FEEDING HUNGRY PLANTS:
THE SECRETED PURPLE ACID PHOSPHATASE
ISOZYMES AtPAP12 AND AtPAP26 PLAY A PIVOTAL ROLE IN
EXTRACELLULAR PHOSPHATE SCAVENGING IN
ARABIDOPSIS THALIANA

by

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Abstract

Orthophosphate (Pi) is a limiting macronutrient in most soils and is essential for plant metabolism. Massive amounts of Pi-fertilizers are applied to agricultural fields to compensate for this limitation. However, Pi-fertilizers are made from non-renewable rock Pi-sources and their application is environmentally destructive. Plants have evolved numerous ways to survive in Pi-deficient (-Pi) soils, including the upregulation and secretion of acid phosphatases (APases). APases catalyze the hydrolysis of phosphate (Pi) from Pi-esters in an acidic environment. The major group of plant secreted APases, purple acid phosphatases (PAPs), have been hypothesized to scavenge Pi from organic-Pi (Po) sources that can compose up to 80% of the total P-content of some soils. Previous biochemical and proteomic studies indicate that AtPAP26 and AtPAP12 are the predominant secretory PAP isozymes upregulated by –Pi Arabidopsis thaliana cell cultures and seedlings. This thesis examines the influence of different Po supplements on the growth, Pi content, secretory APase activity, and secreted AtPAP12 and AtPAP26 polypeptides of wildtype (Col-0) Arabidopsis seedlings. Additionally, this thesis assesses the potential role that AtPAP12 and AtPAP26 play in scavenging Pi from extracellular Po sources by utilizing a homozygous atpap12/atpap26 double knockout mutant. Loss of AtPAP26 and AtPAP12 expression resulted in a 64% decrease in root secreted APase activity of –Pi seedlings. These results corroborate previous findings implying that: (i) Arabidopsis are able to grow on a variety of extracellular Po sources as their sole source of P-nutrition, and (ii) AtPAP12 and AtPAP26 are the principal contributors to secreted APase activity of –Pi Arabidopsis. Total shoot Pi levels, and growth of atpap12/atpap26 Arabidopsis seedlings cultivated in -Pi/+Po media were significantly lower relative to Col-0 controls, but unaffected under Pi sufficient conditions. The atpap12/atpap26 seedlings were unable to grow in a –Pi/+Po soil, whereas the Col-0 seedlings were able to develop. Additionally, both PAPs were strongly upregulated on root surfaces and in shoot cell wall extracts of –Pi seedlings. Taken together, these results strongly suggest that AtPAP12 and AtPAP26 play an important role in the hydrolysis of Pi from extracellular Po and make a large contribution to Pi-recycling and scavenging in –Pi Arabidopsis.
Co-Authorship

With the exception of the literature review and general discussion, all chapters were co-authored with Dr. Plaxton. The work presented in this thesis was derived from collaboration with several important colleagues. Dr. Hue Tran was responsible for crossing the single mutants to generate a homozygous *atap12/atap26* double mutant and provided the results in Appendix 1. Dr. Joonho Park made the *AtPAP12:GUS* and *AtPAP26:GUS* constructs and carried out the confocal microscopy in Fig. 2.3A. Mr. Hernan Del Vecchio and Ms. Jacqui Zins helped perform the cell wall extractions of the seedlings (Fig. 2.3 B, C). Drs. Ketan Patel and Tom McKnight provided the *atap12* single mutant and the anti-AtPAP12 serum.
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## Abbreviations

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
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<tr>
<td>APase</td>
<td>Acid phosphatase</td>
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<tr>
<td>AtPAP</td>
<td><em>Arabidopsis thaliana</em> purple acid phosphatase</td>
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<tr>
<td>β-napthyl-P</td>
<td>Beta-napthyl-phosphate</td>
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<tr>
<td>BCIP</td>
<td>5-Br-4-Cl-3-indoly phosphate</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Linear Alignment Search Tool</td>
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<tr>
<td>CBB</td>
<td>Coomassie brilliant blue</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>Col-0</td>
<td><em>Arabidopsis thaliana</em> ecotype Columbia, accession 0</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>(ELF)-97</td>
<td>Enzyme-labelled florescent</td>
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<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
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<tr>
<td>G1P</td>
<td>Glucose-1-phosphate</td>
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<td>G3P</td>
<td>Glycerol-3-phosphate</td>
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<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
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<tr>
<td>Glc-6-P</td>
<td>Glucose-6-phosphate</td>
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<tr>
<td>HMW</td>
<td>High molecular weight</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>kD</td>
<td>Kilodalton</td>
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<tr>
<td>LMW</td>
<td>Low molecular weight</td>
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</table>
miRNA  micro RNA

Mr  molecular mass

MS  Mass Spectrometry

PAGE  polyacrylamide gel electrophoresis

PAP  purple acid phosphatase

Po  organic-Pi

PEP  phosphoenolpyruvate

PEPC  phosphoenolpyruvate carboxylase

Pi  inorganic phosphate

+Pi and –Pi  Pi sufficient and Pi deficient

pNPP  para-nitrophenyl phosphate

PPI  pyrophosphate

PSI  phosphate starvation inducible

PSR  phosphate starvation response

PVDF  poly(vinylidene difluoride)

RNase  ribonuclease

T-DNA  transferred DNA

U  unit

X-Gluc  5-Br-4-Cl -3-indolyl glucuronide
Chapter 1
Introduction and Literature Review

1.1 General Introduction

Orthophosphate (Pi) plays a central role in a wide range of essential metabolic processes in plants such as photosynthesis and respiration. However, in most natural soils Pi is limited and the soluble Pi concentration ranges from about 1-10 μM. This concentration is considered sub-optimal for plant growth and development. In the agricultural industry, Pi limitation negatively impacts crop production. In order to maximize the productivity of modern crop varieties, large quantities of Pi-fertilizers are applied in massive amounts to increase yields. Pi-fertilizers are produced from non-renewable rock–Pi reserves and it is predicted that within the next 80 years, these reserves will be completely depleted (Vance et al., 2003). Additionally, the application of Pi-fertilizers is environmentally destructive. It is estimated that over 40 million metric tons of Pi-fertilizers are applied each year of which only 20% is utilized by crops. Some of the remaining Pi-fertilizer may runoff into nearby lakes and streams and cause aquatic eutrophication, blooms of toxic cyanobacteria, and other environmentally destructive processes (Vance et al., 2003). The ability to bioengineer Pi-efficient transgenic crops could play a central role in creating sustainable agricultural production that requires less Pi-fertilizer. Designing effective biotechnological strategies to enhance crop Pi acquisition requires a solid understanding of the complex adaptations that Pi-deficient (-Pi) plants have evolved as well as the mechanisms that regulate these adaptations.

1.2 Literature Review

1.2.1 Soil phosphate nutrition
Plant cells can only assimilate exogenous P in its fully oxidized state, $\text{H}_2\text{PO}_4^-$ or $\text{HPO}_4^{2-}$. Globally, soil is the second largest storage pool of Pi (Filippelli, 2008). Despite the high abundance of Pi in soil, Pi is considered one of the least accessible macronutrients due to its affinity to form insoluble complexes with iron and aluminium oxides in acidic soils, rendering it inaccessible for root uptake. Phosphorus deposits in soil are a mix of mineralized Pi, organic Pi (Po), and soluble Pi (Stewart and Tiessen, 1987).

Up to 80% of soil P reserves exist as Po, mainly in the form of Pi-esters (Vance et al., 2003; Fang et al., 2009; Richardson et al., 2009a; Richardson et al., 2009b). Po consists of Pi covalently bound to organic molecules via ester bonds, such as glycerol-3-phosphate (G3P), nucleic acids and various forms of inositol phosphate (IP) including inositol hexaphosphate (IHP, phytic acid) (Stewart and Tiessen, 1987). It is thought that up to 50% of the Po in some soil is IP because it is produced from decaying plant tissues. Currently, there is a poor understanding of the exact Po pools found in soil and which of these pools are accessible to plants. This gap in the literature can mostly be attributed to the complex extractions and harsh chemical conditions required for Pi dissolution and precipitation in addition to the complex nature of the soil environment (Hayes et al., 2000; Richardson, 2001). The rhizosphere is a very active environment with constant fluctuations in the type of P forms present.

Much of the recent research on Po availability has investigated the ability of IHP to act as a nutritional P source for various plant species, including Arabidopsis and tobacco (Hayes et al., 2000; Richardson, 2001; Priya and Sahi, 2008). Fungi, bacteria and some plants have phytases that are able to hydrolyse Pi from IHP. However, studies in general agree that IHP is not able to sustain plant growth as the sole source of nutritional P (Richardson et al., 2001; Mudge et al., 2003; Lung et al., 2005; Priya and Sahi, 2008). Transgenic technologies could potentially
improve the use of IHP as a P source of important crop species by introducing one or more of these known phytase genes (Richardson et al., 2001; Mudge et al., 2003; Lung et al., 2005; Lung and Lim, 2006). *Medicago truncatula* phytase *MtPHY1* expressed in *Arabidopsis* helped to significantly improve the plants` ability to acquire Pi (Xiao et al., 2006). *Arabidopsis* expressing *Bacillus subtilis* β-propeller phytase exhibited enhanced extracellular phytase activity and an increase in Pi uptake from IHP (Lung et al., 2005). *Aspergillus niger* phytase genes expressed in *Arabidopsis* resulted in enhanced phytase activity and a significant increase in plant growth, comparable to that of +Pi seedlings, on IHP supplemented media (Richardson et al., 2001).

However, studies of Po nutrition have not been strictly limited to IHP. *Duo festulolium* has been grown on IHP, ATP, AMP, and glucose-1-P and analyzed for growth development (Priya and Sahi, 2008). In comparison to +Pi controls, G1P and ATP were able to sustain near optimal growth of the duo grass, whereas growth on AMP and IHP was sub-optimal. There was an increase in both acid phosphatase (APase) activity and phytase activity when these plants were grown on Po sources. This observed increase in APase activity was a novel finding because APase activity had previously been predominantly associated with Pi-stress, not Po growth.

White lupin (*Lupinus albus* L.) grown with G3P and phytate as its sole source of P nutrition displayed a significant increase in APase activity in root exudates (Gilbert et al., 1999). Furthermore, growth of *Arabidopsis* seedlings cultivated on agar plates containing DNA/RNA (without any free Pi) was comparable to that of +Pi Col-0 *Arabidopsis* seedlings (Chen et al., 2000). Interestingly, despite the +Pi growth of the shoots, the roots of these seedlings displayed characteristics of –Pi seedlings such as decreased shoot growth and increased secondary root growth.

**1.2.2 Phosphate stress response**
For the most part, current literature focuses on plants grown in +Pi and –Pi conditions with little focus on Po growth. In particular, research in this field has centered on the complex array of morphological, physiological and biochemical adaptations certain plant species have evolved in order to grow in -Pi soil collectively known as the Pi-starvation response (PSR).

