (*myo*-inositol hexaphosphate), an abundant P_o component of certain soils (Richardson, 2009; Richardson *et al.*, 2009, 2011). Hydrolysis of extracellular P_o substrates to release P_i is essential, because P_i anions (primarily $H_2PO_4^-$ or HPO_4^{2-}) are translocated across the root plasmalemma by low- or PSI high-affinity P_i transporters (Plaxton and Tran, 2011). There is no evidence to support direct import of P_o substrates into plant cells, although P_o uptake followed by hydrolysis within the apoplast may occur (Richardson *et al.*, 2009, 2011). PSI APases are also secreted into cell walls where they may contribute to P_i recapture from phosphomonoesters leaked by the $-P_i$ cells (Bieleski and Johnson, 1972; Lefebvre *et al.*, 1990; Barrett-Lennard *et al.*, 1993; Zhang and McManus, 2000; Wasaki *et al.*, 2008; Tran *et al.*, 2010b). Similarly, PSI vacuolar APases appear to be involved in P_i scavenging and remobilization from expendable intracellular phosphomonoesters and anhydrides (Veljanovski *et al.*, 2006; Hurley *et al.*, 2010; Tran *et al.*, 2010b). This is accompanied by a marked reduction in levels of cytoplasmic P_o metabolites during long-term P_i deprivation (Plaxton and Tran, 2011).

Purple acid phosphatases (PAPs), the most important class of plant PSI APases, are characterized by their distinctive purple or pink colour in solution (due to a bimetallic active centre; Tran *et al.*, 2010b). Genome annotation identified 29 PAP genes in the model plant *Arabidopsis thaliana*, several of which are transcriptionally induced during P_i deprivation (del Pozo *et al.*, 1999; Haran *et al.*, 2000; Li *et al.*, 2002; Tran *et al.*, 2010a, b; Wang *et al.*, 2011). These and subsequent studies have demonstrated the complexity and variation of *AtPAP1–29* expression and regulation. The principal PAP isozymes that contribute to extracellular P_i scavenging by $-P_i$ *Arabidopsis* were evaluated recently using a combination of

biochemical and genomic approaches. The results established that *AtPAP12* and *AtPAP26* are the major root- and suspension cell culture secretory APases upregulated by $-P_i$ *Arabidopsis* (Tran *et al.*, 2010a). *AtPAP26* is also the predominant vacuolar APase that functions to recycle intracellular P_i during P_i stress, as well as to remobilize P_i from the P_o pool of senescing leaves (Veljanovski *et al.*, 2006; Hurley *et al.*, 2010; Robinson *et al.*, 2012). The overlapping but non-identical substrate selectivities and pH-activity profiles, and the high specific APase activities of secreted *AtPAP12* and *AtPAP26* (Tran *et al.*, 2010a) support the hypothesis that their combined activities help $-P_i$ *Arabidopsis* to scavenge efficiently P_i from a wide range of extracellular phosphomonoesters over a broad pH range. Analysis of *atpap12* and *atpap26* T-DNA insertional mutants has indicated that *AtPAP12* and *AtPAP26* account for the majority of APase activity secreted by the roots of $-P_i$ *Arabidopsis* (Tran *et al.*, 2010a). Furthermore, *AtPAP10* was shown recently to be associated predominantly with the root surface and to be induced by P_i limitation to help *Arabidopsis* acclimatize to P_i deprivation (Wang *et al.*, 2011). In the present study, analysis of a double *atpap12/atpap26* loss-of-function mutant established conclusively that *AtPAP12* and *AtPAP26* are secreted by $-P_i$ *Arabidopsis* to scavenge P_i from exogenous P_o . The results also revealed that *AtPAP12* and *AtPAP26* are important contributors to the PSI APase activity of the root surface as well as the cell walls of $-P_i$ *Arabidopsis* shoots.

Materials and methods

Plant material and growth conditions

For mutant isolation and routine plant growth, *Arabidopsis* (Col-0 ecotype) seeds were sown in a standard soil mixture (Sunshine Aggregate Plus Mix 1; SunGro) and stratified at 4 °C for 3 d. Plants were cultivated in growth chambers at 23 °C (16/8 h photoperiod at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation) and fertilized twice weekly by subirrigation with 0.25 \times Hoagland's medium (pH 6.0). To assess the influence of P_i deprivation on soil-grown plants, seedlings were established for 7 d in a 24-well microtitre plate (one seedling per well) containing 0.5 ml per well of 0.5 \times Murashige and Skoog (MS) medium supplemented with 1% (w/v) sucrose and 0.2 mM P_i , and then transplanted into a 75–85% sphagnum peat moss/perlite soil mix lacking all nutrients (Sunshine Mix 2; SunGro). Plants were cultivated in growth chambers as described above for an additional 14 d and fertilized twice weekly with 0.25 \times Hoagland's medium containing either 0 or 2 mM KH_2PO_4 . Whenever P_i was eliminated, it was replaced by 2 mM KH_2SO_4 and 0.5 mM MES.

For liquid cultures, 5 mg of seeds were surface sterilized, stratified, and placed in 250 ml Magenta boxes containing 50 ml of 0.5 \times MS medium (pH 5.7) with 1% (w/v) sucrose and 0.2 mM KH_2PO_4 , and placed on an orbital shaker (80 r.p.m.) at 24 °C under continuous illumination (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$). After 7 d, the medium was replaced with fresh medium containing filter-sterilized 0 or 1.5 mM KH_2PO_4 , or 1.5 mM DL-glycerol-3-phosphate (G3P; Sigma Chemical Co.), or 0.6 mg ml^{-1} of DNA. The DNA (crude oligonucleotides from herring sperm; Sigma Chemical Co.) was purified by repeated extractions with phenol/chloroform followed by gel permeation chromatography on a Sephadex G-25 column as described previously (Chen *et al.*, 2000). It was assumed that 0.6 mg ml^{-1} DNA equated to \sim 2 mM total P (Chen *et al.*, 2000). All P_o stocks contained negligible free P_i . The 14-d-old seedlings were blotted dry, snap frozen in liquid N_2 , and stored at -80 °C, whereas growth medium was filtered through 0.45 μm membranes and concentrated over 250-fold using Amicon Ultra-15 centrifugal filter units (30 kDa cut-off). For growth on agar-solidified nutrient medium, stratified

seeds were placed on horizontal or vertically oriented 1% (w/v) agar (Micropropagation Type I Agar; Caisson Laboratories) plates containing 0.5× MS medium and 1% (w/v) sucrose supplemented with 50 μM or 1.5 mM KH₂PO₄, 0.6 mg ml⁻¹ of DNA, 1.5 mM G3P, or 1.5 mM glucose-6-phosphate (Glc-6-P) and cultivated at 24 °C under continuous illumination (100 μmol m⁻² s⁻¹) for 14–21 d.

Extraction of shoot cell-wall proteins

Shoots (2.5 g) of 14 d-old seedlings cultivated in P_i-sufficient (+P_i) or -P_i liquid medium as described above were powdered under liquid N₂ and homogenized (1:15; w/v) using a mortar and pestle in ice-cold buffer [25 mM TES/KOH (pH 7.4) containing 10 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 1% (v/v) Triton X-100, and 1% (w/v) polyvinylpyrrolidone]. The mixture was clarified by centrifugation at 20 000 g at 4 °C for 20 min and the supernatant collected as the soluble cytoplasmic extract. The pellet underwent three more washes by resuspending with homogenizing buffer and recentrifugation as above. The insoluble fraction was extracted with 5 ml of 0.2 M CaCl₂ in 5 mM acetate/NaOH (pH 4.6) and centrifuged at 23 700 g for 15 min. The supernatant was collected as the cell-wall extract (Barrett-Lennard *et al.*, 1993). The pellet was re-extracted with the same buffer and recentrifuged as above. The supernatant was combined with the first cell-wall extract to yield a final volume of ~10 ml. Cytoplasmic and cell-wall extracts were filtered through Miracloth and dialysed overnight against 500 ml of 40 mM Tricine/KOH (pH 7.4) containing 10 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, and 1% (v/v) Triton X-100. Both samples were concentrated ~40-fold as described above to a protein concentration of at least 2 mg ml⁻¹.

APase activity determination

APase activity was routinely measured by coupling the hydrolysis of phosphoenolpyruvate (PEP) to pyruvate to the lactate dehydrogenase reaction at 24 °C and continuously monitoring NADH oxidation at 340 nm using a Molecular Devices Spectromax Plus Microplate spectrophotometer. Optimized assay conditions were: 50 mM sodium acetate (pH 5.6), 5 mM PEP, 10 mM MgCl₂, 0.2 mM NADH, and 3 U of rabbit muscle lactate dehydrogenase in a final volume of 0.2 ml. Assays were corrected for any background NADH oxidation by omitting PEP from the reaction mixture. APase assays were also carried out in an assay mix containing 50 mM sodium acetate (pH 5.6), 5 mM *para*-nitrophenol phosphate (pNPP), and 10 mM MgCl₂ by monitoring the formation of *para*-nitrophenol at 405 nm (ε=18.2 mM⁻¹ cm⁻¹). All APase assays were linear with respect to time and concentration of enzyme assayed. One unit of activity was defined as the amount of enzyme resulting in the hydrolysis of 1 μmol of substrate min⁻¹ at 24 °C.

