The Dual-Targeted Purple Acid Phosphatase Isozyme
AtPAP26 Is Essential for Efficient Acclimation of
Arabidopsis to Nutritional Phosphate Deprivation

Brenden A. Hurley, Hue T. Tran, Naomi J. Marty, Joonho Park, Wayne A. Snedden, Robert T. Mullen, and William C. Plaxton

Department of Biology (B.A.H., H.T.T., J.P., W.A.S., W.C.P.) and Department of Biochemistry (W.C.P.), Queen’s University, Kingston, Ontario, Canada K7L 3N6; and Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1 (N.J.M., R.T.M.)

Induction of intracellular and secreted acid phosphatases (APases) is a widespread response of orthophosphate (Pi)-starved (−Pi) plants. APases catalyze Pi hydrolysis from a broad range of phosphomonoesters at an acidic pH. The largest class of nonspecific plant APases is comprised of the purple APases (PAPs). Although the biochemical properties, subcellular location, and expression of several plant PAPs have been described, their physiological functions have not been fully resolved. Recent biochemical studies indicated that AtPAP26, one of 29 PAPs encoded by the Arabidopsis (Arabidopsis thaliana) genome, is the predominant intracellular APase, as well as a major secreted APase isozyme up-regulated by −Pi Arabidopsis. An atpap26 T-DNA insertion mutant lacking AtPAP26 transcripts and 55-kD immunoreactive AtPAP26 polypeptides exhibited: (1) 9- and 5-fold lower shoot and root APase activity, respectively, which did not change in response to Pi starvation, (2) a 40% decrease in secreted APase activity during Pi deprivation, (3) 35% and 50% reductions in free and total Pi concentration, respectively, as well as 5-fold higher anthocyanin levels in shoots of soil-grown −Pi plants, and (4) impaired shoot and root development when subjected to Pi deficiency. By contrast, no deleterious influence of AtPAP26 loss of function occurred under Pi-replete conditions, or during nitrogen or potassium-limited growth, or oxidative stress. Transient expression of AtPAP26-mCherry in Arabidopsis suspension cells verified that AtPAP26 is targeted to the cell vacuole. Our results confirm that AtPAP26 is a principal contributor to Pi stress-inducible APase activity, and that it plays an important role in the Pi metabolism of −Pi Arabidopsis.

Orthophosphate (Pi) is an essential plant macronutrient required for many pivotal metabolic processes such as photosynthesis and respiration. However, the massive use of Pi fertilizers in agriculture demonstrates how the free Pi level of many soils is suboptimal for plant growth. The world’s reserves of rock phosphate, our major source of Pi fertilizers, are projected to be depleted by the end of this century (Vance et al., 2003). Furthermore, Pi runoff from fertilized fields into nearby surface waters results in environmentally destructive processes such as aquatic eutrophication and blooms of toxic cyanobacteria. Effective biotechnological strategies are needed to engineer Pi-efficient transgenic crops to ensure agricultural sustainability and a reduction in Pi fertilizer overuse. This necessitates a detailed understanding of Pi-starvation-inducible (PSI) gene expression and the complex morphological, physiological, and biochemical adaptations of Pi-deficient (−Pi) plants.

A well-documented component of the plant Pi stress response is the up-regulation of intracellular and secreted acid phosphatases (APases; E.C. 3.1.3.2) that catalyze the hydrolysis of Pi from various phosphate monoesters and anhydrides in the acidic pH range (Tran et al., 2010a). APase induction by −Pi plants has been correlated with de novo APase synthesis in several species, including white lupin (Lupinus albus), tomato (Solanum lycopersicum), and Arabidopsis (Arabidopsis thaliana); Miller et al., 2001; Bozzo et al., 2006; Veljanovski et al., 2006; Bozzo and Plaxton, 2008; and Tran and Plaxton, 2008). The probable function of intracellular APases is to recycle Pi from expendable intracel-
The impact of AtPAP26 loss of function on Pi-starved Arabidopsis

Impact of AtPAP26 Loss of Function on Pi-Starved Arabidopsis

RESULTS AND DISCUSSION

Identification and Validation of an atpap26 Mutant Allele

To assess the contribution of AtPAP26 to intracellular and secreted APase activity during Pi deficiency, as well as its impact on the phenotype of +Pi versus −Pi Arabidopsis, a T-DNA insertion line was identified in the Salk collection (Salk_152821; Alonso et al., 2003). The T-DNA insert was predicted to be located in the seventh intron of the AtPAP26 gene (locus At5g34850; Fig. 1A) and this position was verified by PCR screening of genomic DNA (gDNA) using an AtPAP26 gene-specific primer and a T-DNA left border primer (Fig. 1B). Homozygosity of the T-DNA mutant was confirmed by PCR of gDNA using AtPAP26-specific primers (Fig. 1B). T-DNA insert number was assessed by Southern blotting of gDNA that had been digested with EcoRI, HindIII, or SacI. Each restriction enzyme cleaves a single digestion site within the integrated pBIN-prOK2 vector. All digestions yielded a single strongly hybridizing band (Fig. 1C), indicating a single T-DNA insertion site.

The impact of the T-DNA insertion on AtPAP26 expression was investigated by performing reverse transcription (RT)-PCR using primer pairs to amplify cDNA sequences flanking the second intron of AtPAP26 (Fig. 1A). AtPAP26 transcripts were observed in shoot and root mRNA isolated from wild-type Columbia-0 (Col-0), but not atpap26 mutant plants (Fig. 2A). AtPAP12, AtPAP17, and AtPPCK1 (encodes phosphoenolpyruvate [PEP] carboxylase protein kinase1) were employed as positive controls as their transcript levels are markedly increased in −Pi Arabidopsis (del Pozo et al., 1999; Haran et al., 2000; 2010b). Although the purified intracellular versus secreted AtPAP26 isoforms of −Pi Arabidopsis shared an identical transit peptide cleavage site, they showed significant differences in the nature of the oligosaccharide side chains that are posttranslationally attached to this glycoprotein; glycosylation was therefore suggested to play an important role in the differential targeting of AtPAP26 during Pi stress (Tran et al., 2010b). In this study, we sought to test the hypothesis that AtPAP26 plays an important Pi-scavenging role in Arabidopsis during Pi stress. This was done by taking advantage of the publicly available T-DNA-tagged insertional mutagenized populations of Arabidopsis (Alonso et al., 2003). We identified and characterized a null atpap26 allele that abrogated AtPAP26 expression. This was correlated with the elimination of PSI intracellular APase activity, as well as a significant reduction in secreted APase activity during Pi deprivation. The atpap26 mutant demonstrated impaired development and decreased free and total Pi levels when exposed to Pi deficiency. Our results establish a firm role for AtPAP26 in facilitating the acclimation of Arabidopsis to suboptimal Pi nutrition.