The PSR induces hundreds of Pi-starvation inducible (PSI) genes that help to reprioritize internal Pi use and maximize external Pi acquisition (Hammond et al., 2004; Ticconi and Abel, 2004; Raghothama and Karthikeyan, 2005; Fang et al., 2009). Many elements of the PSR are controlled at the transcriptional level and certain –Pi plants are able to remodel their transcriptome to help them survive Pi-stress conditions. PSI gene expression is highly regulated and controlled in a tissue specific manner (Yuan and Liu, 2008). Only a 25% overlap exists between the genes that are specifically induced in the root and shoot of Arabidopsis. Genes are not strictly induced during PSR; approximately 250-700 genes have decreased transcript accumulation in response to Pi-stress. Again, tissue specificity plays a key role in the repression response with only a 5-10% overlap in repressed gene expression between roots and shoots (Wu et al. 2003; Misson et al., 2005; Morcuende et al., 2007). Transcriptional responses to Pi starvation have a temporal component as well. Some genes are immediately induced by -Pi conditions. These genes are often involved in general stress response, rather than being Pi starvation-specific. On the other hand, some genes can be switched on after prolonged exposure to Pi-stress and are thought to be more specific to the PSR.

Along with changes in the transcriptome, the proteome can also be remodelled during the PSR. An example of PSR proteome remodelling in plants is found in the secretome of -Pi Arabidopsis thaliana suspension cell cultures (Tran and Plaxton, 2008). A total of 18 proteins were upregulated and secreted in –Pi conditions while 6 proteins exhibited downregulation.
Secreted PSR proteins upregulated by the –Pi cells included a ribonuclease (RNase) and a variety of enzymes involved in cell wall modification, proteolysis, pathogen responses, and reactive oxygen species metabolism.

Additionally, the PSR is regulated at a post-transcriptional level. Numerous studies have found that an increase in transcript abundance during Pi-stress does not necessarily translate into an increase in protein accumulation and *vice versa*. This has been shown in proteomic profiling of –Pi corn, rice and *Arabidopsis* (Fukuda *et al.*, 2007; Li *et al.*, 2007; Li *et al.*, 2008, Tran and Plaxton, 2008). Phosphorylation and differential glycosylation are two important post-translational modifications for the control and subcellular targeting of diverse enzymes upregulated by -Pi plants. An example of the important role that post-translational modifications play in regulating PSR proteins can be seen in the examination of phosphoenolpyruvate carboxylase (PEPC) (Duff *et al.*, 1989; Johnson *et al.*, 1996; Udhe-Stone *et al.*, 2003; Vance *et al.*, 2003; Plaxton and Podesta, 2006; Fang *et al.*, 2009; Gregory *et al.*, 2009). The PEPC isozyme AtPPC1 is induced during Pi-stress and has been suggested to provide a metabolic bypass to the ADP-limited cytosolic pyruvate kinase and to increase the synthesis of organic acids such as malic acid and citric acid. PEPC requires *in vivo* phosphorylation in order to increase PEPC activity and help *Arabidopsis* acclimate to –Pi conditions (Gregory *et al.*, 2009). Therefore, it follows that one of the most responsive PSI *Arabidopsis* transcripts include those encoding both PEP carboxylase protein kinase isozymes (AtPPCK1 and AtPPCK2) (Misson *et al.*, 2005; Gregory *et al.*, 2009).

*Morphological adaptations of –Pi plants*
Plants under Pi-stress undergo numerous morphological adaptations to help them survive. A greater Pi absorption from –Pi soil can be achieved by increasing the root’s surface area (Lopez-Bucio et al., 2002). Plants achieve this goal by increasing their root:shoot growth ratio and altering the root architecture by increasing lateral root growth and the number of root hairs to allow for greater topsoil Pi-scavenging (Linkohr et al., 2002; Lopez-Bucio et al., 2002). For example, –Pi Arabidopsis produce elongated root hairs and a five-fold increase in root hair density (Yuan and Liu, 2008). About 90% of all plant species (excluding Cheonpodiaceae, Cruciferae, Cyperaceae, Junaceae, and Proteaceae families) are able to increase Pi acquisition by forming symbiotic associations between their roots and beneficial mycorrhizal fungi such as soil-inhabiting fungi of the order Glomales (Bolan, 1991). This association increases the length and surface area of their roots and enables plants to exploit a larger volume of soil, thereby increasing the uptake of Pi. However, current agricultural practices such as soil tilling disrupt these beneficial mycorrhizal associations.

Many non-mycotrophic plants such as buckwheat (Fagopyrum esculentum), white lupin (Lupinus albus), and harsh hakea (Hakea prostrata) are also able to increase Pi acquisition in –Pi soil through the formation of proteoid roots. Proteoid roots are clusters of short lateral roots that can absorb Pi at a faster rate than non-proteoid roots, thus enhancing Pi uptake (Vance et al., 2003; Grennan, 2008). As a member of the Cruciferae, Arabidopsis does not form mycotrophic associations and is thus an ideal model species for studying the molecular and biochemical adaptations of -Pi plants.

*Biochemical adaptations of –Pi plants*
In conjunction with morphological adaptations, plants are able to utilize a variety of biochemical adaptations to grow in –Pi conditions. During the initial stages of Pi-stress, there is a significant decrease in intracellular Pi concentration of plant cells. In order to maintain cytoplasmic Pi homeostasis, the vacuole is able to decrease its Pi supply in which up to 95% of the cell’s total Pi is stored (Plaxton, 2004). During Pi-stress, there is a decrease in adenylates levels which can be rectified by an upregulation of alternative pathways for cytosolic glycolysis, mitochondrial electron transport, and tonoplast H⁺ pumping to facilitate respiration and vacuolar pH maintenance (Plaxton, 2004; Plaxton and Podesta, 2006; Plaxton and Tran, 2011).

Plants can also switch to using pyrophosphate (PPi) for cellular work in order to conserve ATP and recycle Pi. Glycolytic bypass enzymes such as PPI-dependent phosphofructokinase and PEPC can be switched on to promote intracellular Pi recycling, as Pi is a byproduct of their reactions (Plaxton and Podesta, 2006; Plaxton and Tran, 2011). Enhanced activity of PEPC, malate dehydrogenase and citrate synthase allows for an increase in the synthesis of organic acids from glycolytic metabolites. This is critical for the replenishment of tricarboxylic cycle intermediates during Pi-stress, as well as the root excretion of release of organic acids (which are dissociated into their anionic forms at cytoplasmic pH) to help solubilise mineralized-P (Johnson et al., 1996; Udhe-Stone et al., 2003; Vance et al., 2003; Plaxton and Podesta, 2006; Fang et al., 2009). Other adaptations observed under –Pi conditions include anthocyanin accumulation in the shoots of -Pi plants, which is assumed to protect chloroplasts against photoinhibition (Plaxton, 2004; Yuan and Liu, 2008; Fang et al., 2009). High-affinity Pi transporters of the plasma membrane are also upregulated in times of Pi-stress. This upregulation allows the active assimilation of Pi against a steep concentration gradient as the soil Pi concentration can be 10,000-fold lower than that of root cell cytoplasm (Raghothama and Karthikeyan, 2005). High-
affinity Pi transporters of *Arabidopsis* belong to the nine member PHT1 family and consist of Pi/H+ symporters with 12 transmembrane domains (González et al., 2005). *Arabidopsis* Pht1;4 and Pht1;1 have been shown to be the two major Pi transporters involved in Pi uptake (Mudge et al., 2003; Shin et al., 2004). Consistent with this, knockout mutants of Pht1;4 or Pht1;1 show a significant decrease Pi acquisition during –Pi conditions (Poirier et al., 1991; Shin et al., 2004; Stefanovic et al., 2007).

One of the more studied biochemical adaptation of –Pi plants is the increase in secreted PSI-hydrrolases such as phospholipases, RNases, and APases that help to scavenge Pi from Pi-monoesters (Duff et al., 1994; Bariola et al., 1999; Abel et al., 2000; Plaxton, 2004; Raghothama and Karthikeyan, 2005; Fang et al., 2009). Genes encoding phospholipases are upregulated during Pi-stress and those involved in phospholipid biosynthesis are downregulated (Wu et al., 2003; Misson et al., 2005; Tjellström et al., 2008; Morcuende et al., 2007). The induction of phospholipases during –Pi growth is beneficial because it causes the replacement of membrane phospholipids with amphipathic sulfolipids and galactolipids (Fang et al., 2009; Kobayashi et al., 2009; Okazaki et al., 2009). This replacement allows phospholipids to serve as a P-reserve during Pi starvation. Transgenic *Arabidopsis* seedlings with a phospholipase mutation display a significant decrease in growth in –Pi conditions (Gaude et al., 2008; Kobayashi et al., 2009). In addition to phospholipase induction, RNases, phosphodiesterases, and APases are induced and can be secreted by roots and suspension cell cultures of -Pi plants (Abel et al., 2002; Plaxton, 2004; Fang et al., 2009; Tran et al., 2010). For example, *Arabidopsis* RNase-1 (RNS1) transcripts and secreted protein levels are highly induced in response to Pi-stress (Bariola et al., 1999; Abel et al., 2000; Ticconi and Abel, 2004).
1.2.3 Plant acid phosphatases

The upregulation of intracellular and secreted APase activity has long been recognized as a universal biochemical response to plant Pi deprivation (Duff et al., 1994). APases catalyze the hydrolysis of Pi from a broad range of Pi-esters at an acidic pH (Fig. 1.1). They are expressed in a variety of tissues and species. The majority of plant APases display non-specific substrate selectivity and function in the production, transport, and recycling of Pi (Duff et al., 1994). Plant APases also display considerable variation in terms of native and subunit weight, metal inhibition or activation, subcellular localization, cofactor requirements, pH optima, substrate specificity, and expression determinants (Duff et al., 1994). Intracellular APases are ubiquitous within plant tissues, tend to have a pH optima of 5-6, and are thought to help remobilize and recycle Pi from expendable intracellular Pi-monoesters. Extracellular APases belong to a group of PSI-hydrolases secreted by -Pi plants. In the rhizosphere, they hydrolyze Pi from external Po sources (Ticconi and Abel, 2004; Fang et al., 2009). Induction of intracellular and secreted APase activity has been correlated with de novo APase synthesis in several -Pi plants, including Brassica nigra, tomato, and Arabidopsis suspension cells and seedlings (Duff et al., 1994; Bozzo et al., 2006; Tran et al., 2010a).