Protein electrophoresis and immunoblotting

SDS-PAGE, immunoblotting onto polyvinylidene difluoride membranes and chromogenic detection of antigenic polypeptides using an alkaline phosphatase-tagged secondary antibody were conducted as described previously (Hurley *et al.*, 2010; Tran *et al.*, 2010a). All immunoblot results were replicated a minimum of three times, with representative results shown in the various figures.

Determination of protein, total and free P_i, and anthocyanin concentrations

Protein concentrations were determined using a modified Bradford assay (Bozzo *et al.*, 2002) with bovine γ-globulin as the standard. Total P_i, free P_i, and anthocyanin determinations were carried out as described previously (Hurley *et al.*, 2010).

RNA isolation and semi-quantitative RT-PCR

Total RNA was extracted and purified as described previously (Gregory *et al.*, 2009). RNA samples were assessed for purity via their A₂₆₀/A₂₈₀ ratio and integrity by resolving 1 μg of total RNA on a 1.2% (w/v)

denaturing agarose gel. RNA (5 μg) was reverse transcribed with Superscript III (Invitrogen), and non-competitive RT-PCR was performed with appropriate primers as previously described (Gregory *et al.*, 2009; Hurley *et al.*, 2010; Tran *et al.*, 2010a); all PCR products were sequenced for verification. Conditions were optimized for all RT-PCRs to ensure linearity of response for comparison between samples.

β-Glucuronidase (GUS) analysis

The AtPAP12 and AtPAP26 promoters (2010 and 3853 bp sequences upstream of the start codon of the *AtPAP12* or *AtPAP26* genes, respectively) were amplified from genomic DNA using the following primer pairs: *AtPAP12:GUS12ProFull-InfF*: 5'-TGATTACGCCAAGC TTTTCTCTCCGGTGAAACC-3' and *12ProFull-InfR*: 5'-CCGGG GATCCTCTAGACTTCAAGATTAGTTTCTCTGAATCC-3'; and *AtPAP26:GUS26ProFull-InfF*: 5'-TGATTACGCCAAGCTTATTTGTAATG TCATCACCTCGG-3' and *26ProFull-InfR*: 5'-CCGGGGATCCTCTAG ACACGTCACCAAATCTCGA-3'. The amplified promoter region of *AtPAP12* or *AtPAP26* was mixed at a 3:1 molar ratio with pBI101-N1 linearized by *Hind*III and *Xba*I, incubated with In-Fusion reaction mix, and transformed according to the manufacturer's protocol (Clontech) to yield *AtPAP12:GUS* or *AtPAP26:GUS*. Each construct was transferred into *Agrobacterium tumefaciens* strain LBA4404 and transformed into *Arabidopsis* plants via the floral dip method (Clough and Bent, 1998). Transformed plants were selected on 0.8% (w/v) agar plates containing 0.5× MS medium, 1% (w/v) sucrose, and 30 μg ml⁻¹ of kanamycin, and transferred to soil for self-pollination and propagation. For analysis of mature plants, seeds were planted in soil and grown for 28 d while being fertilized twice weekly with 0.25× Hoagland's medium containing 2 mM P_i.

Histochemical staining of GUS activity was performed as described previously (Jefferson *et al.*, 1987). Tissues were incubated at 37 °C overnight in GUS staining buffer [100 mM NaP_i (pH 7.0), containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.5 mM EDTA, 0.1% (v/v) Triton X-100, 2 mM K₃Fe(CN)₆, and 2 mM K₄Fe(CN)₆]. Cyanide was omitted for *AtPAP12:GUS* staining. The stained tissues were cleared with 70% ethanol prior to imaging using a dissecting microscope.

Isolation and backcross of the *atpap12/atpap26* double-knockout mutant

Homozygous *atpap26* and *atpap12* T-DNA insertion mutants (Salk_152821 and SAIL_1187_A05, respectively) were obtained as reported previously (Hurley *et al.*, 2010; Tran *et al.*, 2010a). Mutant plants had been isolated by PCR-screening using T-DNA left-border and gene-specific primers (Supplementary Fig. S1 at JXB online). All PCR products were sequenced for verification (Centre for Applied Genomics, The Hospital for Sick Children, Toronto, ON, Canada). To generate *atpap26/atpap12* double mutants, the *atpap26* mutant (pollen donor) was crossed into the *atpap12* mutant (pollen receptors). Seeds obtained from these crosses were germinated and grown to obtain F1 seeds. The presence of T-DNA insertions in both *AtPAP12* and *AtPAP26* in the respective F1 plants was verified by PCR-screening. F1 plants were self-pollinated and individual F2 plants were screened on BASTA-containing MS medium. From the BASTA-resistant (for the *atpap12* allele) plants, genomic DNA was extracted and PCR-screened for homozygous double mutants. Of 20 individual F2 plants screened by PCR, three plants were homozygous for both *atpap26* and *atpap12*. To generate backcross lines to restore either *AtPAP12* or *AtPAP26* expression, the *atpap26/atpap12* mutant was crossed with *atpap12* and *atpap26* mutants. F1 plants were self-pollinated and leaf extracts of F2 plants screened using anti-AtPAP12 immunoblot analysis for restoration of AtPAP12 or AtPAP26 expression.

Root-surface APase activity staining

This was conducted using enzyme-labelled fluorescent (ELF)-97 phosphate (Invitrogen) with hydroponically cultivated 14-d-old seedlings. Individual seedlings were rinsed with 75 mM sodium acetate (pH 5.6) and

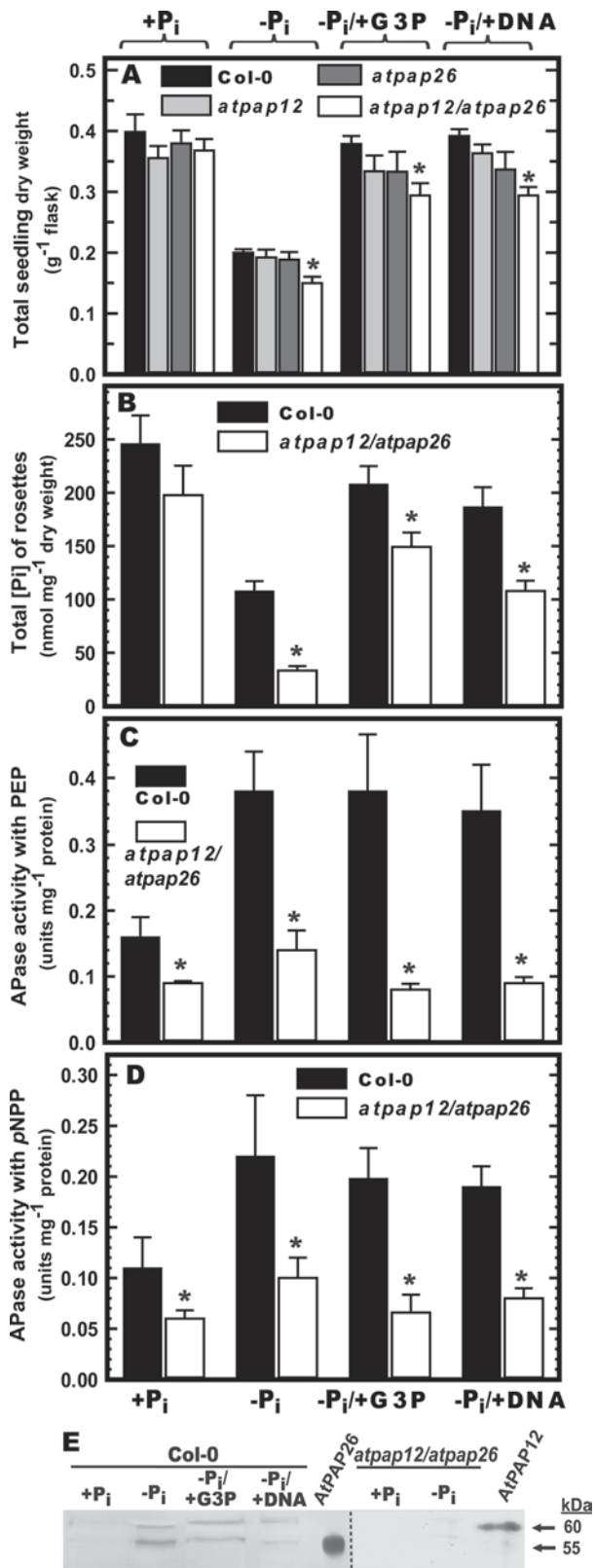


Fig. 1. Influence of different P supplements on biomass accumulation, rosette Pi concentration, secretory APase activity, and secreted AtPAP12 and AtPAP26 polypeptides of Col-0 and mutant Arabidopsis seedlings. Seeds (5mg) of Col-0, atpap12 and atpap26 single mutants, and atpap12/atpap26 double mutants were placed in 50ml of 0.5 MS medium containing 0.2mM Pi and cultivated on an orbital shaker at 24 C under continuous illumination (100 mol m² s⁻¹). After 7 d, the seedlings were

incubated at 23 °C for 1 h with 1 ml of this buffer containing 25 μM ELF-97 phosphate. As a negative control, replicate seedlings were incubated in acetate buffer alone. Roots were washed three times with acetate buffer containing 25 mM EDTA for 15 min. ELF-97, the fluorescent product of APase activity, was imaged using a Zeiss 710 confocal laser scanning microscope equipped with a Zeiss 63× plan apochromat oil-immersion objective and 340 and 450 nm for excitation and emission, respectively. Image processing was carried out using Adobe Photoshop CS (Adobe Systems Inc.). Root-surface APase activity staining was also conducted using β-naphthyl phosphate and 5-bromo-4-chloro-3-indolyl phosphate (BCIP), as previously described (Gilbert et al., 1999; Wang et al., 2011). All root-surface APase activity staining images are representative results obtained from experiments that were replicated at least three times.