RESULTS AND DISCUSSION

Identification and Validation of an atpap26 Mutant Allele

To assess the contribution of AtPAP26 to intracellular and secreted APase activity during Pi deficiency, as well as its impact on the phenotype of +Pi versus −Pi Arabidopsis, a T-DNA insertion line was identified in the Salk collection (Salk_152821; Alonso et al., 2003). The T-DNA insert was predicted to be located in the seventh intron of the AtPAP26 gene (locus At5g34850; Fig. 1A) and this position was verified by PCR screening of genomic DNA (gDNA) using an AtPAP26 gene-specific primer and a T-DNA left border primer (Fig. 1B). Homozygosity of the T-DNA mutant was confirmed by PCR of gDNA using AtPAP26-specific primers (Fig. 1B). T-DNA insert number was assessed by Southern blotting of gDNA that had been digested with EcoRI, HindIII, or SacI. Each restriction enzyme cleaves a single digestion site within the integrated pBIN-prOK2 vector. All digestions yielded a single strongly hybridizing band (Fig. 1C), indicating a single T-DNA insertion site.

The impact of the T-DNA insertion on AtPAP26 expression was investigated by performing reverse transcription (RT)-PCR using primer pairs to amplify cDNA sequences flanking the second intron of AtPAP26 (Fig. 1A). AtPAP26 transcripts were observed in shoot and root mRNA isolated from wild-type Columbia-0 (Col-0), but not atpap26 mutant plants (Fig. 2A). AtPAP12, AtPAP17, and AtPPCK1 (encodes phosphoenolpyruvate [PEP] carboxylase protein kinase1) were employed as positive controls as their transcript levels are markedly increased in −Pi Arabidopsis (del Pozo et al., 1999; Haran et al., 2000; 2010b). Although the purified intracellular versus secreted AtPAP26 isoforms of −Pi Arabidopsis shared an identical transit peptide cleavage site, they showed significant differences in the nature of the oligosaccharide side chains that are posttranslationally attached to this glycoprotein; glycosylation was therefore suggested to play an important role in the differential targeting of AtPAP26 during Pi stress (Tran et al., 2010b). In this study, we sought to test the hypothesis that AtPAP26 plays an important Pi-scavenging role in Arabidopsis during Pi stress. This was done by taking advantage of the publicly available T-DNA-tagged insertional mutagenized populations of Arabidopsis (Alonso et al., 2003). We identified and characterized a null atpap26 allele that abrogated AtPAP26 expression. This was correlated with the elimination of PSI intracellular APase activity, as well as a significant reduction in secreted APase activity during Pi deprivation. The atpap26 mutant demonstrated impaired development and decreased free and total Pi levels when exposed to Pi deficiency. Our results establish a firm role for AtPAP26 in facilitating the acclimation of Arabidopsis to suboptimal Pi nutrition.

RESULTS AND DISCUSSION

Identification and Validation of an atpap26 Mutant Allele

To assess the contribution of AtPAP26 to intracellular and secreted APase activity during Pi deficiency, as well as its impact on the phenotype of +Pi versus −Pi Arabidopsis, a T-DNA insertion line was identified in the Salk collection (Salk_152821; Alonso et al., 2003). The T-DNA insert was predicted to be located in the seventh intron of the AtPAP26 gene (locus At5g34850; Fig. 1A) and this position was verified by PCR screening of genomic DNA (gDNA) using an AtPAP26 gene-specific primer and a T-DNA left border primer (Fig. 1B). Homozygosity of the T-DNA mutant was confirmed by PCR of gDNA using AtPAP26-specific primers (Fig. 1B). T-DNA insert number was assessed by Southern blotting of gDNA that had been digested with EcoRI, HindIII, or SacI. Each restriction enzyme cleaves a single digestion site within the integrated pBIN-prOK2 vector. All digestions yielded a single strongly hybridizing band (Fig. 1C), indicating a single T-DNA insertion site.

The impact of the T-DNA insertion on AtPAP26 expression was investigated by performing reverse transcription (RT)-PCR using primer pairs to amplify cDNA sequences flanking the second intron of AtPAP26 (Fig. 1A). AtPAP26 transcripts were observed in shoot and root mRNA isolated from wild-type Columbia-0 (Col-0), but not atpap26 mutant plants (Fig. 2A). AtPAP12, AtPAP17, and AtPPCK1 (encodes phosphoenolpyruvate [PEP] carboxylase protein kinase1) were employed as positive controls as their transcript levels are markedly increased in −Pi Arabidopsis (del Pozo et al., 1999; Haran et al., 2000; 2010b). Although the purified intracellular versus secreted AtPAP26 isoforms of −Pi Arabidopsis shared an identical transit peptide cleavage site, they showed significant differences in the nature of the oligosaccharide side chains that are posttranslationally attached to this glycoprotein; glycosylation was therefore suggested to play an important role in the differential targeting of AtPAP26 during Pi stress (Tran et al., 2010b). In this study, we sought to test the hypothesis that AtPAP26 plays an important Pi-scavenging role in Arabidopsis during Pi stress. This was done by taking advantage of the publicly available T-DNA-tagged insertional mutagenized populations of Arabidopsis (Alonso et al., 2003). We identified and characterized a null atpap26 allele that abrogated AtPAP26 expression. This was correlated with the elimination of PSI intracellular APase activity, as well as a significant reduction in secreted APase activity during Pi deprivation. The atpap26 mutant demonstrated impaired development and decreased free and total Pi levels when exposed to Pi deficiency. Our results establish a firm role for AtPAP26 in facilitating the acclimation of Arabidopsis to suboptimal Pi nutrition.
Li et al., 2002; Tran and Plaxton, 2008; Gregory et al., 2009; Tran et al., 2010b). All of these Pi-responsive control transcripts showed a similar induction in \(-\)Pi atpap26 mutant and wild-type Col-0 seedlings (Fig. 2A), indicating that the mutant is unimpaired in Pi-starvation signaling.

Consistent with previous studies (Veljanovski et al., 2006; Tran and Plaxton, 2008; Tran et al., 2010b), the amount of 55-kD AtPAP26 immunoreactive polypeptides were about 2-fold greater in root or shoot extracts from the \(-\)Pi relative to +Pi Col-0 seedlings (Fig. 2B), and this was paralleled by the pronounced accumulation of secreted AtPAP26 polypeptides in the media of \(-\)Pi Col-0 seedlings (Fig. 3). By contrast, immunoblotting of clarified shoot or root extracts, or secretome analyses of the +Pi or \(-\)Pi atpap26 mutant failed to reveal any immunoreactive AtPAP26 subunits (Figs. 2B and 3), whereas the up-regulation and secretion of 60-kD AtPAP12 polypeptides during Pi stress was unaffected (Fig. 3). Therefore, atpap26 defines a null allele of AtPAP26, with abrogated expression of AtPAP26 transcript and protein. Results of Fig. 2A corroborate previous studies of Arabidopsis suspension cells and seedlings, documenting the up-regulation of intracellular and secreted AtPAP26 polypeptides in response to Pi starvation, without concomitant changes in AtPAP26 transcript abundance (Veljanovski et al., 2006; Tran and Plaxton, 2008; Tran et al., 2010b). Recent proteomic studies have observed a variety of intracellular and secreted proteins that are also controlled posttranscriptionally at the level of protein accumulation in Arabidopsis, maize (Zea mays), and rice (Oryza sativa) plants responding to changes in environmental Pi availability (Fukuda et al., 2007; Li et al., 2008; Tran and Plaxton, 2008). In contrast to AtPAP26, the up-regulation of Arabidopsis PAPs such as AtPAP12 and AtPAP17 during Pi deprivation appears to be mainly controlled at the transcriptional level (del Pozo et al., 1999; Haran et al., 2000; Li et al., 2002; Tran and Plaxton, 2008; Tran et al., 2010b; Fig. 2A). AtPAP12 is secreted by \(-\)Pi Arabidopsis suspension cells and seedlings (along with AtPAP26) it is expected to play an extracellular Pi-scavenging role (Fig. 3B; Tran and Plaxton, 2008; Tran et al., 2010b). AtPAP17 (formerly known as ACP5) transcripts also accumulate in response to oxidative or salt stress, similar to GmPAP3 (del Pozo et al., 1999; Francisca Li et al., 2008). AtPAP17 may thus function to detoxify reactive oxygen species during general stress rather than play a significant Pi remobilization and scavenging role in \(-\)Pi Arabidopsis.