Purple acid phosphatases

The nomenclature of purple acid phosphatases (PAPs) originates from their purple or pink colour in solution and their insensitivity to tartrate inhibition (Olczak et al., 2003). The distinctive colour of PAPs is due to a charge transfer transition at about 560 nm from the metal-coordinating tyrosine to the metal ligand Fe(III) (Olczak et al., 2003). All PAPs contain five
Figure 1.1. Model showing the intracellular and extracellular roles of APases in plant cells. Intracellular and extracellular APases are highly induced in response to Pi starvation. APases catalyze the hydrolysis of Pi from Pi-ester found in Po. Shown above is an example of secreted APases liberating Pi from two different Po sources. At the top, glycerol-3-phosphate (G3P) is being hydrolyzed into glycerol and Pi by a secreted APase. At the bottom, RNA is being broken down by an RNase and a cyclic nucleotide phosphodiesterase before being hydrolyzed by an APase. In both cases, Pi is absorbed into the cell by high-affinity Pi-transporters. There is no evidence to support direct import of Po substrates into plant cells, although Po uptake followed by hydrolysis within the apoplast may occur.

blocks of conserved metal ligating residues, although the location, number, and identity of the residues differ between the family groups (Li et al., 2002; Olczak et al., 2003). Members of the PAP family contain seven metal ligating residues: DxG-DXXY-GNH(D/E)-VXXH-GHXH; (bold letters indicate metal ligating residues, dashes indicate separation between blocks), which form dimetallic active sites (Li et al., 2002, Olczak et al., 2003). Plant PAPs typically contain a Fe(III)-X(II) active site where X is either Zn$^{2+}$ or Mn$^{2+}$ (Klabunde et al., 1995; Olczak et al., 2003).
The structure of PAP catalytic sites and domains are highly conserved (Klablunde et al., 1995; Olczak et al., 2003). Bacterial, mammalian, and plant PAPs all contain catalytic domains that consist of two sandwiched β-α-β-α-β motifs, which exhibit almost perfect alignment and order of the conserved metal ligating residues (Klablunde et al., 1995, Schenk et al., 2000; Li et al., 2002). Plants possess high molecular weight (HMW) oligomeric PAPs composed of 50-60 kD subunits consisting of an N-terminal non-catalytic domain fused to a C-terminal catalytic domain (Klablunde et al., 1995; Olczak et al., 2003). Although most HMW PAPs appear to exist as homodimers (Duff et al., 1989; Gellatly et al., 1994; Olczak et al., 2003; Olczak and Watorek, 2003; Veljanovski et al., 2006) several HMW PAPs secreted by -Pi plant cells also appear to exist as monomers (Bozzo et al., 2002, Tran et al., 2010b). Dimeric HMW PAPs form either through disulfide bridges or via non-covalent interactions (Olczak et al., 2003; Olczak and Watorek, 2003). Low molecular weight (LMW) plant PAPs are substantially smaller in size compared with HMW plant PAPs (Schenk et al., 2000). Homology modeling shows that LMW plant PAPs lack the equivalent of the NH₂-domain and are hence structurally similar to mammalian PAPs (Schenk et al., 2000).

**Phosphate stress response and purple acid phosphatases**

Pi starvation may cause an upregulation or downregulation of specific PAP expression (Haran et al., 2000; Li et al., 2002; Zimmerman et al., 2004; Misson et al., 2005; Bozzo et al., 2006). During Pi-stress, some PAPs are switched on using transcriptional control and transcription factors while others are controlled post-transcriptionally. Resupplying -Pi plants with Pi has been shown to switch off PSI PAP genes and switch on proteases to break down PSI PAPs (Bozzo et al., 2004; Müller et al., 2004; Veljanovski et al., 2006). Several intracellular and
secreted PAP isozymes have been biochemically characterized in species such as tomato (Bozzo et al., 2002; Bozzo et al., 2004), lupin (Olczak and Watorek, 2003), bean (Liang et al., 2010), and Arabidopsis (del Pozo et al., 1999; Veljanovski et al., 2006; Tran et al., 2010a). Tomato suspension cell cultures grown in –Pi conditions had three main APases upregulated. Two of these PSI PAPs are secreted into the rhizosphere, as 84 and 57 kD monomers. The third PSI PAP is a novel heterodimer of 142 kD composed of an equivalent ratio of 63 and 57 kD subunits and is most likely localized to the cell vacuole (Bozzo et al., 2002; Bozzo et al., 2004).

White lupin secretes copious amounts of APase activity from its proteoid roots when cultivated under -Pi conditions (Miller et al., 2001). One of the more strongly induced APases in white lupin is a glycosylated homodimer composed of 70 kD subunits exhibiting a 63% amino acid sequence identity with AtPAP12. Yellow lupin (Lupinus luteus) secretes from its roots an ortholog to AtPAP26 during times of Pi-stress (Olczak and Watorek, 2003). –Pi bean plants (Phaseolus vulgaris) contain a PvPAP3 in both its shoots and roots (Liang et al., 2010). This 34 kD monomeric PAP had a broad pH-activity profile and was insensitive to tartrate. Its deduced amino acid sequence was highly similar to other PAPs, with AtPAP8 being its closest relative in Arabidopsis.

Arabidopsis thaliana purple acid phosphatases

The Arabidopsis PAP (AtPAP) family is composed of 29 putative PAPs. AtPAPs are classified into three distinct phylogenetic groups according to their deduced amino acid sequences (Li et al., 2002) (Fig. 1.2). Groups I and II are comprised of oligomeric HMW AtPAPs, with group I consisting of PAPs of slightly smaller monomer size than group II. Group III consists of the monomeric low molecular weight mammalian-like AtPAPs. Transcript
profiling of the AtPAP family revealed that while most are expressed in all tissues, seven members are predominantly expressed in flower tissue (Zhu et al., 2005).

AtPAPs have generally been localized to the cell vacuole (AtPAP26) (Carter et al., 2004; Veljanovski et al., 2006), the plastid and mitochondria (AtPAP2) (Sun et al., 2012), the cell wall (AtPAP10, AtPAP12, AtPAP25, AtPAP26) (Irshad et al., 2008; Wang et al., 2011; Del Vecchio et al., unpublished), and the secretome (AtPAP12, AtPAP26) (Haran et al., 2000; Tran et al., 2010). AtPAPs display non-specific substrate specificity and are capable of hydrolyzing Pi from ATP, para-nitrophenyl phosphate (pNPP), and PEP (del Pozo et al., 1999; Haran et al., 2000, Zhu et al., 2005; Veljanovski et al., 2006; Wang et al., 2009, Tran et al., 2010). The subcellular location or function of most of the other AtPAPs remains largely unknown.

AtPAP2, AtPAP15 and AtPAP23 have all been studied and have not been found to be directly involved with the PSR (Olczak et al., 2003; Fang et al., 2009; Sun et al., 2012). AtPAP2 is highly expressed in siliques, stems, flowers, roots and senescing leaves, but its expression is relatively low in leaves and mature seeds (Sun et al., 2012). AtPAP2 is constitutively expressed at the protein level, and its expression does not change under Pi-stress. AtPAP2 has a unique C-terminal hydrophobic motif in Arabidopsis that is required for its localization and dual-targeting to both the plastids and mitochondria (Sun et al., 2012). Overexpression of AtPAP2 resulted in higher seed yield. Metabolite analysis showed that the shoots of AtPAP2 overexpression lines contained higher levels of sugars and tricarboxylic acid metabolites. AtPAP2 is the first PAP shown to modulate carbon metabolism and the first shown to be dual-targeted to both plastids and mitochondria by a C-terminal targeting signal.

AtPAP15:GUS fusion protein showed strong GUS staining at the early stages of seedling growth and pollen germination (Wang et al., 2009). AtPAP15 displays a greater degree of amino
acid identity with soybean (*Glycine max; GmPHY*) and tobacco (*Nicotiana tabacum*) PAP (NtPAP) than the other AtPAPs and is currently the only AtPAP known to possess phytase activity. As discussed in section 1.2.1, IHP is a major component of the soil. Additionally, IHP is the primary Pi storage reserve in seeds. *AtPAP15* T-DNA insertion lines exhibited lower phytase and APase activity in germinating seeds and pollen and lower pollen germination rate compared to Col-0. Constitutive overexpression of *AtPAP15* containing an extracellular targeting peptide in soybean plants significantly improved the growth and P efficiency of the resulting transformants when cultivated on sand containing phytate as the sole source of external P (Wang *et al.*, 2009), confirming the phytase activity of AtPAP15. AtPAP15 not regulated by Pi-stress and therefore is not thought to play a significant role in Pi acquisition except in mobilizing Pi from IHP in germinating pollen and seeds.

*AtPAP23* is highly transcribed in flowers. *AtPAP23*:GUS showed high expression in flower apical meristems, but became restricted to petals and anther filaments in fully developed flowers (Zhu *et al.*, 2005). AtPAP23 displays a high APase activity; however its function is still unknown. T-DNA knock-out and overexpression lines of *AtPAP23* were indistinguishable from Col-0 plants in the development of flowers (or other organs).

*Arabidopsis purple acid phosphatases induced during Pi-stress*

Many PSI induced AtPAPs have been characterized and studied. AtPAP10 is a PSI APase predominantly associated with the root surface (Wang *et al.*, 2011). AtPAP10 exhibits activity against a broad variety of substrates. Expression of AtPAP10 is specifically induced by Pi limitation at the transcript and protein level. Functional analyses of multiple *atpap10* mutant
alleles and overexpressing lines indicated that AtPAP10 plays a role in helping *Arabidopsis* acclimate to Pi-stress.

AtPAP17 was purified and sequenced and found to be strongly induced by Pi-stress. AtPAP17 displays high peroxidase activity and APase activity. GUS analysis showed that it is not only responsive to –Pi conditions but also to ABA and salt stress. It is also highly expressed in senescing leaves and during oxidative stress (del Pozo *et al.*, 1999).

AtPAP25 was purified from the cell wall of -Pi *Arabidopsis* suspension cells (Del Vecchio *et al.*, unpublished). Biochemical characterization of AtPAP25 showed that it is a monomeric protein containing three highly complex NXS/T glycosylation motifs. These motifs are hypothesized to play a role in AtPAP25 signalling. Immunoblot and semi-quantitative RT-PCR transcript analysis showed that AtPAP25 is *de novo* synthesized under –Pi conditions.

Enzyme activity assays and immunoblotting revealed a pair of secreted AtPAP isoforms in –Pi *Arabidopsis* suspension cell cultures (Tran and Plaxton, 2008). These two isoforms were later found to be secreted by –Pi *Arabidopsis* seedlings as well and were biochemically characterized (Tran *et al.*, 2010). One of the two isoforms was identified as AtPAP12. AtPAP12 is a homodimer composed of a 60-kD subunit and exhibits a broad pH-activity profile and broad substrate specificities. Semiquantitative RT-PCR of seedling mRNAs showed that *AtPAP12* transcripts are upregulated in roots and shoots of –Pi *Arabidopsis*. *AtPAP12* transcripts correlated well with relative levels of secreted AtPAP12 protein (Haran *et al.*, 2000; Tran *et al.*, 2010).

The other secreted AtPAP isoform discovered in both –Pi suspension cell cultures and secreted seedling media of *Arabidopsis* was identified as AtPAP26. AtPAP26 was first discovered as a vacuolar APase that accumulates during Pi-stress of *Arabidopsis* suspension cells.
Figure 1.2. A classification scheme for *Arabidopsis* PAPs based on clustering analysis of amino acid sequences. The clustering analysis used amino acid sequences of 19 predicted purple acid phosphatases and those of 10 PAPs (AtPAP3, AtPAP7–AtPAP13, AtPAP17, AtPAP18) derived from cDNA analysis. The main groups (groups I, II, and III) are further divided to yield subgroups (second column). The bootstrap values for the three main groups are boxed, whereas those for the subgroups are indicated by arrows. The predicted molecular masses of the deduced polypeptides are listed in the third column. Figure modified from Li et al. 2002.
(Veljanovski et al., 2006). Transient expression of 35S:AtPAP26-mCherry in Arabidopsis suspension cells verified that AtPAP26 is targeted to the cell vacuole (Hurley et al., 2010).