Statistics

All values are presented as means ±standard error (SE). Data were analysed using a one-tailed Student's *t*-test, and deemed significant for *P* < 0.05.

Results and discussion

Influence of different P supplements on growth, total Pi concentration, secretory APase activity, and secreted AtPAP12 and AtPAP26 polypeptides of wild-type Arabidopsis seedlings

The ability of exogenous P_i, G3P, or purified herring sperm DNA to support growth and P nutrition of wild-type (Col-0) Arabidopsis seedlings was compared. G3P and nucleic acids are common soil P_o components (Tarafdar and Claassen, 1988; Ticconi and Abel, 2004; Richardson et al., 2009), whereas G3P is an effective *in vitro* substrate for native AtPAP12 and AtPAP26 purified from the secretome of -P_i Arabidopsis suspension cells (Tran et al., 2010a). Seedling dry weight biomass and total P_i concentration of rosette leaves of 14-d-old Col-0 seedlings cultivated over the previous 7 d in liquid medium containing 1.5 mM P_i and 1.5 mM G3P (-P_i+G3P) or 0.6 mg ml⁻¹ DNA (-P_i+DNA) (equivalent to ~2 mM total P_i) were generally comparable, whereas biomass and total shoot P_i concentration of -P_i seedlings were both reduced by ~50% (Fig. 1A, B). These

transferred into fresh medium containing 0 or 1.5mM Pi (Pi and +Pi, respectively), 1.5mM G3P (Pi+G3P), or 0.6mg ml⁻¹ DNA (Pi+DNA) and cultured for an additional 7 d. (A) Seedling dry weight per flask. (B) Total Pi concentration of rosette leaves. (C, D) Secreted APase activity of concentrated seedling culture filtrates of Col-0 and atpap12/atpap26 plants. Spectrophotometric APase activity assays were conducted using 5mM PEP (C) or 5mM pNPP (D) as described in Materials and methods. All values represent means SE of duplicate determinations for three biological replicates; asterisks indicate values that are significantly different from those of Col-0 (*P* < 0.01). (E) Concentrated secreted proteins (15 g per lane) of Col-0 and atpap12/atpap26 mutant seedlings, and secretory AtPAP12 and AtPAP26 (25ng each) purified from the culture medium of Pi Arabidopsis suspension cells (Tran et al., 2010a) were resolved by SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane. Blots were probed with anti-AtPAP12 immune serum (Tran et al., 2010a) and immunoreactive polypeptides were detected as described in Materials and methods.

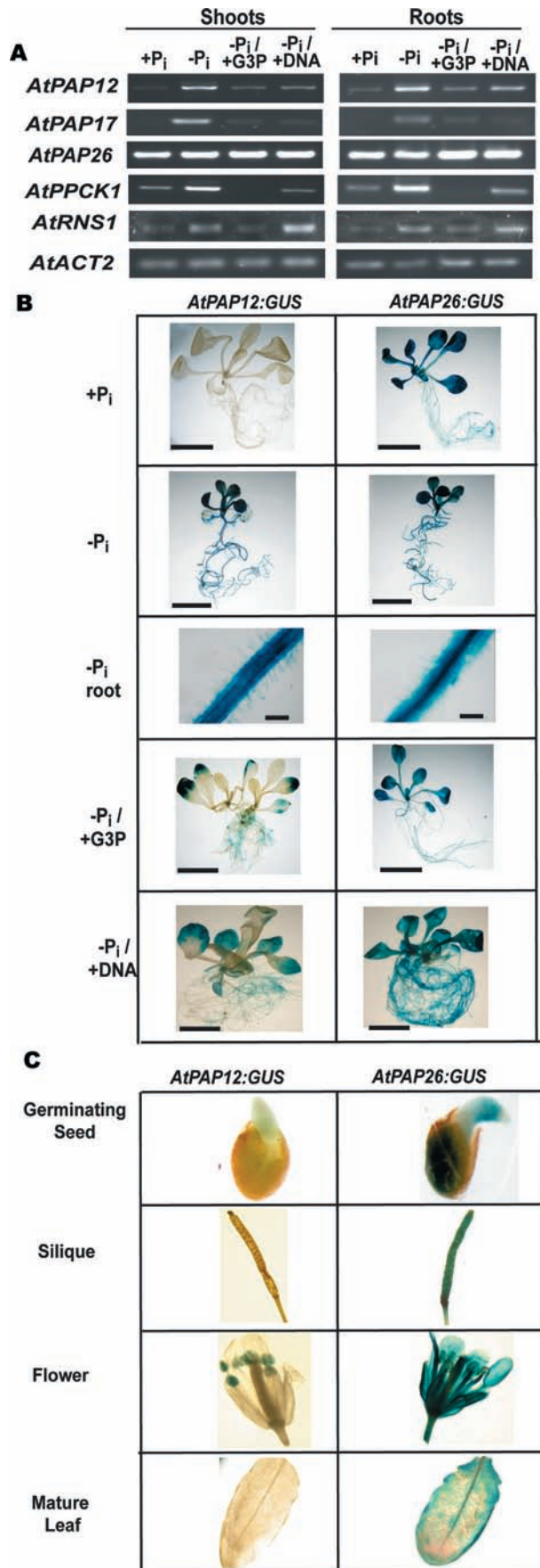


Fig. 2. Analysis of *AtPAP12* and *AtPAP26* gene expression. (A) Levels of mRNA were analysed by semi-quantitative RT-PCR using gene-specific primers for *AtPAP12*, *AtPAP17*, *AtPAP26*,

results agree with previous studies showing that plants cultivated in sterile culture were able to use exogenous P_o substrates, such as G3P, glucose-1-phosphate, ATP, or nucleic acids as equivalent sources to P_i for growth (Ticconi and Abel, 2004; Richardson *et al.*, 2009; Liang *et al.*, 2010; Richardson *et al.*, 2011). Our results also corroborate previous studies demonstrating that *Arabidopsis* seedlings and tomato cell cultures efficiently scavenge P_i from exogenous nucleic acids as their sole source of P nutrition owing to secretion of PSI nucleases, phosphodiesterases, and APases (Abel *et al.*, 2000; Chen *et al.*, 2000; Ticconi and Abel, 2004).

We next assessed whether the capacity of Col-0 seedlings to scavenge P_i from G3P or DNA was correlated with secretory APase activity or immunoreactive AtPAP12 or AtPAP26 polypeptides. APase activities were determined using both 5 mM PEP and 5 mM *p*NPp as substrates. Irrespective of which substrate was used, the growth medium of Col-0 seedlings cultivated under -P_i, -P_i/+G3P, or -P_i/+DNA conditions exhibited a significant increase in secreted APase activity relative to +P_i seedlings (Fig. 1C, D). Immunoblotting using anti-AtPAP12 immune serum (which cross-reacts with both AtPAP12 and AtPAP26; Tran *et al.*, 2010a) indicated that 60 kDa AtPAP12 and 55 kDa AtPAP26 immunoreactive polypeptides were upregulated in the growth medium of the -P_i, -P_i/+G3P, and -P_i/+DNA Col-0 seedlings (Fig. 1E). These results suggested that AtPAP12 and AtPAP26 were secreted into the medium in order to hydrolyse P_i from the exogenous P_o sources.