AtPAP26 Is the Predominant Intracellular and a Major Secreted APase Isozyme Up-Regulated by \(-\)Pi Arabidopsis

Pi deprivation of Col-0 seedlings resulted in 2- to 3-fold increases in shoot, root, and secreted APase activities (Figs. 2C and 3), as previously reported (Zakhleniuk et al., 2001; Veljanovski et al., 2006; Tran and Plaxton, 2008; Tran et al., 2010b). However, the \(-\)Pi atpap26 mutant exhibited 9- and 5-fold lower shoot and root APase activities, respectively (Fig. 2C), as well as a 40% reduction in secreted APase activity relative to Col-0 (Fig. 3). It is notable that no increase in intracellular APase activity was detected in response to Pi starvation of the atpap26 mutant (Fig. 2C). APase assays employing 5 mM para-nitrophenol-P (pNPP) rather than 5 mM PEP as the substrate were also performed with shoot and root extracts of \(-\)Pi atpap26 and Col-0 seedlings. Consistent with the PEP-based assays (Fig. 2C), extracts from \(-\)Pi shoots and roots of atpap26 seedlings, respectively, exhibited about 4- and 6-fold lower pNPP-hydrolyzing activity relative to Col-0 controls (shoots = 65 ± 7 and 265 ± 20 nmol pNPP hydrolyzed min\(^{-1}\) mg\(^{-1}\) protein, respectively; roots = 55 ± 3 and 202 ± 33 nmol pNPP hydrolyzed min\(^{-1}\) mg\(^{-1}\) protein, respectively; means ± se of n = 4 biological replicates).

Clarified extracts of \(-\)Pi shoots were also resolved by nondenaturing PAGE and subjected to in-gel APase activity staining using \(\beta\)-naphthyl-P as the substrate, and parallel immunoblotting with anti-AtPAP26 immune serum (Fig. 2D). Shoot extracts of \(-\)Pi Col-0 yielded several APase activity-staining bands on nondenaturing gels, in agreement with previous results (Tomcska et al., 2004). However, an abundant high molecular mass APase activity-staining band that
strongly cross-reacted with the anti-AtPAP26 immune serum was absent in the atpap26 mutant (Fig. 2D). Similarly, nondenaturing PAGE of concentrated secretome proteins of −Pi Col-0 seedlings revealed a prominent PSi APase activity-staining band comigrating with purified AtPAP26 that was missing in the secretome of the −Pi atpap26 mutant. Collective results of Figures 2 and 3 support earlier biochemical studies (Veljanovski et al., 2006; Tran et al., 2010b), and confirm that AtPAP26 is a principal contributor of intracellular and secreted APase activity of −Pi Arabidopsis. Similar results were obtained by Tomscha and coworkers (2004) who characterized an Arabidopsis phosphatase underproducer (pup3) ethylmethane sulfonate mutant that exhibited about 40% lower APase activity in shoot and root extracts. Although immunoblotting using antiserum raised against recombinant AtPAP12 led them to conclude that AtPAP12 was one of the AtPAP isozymes defective in pup3, AtPAP26 was also implicated since the pup3 mutation mapped to a 2.7 Mb sequence of chromosome 5 within the Arabidopsis genome that encompasses AtPAP26 (Tomscha et al., 2004). That pup3 may have been defective in AtPAP26 rather than AtPAP12 is supported by the observations that: (1) AtPAP12 transcript levels were unaffected in pup3 (Tomscha et al., 2004), whereas (2) the same anti-recombinant AtPAP12 immune serum employed by Tomscha et al. (2004) effectively cross-reacts with both AtPAP12 and AtPAP26 (Fig. 3A; Tran et al., 2010b). By contrast, no detectable influence on total APase activity was obtained with several other AtPAP isozyme loss-of-function mutants. For example, no alteration in extractable APase activity was reported in atpap23 T-DNA mutants (Zhu et al., 2005). Similarly, extracts of atpap15 T-DNA mutants contained 6-fold lower phytase activity, but unaltered pNPP hydrolytic activity relative to wild-type controls, possibly due to AtPAP15’s specificity as a phytase (Zhang et al., 2008) and/or its low abundance relative to AtPAP26.

Figure 2. AtPAP26 is the predominant intracellular APase isozyme up-regulated by −Pi Arabidopsis and whose expression is nullified in atpap26 mutant seedlings. RNA and soluble proteins were isolated from seedlings cultivated in liquid media containing 0.2 mM Pi for 7 d prior to transfer into media containing 0 (−Pi) or 1.5 mM Pi (+Pi) for an additional 7 d. A, Levels of mRNA were analyzed by semiquantitative RT-PCR using gene-specific primers for AtPAP12, AtPAP17, AtPAP26, AtPPCK1, and AtACT2. AtACT2 was used as a reference to ensure equal template loading. Control RT-PCR reactions lacking reverse transcriptase did not show any PCR product. B, Purified native AtPAP26 from −Pi Arabidopsis suspension cells (50 ng/lane; Veljanovski et al., 2006) and clarified extract proteins from shoots (2 µg/lane) and roots (4 µg/lane) of the +Pi and −Pi seedlings were resolved by SDS-PAGE and electroblotted onto a poly(vinylidene difluoride) membrane. Following oxidation of antigenic glycosyl groups with sodium-periodate (Laine, 1988), blots were probed with a 1,000-fold dilution of anti-native AtPAP26-immune serum and immunoreactive polypeptides detected using an alkaline-phosphatase-linked secondary antibody and chromogenic detection (Veljanovski et al., 2006). C, APase activity of clarified extracts represent means (±SE) of duplicate assays on n = 3 biological replicates; asterisks denote values that are significantly different from Col-0 (P < 0.01). D, Clarified extracts from −Pi shoots of Col-0 and atpap26 were resolved by nondenaturing PAGE and subjected to in-gel APase activity staining (50 µg protein/lane) or immunoblotting with anti-native AtPAP26-immune serum (7 µg protein/ lane). O, Origin; TD, tracking dye front.
AtPAP26 Is Essential for Efficient Acclimation of Arabidopsis to Pi Starvation

The influence of Pi nutrition on the development of Col-0 versus atpap26 plants was initially compared by examining their growth on +Pi and −Pi Murashige and Skoog agar media. No differences were noted in the appearance or average fresh weight of shoots of 14-d-old +Pi Col-0 versus atpap26 seedlings (Fig. 4A; data not shown). However, atpap26 shoot development was significantly disrupted during growth on −Pi media, exhibiting a 30% decrease in fresh weight relative to Col-0 (Fig. 4A). The development of atpap26 mutants was also characterized in plants cultivated on +Pi or −Pi agar media, as well as on +Pi media deficient in nitrogen or potassium, or supplemented with 1 μM paraquat. Similar to 14-d-old plants (Fig. 4B), rosette fresh weight of 3-week-old −Pi atpap26 seedlings was reduced by about 25% relative to −Pi Col-0, but unaffected under +Pi conditions (Fig. 4B). Likewise, no phenotypic differences relative to Col-0 were apparent when +Pi atpap26 seedlings were sub-