Vacuolar AtPAP26 is a 100-kD homodimer composed of 55-kD glycosylated subunits and contains a 30-amino acid signal peptide that is cleaved from the AtPAP26 preprotein during its translocation into the vacuole (Veljanovski et al., 2006). Semiquantitative RT-PCR indicated that +Pi, -Pi, and Pi-resupplied cells contain similar amounts of AtPAP26 transcripts (Veljanovski et al., 2006, Hurley et al., 2010). APase activity and AtPAP26 protein levels were found to be significantly upregulated in both shoots and roots of -Pi Arabidopsis seedlings. Vacuolar AtPAP26 exhibits alkaline peroxidase and APase activity.

In addition to the vacuolar AtPAP26, two secreted AtPAP26 glycoforms were identified (Tran et al., 2010). Differential glycosylation of secreted AtPAP26 is hypothesized to influence the subcellular targeting and substrate selectivity of AtPAP26. Similar to the vacuolar AtPAP26, transcriptional controls exerted very little influence on AtPAP26 upregulation and secretion during Pi-stress. A T-DNA atpap26 insertional mutant exhibited a large decrease in shoot and root APase activity of Arabidopsis seedlings and a 40% decrease in secreted APase activity during Pi deprivation (Hurley et al., 2010). The mutant also displayed impaired shoot and root development when subjected to Pi deficiency.

**Purple acid phosphatases and the cell wall proteome**

As mentioned above, several AtPAPs have been localized to the cell wall during Pi-stress (Irshad et al., 2008; Wang et al., 2011; Del Vecchio et al., unpublished). The cell wall of plants acts as a supportive cellulosic wall that aids in shape and structure and helps to provide an environment for extracellular interactions. The cell wall is composed mainly of carbohydrates.
and contains a framework of cellulose microfibrils embedded in a matrix of hemicellulose and pectins (Bayer et al., 2006). Proteins on the other hand only contribute about 10% to the cell wall mass. Within this 10% are hundreds of different proteins that help to regulate both the physical and biological functions of the plant cell wall (Bayer et al., 2006).

It has been hypothesized that the role of AtPAPs secreted into the rhizosphere is to scavenge Pi from Po sources in the soil, while AtPAPs secreted into the cell wall are hypothesized to play a role in recycling Pi from leaked esterified-Pi back into plant cells (Barrett-Lennard et al., 1993). The latter hypothesis is based on the fact that cell walls likely become leaky under Pi-stress as a result of membrane phospholipids being replaced by non-P containing galacto and sulfonyl lipids (Barrett-Lennard et al., 1993). Recent studies have found AtPAP12, AtPAP25, and AtPAP26 in the cell wall proteome of Arabidopsis suspension cell cultures (Del Vecchio et al., unpublished). Furthermore, a significant increase in APase activity was observed in the cell walls of -Pi Arabidopsis suspension cells compared to +Pi cells. However, it is currently unknown if these same cell wall PAP isozymes are upregulated in planta during Pi-stress.

Discovering and characterizing the cell wall proteome is not an easy task. Among other obstacles, many cell wall proteins are tightly bound and their separation entails strong denaturing agents. Recently, a variety of techniques have been examined in order to extract and purify cell wall proteins. The main approach for Arabidopsis has been the use of ionic solutions (CaCl₂ in particular) to elute loosely-bound cell wall proteins from suspension cells (Bayer et al., 2006; Feiz et al., 2006). This method has been applied successfully to Arabidopsis cell culture with little to no cytoplasmic contamination (Del Vecchio et al., unpublished). However, this method has not yet been tested with seedling tissue. Analyzing this subcellular compartment is becoming
an increasingly important requirement in the study of Pi-stress as a number of AtPAPs have been targeted and highly expressed in cell walls during Pi-limitation.

1.3 Thesis Objectives

AtPAP12 and AtPAP26 have been identified as the main PAPs upregulated and secreted during Pi-stress in both *Arabidopsis* suspension cell cultures and seedlings (Tran and Plaxton, 2008). Furthermore, *Arabidopsis* seedlings have been shown to grow well on Po sources such as G3P, ATP, glucose-1-P and nucleic acids, as equivalent P sources for +Pi growth (Ticconi and Abel, 2004; Richardson *et al.*, 2009b; Liang *et al.*, 2010; Richardson *et al.*, 2011). However, exactly which pools of Po can be utilized by roots and which enzymes play a role in hydrolyzing these Po to produce Pi anions remains elusive (Vance *et al.*, 2003; Plaxton and Tran, 2011). It is the primary objective of this thesis to test the hypotheses that (i) *Arabidopsis* seedlings are able to grow on a variety of extracellular Po sources, and (ii) AtPAP26 and AtPAP12 play a key role in scavenging Pi from extracellular Po sources. In addition, this thesis examines the contribution of AtPAP12 and AtPAP26 to the cell wall PSI APase activity of *Arabidopsis* shoots and root surface. These objectives were carried out primarily by using a reverse genetic approach capitalizing on the publicly available T-DNA insertion lines of *Arabidopsis* (Alonso *et al.*, 2003). This study also highlights the importance of using biochemical and proteomic analyses to identify responsive genes, since AtPAP26 would never have been identified as an important player in the *Arabidopsis* PSR via transcript profiling. Work presented here provides insight into a fundamental biochemical adaptation of -Pi and Po grown *Arabidopsis* and helps to provide targets for bioengineering Pi-efficient transgenic crops.
Chapter 2

The secreted purple acid phosphatase isozymes AtPAP12 and AtPAP26 play a pivotal role in extracellular phosphate-scavenging by *Arabidopsis thaliana*¹

(submitted for publication)

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2.1 SUMMARY

Orthophosphate (Pi) is an essential, but limiting macronutrient for plant growth. Extensive soil P reserves exist in the form of organic-Pi (Po) which is unavailable for root uptake until hydrolyzed by secretory acid phosphatases (APases). The predominant purple APase (PAP) isozymes secreted by roots of Pi-deficient (-Pi) Arabidopsis thaliana were recently identified as AtPAP12 (At2g27190) and AtPAP26 (At5g34850). The present study demonstrates that exogenous Po compounds such as glycerol-3-phosphate or herring sperm DNA: (1) effectively substituted for Pi in supporting P nutrition of Arabidopsis seedlings, and (2) caused upregulation and secretion of AtPAP12 and AtPAP26 into the growth media. However, when cultivated under –Pi conditions or supplied with Po 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 Pi content, respectively. Development of the atpap12/atpap26 mutant was unaffected during growth on Pi-replete media, but was completely arrested when 7-day-old Pi-sufficient seedlings were transplanted into a –Pi, Po-containing soil mix. Both PAPs were also strongly upregulated on root surfaces and in shoot cell wall extracts of –Pi seedlings. Our results establish that AtPAP12 and AtPAP26 have an important function to scavenge Pi from extracellular Po. The susceptibility of soil Po to enzymatic hydrolysis is a probable constraint for crop Pi acquisition. Thus, AtPAP12 and AtPAP26 are promising targets for engineering P-efficient crops needed to reduce the input of non-renewable and polluting Pi fertilizers in agriculture.
2.2 INTRODUCTION

Acid phosphatases (APases; EC 3.1.3.2) catalyze the hydrolysis of orthophosphate (Pi) from a broad range of phosphomonoesters and anhydrides with an acidic pH optimum. They function in the production, transport, and recycling of Pi, a critical macronutrient for cellular metabolism and bioenergetics. The induction of extra- and intracellular APases appears to be a ubiquitous plant response to nutritional Pi-deprivation, a common abiotic stress that frequently limits plant growth in natural ecosystems (Vance et al., 2003; Plaxton and Tran, 2011). Extracellular APases belong to a group of Pi-starvation inducible (PSI) phosphohydrolases secreted by roots of Pi-deficient (–Pi) plants to hydrolyze Pi from external phosphomonoesters and phosphodiesters derived from decomposing biomaterial, referred to as organic-Pi (Po). For example, the combined activities of secreted nucleases, phosphoesterases, and APases allows –Pi plants to efficiently scavenge extracellular nucleic acids as their sole source of P nutrition (Abel et al., 2000; Chen et al., 2000; Ticconi and Abel, 2004). Po generally accounts for around 50% of the soil’s total P content (Richardson et al., 2009). Owing to microbial activity, extended periods of Pi fertilizer application increases the proportion of applied P that accumulates in agricultural soils as labile Po (George et al., 2007). Given the abundance of Po in most soils and its steady accumulation under various Pi fertilizer regimes, soil Po makes an important contribution to plant P nutrition and overall efficiency of crop Pi uptake from applied fertilizers (Richardson et al., 2009; Richardson et al., 2011).

Soils have demonstrable APase activity and substantial increases in APase activity have been documented in the rhizosphere of -Pi plants, with several studies showing this to be associated with soil Po depletion (Tarafdar and Claassen, 1988; Miller et al., 2001; Richardson et al., 2009). However, which soil Po pools are accessible to roots remains unclear, and most plants
have a limited capacity to obtain Pi from phytate (*myo*-inositol hexaphosphate), an abundant Po component of certain soils (Richardson *et al.*, 2009). Hydrolysis of extracellular Po substrates to release Pi is essential, because Pi anions (primarily $\text{H}_2\text{PO}_4^-$ or $\text{HPO}_4^{2-}$) are translocated across the root plasmalemma by low- or PSI high-affinity Pi transporters (Vance *et al.*, 2003; Plaxton and Tran, 2011). There is no evidence to support direct import of Po substrates into plant cells, although Po uptake followed by hydrolysis within the apoplast may occur (Richardson *et al.*, 2011). PSI APases are also secreted into cell walls where they may contribute to Pi recapture from phosphomonoesters leaked by the –Pi 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 Pi 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 Po metabolites during long-term Pi deprivation (Vance *et al.*, 2003; Plaxton and Tran, 2011).

Purple acid phosphatases (PAPs), the most important class of plant PSI APases, are characterized by their distinctive purple or pink color in solution (due to a bimetallic active center) (Tran *et al.*, 2010b). Identification and characterization of PSI secretory PAPs is required to pinpoint targets for improving crop Pi acquisition from the soil’s abundant Po pool. Genome annotation identified 29 PAP genes in the model plant *Arabidopsis thaliana*, several of which are transcriptionally induced during Pi-deprivation (del Pozo *et al.*, 1999; Haran *et al.*, 2000; Li *et al.*, 2002; Tran *et al.*, 2010a; Tran *et al.*, 2010b). These and subsequent studies have demonstrated the complexity and variation of *AtPAPI-29* expression and regulation. The principal AtPAP isozymes that contribute to extracellular versus intracellular Pi scavenging were
recently evaluated using a combination of biochemical and genomic approaches. AtPAP12 and AtPAP26 are the major root- and suspension cell culture secretory APases upregulated by -Pi Arabidopsis, whereas the dual-targeted AtPAP26 is the predominant vacuolar APase that appears to function in intracellular Pi recycling during Pi-stress (Veljanovski et al., 2006; Hurley et al., 2010; Tran et al., 2010a; Tran et al., 2010b). The overlapping but non-identical substrate selectivities and pH-activity profiles, and high specific APase activities of secreted AtPAP12 and AtPAP26 (Tran et al., 2010a) supports the hypothesis that their combined activities helps –Pi Arabidopsis to efficiently scavenge Pi from a wide range of extracellular phosphomonoesters over a broad pH range. Analysis of atpap12 and atpap26 T-DNA insertional mutants demonstrated that AtPAP12 and AtPAP26 account for the majority of APase activity secreted by roots of –Pi Arabidopsis (Tran et al., 2010a). Furthermore, AtPAP10 was recently shown to be predominantly associated with the root surface and to be induced by Pi limitation to help Arabidopsis acclimatize to Pi deprivation (Wang et al., 2011). In the present study, analysis of a double atpap12/atpap26 loss-of-function mutant established that AtPAP12 and AtPAP26 are secreted by -Pi Arabidopsis to scavenge Pi from exogenous Po. Our 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 –Pi Arabidopsis shoots.