Influence of inorganic versus organic phosphate supply on AtPAP12 and AtPAP26 gene expression

Semi-quantitative RT-PCR was used to assess the relationship between exogenous P source and the relative shoot versus root expression of several PSI genes. The results of Fig. 2A confirmed previous studies documenting the constitutive expression of *AtPAP26*, whereas *AtPAP12*, *AtPAP17*, *RNS1*, and *AtPPCK1* transcripts are significantly induced in shoots and roots of -P_i *Arabidopsis* (del Pozo *et al.*, 1999; Haran *et al.*, 2000; Veljanovski *et al.*, 2006; Gregory *et al.*, 2009; Hurley *et al.*, 2010; Tran *et al.*, 2010a). *AtPAP12* was also induced in both shoots and roots when the seedlings were grown on -P_i/+G3P

RNS1, and *AtPPCK1*. *AtACT2* was used as a reference to ensure equal template loading. Seedlings were cultivated as described in the legend for Fig. 1. All PCR products were taken at cycle numbers determined to be non-saturating. Control RT-PCRs lacking reverse transcriptase did not produce any bands. (B) *AtPAP12:GUS* and *AtPAP26:GUS* transgenic lines were cultivated in 24-well microtitre plates in liquid MS medium containing 0.2 mM P_i for 7 d, before being transferred into medium containing 0 or 1.5 mM KH₂PO₄ (-P_i and +P_i, respectively), 1.5 mM G3P (-P_i/+G3P), or 0.6 mg ml⁻¹ DNA (-P_i/+DNA) for another 7 d. Bars, 1 cm, except for '-P_i root' (bar, 100 μm). (C) *AtPAP12:GUS* and *AtPAP26:GUS* expression was also examined in several aerial tissues of 4-week-old +P_i plants that had been cultivated in soil under a regular light/dark diurnal cycle. 'Germinating seed' is a representative image of seeds that had been placed on moist filter paper and allowed to germinate for 1 d before GUS staining.

or $-P_i/+DNA$, whereas transcripts for *AtPAP17* or *AtPPCK1* were either undetectable or expressed at a lower level relative to plants grown on $-P_i$ medium (Fig. 2A). *AtPAP17* was the first PSI PAP to be characterized in *Arabidopsis* (del Pozo *et al.*, 1999), although its cellular location and biological function(s) remain elusive. *AtPPCK* encodes a protein kinase that specifically phosphorylates and thereby activates the cytosolic enzyme PEP carboxylase (PEPC) in $-P_i$ *Arabidopsis* (Gregory *et al.*, 2009). *RNS1* encodes a nuclease that is upregulated and secreted by roots of $-P_i$ *Arabidopsis*, or during cultivation on exogenous RNA or DNA as the sole source of nutritional P (Chen *et al.*, 2000). *RNS1* transcripts were induced in both shoots and roots when the seedlings were grown on $-P_i$ and $-P_i/+DNA$ but not $-P_i/+G3P$. These findings suggest a selective upregulation of genes based on the type of P_o supplied to the seedlings. A challenging yet intriguing aspect for future studies will be to delineate the respective signal transduction pathways that appear to result in differential expression of secretory hydrolases such as *AtPAP12*, *AtPAP26*, and *RNS1* during *Arabidopsis* growth on exogenous P_o sources such as G3P and DNA.

To determine the tissue specificity of *AtPAP12* and *AtPAP26* expression, promoter:GUS reporter gene fusions were generated. The expression of GUS activity was examined in 12 *AtPAP12:GUS* and five *AtPAP26:GUS* independent transgenic lines, which all exhibited similar tissue-specific expression patterns. The *GUS* expression patterns of representative lines are reported here. In agreement with the results of Fig. 2A: (i) the *AtPAP26:GUS* plants showed widespread GUS activity in all tissues, irrespective of the plant's age or P status, whereas (ii) GUS activity was generally undetectable in $+P_i$ *AtPAP12:GUS* tissues (other than in anthers) but was prevalent in shoots and roots of seedlings cultivated on $-P_i$ or $-P_i/G3P$ medium (Fig. 2B, C). *AtPAP12* induction in shoots and roots of $-P_i$ *Arabidopsis* seedlings has been well documented (Haran *et al.*, 2000; Tran *et al.*, 2010a). To the best of our knowledge, however, the present study is first to observe the induction of a PSI PAP isozyme such as *AtPAP12* during plant growth on medium in which the only accessible form of P nutrition is exogenous P_o .

Identification and validation of an *atpap12/atpap26* double mutant

To assess further the role that secreted *AtPAP12* and *AtPAP26* play in scavenging extracellular P_o , a double *atpap12/atpap26* knockout mutant was isolated by crossing homozygous *atpap12* and *atpap26* T-DNA insertion lines (Salk_152821 and SAIL_1187_A05, respectively) (Hurley *et al.*, 2010; Tran *et al.*, 2010a). Confirmation of loss of *AtPAP12* and/or *AtPAP26* gene expression in the *atpap12*, *atpap26*, and *atpap12/atpap26* mutants was confirmed by PCR of genomic DNA using *AtPAP12*- and *AtPAP26*-specific primers (Supplementary Fig. S1). Immunoblotting indicated that *AtPAP12* or *AtPAP26* polypeptides were absent in the concentrated secretome of $+P_i$ or $-P_i$ *atpap12/atpap26* seedlings (Fig. 1E). This correlated with a >60% reduction in secreted APase activity during P_i deprivation (Fig. 1C, D). These results agreed with our earlier study of *atpap12* and *atpap26* single mutants, which concluded that

AtPAP12 and *AtPAP26* account for most of the APase activity secreted by roots of $-P_i$ *Arabidopsis* seedlings (Tran *et al.*, 2010a).

AtPAP12 and *AtPAP26* are major cell-wall acid phosphatases upregulated by P_i -deprived *Arabidopsis*

P_i -starvation-inducible root surface and/or cell-wall APase activities have been reported for numerous plant species including *Arabidopsis* (Lefebvre *et al.*, 1990; Duff *et al.*, 1991; Barrett-Lennard *et al.*, 1993; Gilbert *et al.*, 1999; Wasaki *et al.*, 2000, 2008; Zhang and McManus, 2000; Kaida *et al.*, 2008; Richardson *et al.*, 2009; Tran *et al.*, 2010b; Wang *et al.*, 2011). For example, *AtPAP10* is a PSI-secreted PAP that is predominantly associated with the surface of root epidermal cells (but undetectable in culture medium), and that functions in the acclimation of *Arabidopsis* to P_i limitation (Wang *et al.*, 2011). Cell-wall-associated PSI APases have been hypothesized to facilitate maintenance of the plant's P status either by scavenging P_i from P_o compounds present in the rhizosphere or by recycling P_i from endogenous phosphomonoesters that have been leaked from the cytoplasm across the plasma membrane (Lefebvre *et al.*, 1990; Barrett-Lennard *et al.*, 1993; Zhang and McManus, 2000; Tran *et al.*, 2010b; Wang *et al.*, 2011). Classic studies by Bieliski's group with the small aquatic plant *Spirodela oligorrhiza* demonstrated that significant levels of phosphomonoesters can be leaked during $-P_i$ growth, and that failure to recapture this lost P can seriously compromise the overall P economy of the plant (Bieliski and Johnson, 1972).

Histochemical localization using ELF-97 phosphate as a substrate was applied to root samples of hydroponically cultivated seedlings. ELF-97 phosphate produces a fluorescent precipitate at the site of enzymatic hydrolysis, thus localizing active APases when viewed by fluorescence microscopy (Wasaki *et al.*, 2008). Strong PSI APase activity was observed on the root surface and particularly at the root meristematic (tip) region of $-P_i$ Col-0 seedlings. This activity was noticeably diminished in the *atpap12* and *atpap26* single mutants, and almost negligible in the *atpap12/atpap26* double mutant (Fig. 3A). The *atpap12/atpap26* plants also showed decreased root-surface APase staining when incubated with β -naphthyl phosphate (Supplementary Fig. S2 at JXB online), an excellent *in vitro* substrate for purified *AtPAP12* and *AtPAP26* (Tran *et al.*, 2010a). However, when incubated with BCIP instead of ELF-97 phosphate or β -naphthyl phosphate there was no obvious decrease in root-surface APase activity staining between Col-0 and the *atpap12/atpap26* mutant (Supplementary Fig. S2). This result can be explained by the very low *in vitro* activity of purified native *AtPAP12* and *AtPAP26* with BCIP (H. Del Vecchio and W. Plaxton, unpublished data), coupled with *AtPAP10*'s known contribution to the BCIP-dependent APase activity of $-P_i$ *Arabidopsis* root surfaces (Wang *et al.*, 2011). Our ELF-97 phosphate and β -naphthyl phosphate results indicated that *AtPAP12* and *AtPAP26* account for a substantial proportion of root-surface-localized PSI APase activity. The results of Fig. 3A, coupled with the transcriptional activation of *AtPAP12* in $-P_i$ *Arabidopsis* shoots (Fig. 2), prompted us to investigate