Figure 3. Immunological AtPAP detection, in-gel APase activity staining, and corresponding APase-specific activities of proteins secreted into the media by +Pi and −Pi Col-0 and atpap26 seedlings. Growth medium containing secreted proteins was passed through a 0.45 μm filter and concentrated 250-fold with Amicon Ultra-15 ultrafiltration devices (30,000 Mr cutoff; Millipore) at room temperature. A and B, Concentrated proteins (15 μg/lane) as well as homoimmune AtPAP12 and AtPAP26 from −Pi Arabidopsis suspension cells (Tran et al., 2010b; 20 ng each) were subjected to SDS-PAGE and immunoblotting as described in the legend for Figure 2, except that the immunoreactive polypeptides were visualized using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescent detection (ECL Plus, GE Healthcare). C, Concentrated secreted proteins (30 μg/lane) and nonsecreted AtPAP26 (1 μg; Tran et al., 2010b) were subjected to nondenaturing PAGE and in-gel APase activity staining as described in the legend for Figure 2D. APase activity was also assayed from the concentrated liquid media collected from seedlings cultivated as described in the legend for Figure 2. All APase activities represent the means of duplicate determinations on n = 3 biological replicates and are reproducible to within ±15% of the mean value.

Figure 4. Influence of nutrient deprivation or oxidative stress on growth of atpap26 and Col-0 seedlings. A, Shoot fresh weight (FW) of seedlings cultivated for 14 d under continuous illumination (100 μmol m⁻² s⁻¹ photosynthetically active radiation) on agar-solidified 0.5× Murashige and Skoog media containing 1% Suc and 1.5 mM or 50 μM Pi. B, Shoot fresh weight of seedlings cultivated on agar media containing 1.5 mM Pi for 7 d, then grown for an additional 14 d on media containing 1.5 mM or 50 μM Pi, or on +Pi media lacking nitrogen (−N) or potassium (−K), or containing 1 μM paraquat (PQ). C, Primary root length of seedlings cultivated on vertically oriented plates for 6, 9, or 12 d as described in A. All values in A to C represent means ± SE of n = 16 seedlings from four different plates; asterisks denote values that are significantly different from Col-0 (P < 0.01).
jected to nitrogen or potassium deficiency, or paraquat treatment (Fig. 4B; data not shown). Loss of AtPAP26 function also failed to influence the sensitivity of Arabidopsis to paraquat-mediated oxidative stress, suggesting that the in vitro alkaline peroxidase activity of purified AtPAP26 (Veljanovski et al., 2006) has little in vivo relevance during Pi stress. This is consistent with AtPAP26’s vacuolar localization (Carter et al., 2004; and see below) in which an acidic pH of about pH 5.5 closely aligns with the enzyme’s APase pH-activity optimum of pH 5.6, but is far below AtPAP26’s peroxidase pH-activity optimum of pH 9.0 (Veljanovski et al., 2006). It therefore appears that while AtPAP26 is indispensable for the acclimation of Arabidopsis to Pi deprivation, it is expendable in +Pi Arabidopsis, or during other macronutrient deficiencies or oxidative stress.

Root development of the atpap26 mutant was examined by cultivating seedlings on vertically orientated agar plates for 12 d. Decreasing the Pi concentration in the growth media resulted in a substantial reduction in primary root growth of atpap26 and Col-0 seedlings (Fig. 4D), as well as increased root hair proliferation (Supplemental Fig. S1B). However, primary roots of the atpap26 mutant were about 20% shorter than those of Col-0 during growth on the –Pi media (Fig. 4D; Supplemental Fig. S1A). By contrast, there was no obvious influence of the loss of AtPAP26 expression on root structure architecture of +Pi or –Pi seedlings, or root hair number within 5 mm of the root tip (Supplemental Fig. S1).

Figure 5. Effect of Pi deprivation on soil-grown atpap26 and Col-0 seedlings. A, Seedlings were cultivated for 7 d on Pi-fertilized soil, then transplanted into a Pi-deficient soil mixture and grown for an additional 14 d; fertilization occurred biweekly with 0.25× Hoagland media containing 2 mM Pi (+Pi) or 0 mM Pi (–Pi). Pots shown are representative of 10 replicates. B, Rosette dry weight of seedlings cultivated as in A. C to E, Anthocyanin (C), and free and total Pi concentrations (D and E) of rosette leaves of seedlings cultivated as in A. All values are means ± SE (n = 20 for B, 5 for C, and 10 for D and E); asterisks denote values that are significantly different from Col-0 (P < 0.01).
Although altered root development is a common phenomenon within functional analyses of Arabidopsis PSI genes, this response can be grouped into two categories based on the function of the PSI gene in question. Disruption of genes involved in transcriptional reorganization or hormone-mediated responses to Pi starvation generally causes a shift in root structure architecture in addition to decreased primary root growth. For example, knockout of PSI transcription factors has been reported to reduce primary root growth with concomitant increases in lateral root and root hair development (Devaiah et al., 2007a, 2007b). Similar results have been obtained with genes that mediate signal transduction in −Pi Arabidopsi, such as phospholipase Dζ1 and Dζ2, in which a double knockout exhibited reduced phosphatidic acid levels and altered root structure architecture during Pi starvation (Li et al., 2006). By contrast, disruption of PSI genes encoding metabolic enzymes often results in a reduction of total root growth during Pi stress without obvious changes in root structure architecture. For example, knockout of monogalactosyldiacylglycerol synthase2 resulted in an overall 20% decrease in root growth, without influencing root structure architecture (Kobayashi et al., 2009). The observed up-regulation of three well-documented PSI genes (Fig. 2A) and overall reduced root growth without concomitant alterations in root structure architecture are consistent with ATPAP26’s function in Pi scavenging and recycling, rather than Pi signaling, during Pi starvation.

PSI gene expression is influenced by sugar levels (Karthikeyan et al., 2006), while exogenous Suc may exacerbate Pi starvation through increased cell proliferation signaling (Lai et al., 2007). As plant cultivation under sterile conditions in the presence of exogenous Suc may generate phenotypes not seen under more physiological conditions, we examined whether soil-grown atpap26 plants displayed any phenotype. The atpap26 mutant exhibited markedly impaired development during cultivation for 14 d under −Pi conditions on a nutrient-depleted soil mixture (Fig. 5A), as reflected by the 25% reduction in rosette dry weight relative to Col-0 control plants (Fig. 5B). By contrast, atpap26 plants showed a small, but significant 16% increase in rosette dry weight during their cultivation on +Pi soil. Similar to seedlings cultivated in sterile liquid media (Fig. 2C), the extractable APase activity of soil-grown atpap26 mutant plants was much lower than that of Col-0 controls. Clarified extracts prepared from fully expanded leaves of 3-week-old −Pi atpap26 plants exhibited about 4- and 11-fold lower PEP- and pNPP-hydrolyzing activity, respectively, relative to Col-0 (60 ± 6 and 212 ± 37 nmol PEP versus 36 ± 4 and 530 ± 44 nmol pNPP hydrolyzed min⁻¹ mg⁻¹ protein, respectively; means ± se of n = 4 biological replicates).