2.3 RESULTS AND DISCUSSION

2.3.1 Influence of different P supplements on growth, Pi content, secretory APase activity, and secreted AtPAP12 and AtPAP26 polypeptides of wild-type Arabidopsis seedlings
The ability of exogenous Pi, glycerol-3-P (G3P), or purified herring sperm DNA to support growth and Pi nutrition of wild-type (Col-0) *Arabidopsis* seedlings was compared. G3P and nucleic acids are common soil Po 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 –Pi *Arabidopsis* suspension cells (Tran *et al.*, 2010a). Seedling dry weight biomass and total Pi content of rosette leaves of 14-d-old Col-0 seedlings cultivated over the previous 7-d in liquid media containing 1.5 mM Pi, 1.5 mM G3P (-Pi/+G3P), or 0.6 mg mL\(^{-1}\) DNA (-Pi/+DNA) (equivalent to ~2 mM total Pi) were quite comparable, whereas biomass and total shoot Pi concentration of –Pi seedlings were both reduced by ~50% (Fig. 2.1A and 2.1B). These results agree with previous studies showing that plants cultivated in sterile culture were able to use Po substrates, such as G3P and glucose-1-phosphate, as equivalent sources to Pi for growth (Richardson *et al.*, 2009; Liang *et al.*, 2010; Richardson *et al.*, 2011). Our results also support those of Chen and coworkers (Chen *et al.*, 2000) who demonstrated that *Arabidopsis* seedlings efficiently scavenge Pi from exogenous nucleic acids as their sole source of P nutrition owing to root secretion of nucleases, phosphodiesterases, and APases.

We next assessed whether the capacity of Col-0 seedlings to scavenge Pi from G3P or DNA was correlated with secretory APase activity or immunoreactive AtPAP12 or AtPAP26 polypeptides. APase activities were determined using both 5 mM phosphoenolpyruvate (PEP) and 5 mM para-nitrophenol-P (pNPP) as substrates. Irrespective of which substrate was used, the growth media of Col-0 seedlings cultivated under –Pi, –Pi/+G3P, or –Pi/+DNA conditions exhibited a significant increase in secreted APase activity relative to Pi-sufficient (+Pi) seedlings (Fig. 2.1C and 2.1D). Immunoblotting using anti-AtPAP12 immune serum (which cross-reacts
with both AtPAP12 and AtPAP26 (Tran et al., 2010a)) indicated that 60-kD AtPAP12 and 55-kD AtPAP26 immunoreactive polypeptides were upregulated in the growth media of the –Pi, –Pi/+G3P, and –Pi/+DNA Col-0 seedlings (Fig. 2.1E). These results suggest that AtPAP12 and AtPAP26 were secreted into the media in order to hydrolyze Pi from the exogenous Po sources.

2.3.2 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. Results of Fig. 2.2A confirm previous studies documenting the constitutive expression of AtPAP26, whereas AtPAP12, AtPAP17, and AtPPCK1 transcripts are significantly induced in shoots and roots of –Pi Arabidopsis (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 cultivated on –Pi/+G3P or –Pi/+DNA, whereas transcripts for AtPAP17 or AtPPCK1 were either undetectable or expressed at a lower level relative to plants grown on –Pi media (Fig. 2.2A). 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 –Pi Arabidopsis (Gregory et al., 2009). Our results indicate that an independent signal transduction pathway may exist during growth on exogenous Po that selectivity leads to upregulation of secretory hydrolases such as AtPAP12 and AtPAP26 needed to access Pi from those sources, even though the total biomass accumulation and total shoot Pi
contents of –Pi/+G3P and –Pi/+DNA cultivated seedlings were comparable to those of +Pi plants (Fig. 2.1A and 2.1B).

To determine the tissue specificity of AtPAP12 and AtPAP26 expression, promoter reporter gene fusions were generated. The promoter:GUS fusion transgenics were developed by amplifying the promoter regions of AtPAP12 and AtPAP26 and fusing them to the coding region of the GUS reporter gene, followed by transformation into Col-0 plants. Transgenic plants were screened for kanamycin resistance, and the expression of GUS activity was examined in 12 AtPAP12:GUS and 5 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. 2.2A: (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 +Pi AtPAP12:GUS tissues (other than in anthers), but prevalent in shoots and roots of seedlings cultivated on –Pi or –Pi/G3P media (Fig. 2.2B and 2.2C). AtPAP12 induction in shoots and roots of –Pi 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 upregulation of a PSI PAP isozyme such as AtPAP12 during plant growth on media in which the only accessible form of P nutrition is exogenous Po.

2.3.3 Identification and validation of an atpap12/atpap26 double mutant

To further assess the role that secreted AtPAP12 and AtPAP26 play in scavenging extracellular Po, 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 gDNA using AtPAP12- and AtPAP26-specific primers (Appendix 1). Immunoblotting indicated that AtPAP12 or AtPAP26 polypeptides were also absent in the concentrated secretome of +Pi or –Pi atpap12/atpap26 seedlings (Fig. 2.1E). This was correlated with a >60% reduction in secreted APase activity during Pi deprivation (Fig. 2.1C and 2.1D). These results agree with our earlier study of the atpap12 and atpap26 single mutants which concluded that AtPAP12 and AtPAP26 account for most of the APase activity secreted by roots of –Pi Arabidopsis seedlings (Tran et al., 2010a).

2.3.4 AtPAP12 and AtPAP26 are major cell wall acid phosphatases upregulated by Pi-deprived Arabidopsis

Pi-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; Wasaki et al., 2000; Vance et al., 2003; Irshad et al., 2008; Kaida et al., 2008; Wasaki et al., 2008; Richardson et al., 2009; Tran et al., 2010b; Plaxton and Tran, 2011; Wang et al., 2011; Zhang and McManus, 2000). For example, AtPAP10 is a PSI secreted PAP that is predominantly associated with the surface of root epidermal cells (but undetectable in culture media), and that functions in the acclimation of Arabidopsis to Pi limitation (Wang et al., 2011). Cell wall associated PSI APases have been hypothesized to facilitate maintenance of the plant’s P status by either scavenging Pi from Po compounds present in the rhizosphere or by recycling Pi from endogenous phosphomonoesters that have been leaked from the cytoplasm across the plasma membrane (Lefebvre et al., 1990; Barrett-Lennard et al., 1993; Tran et al., 2010b). Classic studies by Bieliski’s group with the small aquatic plant Spirodea oligorrhiza...
demonstrated that significant levels of phosphomonoesters can be leaked during -Pi growth, and that failure to recapture this lost P could seriously compromise the overall P economy of the plant (Bieleski and Johnson, 1972).

Histochemical localization using enzyme-labeled fluorescence (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 -Pi 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. 2.3A). Roots of the atpap12/atpap26 double mutant stained with β-napthyl-P also showed decreased APase staining relative to Col-0 roots (Appendix 5). β-napthyl-P has been shown to be an excellent in vitro substrate for purified AtPAP12 and AtPAP26 (Tran et al., 2010). However, there was no decrease in 5-Br-4-Cl-3-indolyl phosphate (BCIP) in vivo root staining between Col-0 and atpap12/atpap26 double mutant (Appendix 5). This negative result can be explained by the low activity of cell wall AtPAP12 and AtPAP26 with BCIP (Del Vecchio et al., unpublished). The ELF-97 and β-napthyl-P in vivo root surface APase staining indicates that AtPAP12 and AtPAP26 are localized to the root surface and that they account for the majority of root surface localized PSI APase activity. The results of Fig. 2.3, coupled with the transcriptional activation of AtPAP12 in –Pi Arabidopsis shoots (Fig. 2.2) prompted us to investigate the influence of Pi 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 +Pi and –Pi 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-kD immunoreactive PEPC polypeptides in the cell wall, but not corresponding cytoplasmic fractions (Appendix 2). Comparison of the cytoplasmic and cell wall fractions on protein-stained SDS gels indicated clear differences in their respective proteomes (Appendix 2). The –Pi Col-0 seedlings exhibited a large increase in shoot cell wall APase activity compared to +Pi seedlings; this was correlated with the upregulation of immunoreactive 60-kD AtPAP12 and 55-kD AtPAP26 polypeptides (Fig. 2.3B and 2.3C). By contrast, immunoreactive AtPAP12 and AtPAP26 polypeptides were absent on immunoblots of cell wall extracts prepared from the +Pi or –Pi atpap12/atpap26 mutants (Fig. 2.3C). This was paralleled by a >70% reduction in extractable cell wall APase activity of –Pi atpap12/atpap26 shoots relative to Col-0, irrespective of whether PEP or pNPP was used as the APase assay substrate (Fig. 2.3B). These results demonstrate that AtPAP12 and AtPAP26 account for most of the APase activity secreted into the cell walls of –Pi Arabidopsis shoots. Pi recycling by PSI cell wall targeted AtPAP12 and AtPAP26 could be critical in maintaining cytoplasmic Pi and thus photosynthetic metabolism in leaves of -Pi plants.

2.3.5 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 +Pi seedlings for an additional 7-d on +Pi, -Pi, -Pi/+G3P, and -Pi/+DNA liquid media. No differences were noted in the growth or appearance of +Pi plants (Figs. 2.1A and 2.4). However, under -Pi, -Pi/+G3P, or -Pi/+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. 2.1A and 2.4). This suggests that the absence of AtPAP12 was largely compensated for by AtPAP26 and *vice versa*, during growth of the single mutants in -Pi, -Pi/+G3P, or -Pi/+DNA liquid media. However, when expression of *AtPAP12* and AtPAP26 was eliminated in the *atpap12/atpap26* mutant, their absence could not be fully compensated by other extracellular PSI PAP isozymes such as AtPAP10 (Wang *et al.*, 2011). Diminished growth of the -Pi, -Pi/+G3P, and -Pi/+DNA *atpap12/atpap26* seedlings was likely due to the marked reductions in their total Pi content, particularly during -Pi growth (Fig. 2.1B). The reduced biomass accumulation of -Pi *atpap12/atpap26* seedlings relative to Col-0 appears to be specific to Pi deprivation as no phenotypic differences were apparent when +Pi seedlings were subjected to nitrogen or potassium deficiency, or oxidative stress imposed by paraquat treatment (Appendix 3).