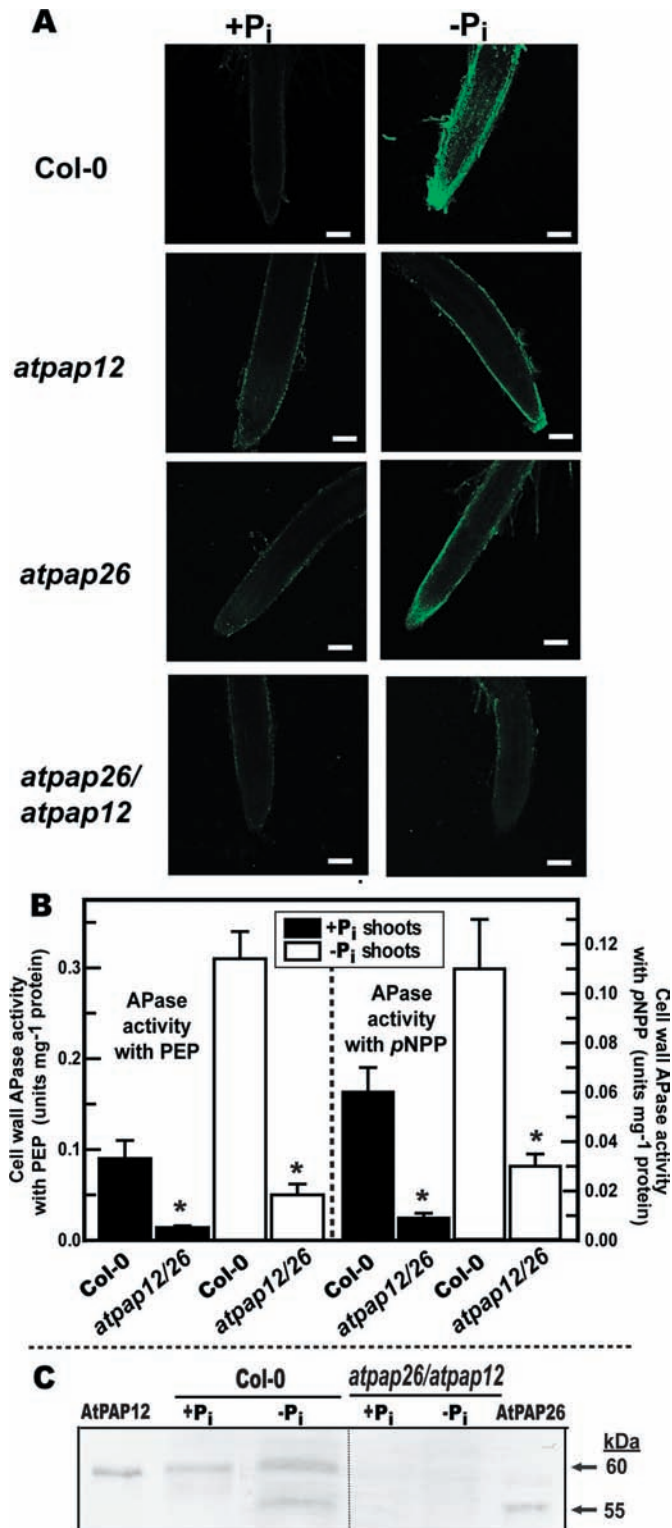


Fig. 3. AtPAP12 and AtPAP26 make an important contribution to P_i-starvation-inducible APase activity of *Arabidopsis* root surfaces and shoot cell walls. (A) Histochemical staining of root-surface APase activity of Col-0, *atpap12*, *atpap26* and *atpap26/atpap12* seedlings using ELF-97 phosphate as a substrate. Green fluorescent precipitates of the APase product ELF-97 were observed using a confocal-laser scanning microscope. Bars, 100 μm. Seedlings were cultivated as described in the legend for Fig. 2B. (B) Concentrated cell-wall proteins extracted from

the influence of P_i deprivation on extractable APase activity and immunoreactive AtPAP12 and AtPAP26 polypeptides of shoot cell-wall extracts of hydroponically cultivated Col-0 and *atpap12/atpap26* plants.

The complement of ionically bound (0.2 M CaCl₂-extractable) cell-wall proteins in shoots of +P_i and -P_i Col-0 seedlings was compared. The effectiveness of our extraction procedure was evaluated by testing for cytoplasmic contamination of the cell-wall fraction, using PEPC as a cytoplasmic marker enzyme. Immunoblots probed with anti- (castor bean PEPC) IgG demonstrated a lack of cytoplasmic contamination in the concentrated cell-wall fraction, as reflected by the absence of 107 kDa immunoreactive PEPC polypeptides in the cell wall but not in corresponding cytoplasmic fractions (Supplementary Fig. S3A at JXB online). Comparison of the cytoplasmic and cell-wall fractions on protein-stained SDS gels indicated clear differences in their respective proteomes (Supplementary Fig. S3B). The -P_i Col-0 seedlings exhibited a large increase in shoot cell-wall APase activity compared with +P_i seedlings; this was correlated with the upregulation of immunoreactive 60 kDa AtPAP12 and 55 kDa AtPAP26 polypeptides (Fig. 3B, C). By contrast, immunoreactive AtPAP12 and AtPAP26 polypeptides were absent on immunoblots of cell-wall extracts prepared from the +P_i or -P_i *atpap12/atpap26* mutant (Fig. 3C). This was paralleled by a >70% reduction in extractable cell-wall APase activity of -P_i *atpap12/atpap26* shoots relative to Col-0, irrespective of whether PEP or pNPP was used as the APase substrate (Fig. 3B). These results demonstrated that AtPAP12 and AtPAP26 account for most of the APase activity secreted into the cell walls of -P_i *Arabidopsis* shoots. P_i recycling by PSI cell-wall-targeted AtPAP12 and AtPAP26 could be critical in maintaining cytoplasmic P_i and thus photosynthetic metabolism in the leaves of -P_i plants.

It is likely that the residual extracellular APase activity of -P_i *atpap12/atpap26* seedlings (Figs 1C, D, and 3B) is at least partially due to a low-molecular-mass APase that has been shown previously to be upregulated and secreted by roots of -P_i *Arabidopsis* (Hurley *et al.*, 2010). This APase may correspond to AtPAP17, a PSI ~35 kDa PAP isozyme that is also induced (with AtPAP26) during leaf senescence (del Pozo *et al.*, 1999; Robinson *et al.*, 2012). However, the remaining extracellular APase activity of *atpap12/atpap26* seedlings was unable to fully compensate for the loss of AtPAP12 and AtPAP26 function, as overall seedling growth and P_i acquisition efficiency was clearly compromised during cultivation on -P_i or -P_i/+P_o medium (Figs. 1A and 4).

shoots of Col-0 and *atpap26/atpap12* seedlings were assayed for APase activity using 5 mM PEP or 5 mM pNPP as substrate. Values represent means ±SE of duplicate determinations on three biological replicates; asterisks indicate values that are significantly different from those of Col-0 (*P* < 0.01). Seedlings were cultivated as described in the legend for Fig. 1. (C) Concentrated shoot cell-wall proteins (15 μg per lane) and purified native AtPAP26 and AtPAP12 (25 ng per lane) (Tran *et al.*, 2010a) were subjected to immunoblot analysis with anti-AtPAP12 immune serum.

Secreted AtPAP12 and AtPAP26 scavenge phosphate from extracellular organic phosphates

The growth of Col-0 versus *atpap12*, *atpap26*, and *atpap12/atpap26* mutant plants was examined by cultivating 7 d +P_i seedlings for an additional 7 d on +P_i, -P_i, -P_i/+G3P, or -P_i/+DNA liquid medium. No differences were noted in the growth or appearance of +P_i plants (Figs 1A and 4). However, under -P_i, -P_i/+G3P, or -P_i/+DNA conditions, biomass yield of *atpap12/atpap26* plants was significantly reduced (by up to ~25%) relative to the Col-0, or *atpap12* and *atpap26* single mutant plants (Figs 1A and 4). This suggests that the absence of AtPAP12 was largely compensated for by AtPAP26 and vice versa during cultivation of the single mutants in -P_i, -P_i/+G3P, or -P_i/+DNA liquid medium. However, when expression of both PAP isozymes was eliminated in the *atpap12/atpap26* mutant, their absence could not be fully compensated by other PSI PAP isozymes such as AtPAP10 or AtPAP17 (del Pozo et al., 1999; Wang et al., 2011). Diminished growth of the -P_i, -P_i/+G3P, and -P_i/+DNA *atpap12/atpap26* seedlings was probably due to the marked reductions in their total P_i concentration, particularly during -P_i growth (Fig. 1B). The reduced biomass accumulation of -P_i *atpap12/atpap26* seedlings relative to Col-0 appeared to be specific to P_i deprivation, as no phenotypic differences were apparent when +P_i seedlings were subjected to nitrogen or potassium deficiency, or oxidative stress imposed by paraquat treatment (Supplementary Fig. S4 at JXB online).

The impaired development of *atpap12/atpap26* seedlings during growth on -P_i, -P_i/+G3P, or -P_i/+DNA medium was also evident during their cultivation on vertically oriented agar plates (Fig. 4, right panels). Similar results were obtained when the plants were cultured in -P_i liquid medium or vertical agar plates supplemented with 1.5 mM Glc-6-P, which like G3P is also efficiently hydrolysed by the native AtPAP12 or AtPAP26 purified from the secretome of -P_i *Arabidopsis* (Tran et al., 2010a). It was notable that Col-0 or *atpap12/atpap26* plants cultivated on -P_i agar plates supplemented with G3P, DNA, or Glc-6-P showed typical root architectural adaptations to P_i limitation (e.g. decreased primary root growth and increased lateral branching; Williamson et al., 2001), even though total biomass accumulation and shoot P_i concentration of Col-0 plants paralleled that of the respective +P_i seedlings (Figs 1A, B and 4). A rationale for this observation is that intracellular P_i status appears to be irrelevant to the reprogramming of root architecture in -P_i *Arabidopsis*, whereas low extracellular P_i in the area surrounding the root tip appears to trigger this response (Svistonoff et al., 2007). Presumably, the root-cap P_i sensor complex that mediates adaptive modifications in root structure to P_i limitation does not perceive exogenous P_o sources such as G3P, DNA, or Glc-6-P as a potential source of P nutrition, despite the fact that these compounds supported growth and P_i assimilation typical of P_i-fertilized plants.