One of the most obvious symptoms of plant Pi stress is anthocyanin accumulation in shoots that is believed to protect chloroplasts against photo-inhibition (Vance et al., 2003). Since petioles of soil-cultivated −Pi atpap26 plants were purple in color (Fig. 5A) their leaf anthocyanin content was quantified. Although anthocyanins accumulated in Col-0 and atpap26 leaves during growth on the −Pi soil, the anthocyanin level of atpap26 leaves was about 5-fold greater than that of Col-0 (Fig. 5C). The stunted development of −Pi atpap26 seedlings coupled with their drastically elevated shoot anthocyanin levels suggested that the mutant was suffering from more pronounced nutritional Pi deficiency relative to Col-0. Indeed, a 35% and 50% reduction in free and total Pi concentration, respectively, was quantified in leaves of −Pi atpap26 plants, relative to Col-0 controls (Fig. 5D). This adds further support for ATPAP26’s critical role in the Pi metabolism of −Pi Arabidopsis. The peat-vermiculite soil mix used in these experiments contained 12.8 ± 0.5 μmol total Pi g⁻¹ dry weight (mean ± se, of n = 3 determinations). However, all Pi present in this soil was in the form of organic P, as its free Pi content was undetectable. Since AtPAP26 is also secreted by Arabidopsis roots during Pi stress (Fig. 3; Tran and Plaxton, 2008; Tran et al., 2010b) it is possible that decreased hydrolysis of soil-localized organic P reduced the total amount of Pi made available to the atpap26 mutant.

Figure 6. ATPAP26-mCherry localizes to lytic vacuoles of transiently transformed Arabidopsis suspension cells. Heterotrophic suspension cells were transiently cotransformed via biolistic bombardment with ATPAP26-mCherry and sporamin NTPP-GFP. Following bombardment, cells were incubated for 8 h to allow for gene expression and protein sorting, then fixed in formaldehyde and viewed using epifluorescence microscopy. Note that the fluorescence patterns attributable to coexpressed ATPAP26-mCherry (A) and sporamin NTPP-GFP (B) colocalize in the same cell, as evidenced by yellow color in the merged image (C). Also shown (D), is the differential interference contrast (DIC) image of the cell depicted in A to C. This result is representative of ≥25 cells from at least two independent biolistic bombardments. Bar = 10 μm.
during its cultivation on the Pi-deficient soil. In contrast to –Pi conditions, the atpap26 leaves accumulated about 25% more free and total Pi relative to Col-0 during their growth on Pi-fertilized soil (Fig. 5D). This provides a feasible basis for the small increase in rosette dry weight of soil-cultivated +Pi atpap26 plants (Fig. 5B), and indicates that AtPAP26 may also function in intracellular Pi homeostasis of +Pi plants.

**Vacuolar Localization of AtPAP26-mCherry**

To determine the subcellular localization of AtPAP26, its coding region was fused with the 5’ end of a mCherry reporter gene and transiently expressed via biolistic bombardment in Arabidopsis suspension cells under the control of the cauliflower mosaic virus 35S promoter. Epifluorescence microscopy demonstrated that AtPAP26-mCherry was targeted to lytic vacuoles (Fig. 6A) as evidenced by its colocalization with coexpressed sporamin N-terminal propeptide (NTPP)-GFP (Fig. 6, B and C), serving as a well-characterized lytic vacuole marker fusion protein (Jin et al., 2001; Kim et al., 2001). These results are consistent with those of Carter and coworkers (2004) who reported that the protein encoded by gene locus At5g34850 (i.e. AtPAP26) is a member of the vacuolar proteome of Arabidopsis leaves.

**CONCLUSION**

Results of this study corroborate parallel biochemical analyses, indicating that AtPAP26 encodes the principal vacuolar, as well as a major secreted APase isozyme up-regulated by –Pi Arabidopsis (Veljanovski et al., 2006; Tran and Plaxton, 2008; Tran et al., 2010b). However, in contrast to PSI enzymes such as AtPAP12 and AtPAP17, AtPAP26’s enhanced synthesis during Pi deprivation is under posttranscriptional control (Fig. 3). Recent proteomic studies established that transcriptional controls exert little influence on levels of certain intracellular and secreted proteins up-regulated by –Pi plants relative to translational and posttranslational controls that influence protein synthesis and degradation (see Tran and Plaxton 2008 and refs. therein). This highlights the need to integrate transcriptomics with parallel biochemical and proteomic analyses of plant stress responses, as the combined datasets will provide a far better depiction of how alterations in gene expression may be linked to adaptive changes in plant metabolism. As the predominant APase isozyme up-regulated by –Pi Arabidopsis, it was not unexpected that the development of atpap26 seedlings was specifically disrupted during their cultivation on –Pi media (Figs. 4 and 5). Shoots of soil-grown atpap26 mutants accumulated significantly less free and total Pi relative to Col-0 during Pi stress (Fig. 5). This supports the hypothesis that AtPAP26 functions to recycle and scavenge Pi from intracellular and extracellular P-ester pools, respectively, so as to enhance the overall Pi utilization efficiency of –Pi Arabidopsis. Eliminating the expression of genes encoding PSI metabolic enzymes has been reported to disrupt the growth of –Pi Arabidopsis, albeit in the context of altered Pi recycling from membrane phospholipids due to loss of the PSI phospholipase-C isoform NPC5, or monogalactosyldiacylglycerol synthase2 (Gaude et al., 2008; Kobayashi et al., 2009). Similarly, loss of PSI high-affinity Pi transporters disrupted the shoot development of –Pi, but not +Pi Arabidopsis (Shin et al., 2004). However, to our knowledge, this is the first study showing that elimination of a single member of the AtPAP family simultaneously exerts a significant inhibitory effect on nonspecific APase activity and growth of –Pi Arabidopsis.

Several studies have reported that overexpression of secreted PAPs can improve plant biomass and Pi accumulation (Xiao et al., 2006; Hur et al., 2007; Ma et al., 2009; Wang et al., 2009). However, these attempts have either focused around high-specificity phytases or PAPs with extracellular roles, but unclear intracellular function. On the other hand, AtPAP26 not only has a demonstrated vacuolar localization (Fig. 6; Veljanovski et al., 2006), but is a dual-targeted enzyme that is also secreted by –Pi Arabidopsis (Fig. 3; Tran and Plaxton, 2008; Tran et al., 2010b). Although differential glycosylation appears to be involved in AtPAP26’s dual targeting to the vacuole versus extracellular space of –Pi Arabidopsis, the precise molecular and cellular mechanisms underlying this phenomenon remain to be investigated. Intracellular and secreted AtPAP26 glycoforms of –Pi Arabidopsis are highly active against a wide range of P-ester substrates over a broad pH range (Veljanovski et al., 2006; Tran et al., 2010b), making AtPAP26 a promising candidate for biotechnological strategies aimed at improving crop Pi acquisition and utilization. It will thus be of interest to examine the Pi metabolism and growth characteristics of AtPAP26 overexpressors cultivated on unfertilized soil and/or with various P-esters as their sole source of exogenous P.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