The impaired development of *atpap12/atpap26* seedlings during growth on -Pi, -Pi/+G3P, or -Pi/+DNA media was also evident during their cultivation on vertically oriented agar plates (Fig. 2.4B). Similar results were obtained when the plants were cultured in -Pi liquid media or vertical agar plates supplemented with 1.5 mM glucose-6-phosphate (Glc-6-P), which is also efficiently hydrolyzed by the native AtPAP12 or AtPAP26 purified from the secretome of -Pi *Arabidopsis* suspension cells (Tran *et al.*, 2010a). It is notable that Col-0 or *atpap12/atpap26* plants cultivated on -Pi agar plates supplemented with G3P, DNA, or Glc-6-P showed typical
root architectural adaptations to Pi limitation (e.g. decreased primary root growth and increased lateral branching (Williamson et al., 2001)), even though total biomass accumulation and shoot Pi contents of Col-0 plants paralleled that of the respective +Pi seedlings (Fig. 2.1A and 2.1B, Fig. 2.4). A rationale for this observation is that intracellular Pi status appears to be irrelevant to the reprogramming of root architecture in –Pi Arabidopsis, whereas low extracellular Pi in the area surrounding the root tip appears to trigger this response (Svistoonoff et al., 2007). Presumably, the root cap Pi sensor complex that mediates adaptive modifications in root structure in response to Pi limitation does not perceive exogenous Po 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 Pi assimilation typical of Pi-fertilized plants.

As Arabidopsis growth under sterile conditions in the presence of exogenous sucrose could generate phenotypes not observed under more physiologically relevant conditions we also examined the phenotype of soil-grown plants. Seedlings were cultivated in +Pi liquid media 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 Pi present in the peat-vermiculite soil mix used for these experiments was in the form of Po; it contained 12.8 ±0.5 μmol total Pi g⁻¹ dry weight, but undetectable free Pi. No obvious phenotypic differences were noted when any of the soil-grown plants were provided with a regular Pi fertilizer treatment (Fig. 2.5). However, the growth of the atpap12 and atpap26 single mutants was obviously compromised during their cultivation on the –Pi soil, as reflected by the ~50% reduction in their rosette dry weights relative to Col-0 plants (Fig. 2.5). Impaired development of atpap26 mutant seedlings on a –Pi soil mixture was previously noted (Veljanovski et al., 2006; Hurley et al., 2010). It is remarkable, however, that development of atpap12/atpap26 plants was completely
arrested when +Pi seedlings were transplanted into the -Pi soil mix (Fig. 2.5). In addition, shoots of soil grown -Pi atpap12/atpap26 plants rapidly turned purple, as reflected by their anthocyanin accumulation, a typical symptom of severe Pi-stress (Vance et al., 2003; Plaxton and Tran, 2011); the leaf anthocyanin concentration of the soil grown -Pi Col-0 and atpap12/atpap26 plants was 70 ±8 and 900 ±12 nmol.mg⁻¹ FW, respectively (means ±SE of duplicate determinations on n = 3 biological replicates). Shoots of soil grown -Pi atpap12/atpap26 plants also contained significantly less free Pi; the leaf free Pi concentration of the soil grown -Pi Col-0 and atpap12/atpap26 plants was 1.7 ±0.2 and 0.38 ±0.09 µmol.g⁻¹ FW, respectively (means ±SE of duplicate determinations on n = 3 biological replicates). It is important to note that the impaired growth of atpap12/atpap26 plants was obviously improved when soil cultivated 21-d old -Pi plants were resupplied with 2 mM Pi for 5 d (Appendix 6). This was paralleled by rapid conversion of purple leaves to green leaves.

Backcrossing atpap12/atpap26 plants with each of the atpap12 and atpap26 single mutants restored AtPAP12 or AtPAP26 expression (Appendix 4), as well as the –Pi soil growth phenotype characteristic of the respective single mutants (Fig. 2.5). This supports the ability of AtPAP12 to partially compensate for the absence AtPAP26 and vice versa. It is hypothesized that decreased scavenging of soil-localized Po reduced the amount of Pi assimilated by the atpap12 and atpap26 mutants, and that this was particularly exacerbated in the atpap12/atpap26 double mutant. As both PAPs were markedly upregulated in cell walls of –Pi Arabidopsis shoots (Fig. 2.3B), diminished Pi recapture from leaked phosphomonoesters could also contribute to the prominent phenotype of atpap12/atpap26 mutant plants cultivated on the -Pi soil.

2.3.6 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 –Pi plants (Vance et al., 2003; Tran et al., 2010b; Plaxton and Tran, 2011). Conversely, the molecular identities, biochemical properties, and genetic control of PSI-secreted APases are not well understood. However, such an understanding will be a crucial step towards exploiting biotechnological strategies for improving crop P acquisition from the abundant Po sources prevalent in agricultural soils (Richardson, 2009). Results of the current study corroborate our earlier report indicating that AtPAP12 and AtPAP26 are the predominant secretory APases of –Pi Arabidopsis seedlings (Tran et al., 2010a). Their upregulation and secretion during growth of the Col-0 seedlings on –Pi/+Po media clearly contributes to Arabidopsis’ ability to exploit exogenous Po compounds such as G3P, Glc-6-P, and DNA as an alternative source of P nutrition (Fig. 2.4). AtPAP12 and AtPAP26 were also upregulated in shoot cell walls and on the root surface of –Pi plants (Fig. 2.3). This indicates that they may have an additional function to recycle Pi 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; Miller et al., 2001; Bozzo et al., 2002; Vance et al., 2003; Bozzo et al., 2006; Xiao et al., 2006; Kaida et al., 2008). To the best of our knowledge, however, the involvement of AtPAP26 orthologues in scavenging Pi from extracellular Po has not yet been reported in any other species.

During the cultivation of atpap12 and atpap26 single mutants on sterile –Pi/+G3P or –Pi/+DNA liquid media it was apparent that AtPAP12 could compensate for the absence of AtPAP26 and vice versa (Fig. 2.1A). However, this was not evident when either of the single mutants was cultivated on a more physiologically relevant –Pi, Po-containing soil mix, as both
groups showed poorer growth relative to Col-0 control plants (Fig. 2.5). It was particularly noteworthy that development of the atpap12/atpap26 double mutant was totally blocked when seedlings were transplanted into the –Pi soil mixture. This highlights the fundamental importance of AtPAP12 and AtPAP26 in facilitating acclimation of Arabidopsis to nutritional Pi deprivation. AtPAP10, AtPAP12, and AtPAP26 are closely related high molecular weight PSI PAPs that comprise subgroup Ia-2 of the Arabidopsis PAP family (Fig. 1.2) (Li et al., 2002; Tran et al., 2010b). Evolution of this PAP subgroup appears to have endowed Arabidopsis with an efficient hydrolytic machinery for scavenging Pi from exogenous Po compounds prevalent in the Pi-deficient soils typical of most ecosystems (Tran et al. 2010a; Wang et al., 2011). As the susceptibility of soil Po to enzymatic hydrolysis is a probable constraint for crop Pi acquisition, AtPAP12 and AtPAP26 are promising candidates for engineering P-efficient crops needed to reduce the use of Pi fertilizers in agriculture.

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2.5 EXPERIMENTAL PROCEDURES

2.5.1 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, Vancouver, Canada)
and stratified at 4 °C for 3-d. Plants were cultivated in growth chambers at 23 °C (16/8 h photoperiod at 100 µmol m⁻² s⁻¹ PAR) and fertilized biweekly by subirrigation with 0.25x Hoaglands media. To assess the influence of Pi deprivation on soil-grown plants, seedlings were grown for 7-d in a 24-well microtitre plate (1 seedling per well) containing 0.5 mL well⁻¹ of 0.5x MS media supplemented with 1% (w/v) sucrose and 0.2 mM Pi, then transplanted into a 75% to 85% sphagnum peat moss/perlite soil mix lacking all nutrients (Sunshine Mix 2; SunGro). Plants were grown in aforementioned growth chambers for an additional 14-d and fertilized biweekly with 0.25x Hoaglands media (pH 6.0) containing containing either 0 or 2 mM KH₂PO₄. Whenever Pi was eliminated, it was replaced by 2 mM KH₂SO₄ 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 0.5x Murashige-Skoog (MS) media, pH 5.7, containing 1% (w/v) sucrose and 0.2 mM KH₂PO₄, and placed on an orbital shaker (80 r.p.m.) at 24 °C under continuous illumination (100 µmol m⁻² s⁻¹). After 7-d the medium was replaced with fresh media containing filter-sterilized 0 or 1.5 mM KH₂PO₄, or 1.5 mM DL-glycerol-3-P (G3P; Sigma Chemical Co.), or 0.6 mg mL⁻¹ 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 previously described (Chen et al. 2000). It was assumed that 0.6 mg mL⁻¹ DNA equates to ~2 mM total P (Chen et al. 2000). All Po stocks were found to contain negligible free Pi. The 14-d-old seedlings were blotted dry, snap frozen in liquid N₂, and stored at -80 °C, whereas growth media was filtered through 0.45 µm membranes and concentrated over 250-fold using Amicon Ultra-15 centrifugal filter units (30-kD cut-off). For growth on agar-solidified nutrient media, stratified seeds were placed on horizontal or vertically oriented 1% (w/v) agar (Micropropagation Type I Agar from Caisson
Labs, Cat. #A038) plates containing 0.5x MS media and 1% (w/v) sucrose supplemented with 50 μM or 1.5 mM KH$_2$PO$_4$, 0.6 mg mL$^{-1}$ DNA, 1.5 mM G3P, or 1.5 mM Glc-6-P and cultivated at 24 °C under continuous illumination (100 μmol m$^{-2}$ s$^{-1}$) for 14- to 21-d.

2.5.2 Extraction of Shoot Cell Wall Proteins

Shoots (2.5 g) of Arabidopsis seedlings cultivated in +Pi or –Pi liquid media as described above were powdered under liquid N$_2$ 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 MgCl$_2$, 1 mM EDTA, 1 mM dithiothreitol, 1% (v/v) Triton X-100), and 1% (w/v) polyvinylpolypyrrolidone). The mixture was clarified by centrifugation at 20000 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 re-centrifugation as above. The insoluble fraction was extracted with 5 mL of 0.2 M CaCl$_2$ in 5 mM acetate-NaOH (pH 4.6), and centrifuged at 23700 g for 15 min. The supernatant was collected as the cell wall extracts (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 dialyzed overnight against 0.5 L of 40 mM Tricine-KOH (pH 7.4) containing 10 mM MgCl$_2$, 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$^{-1}$.

2.5.3 Acid Phosphatase Activity Determination

APase activity was routinely measured by coupling the hydrolysis of 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 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 any background NADH oxidation by omitting PEP from the reaction mixture. APase assays were also carried out in an assay mix containing 50 mM Na-acetate (pH 5.6), 5 mM pNPP, and 10 mM MgCl₂ by monitoring the formation of para-nitrophenol at 405 nm \( (\varepsilon = 18.2 \text{ mM}^{-1} \text{ cm}^{-1}) \). 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 \( \mu \text{mol} \) of substrate \( \text{min}^{-1} \) at 24 °C.

### 2.5.4 Protein Electrophoresis and Immunoblotting

SDS-PAGE, immunoblotting onto poly(vinylidene difluoride) membranes and chromogenic detection of antigenic polypeptides using an alkaline phosphatase-tagged secondary antibody were conducted as previously described (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.

### 2.5.5 Determination of Protein, Total Pi, Free Pi and Anthocyanin Concentrations

Protein concentrations were determined using a modified Bradford assay (Bozzo et al., 2002) with bovine \( \gamma \)-globulin as the standard. Total Pi, free Pi, and anthocyanin determinations were carried out as previously described (Hurley et al., 2010).
2.5.6 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 $A_{260}/A_{280}$ 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, Carlsbad, CA, USA), and noncompetitive RT-PCR 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-PCR reactions to ensure linearity of response for comparison between samples.