We also examined the phenotype of soil-grown plants. Seedlings were cultivated in +P_i liquid medium for 7 d, before being transferred into a nutrient-depleted soil mixture and cultivated in growth cabinets under a regular light/dark regime for an additional 14 d. All P_i present in the peat/vermiculite soil mix

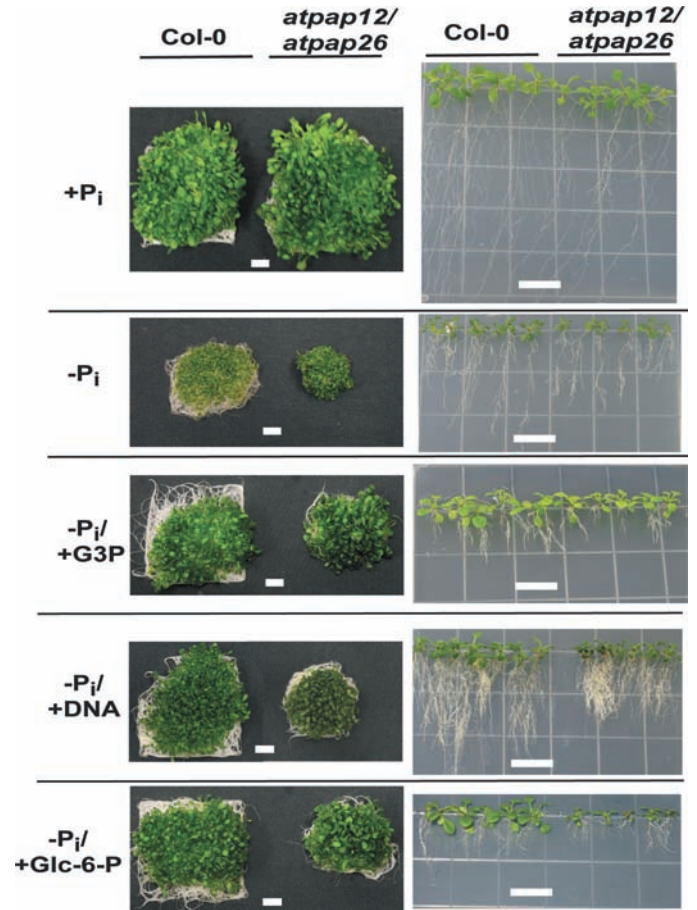


Fig. 4. Impact of different P sources on appearance and root morphology of Col-0 and *atpap26/atpap12* mutant seedlings. Left panels: seedlings were cultivated for 14 d in liquid medium as described in the legend for Fig. 1. Right panels: plants were cultivated for 21 d on vertically oriented agar plates containing 0.5× MS medium, 1% (w/v) sucrose, and 50 μM or 1.5 mM P_i (-P_i and +P_i, respectively), 1.5 mM G3P (-P_i/+G3P), 0.6 mg ml⁻¹ DNA (-P_i/+DNA), or 1.5 mM Glc-6-P (-P_i/Glc-6-P). Images are representative of at least five replicates. Bars, 1 cm.

used for these experiments was in the form of P_o; it contained 12.8±0.5 μmol total P_i g⁻¹ of dry weight but undetectable free P_i. No obvious phenotypic differences were noted when any of the soil-grown plants were provided with a regular P_i fertilizer treatment (Fig. 5A). However, the growth of the *atpap12* and *atpap26* single mutants was obviously compromised during their cultivation on the -P_i soil, as reflected by the ~50% reduction in their rosette dry weights relative to Col-0 plants (Fig. 5B). Impaired growth of *atpap26* seedlings on a -P_i soil mixture has been noted previously (Hurley et al., 2010). It is remarkable, however, that development of *atpap12/atpap26* plants was completely arrested when +P_i seedlings were transplanted into the -P_i soil mix (Figs. 5 and 6). In addition, shoots of soil-grown -P_i *atpap12/atpap26* plants rapidly turned purple, reflecting their anthocyanin accumulation, a typical symptom of severe P_i stress (Plaxton and Tran, 2011); the leaf anthocyanin concentration of the soil-grown -P_i Col-0 and *atpap12/atpap26*

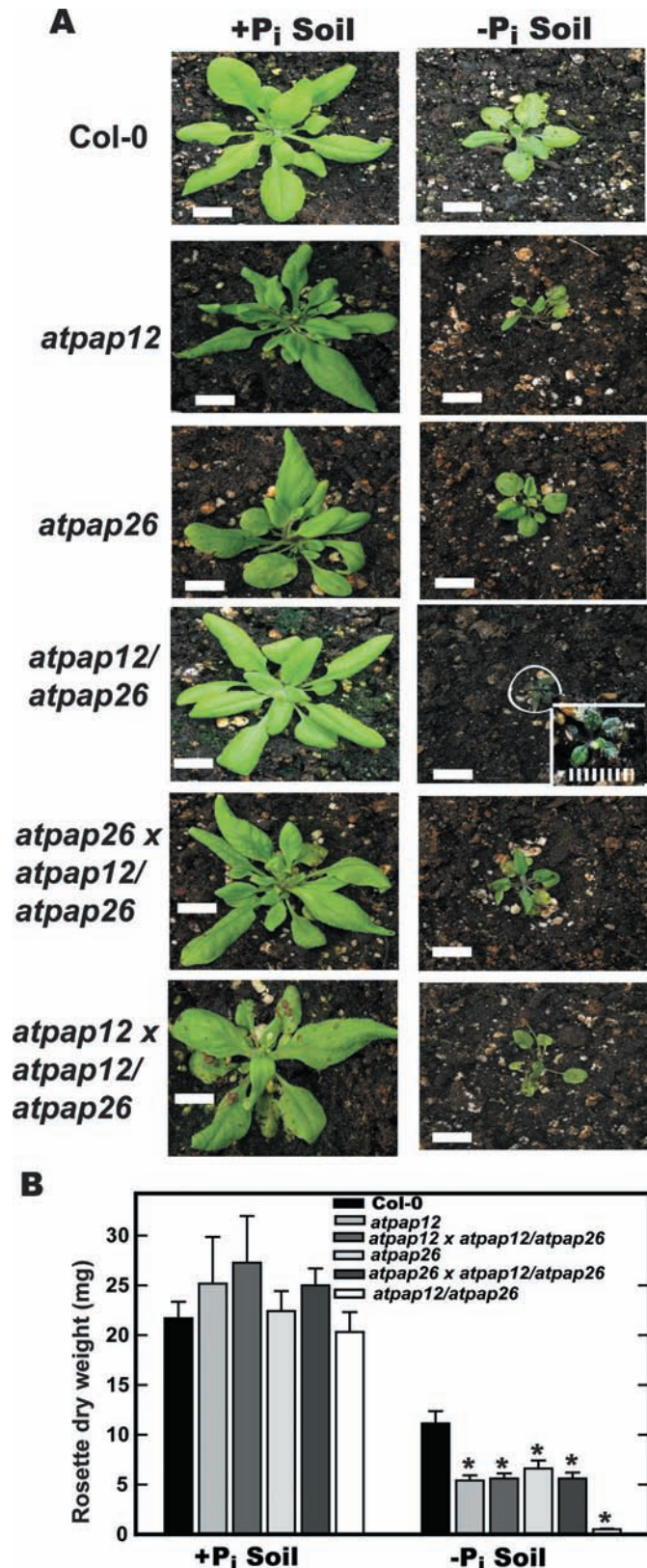


Fig. 5. Effect of P_i deprivation on appearance and shoot biomass accumulation of soil-grown Col-0 and mutant *Arabidopsis* seedlings. (A) Seedlings were cultivated for 7 d in liquid medium containing 0.2 mM P_i, then transplanted into a P_i-deficient soil mix and grown for an additional 14 d. Fertilization occurred twice weekly with 0.25× Hoagland's medium containing 0 or 2 mM P_i (–P_i;

plants was 70±8 and 900±12 nmol mg⁻¹ of fresh weight, respectively (means ±SE of duplicate determinations on three biological replicates). Shoots of soil-grown –P_i *atpap12/atpap26* plants also contained significantly less free P_i; the free P_i concentration of leaves of the –P_i Col-0 and *atpap12/atpap26* plants was 1.7±0.2 and 0.38±0.09 μmol g⁻¹ of fresh weight, respectively (means ±SE of duplicate determinations on three biological replicates). The arrested development of soil-cultivated 21-d-old –P_i *atpap12/atpap26* plants was quickly reversed when they were fertilized with medium containing 2 mM P_i and cultivated for an additional 5 d; this was paralleled by rapid leaf colour conversion from purple to green (Fig. 6).