For mutant isolation and routine plant growth Arabidopsis (Arabidopsis thaliana; Col-0 ecotype) seeds were sown in a standard soil mixture (Sunshine Aggregate Plus Mix 4; SunGro) and stratified at 4°C for 2 d. Plants were cultivated in growth chambers at 22°C (16/8 h photoperiod at 100 μmol m⁻² s⁻¹), and fertilized biweekly by subirrigation with 0.25× Hoagland media. To assess the influence of Pi deprivation on the development of soil-grown plants, 7-d-old seedlings were transplanted into a 75% to 85% sphagnum peat:vermiculite soil mix lacking all nutrients (Sunshine Mix 2; SunGro) at a density of four seedlings per pot. Plants were fertilized biweekly with 0.25× Hoagland media containing either 2 or 0 mM Pi. At 3 weeks post germination entire rosettes were harvested and either dried for 48 h at 65°C, or snap frozen in liquid N₂, and stored at –80°C for later analysis. Whenever Pi was reduced or eliminated from the growth medium, Pi salts were replaced with appropriate sulfate salts, so that the conjugate cation remained constant.
For liquid culture, approximately 100 seeds were surface sterilized and stratified for 2 d at 4°C, then placed in 250 mL magenta boxes containing 50 mL of 0.5% Murashige and Skoog media, pH 5.7, 1% (w/v) Suc, and 0.2 mM Pi, and cultured at 24°C under continuous illumination (100 μmol m⁻² s⁻¹) on an orbital shaker set at 80 rpm. After 7 d the seedlings were transferred into fresh media containing 1.5 or 0 mM Pi for an additional 7 d. The 14-d-old seedlings were blotted dry, snap frozen in liquid N₂, and stored at −80°C, whereas growth media containing secreted proteins was passed through a 0.45 μm filter and concentrated over 250-fold with an Amicon Ultra-15 ultrafiltration device (30,000 M, cutoff; Millipore) at room temperature (23°C).

**Mutant Isolation**

A potential atpap26 Salk_152821 mutant line was identified from the Salk T-DNA lines (Alonzo et al., 2003) via analyses of the SinGAL database (http://signal.salk.edu/cgi-bin/tdnaexpress). Seeds were obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University. Heterozygous mutant plants were isolated by PCR screening of gDNA from the T4 generation using primers mapped on Figure 1A and described in Supplementary Table S1. All PCR products were sequenced for verification (Genome Québec). Southern blotting was performed on atpap26 gDNA to determine T-DNA insert number. gDNA was isolated according to Zhang and Zeecaart (1999) except that isopropanol–precipitated nucleic acids were pelleted by centrifugation at 15,000 × g for 10 min, followed by two washes with 70% (v/v) ethanol; DNA pellets were dissolved in 10 mM Tris-HCl (pH 8.0). Gel-blot analysis was performed as described (Quan et al., 2007). The 795 bp neomycin phosphotransferase coding sequence (NPTII CDS) was used to generate a digoxigenin (dig)-labeled probe using Roche’s dig labeling kit. Southern blot hybridization was performed at 70°C for 18 h using hybridization solution (250 mM NaPi, pH 7.2, 1 mM EDTA, 20% [w/v] SDS, 0.5% [w/v] milk powder) with dig-labeled NPTII CDS probes followed by three 20 min washes at 60°C in 20 mM NaPi (pH 7.2) containing 1 mM EDTA and 1% (w/v) SDS. The blot was incubated for 90 min in 1% (w/v) milk powder and probed for 30 min with 1:5,000 anti-dig- IgG conjugated to alkaline phosphatase (Roche), followed by two 15 min washes in detection wash solution. Bands were visualized by chemiluminescence using CDP-Star (Roche).

**RNA Isolation and Semi-Quantitative RT-PCR**

Total RNA was extracted and purified as described previously (Veljanovski et al., 2006). 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. Normalization of RNA for RT was performed for each sample by density measurement of 28S ribosomal RNA bands from the above gel (scanned using ImageJ software from the National Institutes for Health). RNA (5 μg) was reverse transcribed with Superscript III (Invitrogen) and non-competitive RT-PCR was performed as described in Genndiaakis et al. (2007). Primers used to amplify AtPAP12, AtPAP17, AtPAP26, and AtACT2 were previously described (Veljanovski et al., 2006; Gregory et al., 2009). Transcripts for AtPAP12 were amplified using gene-specific primers (Supplemental Table S1); all PCR products were sequenced for verification. Conditions were optimized for all semi-quantitative RT-PCR reactions to ensure linearity of response for comparison between samples.

**Protein Extraction, APase Assays, and Determination of Protein Concentration**

Tissues were homogenized (1:2; v/w) in ice-cold extraction buffer composed of 20 mM sodium acetate (pH 5.6), 1 mM EDTA, 1 mM diethyithiolate, 1 mM phenymethylsulfonyl fluoride, 5 mM thiosurea, and 1% (w/v) insoluble polyvinyl (polypropyridrone). Homogenates were centrifuged at 4°C and 14,000g for 5 min, and the supernatants were reserved as clarified extract. APase activity was routinely measured at 25°C by coupling the hydrolysis of PEP to pyruvate to the lactate dehydrogenase reaction and continuously monitoring NADH oxidation at 340 nm using a Molecular Devices Spectromax Plus microplate spectrophotometer and the following optimized assay conditions: 50 mM Na acetate (pH 5.6), 5 mM PEP, 10 mM MgCl₂, 0.2 mM NADH, and 3 units of rabbit muscle lactate dehydrogenase in a final volume of 0.2 mL. Assays were corrected for 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 pNPP, and 10 mM MgCl₂ by monitoring the formation of para-nitrophenol at 405 nm (ε = 18.2 mΜ⁻¹ cm⁻¹). All APase assays were linear with respect to time and concentration of enzyme assayed. One unit of activity is defined as the amount of enzyme resulting in the hydrolysis of 1 μmol of substrate min⁻¹ at 25°C. Protein concentrations were determined using a modified Bradford assay (Boozato et al., 2002) with bovine γ-globulin as the standard.

**Protein Electrophoresis and Immunoblotting**

SDS-PAGE, immunoblotting onto poly(vinylidene difluoride) membranes (Immobilon; Millipore) and visualization of antigenic polypeptides using an alkaline-phosphatase-tagged secondary antibody were conducted as previously described (Veljanowski et al., 2006; Tran and Plexton, 2008). Densitometric analysis of immunoblots was performed using an LKB Ultrascan XL laser densitometer and GELSAN software (version 2.1; Pharmacia LKB Biotech). Derived A₆₀₀ values were linear with respect to the amount of the immunoblotted extract. All immunoblot results were replicated a minimum of three times, with representative results shown in the various figures. Nondenaturing PAGE was carried out using 7% separating gels (Gennidakis et al., 2007). In-gel APase activity staining was performed using the following procedure. Samples were blotted onto nitrocellulose (Schleicher & Schuell, Keene, NH) and visualized using the following antibody cocktail: 1:5000 anti-dig-IgG conjugated to alkaline phosphatase (Roche), followed by 1:5000 anti-dig-γ- glutaraldehyde conjugated to alkaline phosphatase (Roche), followed by 1:5000 anti-dig-β- steroid isopropyl phosphate (Roche). The blots were imaged using an Odyssey (LI-COR, Lincoln, NE).