2.5.7 GUS Analysis

The AtPAP12 and AtPAP26 promoters (2010 bp and 3853 bp sequences upstream of the start codon of the AtPAP12 or AtPAP26 genes, respectively) were amplified from genomic DNA using the following primers. Primer for AtPAP12:GUS 12ProFull-InfF: TGATTACGCCAAGCTTTTTCTTCTCCGGTGAAACC and 12ProFull-InfR: CCGGGGATCTCTCTAGACTTCAAGATTAGTTTCTCTGAATCC. Primer for AtPAP26:GUS 26ProFull-InfF: TGATTACGCCAAGCTTATTTGTAATGTCATCACCTCGG and 26ProFull-InfR: CCGGGGATCTCTCTAGACACGTCACCAAATCTCGA. Amplified promoter region of AtPAP12 or AtPAP26 was mixed in 3:1 molar ratio with pBI101 N1 linearized by HindIII and XbaI, incubated with In-Fusion reaction mix, and transformed according to manufacturer’s (Clontech) protocol 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) +Pi agar plates containing 0.5x MS media, 1% (w/v) sucrose, and 30 µg mL$^{-1}$ 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 bi-weekly with 0.25x Hoaglands media containing 2 mM Pi.

### 2.5.8 atpap12/atpap26 Double Knockout Mutant Isolation and Backcross

Homozygous atpap26 and atpap12 T-DNA insertion mutants (Salk_152821 and SAIL_1187_A05, respectively) were obtained as previously reported (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 (Appendix 1). All PCR products were sequenced for verification (Centre for Applied Genomics, The Hospital for Sick Children, Toronto, ON). 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 media. From the BASTA-resistant (for the atpap12 allele) plants, gDNA 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.

### 2.5.9 Histochemistry and Microscopy
Histochemical staining of GUS activity was performed as previously described (Jefferson et al., 1987). Tissues were incubated at 37 °C overnight in GUS staining buffer (100 mM NaPi, pH 7.0, containing 1 mM 5-Br-4-Cl-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.

Root surface APase activity staining using β-napthyl-P and BCIP was carried as described in Appendix 5 (Gilbert et al., 1999; Wang et al., 2011). Root surface APase activity staining using enzyme labeled fluorescent (ELF)-97 phosphate (Invitrogen) was conducted using hydroponically cultivated 14-d-old seedlings. Individual seedlings were rinsed with 75 mM Na-acetate (pH 5.6) and 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 63x 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.). All micrographs shown in Fig. 2.3A are representative images obtained from experiments that were replicated at least three times.

2.5.10 Statistics

All values are presented as means ±SE. Data were analyzed using the one-tailed Student's t test, and deemed significant if P < 0.05.
Figure 2.1 Influence of different P supplements on biomass accumulation, rosette Pi content, secretory APase activity, and secreted AtPAP12 and AtPAP26 polypeptides of Col-0 and mutant Arabidopsis seedlings. Seeds (5 mg) of Col-0, atpap12 and atpap26 single mutants, and atpap12/atpap26 double mutants were placed in 50 mL of 0.5x Murashige-Skoog (MS) media containing 0.2 mM Pi and cultivated on an orbital shaker at 24 °C under continuous illumination (100 μmol m⁻² s⁻¹). After 7-d, the seedlings were transferred into fresh media containing 0 or 1.5 mM Pi (-Pi and +Pi, respectively), 1.5 mM G3P (-Pi/+G3P), or 0.6 mg mL⁻¹ DNA (-Pi/+DNA) and cultured for an additional 7-d. A. Seedling dry weight per flask. Total Pi content of rosette leaves (B) and secreted APase activity of concentrated seedling culture filtrates (C and D) of Col-0 and atpap12/atpap26 plants were also determined. Spectrophotometric APase activity assays were conducted using both 5 mM PEP (C) and 5 mM pNPP (D) as described in the Materials and Methods. All values in panels A - D represent means ±SE of duplicate determinations on n = 3 biological replicates; asterisks denote values that are significantly different from Col-0 (P < 0.01). E. Concentrated secreted proteins (15 µg/lane) of Col-0 and atpap12/atpap26 mutant seedlings, and secretory AtPAP12 and AtPAP26 (25 ng each) purified from culture media of –Pi Arabidopsis suspension cells (Tran et al., 2010a) were resolved by SDS-PAGE and electroblotted onto a poly(vinylidene difluoride) membrane. Blots were probed with rabbit anti-AtPAP12 immune serum (Tran et al., 2010a) and immunoreactive polypeptides detected using an alkaline-phosphatase linked secondary antibody and chromogenic detection.
Figure 2.2 Analysis of *AtPAP12* and *AtPAP26* gene expression. A. Levels of mRNA were analyzed by semi-quantitative RT-PCR using gene specific primers for *AtPAP12*, *AtPAP17*, *AtPAP26*, *AtPPCK1*, and *AtACT2*. *AtACT2* was used as a reference to ensure equal template loading. Seedlings were cultivated as described in the legend for Fig. 2.1. All PCR products were taken at cycle numbers determined to be nonsaturating. Control RT–PCR reactions lacking reverse transcriptase did not show any bands. B. *AtPAP12:GUS* and *AtPAP26:GUS* transgenic lines were cultivated in 24 well microtitre plates in liquid MS media containing 0.2 mM Pi for 7-d, before being transferred into media containing 0 or 1.5 mM KH₂PO₄ (-Pi and +Pi, respectively), 1.5 mM G3P (-Pi/+G3P), or 0.6 mg mL⁻¹ DNA (-Pi/+DNA) for another 7-d. Scale bar = 1 cm, except for ‘-Pi root’ for which the scale bar = 100 µm. C. *AtPAP12:GUS* and *AtPAP26:GUS* expression was also examined in several aerial tissues of 4 week old +Pi 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.
Figure 2.3 AtPAP12 and AtPAP26 make an important contribution to Pi-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; scale bar represents 100 µm. Seedlings were cultivated as described in the legend for Fig. 2.2B. B. Concentrated cell wall proteins extracted from shoots of Col-0 and atpap26/atpap12 seedlings were assayed for APase activity using 5 mM PEP or 5 mM pNPP as substrates; all values represent means ±SE of duplicate determinations on n = 3 biological replicates; asterisks denote values that are significantly different from Col-0 (P < 0.01). Seedlings were cultivated as described in the legend for Fig. 2.1. C. Concentrated shoot cell wall proteins (15 µg/lane) and purified native AtPAP26 and AtPAP12 (25 ng/lane) (Tran et al., 2010a) were resolved by SDS-PAGE, electroblotted onto a poly(vinylidene difluoride) membrane, and probed with anti-AtPAP12 immune serum as described in the legend for Fig. 2.1E.
Figure 2.4 Impact of different P sources on appearance and root morphology of Col-0 and atpap26/atpap12 mutant seedlings. A. Seeds (5 mg) were placed into 50 mL of 0.5x MS media containing 1% (w/v) sucrose and 0.2 mM Pi and cultivated on an orbital shaker at 24 °C under continuous illumination (100 μmol m⁻² s⁻¹). After 7-d, the seedlings were transferred into fresh media containing 0 or 1.5 mM Pi (-Pi and +Pi, respectively), 1.5 mM G3P (-Pi/+G3P), 0.6 mg mL⁻¹ DNA (-Pi/+DNA), or 1.5 mM Glc-6-P (-Pi/+Glc-6-P) and cultured for an additional 7-d. B. Plants were cultivated for 3 weeks on vertically oriented agar plates containing 0.5x MS media, 1% (w/v) sucrose, and 50 μM or 1.5 mM Pi (-Pi and +Pi, respectively), 1.5 mM G3P (-Pi/+G3P), 0.6 mg mL⁻¹ DNA (-Pi/+DNA), or 1.5 mM Glc-6-P (-Pi/Glc-6-P). Images shown in panel A and B are representative of at least five replicates; scale bar = 1 cm.
Figure 2.5 Effect of Pi deprivation on appearance and shoot biomass accumulation of soil-grown Col-0 and mutant Arabidopsis seedlings. A and B. Seedlings were grown for 7-d in liquid media containing 0.2 mM Pi, then transplanted into a Pi-deficient soil mix and grown for an additional 14-d. Fertilization occurred biweekly with 0.25x Hoaglands media containing 0 or 2 mM Pi (-Pi and +Pi, respectively); solid scale bars = 1 cm; individual dash scale bar = 0.1 cm. B. Rosette dry weights of soil grown seedlings. All values represent means ±SE of n = 10 different seedlings; asterisks denote values that are significantly different from Col-0 (P < 0.01) and † denotes values that are significantly different from single and backcross mutants (P < 0.01).
Chapter 3

General Discussion

3.1 Overview

Plants are faced with a variety of biotic and abiotic stresses that affect their growth, survival, and reproduction. Unlike mobile organisms, plants are forced to deal and adapt with the environment that surrounds them. Current literature focuses on a variety of environmental factors that impact plant growth such as drought, pests, and soil nutrient content. Nutrient limitation is a ubiquitous abiotic stress. Pi is one of the main limiting nutrients in most soils and is of particular interest because (i) Pi is a critical macronutrient required for optimal plant growth, and (ii) global rock-Pi reserves needed for Pi-fertilizer production are a non-renewable resource that will be mostly exhausted by the end of this century (Vance et al., 2003). Plants have evolved a multitude of biochemical, molecular and physiological adaptations that allow them to grow in –Pi soil. One of the main adaptations is the upregulation and secretion of PAPs to help recycle and scavenge Pi from extracellular Pi-monoesters.

This thesis demonstrated the ability of Arabidopsis to grow on a variety of extracellular Po sources and the pivotal role that AtPAP12 and AtPAP26 play in scavenging Pi from these Po sources (Fig. 2.1 and 2.4). Col-0 Arabidopsis seedlings were found to grow just as well in media containing no free Pi and Po sources such as G3P, Glu-6-P, and DNA relative to Col-0 Arabidopsis grown in +Pi media. The shoot growth and total Pi content of plants cultivated on these sources was comparable to that of +Pi plants (Fig. 2.1). These findings support the idea that Arabidopsis is able to secrete enzymes that hydrolyze Pi from extracellular Po. However, the root architecture of Po grown plants was similar to that of –Pi plants as they showed a decrease in primary root growth and an increase in secondary root growth (Fig. 2.4). This observation was one that had been noted in previous studies (Chen et al., 2000) and suggests that perhaps low
extracellular Pi concentrations around the root tip triggers the root alteration in architecture growth and that the intracellular Pi content is not directly involved in this response (Svistoonoff et al., 2007).