Backcrossing *atpap12/atpap26* plants with each of the *atpap12* and *atpap26* single mutants restored AtPAP12 or AtPAP26 expression (Supplementary Fig. S5 at JXB online), as well as the –P_i soil growth phenotype characteristic of the respective single mutants (Fig. 5). This supports the ability of

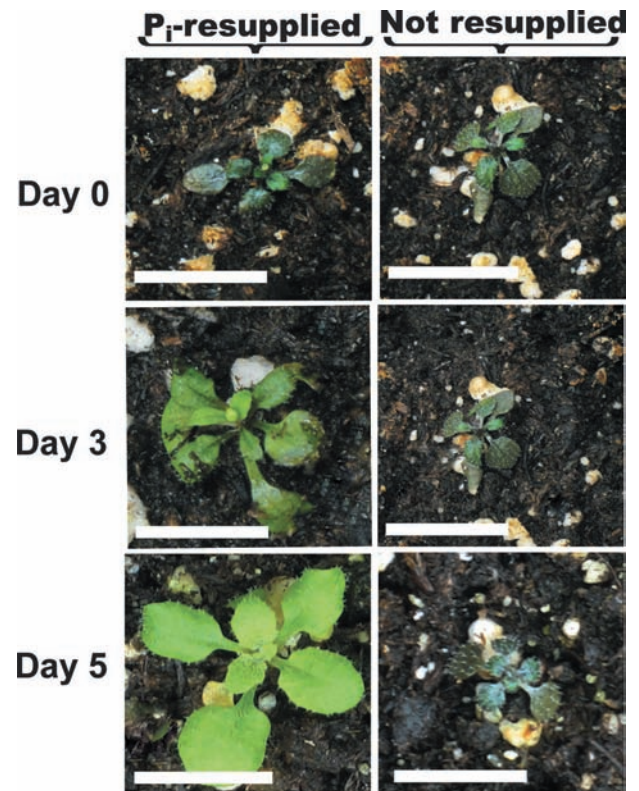


Fig. 6. Influence of P_i resupply on appearance of soil-grown –P_i *atpap12/atpap26* plants. Seedlings were grown for 7 d in liquid medium containing 0.2 mM P_i and then transplanted into the –P_i soil mix and grown for an additional 14 d. Seedlings were then fertilized with 0.25× Hoagland's medium containing 0 or 2 mM P_i and cultivated for an additional 5 d. Bars, 1 cm.

and +P_i, respectively). Solid bars, 1 cm; dashed bar, 0.5 cm. (B) Rosette dry weights of soil grown seedlings. All values represent means ±SE of ten different seedlings; asterisks indicate values that were significantly different from those of Col-0 (*P* < 0.01).

AtPAP12 to partially compensate for the absence of AtPAP26 and vice versa. It is hypothesized that decreased scavenging of soil-localized P_o reduced the amount of P_i assimilated by the *atpap12* or *atpap26* mutants, and that this was particularly exacerbated in the *atpap12/atpap26* double mutant. It is important to note that direct hydrolysis of rhizosphere P_o and subsequent assimilation of released P_i by APase-secreting roots has been demonstrated in soil-grown plants (Richardson *et al.*, 2009, 2011). However, while both monoester and diester (e.g. nucleic acid) P_o pools were depleted, (i) the precise chemical nature of the specific P_o substrates remains unclear, and (ii) the relative contributions of APases secreted by roots of $-P_i$ plants relative to those secreted by soil-dwelling bacteria remain to be established. Nevertheless, mineralization of soil P_o by plant and microbial APases does occur in the rhizosphere and appears to make an important contribution to the P_i nutrition of $-P_i$ plants (Richardson *et al.*, 2009, 2011). As both AtPAP12 and AtPAP26 were also markedly upregulated in the cell walls of $-P_i$ Col-0 *Arabidopsis* shoots (Fig. 3B and 3C), diminished P_i recapture from leaked phosphomonoesters is also suggested to contribute to the prominent phenotype of *atpap12/atpap26* mutant plants cultivated on $-P_i$ soil.

Concluding remarks

The *de novo* synthesis and secretion of APases by roots or suspension cell cultures has long been recognized as a widespread response of $-P_i$ plants (Tran *et al.*, 2010b; Plaxton and Tran, 2011). Conversely, the molecular identities, biochemical properties, and genetic control of PSI-secreted APases are not fully understood. However, such an understanding is likely to contribute towards exploiting biotechnological strategies for improving crop P acquisition from the abundant P_o sources prevalent in agricultural soils (Richardson, 2009). The results of the current study corroborate our earlier report indicating that AtPAP12 and AtPAP26 are the predominant secretory APases of $-P_i$ *Arabidopsis* seedlings (Tran *et al.*, 2010a). Their upregulation and secretion during growth on $-P_i/+P_o$ medium clearly helps *Arabidopsis* to exploit exogenous P_o compounds such as G3P, Glc-6-P, and DNA as alternative sources of P nutrition (Figs 1 and 4). AtPAP12 and AtPAP26 were also upregulated in shoot cell walls and on the root surface of $-P_i$ plants (Fig. 3), indicating that they have an additional function to recycle P_i from leaked phosphomonoesters. Cell-wall-localized or root secretory PSI AtPAP12 orthologues have been described in a variety of plant species including white lupin, tobacco, barrel medic, and tomato (Wasaki *et al.*, 2000, 2008; Miller *et al.*, 2001; Bozzo *et al.*, 2002, 2006; Xiao *et al.*, 2006; Kaida *et al.*, 2008). Wasaki *et al.* (2009) recently overexpressed a secreted AtPAP12 orthologue (LaSAP2) in tobacco; the transgenic plants exhibited enhanced P_i uptake and growth during cultivation on $-P_i$ soils. To the best of our knowledge, however, the involvement of AtPAP26 orthologues in scavenging P_i from extracellular P_o has not yet been reported in any other species.

During the cultivation of *atpap12* and *atpap26* single mutants on sterile $-P_i/+G3P$ or $-P_i/+DNA$ liquid medium, it was apparent that AtPAP12 could compensate for the absence of AtPAP26 and vice versa (Fig. 1A). However, this was not evident when either of

the single mutants was cultivated on a more physiologically relevant $-P_i$, P_o -containing soil mix, as both groups showed poorer growth relative to Col-0 control plants (Fig. 5). It was particularly noteworthy that development of the *atpap12/atpap26* double mutant was totally blocked when seedlings were transplanted into the $-P_i$ soil. This highlights the critical role that AtPAP12 and AtPAP26 have in facilitating acclimation of *Arabidopsis* to nutritional P_i deprivation. AtPAP10, AtPAP12, and AtPAP26 are closely related high-molecular-mass PSI PAPs that comprise subgroup Ia-2 of the *Arabidopsis* PAP family (Supplementary Fig. S6 at JXB online) (Li *et al.*, 2002; Tran *et al.*, 2010b). Evolution of this PAP subgroup appears to have endowed *Arabidopsis* with an effective hydrolytic machinery for scavenging P_i from exogenous P_o compounds prevalent in the $-P_i$ soils typical of most ecosystems (Tran *et al.*, 2010a; Wang *et al.*, 2011). As the susceptibility of soil P_o to enzymatic hydrolysis is a probable constraint for crop P_i acquisition (Richardson, 2009), it will be of interest to determine whether AtPAP12 and/or AtPAP26 overexpression could facilitate the production of P-use-efficient crops needed to reduce the use of P_i fertilizers in agriculture.

Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1. Confirmation of T-DNA insert location and loss of *AtPAP12* and/or *AtPAP26* gene expression in *atpap12*, *atpap26*, and *atpap26/atpap12* mutants.

Supplementary Fig. S2. Histochemical staining of root-surface APase activity in Col-0 and *atpap12/atpap26* seedlings using β -naphthyl phosphate or BCIP.

Supplementary Fig. S3. Immunoblot and SDS-PAGE analysis of cytoplasmic and cell-wall extracts isolated from shoots of $+P_i$ versus $-P_i$ Col-0 *Arabidopsis* seedlings.

Supplementary Fig. S4. Influence of nutrient deprivation or oxidative stress on growth of *atpap26/atpap12* and Col-0 seedlings.

Supplementary Fig. S5. Immunoblot analysis of AtPAP12 and AtPAP26 polypeptides in clarified rosette extracts of 21-d-old *Arabidopsis* plants cultivated in $-P_i$ soil.

Supplementary Fig. S6. A classification scheme for *Arabidopsis* PAPs based on clustering analysis of amino acid sequences.

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References

- Abel S, Nurnberger T, Ahnert V, Krauss GJ, Glund K. 2000. Induction of an extracellular cyclic nucleotide phosphodiesterase as an accessory ribonucleolytic activity during phosphate starvation of cultured tomato cells. *Plant Physiology* **122**, 543–552.