**Quantification of Total and Soluble Pi**

Total Pi determinations were carried out using dried leaf tissue that had been flamed to ash by heating at 500°C for 3 h. The ash was dissolved in 30% (v/v) HCl containing 10% (v/v) HNO₃, and centrifuged at 14,000g for 10 min. The supernatant was diluted 50-fold in water and its Pi concentration quantified as previously described (Boozato et al., 2006). Diluted sample (800 μL) was mixed with 200 μL of a Pi assay reagent and incubated at 45°C for 20 min. Pi assay reagent consisted of four parts of freshly prepared 10% (v/v) ascorbic acid plus one part 10 mM ammonium molybdate containing 15 mM zinc acetate (pH 5.0). Total Pi content was measured at A₆₆₀ using appropriate Pi standards and is expressed as μmol Pi mg⁻¹ dry weight. Soluble Pi was determined by extracting frozen tissues (1:5; w/v) with 1% glacial acetic acid. Samples were centrifuged at 14,000g and supernatants assayed for Pi as described above. For available soil Pi, 0.5 g of soil was suspended in 10 mL of deionized water and the eluate removed for free Pi determination. To determine the total amount of Pi in the soil, 1 g was converted to ash as described above prior to Pi determination.

**Determination of Anthocyanin Concentration**

Frozen leaf material was transferred to extraction buffer (18% [v/v] 1-propanol containing 1% [v/v] concentrated HCl), and then the extraction vials were placed in a boiling water bath for 3 min. Extracts were centrifuged at 14,000g for 10 min and the absorbance was measured at 532 and 653 nm. Subtraction of 0.2A₅₃₂ compensated for the small overlap in A₅₃₂ by the chlorophylls (Schmidt and Mohr, 1981). Anthocyanin concentration was determined using the corrected absorbance (ε = 38,000 μM⁻¹ cm⁻¹).

**Subcellular Localization of AtPAP26-mCherry Fusion Protein**

An AtPAP26 cDNA clone (U11049) was obtained from ABRC, and amplified using PCR and appropriate oligonucleotide primers (Supplemental Table S1). The resulting PCR fragment, containing the entire open reading frame of AtPAP26, was inserted into the Xhol of pSAT4A-mCherry-N1 (ABRC) to yield pSAT4A-AtPAP26-mCherry. The plasmid, p35S-NTPP-GFP, encoding the Arabidopsis basic chitinase N-terminal signal sequence, followed by the 16-amino-acid long sweet potato (Ipomoea batatas) sporamin NTPP fused to the GFP was a gift from Christopher Trebaker and John Greenwood (University of Guelph, Canada) and was constructed in the following manner. First, the plasmid p35S-mGFP5-HDEL (provided by Jim Haseloff, University of Cambridge, United Kingdom) containing the 35S cauliflower mosaic virus promoter driving the expression of a fusion protein consisting of the Arabidopsis basic chitinase signal sequence fused to GFP and a C-terminal HDEL endoplasmic reticulum retrieval sequence (Haseloff et al., 1997) was modified (using the Quikchange PCR-based site-directed muta-
inducible tomato purple acid phosphatase isozymes. FEBS Lett 573: 51–54
Carter C, Pan S, Zouhar J, Avila EL, Girke T, Raikhel NV (2004) The vegetative vacuole proteome of Arabidopsis thaliana reveals predicted and unexpected proteins. Plant Cell 16: 3285–3303
del Pozo JC, Allona I, Rubio V, Leyva A, de la Pena A, Aragoncillo C, Paez-Ares J (1999) A type 5 acid phosphatase gene from Arabidopsis thaliana is induced by phosphate starvation and by some other types of phosphate metabolism/oxidative stress conditions. Plant J 19: 579–589
Devaiah BN, Karthikeyan AS, Raghothama KG (2007a) WRKY75 transcription factor is a modulator of phosphate acquisition and root development in Arabidopsis. Plant Physiol 143: 1789–1801
Devaiah BN, Nagarajan VK, Raghothama KG (2007b) Phosphate homeostasis and root development in Arabidopsis are synchronized by the zinc finger transcription factor ZAT6. Plant Physiol 145: 147–159
Francisca Li WY, Shao G, Lam H (2008) Ectopic expression of GnPAL3 alleviates oxidative damage caused by salinity and osmotic stresses. New Phytol 178: 80–91
Fukuda T, Saito A, Wasaki J, Shinano T, Osaki M (2007) Metabolic alterations proposed by proteome in rice roots grown under low P and high Al concentration under low pH. Plant Sci 172: 1157–1165
Guade N, Nakamura Y, Scheible W, Ohta H, Dürmann P (2008) Phospholipase C5 (NPC5) is involved in galactolipid accumulation during phosphate limitation in leaves of Arabidopsis. Plant J 56: 28–39
Gennidakis S, Rao SK, Greenham K, Uhrig RG, O’Leary BM, Snedden WA, Lu C, Plaxton WC (2007) Bacterial- and plant-type phosphoenolpyruvate carboxylase polypeptides interact in the hetero-oligomeric class-2 PEPC complex of developing castor oil seeds. Plant J 52: 839–849
Gregory AL, Hurley BA, Tran HT, Valentine AJ, She YM, Knowles VL, Plaxton WC (2009) In vivo regulatory phosphorylation of the phosphoenolpyruvate carboxylase ATPC1 in phosphate starved Arabidopsis thaliana. Biochem J 420: 57–65
Haran S, Logendra S, Seshar M, Bratanov M, Raskin I (2009) Characterization of Arabidopsis acid phosphatase promoter and regulation of acid phosphatase expression. Plant Physiol 124: 615–626
Haseloff J, Siemering KR, Prasher DC, Hodge S (1997) Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly. Proc Natl Acad Sci USA 94: 2122–2127
Hur YJ, Lee HG, Jeon EJ, Lee YY, Nam MH, Yi G, Eun MY, Nam J, Lee JH, Kim DH (2007) A phosphate starvation-induced acid phosphatase from Orgyia sativa: transcription regulation and transgenic expression. Biotechnol Lett 29: 829–835
Jin JB, Kim YA, Kim SJ, Lee SH, Kim DH, Cheong GW, Hwang I (2001) A new dynamin-like protein, ADL6, is involved in trafficking from the trans-Golgi network to the central vacuole in Arabidopsis. Plant Cell 13: 1511–1525
Karthikeyan AS, Varadarajan DK, Jain A, Held MA, Carpita NC, Raghothama KG (2006) Phosphate starvation responses are mediated by sugar signalling in Arabidopsis. Planta 225: 907–918
Kim DH, Eu YJ, Yoo CM, Kim YW, Pih KT, Jin JB, Kim SJ, Stenmark H, Hwang I (2001) Trafficking of phosphatidylinositol 3-phosphate from the trans-Golgi network to the lumen of the central vacuole in plant cells. Plant Cell 13: 287–301
Kobayashi A, Awai K, Nakamura M, Nagatani A, Masuda T, Ohta H (2009) Type-B monogalactosyldiacylglycerol synthases are involved in phosphate starvation-induced lipid remodelling and are crucial for low-phosphate adaptation. Plant J 57: 322–331
Koide Y, Hiranoh S, Matsuoka K, Nakamura K (2007) The N-terminal propeptide of the precursor to sporation acts as a vacuole-targeting signal even at the C terminus of the mature part in tobacco cells. Plant Physiol 114: 863–870
Lai F, Thacker J, Li Y, Doemer P (2007) Cell division activity determines the magnitude of phosphate starvation responses in Arabidopsis. Plant J 50: 545–556
Laine AC (1988) Significant immunological cross-reactivity of plant glycoconjugates. Electrophoresis 9: 841–844
Li D, Zhu H, Liu K, Liu X, Leggewie G, Udvardi M, Wang D (2002) Purple acid phosphatases of Arabidopsis thaliana: comparative analysis and differential regulation by phosphate deprivation. J Biol Chem 277: 27772–27781
Li K, Xu C, Li Z, Zhang K, Yang A, Zhang J (2008) Comparative proteome analyses of phosphorus responses in maize (Zea mays L.) roots of wild-