Growth of *Arabidopsis* seedlings on Po was accompanied by an upregulation and secretion of AtPAP12 and AtPAP26 (Fig. 2.1). Known Pi-starved responsive genes, *AtPPCK1* and *AtPAP17*, were not switched on during growth on Po sources, as indicated by semi-quantitative RT-PCR (Fig. 2.2), which suggests selective upregulation of AtPAP12 and AtPAP26. This is the first time to our knowledge that it appears genes involved in the PSR can be selectively upregulated and plants do not have to undergo a full PSR. In order to determine the possible role that AtPAP26 and AtPAP12 play in scavenging Pi from extracellular Po sources, the publicly available T-DNA insertion knockout lines for *Arabidopsis* were utilized. Once *AtPAP26* and *AtPAP12* null alleles were identified, they were crossed and screened for an *Arabidopsis* line with nullified expression of both the AtPAP26 and AtPAP12 (Appendix 1). Elimination of *AtPAP26* and *AtPAP12* expression resulted in a 64% decrease in secreted APase activity in response to Pi starvation. The secreted APase activity of seedlings grown with Po as their sole source of P also significantly decreased in the *atpap12/atpap26* compared to Col-0. Ionically-bound (0.2 M CaCl₂-extractable) cell wall proteins were extracted and isolated from shoots of seedlings grown in liquid culture and lacked any cytoplasmic contamination as determined by an absence of the cytoplasmic marker PEPC protein (Appendix 2). Shoots of *atpap12/atpap26* mutants showed a significant decrease in cell wall APase activity compared to Col-0 (Fig. 2.3). Absence of *AtPAP26* and *AtPAP12* expression resulted in compromised growth of shoots and roots during Pi starvation and growth on extracellular Po sources (Fig. 2.4). This reduction in growth was specific to Pi-stress and Po growth as no phenotype was observed on
+Pi media or during growth limited by nitrogen or potassium, and under oxidative stress conditions (Appendix 3). In addition to liquid culture and agar plate growth, Col-0 seedlings and atpap12/atpap26 mutant seedlings were grown in soil containing only Po and no free Pi. Col-0 seedlings were able to grow and develop in this soil, whereas atpap12/atpap26 mutants were completely arrested (Fig 2.5). This thesis further demonstrated the importance of these two PAPs by rescuing the expression of AtPAP12 or AtPAP26 using a backcross. The restoration of either one of these two proteins resulted in the elimination of the atpap12/atpap26 phenotype in –Pi/+Po soil. This suggests that AtPAP26 is able to compensate for AtPAP12 in atpap12 single knockout mutant and vice versa. However, when the expression of both of these PAPs is eliminated, plants are not able to grow and develop in soil containing only Po.

3.2 Future Directions

For the most part, it is still unknown which PAP isozymes contribute to overall APase activity in the different cellular compartments. AtPAP10, AtPAP12, AtPAP25, and AtPAP26 have all been localized to the cell wall of –Pi Arabidopsis (del Pozo et al., 1999; Wang et al., 2011; Del Vecchio et al., unpublished). AtPAP10 was recently found to be associated with the root surface and to help Arabidopsis acclimate to Pi-stress (Wang et al., 2011). However, this enzyme has yet to be biochemically characterized in planta. AtPAP12, AtPAP26, and AtPAP25 have been found to be the predominant APase in cell walls of –Pi Arabidopsis cell cultures (Del Vecchio et al., unpublished).

In order to establish the in planta function of these cell wall PAPs, AtPAP12, AtPAP26, AtPAP10 and AtPAP25 should all have their expression nullified in a quadruple knockout. Since the individual single knockouts are all available, a cross experiment should be performed and screened using RT-PCR to verify a homozygous atpap12/atpap26/atpap10/atpap25 mutant
expressing no corresponding transcripts. Measurement of APase activity in the secretome, root and shoot cell wall extracts of the –Pi quadruple mutants would determine the extent these four proteins contribute to the secreted and cell wall APase activity of Arabidopsis. Root-surface staining of Col-0 and atpap12/atpap26/atpap10/atpap25 with BCIP, β-napthyl-P and ELF-97, followed by immunoblotting of intact roots using specific antibodies would help to determine how much these APase contribute to the cell wall associated APase activity (Bozzo et al., 2006). Measuring the total and free Pi concentrations, as well as anthocyanin accumulation of -Pi soil grown atpap12/atpap26/atpap10/atpap25 mutant plants would help elucidate the role of these PAPs in Pi scavenging. Col-0 and atpap12/atpap26/atpap10/atpap25 mutant plants grown on agar plates, liquid media, and –Pi/+Po soil will help determine if there is a phenotype associated with this quadruple mutant. It is hypothesized that this quadruple mutant will not be able to scavenge Po from the extracellular environment or salvage leaked esterified-Pi and thus will display a strong phenotype when grown on –Pi /+Po soil.

Overexpressing these PAPs in Arabidopsis or in crop plants will be crucial to further determine their role in helping plants scavenge extracellular Pi and perhaps aid in increasing the Pi-use efficiency of different crop species. AtPAP10 has already been successfully overexpressed in Arabidopsis and shown to help plants survive Pi limitation (Wang et al., 2011). Characterizing the growth and Pi acquisition of the AtPAP12, AtPAP25 and AtPAP26 overexpressors in Arabidopsis or a crop plant on –Pi and +Po media will allow their roles in Pi scavenging to be fully elucidated. According to our hypothesis, we would expect to see increased levels of Pi uptake in both the AtPAP12 and AtPAP26 overexpressors. This increase in Pi should allow for greater growth under –Pi and –Pi/+Po growth conditions.
This thesis also found a strong induction of AtPAP26 expression in the secretome and cell wall during *Arabidopsis* growth on Po and –Pi conditions; however the transcripts were constitutively expressed. This finding strongly suggests that post-transcriptional control mediates changes in AtPAP26 gene expression. AtPAP26 accumulation may be regulated at the level of translation or protein stability. The upregulation of a specific protease or group of proteases may play a role in controlling the protein stability of AtPAP26. This possibility could be investigated using *Arabidopsis* seedlings that are subjected to Pi-stress and then resupplied with Pi. We hypothesize that by adding Pi to the seedlings, Pi-inducible secreted proteases that are targeted to PSI extracellular APases will be upregulated. This will allow for the purification and characterization of these proteases. Secreted PSI PAPs in tomato have been shown to be selectively degraded by proteases expressed following Pi resupply (Bozzo et al., 2004b). Learning about the proteases and other post-transcriptional controls will increase our understanding of the regulation and control of AtPAP26 and other PSI secreted proteins such as RNases and phosphodiesterases.

The aforementioned work will greatly expand our knowledge of the function and regulation of PSI APases. This data will help develop targets and strategies for biotechnological efforts in producing crop species with increased Pi efficiency, which are needed to minimize the input of unsustainable and polluting Pi fertilizers in crop production.
Literature Cited


in soil extracts that are hydrolyzed by phytase and acid phosphatase. *Biology and Fertility of Soils*, 32, 279-286.


APPENDIX 1

**Confirmation of T-DNA insert location and loss of AtPAP12 and/or AtPAP26 gene expression in atpap12, atpap26, and atpap12/atpap26 mutants.** A. Schematic representation of the AtPAP26 and AtPAP12 genes; white boxes and solid lines represent exons and introns, respectively. T-DNA insertion locations are indicated by atpap12 and atpap26 T-DNA, while arrows represent primers (B) used for RT-PCR and genotyping. C. Assessment of T-DNA location and homozygosity of mutants via PCR-based screening of gDNA template isolated from leaves of +Pi soil grown seedlings. PCR products were amplified using AtPAP26-specific primers (primer pair A+B) and AtPAP12-specific primers (primer pair C+D) from the Col-0, atpap26, atpap12, and atpap26/atpap12 gDNA. The lack of the AtPAP26 DNA band in atpap26 and atpap26/atpap12 demonstrates that the mutant is homozygous. Comparable results were obtained with the atpap12 mutant, where the AtPAP12 DNA band was absent in both the atpap12 and atpap26/atpap12 mutants. T-DNA insertion positions were confirmed using a left-border primer (LBb1.3 and LB3) and gene specific primers (A and C) respectively for atpap26 and atpap12 mutants.
APPENDIX 2

Immunoblot and SDS-PAGE analysis of cytoplasmic and cell wall extracts isolated from shoots of Pi-sufficient versus Pi-deficient Col-0 Arabidopsis seedlings. Seedlings were cultivated for 7-d in liquid 0.5x MS media containing 0.2 mM Pi before being transferred into 0 or 2.5 mM Pi (-Pi and +Pi, respectively) for an additional 7-d. Soluble cytoplasmic and concentrated cell wall proteins extracted from rosette leaves of the –Pi and +Pi seedlings were resolved using 10% SDS-PAGE mini-gels (30 µg protein/lane) and (A) subjected to immunoblot analysis using affinity-purified rabbit anti-(castor bean PEPC)-IgG, or (B) stained with Coomassie Blue R-250 to visualize total protein. In panel A the immunoreactive PEPC polypeptides were detected chromogenically using an alkaline phosphatase tagged anti-rabbit secondary antibody; ‘Pure PEPC’ represents 100 ng of homogeneous PEPC isolated from endosperm of developing castor beans. M, molecular mass standards; TD, tracking dye front.
Influence of nutrient deprivation or oxidative stress on growth of *atpap12/atpap26* and Col-0 seedlings. Shoot fresh weight of seedlings cultivated on horizontal agar plates containing 1.5 mM Pi for 7-d, then grown for an additional 7-d on media containing 1.5 mM or 50 μM Pi (+Pi and –Pi, respectively), or on +Pi media lacking nitrogen (-N) or potassium (-K), or containing 1 μM paraquat (PQ). All values represent means ±SE of n = 12 different seedlings from four different plates; asterisks denote values that are significantly different from Col-0 (P < 0.01).
Immunoblot analysis of AtPAP12 and AtPAP26 polypeptides in clarified rosette extracts of 21-d-old Arabidopsis plants cultivated in –Pi soil. The atpap12/atpap26 double mutant was backcrossed with atpap12 and atpap26 single mutants to restore the expression of AtPAP26 and AtPAP12, respectively (for details see text and legend for Fig. 5). Protein extracts (5 μg/lane) as well as homogeneous native AtPAP12 and AtPAP26 (50 ng each) purified from secretome of –Pi Arabidopsis suspension cells (Tran et al., 2010a) were resolved by SDS-PAGE, electroblotted onto a PVDF membrane, and immunoblotted using the anti-AtPAP12 immune serum as described in the legend for Fig. 1E.
In vivo APase staining of *Arabidopsis* root surfaces in Col-0 and atpap12/atpap26 seedlings.

Plants were cultivated for 2 weeks on vertically oriented agar plates containing 0.5x MS media, 1% (w/v) sucrose, and 50 μM Pi before staining. A. Roots were rinsed in sodium acetate buffer (0.1 mM, pH 5.0) and covered with an agarose substrate mixture containing 200 mM acetate buffer, pH 5.0, β-naphthyl-P (0.2% w/v), and diazo blue B (o-dianisidine, textrazotied 0.2% w/v). A dark purple/red colour indicated APase activity in the roots and root exudates (Gilbert et al., 1999). B. Root segments were embedded in an agarose substrate mixture containing 0.01% w/v BCIP (Wang et al., 2011). The dark blue colour indicates APase activity. Images shown in panel A and B are representative of at least three replicates; scale bar = 1 cm.
Soil grown – Pi atpap12/atpap26 resupplied with Pi. Seedlings were grown for 7-d in liquid media containing 0.2 mM Pi, then transplanted into a Pi-deficient soil mix and grown for an additional 14-d. Seedlings were then fertilized with 0.25x Hoaglands media containing 0 or 2 mM Pi and cultivated for an additional 5 d; scale bar = 1 cm.