- Barrett-Lennard EG, Dracup M, Greenway H.** 1993. Role of extracellular phosphatases in the phosphorus-nutrition of cover. *Journal of Experimental Botany* **44**, 1595–1600.
- Bialeski RL, Johnson PN.** 1972. External location of phosphatase-activity in phosphorus-deficient *Spirodela oligorrhiza*. *Australian Journal of Biological Sciences* **25**, 707–720.
- Bozzo GG, Dunn EL, Plaxton WC.** 2006. Differential synthesis of phosphate-starvation inducible purple acid phosphatase isozymes in tomato (*Lycopersicon esculentum*) suspension cells and seedlings. *Plant Cell and Environment* **29**, 303–313.
- Bozzo GG, Raghothama KG, Plaxton WC.** 2002. Purification and characterization of two secreted purple acid phosphatase isozymes from phosphate-starved tomato (*Lycopersicon esculentum*) cell cultures. *European Journal of Biochemistry* **269**, 6278–6286.
- Chen DL, Delatorre CA, Bakker A, Abel S.** 2000. Conditional identification of phosphate-starvation-response mutants in *Arabidopsis thaliana*. *Planta* **211**, 13–22.
- Clough SJ, Bent AF.** 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16**, 735–743.
- del Pozo JC, Allona I, Rubio V, Leyva A, de la Pena A, Aragoncillo C, Paz-Ares J.** 1999. 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. *The Plant Journal* **19**, 579–589.
- Duff SMG, Lefebvre DD, Plaxton WC.** 1991. Purification, characterization, and subcellular-localization of an acid-phosphatase from black mustard cell-suspension cultures: comparison with phosphoenolpyruvate phosphatase. *Archives of Biochemistry and Biophysics* **286**, 226–232.
- George TS, Simpson RJ, Hadobas PA, Marshall DJ, Richardson AE.** 2007. Accumulation and phosphatase-lability of organic phosphorus in fertilised pasture soils. *Australian Journal of Agricultural Research* **58**, 47–55.
- Gilbert GA, Knight JD, Vance CP, Allan DL.** 1999. Acid phosphatase activity in phosphorus-deficient white lupin roots. *Plant, Cell & Environment* **22**, 801–810.
- Gregory AL, Hurley BA, Tran HT, Valentine AJ, She YM, Knowles VL, Plaxton WC.** 2009. *In vivo* regulatory phosphorylation of the phosphoenolpyruvate carboxylase AtPPC1 in phosphate-starved *Arabidopsis thaliana*. *Biochemical Journal* **420**, 57–65.
- Haran S, Logendra S, Seskar M, Bratanova M, Raskin I.** 2000. Characterization of *Arabidopsis* acid phosphatase promoter and regulation of acid phosphatase expression. *Plant Physiology* **124**, 615–626.
- Hurley BA, Tran HT, Marty NJ, Park J, Snedden WA, Mullen RT, Plaxton WC.** 2010. The dual-targeted purple acid phosphatase AtPAP26 is essential for efficient acclimation of *Arabidopsis thaliana* to nutritional phosphate deprivation. *Plant Physiology* **153**, 1112–1122.
- Jefferson RA, Kavanagh TA, Bevan MW.** 1987. Gus fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher-plants. *EMBO Journal* **6**, 3901–3907.
- Kaida R, Hayashi T, Kaneko TS.** 2008. Purple acid phosphatase in the walls of tobacco cells. *Phytochemistry* **69**, 2546–2551.
- Lefebvre DD, Duff SMG, Fife CA, Julien-Inalsingh C, Plaxton WC.** 1990. Response to phosphate deprivation in *Brassica nigra* suspension cells: enhancement of intracellular, cell-surface, and secreted phosphatase-activities compared to increases in Pi-absorption rate. *Plant Physiology* **93**, 504–511.
- Li DP, Zhu HF, Liu KF, Liu X, Leggewie G, Udvardi M, Wang DW.** 2002. Purple acid phosphatases of *Arabidopsis thaliana*: comparative analysis and differential regulation by phosphate deprivation. *Journal of Biological Chemistry* **277**, 27772–27781.
- Liang C, Tian J, Lam H, Lim BL, Yan X, Liao H.** 2010. Biochemical and molecular characterization of PvPAP3, a novel purple acid phosphatase isolated from common bean enhancing extracellular ATP utilization. *Plant Physiology* **152**, 854–865.
- Miller SS, Liu J, Allan DL, Menzhuber CJ, Fedorova M, Vance CP.** 2001. Molecular control of acid phosphatase secretion into the rhizosphere of proteoid roots from phosphorus-stressed white lupin. *Plant Physiology* **127**, 594–606.
- Nuruzzaman M, Lambers H, Bolland MDA.** 2006. Distribution of carboxylates and acid phosphatase and depletion of different phosphorus fractions in the rhizosphere of a cereal and three grain legumes. *Plant and Soil* **281**, 109–120.
- Plaxton WC, Tran HT.** 2011. Metabolic adaptations of phosphate-starved plants. *Plant Physiology* **156**, 1006–1015.
- Richardson AE.** 2009. Regulating the phosphorus nutrition of plants: molecular biology meeting agronomic needs. *Plant and Soil* **322**, 17–24.
- Richardson AE, Hocking PJ, Simpson RJ, George TS.** 2009. Plant mechanisms to optimise access to soil phosphorus. *Crop and Pasture Science* **60**, 124–143.
- Richardson AE, Lynch JP, Ryan PR, et al.** 2011. Plant and microbial strategies to improve the phosphorus efficiency of agriculture. *Plant and Soil* **349**, 121–156.
- Robinson WD, Carson I, Ying S, Ellis K, Plaxton WC.** 2012. Eliminating the purple acid phosphatase AtPAP26 in *Arabidopsis thaliana* delays leaf senescence and impairs phosphorus remobilization. *New Phytologist* (in press, doi: 10.1111/nph.12006).
- Svistonoff S, Creff A, Reymond M, Sigoillot-Claude C, Ricaud L, Blanchet A, Nussaume L, Desnos T.** 2007. Root tip contact with low-phosphate media reprograms plant root architecture. *Nature Genetics* **39**, 792–796.
- Tarafdar JC, Claassen N.** 1988. Organic phosphorus-compounds as a phosphorus source for higher-plants through the activity of phosphatases produced by plant-roots and microorganisms. *Biology and Fertility of Soils* **5**, 308–312.
- Ticconi CA, Abel S.** 2004. Short on phosphate: plant surveillance and countermeasures. *Trends in Plant Science* **9**, 548–555.
- Tran HT, Qian W, Hurley BA, She Y, Wang D, Plaxton WC.** 2010a. Biochemical and molecular characterization of AtPAP12 and AtPAP26: the predominant purple acid phosphatase isozymes secreted by phosphate-starved *Arabidopsis thaliana*. *Plant Cell and Environment* **33**, 1789–1803.
- Tran HT, Hurley BA, Plaxton WC.** 2010b. Feeding hungry plants: the role of purple acid phosphatases in phosphate nutrition. *Plant Science* **179**, 14–27.

- Veljanovski V, Vanderbeld B, Knowles VL, Snedden WA, Plaxton WC.** 2006. Biochemical and molecular characterization of AtPAP26, a vacuolar purple acid phosphatase up-regulated in phosphate-deprived *Arabidopsis* suspension cells and seedlings. *Plant Physiology* **142**, 1282–1293.
- Wang L, Li Z, Qian W, et al.** 2011. The Arabidopsis purple acid phosphatase AtPAP10 is predominantly associated with the root surface and plays an important role in plant tolerance to phosphate limitation. *Plant Physiology* **157**, 1283–1299.
- Wasaki J, Kojima S, Maruyama H, Haase S, Osaki M, Kandeler E.** 2008. Localization of acid phosphatase activities in the roots of white lupin plants grown under phosphorus-deficient conditions. *Soil Science and Plant Nutrition* **54**, 95–102.
- Wasaki J, Maruyama H, Tanaka M, Yamamura T, Dateki H, Shinano T, Susumu I, Osaki M.** 2009. Overexpression of the *LASAP2* gene for secretory acid phosphatase in white lupin improves the phosphorus uptake and growth of tobacco plants. *Soil Science and Plant Nutrition* **55**, 107–113.
- Wasaki J, Omura M, Ando M, Dateki H, Shinano T, Osaki M, Ito H, Matsui H, Tadano T.** 2000. Molecular cloning and root specific expression of secretory acid phosphatase from phosphate deficient lupin (*Lupinus albus* L.). *Soil Science and Plant Nutrition* **46**, 427–437.
- Williamson LC, Ribrioux SPCP, Fitter AH, Leyser HMO.** 2001. Phosphate availability regulates root system architecture in *Arabidopsis*. *Plant Physiology* **126**, 875–882.
- Xiao K, Harrison M, Wang ZY.** 2006. Cloning and characterization of a novel purple acid phosphatase gene (*MtPAP1*) from *Medicago truncatula* Barrel Medic. *Journal of Integrative Plant Biology* **48**, 204–211.
- Zhang C, McManus MT.** 2000. Identification and characterisation of two distinct acid phosphatases in cell walls of roots of white clover. *Plant Physiology and Biochemistry* **38**, 259–270.