LITERATURE CITED
Alonso JM, Stepanova A, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al. (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science 301: 655–657
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 Environ 29: 303–313
Bozzo GG, Plaxton WC (2008) The role of intracellular and secreted pur-
ple acid phosphatases in tomato phosphate nutrition. In V Preddy, R Watson, eds. Tomatoes and Tomato Products. Science Publishers, Enfield, NH, pp 216–233
Bozzo GG, Raghothama KG, Plaxton WC (2002) Purification and charac-
terization of two secreted purple acid phosphatase isozymes from phosphate-starved tomato (Lycopersicon esculentum) cell cultures. Eur J Biochem 269: 6278–6286
Bozzo GG, Raghothama KG, Plaxton WC (2004a) Structural and kinetic properties of a novel purple acid phosphatase from phosphate-starved tomato (Lycopersicon esculentum) cell cultures. Biochem J 377: 419–428
Bozzo GG, Singh VK, Plaxton WC (2004b) Phosphate or phosphate addition promotes the proteolytic turnover of phosphate-starvation
type and a low-P-tolerant mutant reveal root characteristics associated with phosphorus efficiency. Plant J 55: 927–939
Li M, Qin C, Weli R, Wang X (2006) Double knockouts of phospholipases D1 and D2 in Arabidopsis affect root elongation during phosphate-limited growth but do not affect root hair patterning. Physiol Plant 140: 761–770
Lingard MJ, Gidda SK, Bingham S, Rothstein SJ, Mullen RT, Trelease RN (2008) Arabidopsis PEROX111c-e, FISISSION1b, and DYNAMIN-RELATED PROTEIN3A cooperate in cell cycle-associated replication of peroxisomes. Plant Cell 20: 1567–1585
Ma XF, Wright E, Ge Y, Bell J, Xu Y, Bouton JH, Wang ZY (2009) Improving phosphorus acquisition of white clover (Trifolium repens L.) by transgenic expression of plant-derived phytase and acid phosphatase genes. Plant Sci 176: 479–488
Matsuoka K, Nakamura K (1991) Peptide of a precursor to a plant vacuolar protein required for vacuolar targeting. Proc Natl Acad Sci USA 88: 834–838
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 Physiol 127: 594–606
Missen J, Raghorthama KG, Jain A, Jouhet J, Block MA, Bligny R, Ortel P, Creff A, Somerville S, Doumas P, et al. (2005) A genome-wide transcriptional analysis using Arabidopsis thaliana Affymetrix gene chips determined plant responses to phosphate deprivation. Proc Natl Acad Sci USA 102: 11934–11939
Müller R, Nilsson L, Krinitel C, Nielsen TH (2004) Gene expression during recovery from phosphate starvation in roots and shoots of Arabidopsis thaliana. Physiol Plant 122: 233–243
Quan R, Lin HM, Mendoza I, Zhang Y, Cao W, Yang Y, Shan M, Chen S, Pardo JM, Guo Y (2007) SCABFS/CBL10, a putative calcium sensor, interacts with the protein kinase SOS2 to protect Arabidopsis shoots from salt stress. Plant Cell 19: 1415–1431
Rubio V, Linhares F, Solano R, Martin AC, Iglesias J, Leyva A, Paz-Ares J (2001) A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. Genes Dev 15: 2122–2133
Schmidt R, Mohr H (1981) Time-dependent changes in the responsiveness to light of phytochrome-mediated anthocyanin synthesis. Plant Cell Environ 4: 433–437
Shin H, Shin HS, Dewbre GR, Harrison MJ (2004) Phosphate transport in Arabidopsis: Pht1;1 and Pht1;4 play a major role in phosphate acquisition from both low- and high-phosphate environments. Plant J 39: 629–642
Tomscha JL, Trull MC, Deikman J, Lynch JP, Guijtinan MJ (2004) Phosphatase under-producer mutants have altered phosphorus relations. Plant Physiol 135: 334–345
Tran HT, Hurley BA, Plaxton WC (2010a) Feeding hungry plants: the role of purple acid phosphatases in phosphate nutrition. Plant Sci 179: 14–27
Tran HT, Plaxton WC (2008) Proteomic analysis of alterations in the secretome of Arabidopsis thaliana suspension cells subjected to nutritional phosphate deficiency. Proteomics 8: 4317–4326
Tran HT, Qian W, Hurley BA, She Y, Wang D, Plaxton WC (2010b) Biochemical and molecular characterization of AtPAP12 and AtPAP26: the predominant purple acid phosphatase isozymes secreted by phosphate-starved Arabidopsis thaliana. Plant Cell Environ (in press)
Vance CP, Uhde-Stone C, Allan DL (2003) Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. New Phytol 157: 423–447
Veljanovski V, Vanderbeek 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 Physiol 142: 1282–1293
Wang X, Wang Y, Tian J, Lim BL, Yan X, Liao H (2009) Overexpressing AtPAP15 enhances phosphorus efficiency in soybean. Plant Physiol 151: 233–240
Wu P, Ma L, Hou X, Wang M, Wu Y, Liu F, Deng X (2003) Phosphate starvation triggers distinct alterations of genome expression in Arabidopsis roots and leaves. Plant Physiol 132: 1260–1271
Xiao K, Katagi H, Harrison M, Wang ZY (2006) Improved phosphorus acquisition and biomass production in Arabidopsis by transgenic expression of a purple acid phosphatase gene for M. trunculata. Plant Sci 170: 191–202
Zakhleniuk OV, Raines CA, Lloyd JC (2001) pho3: a phosphorus-deficient mutant of Arabidopsis thaliana (L.) Heynh. Planta 212: 529–534
Zhang HX, Zeevaart JAD (1999) An efficient transformation protocol for Arabidopsis thaliana (L.) Heynh. Plant J 17: 212–220
Zhang H, Zeevaart JAD (1999) An efficient Agrobacterium tumefaciens mediated transformation and regeneration system for cotyledons of spinach (Spinacia oleracea L.). Plant Cell Rep 18: 640–645
Zhang W, Gruszewski HA, Chevone BI, Nessler CI (2008) An Arabidopsis purple acid phosphatase with phytase activity increases foliar ascorbate. Plant Physiol 146: 431–440
Zhu H, Qian W, Xuzhong L, Li D, Liu X, Liu K, Wang D (2005) Expression patterns of purple acid phosphatase genes in Arabidopsis organs and functional analysis of AtPAP23 predominantly transcribed in flower. Plant Mol Biol 59: 581–594
Zimmerman P, Regrierer B, Kossmann J, Amrhein N, Bucher M (2004) Differential expression of three purple acid phosphatases from potato. Plant Biol 6: 519–528