THE INFLUENCE OF NUTRITIONAL PHOSPHATE DEPRIVATION
ON THE SECRETED PROTEOME OF ARABIDOPSIS THALIANA

by

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Abstract

This thesis examines the influence of nutritional phosphate (Pi) deprivation on extracellular proteins secreted by the model plant *Arabidopsis thaliana*. Initial studies compared the secretome of Pi-sufficient (+Pi) versus Pi-deficient (-Pi) Arabidopsis cell cultures by 2-dimensional gel electrophoresis. Mass spectrometry identified 18 different secreted proteins that were upregulated by at least 2-fold by -Pi Arabidopsis. They were predicted to function in Pi scavenging, cell wall and ROS metabolism, proteolysis, and pathogen responses. The relationship between mRNA levels and relative amounts of selected secretome proteins was assessed. The results indicate that transcriptional control is but one of many factors contributing to Arabidopsis Pi starvation responses and highlight the importance of parallel biochemical and proteomic studies of -Pi plants. Three purple acid phosphatase (APase) isoforms were fully purified from the culture media of -Pi Arabidopsis cells and identified as AtPAP12 (At2g27190) and two AtPAP26 (At5g34850) glycoforms. As each purple APase exhibited broad substrate specificities and pH-activity profiles, it is hypothesized that their combined activities facilitate Pi scavenging from soil-localized organophosphates during nutritional Pi deprivation. AtPAP26 is dual-targeted during Pi stress since an earlier report demonstrated that it is also the principal intracellular (vacuolar) APase upregulated by -Pi Arabidopsis. The results indicate that differential glycosylation influences AtPAP26’s substrate specificity and subcellular targeting. An *atpap26* T-DNA insertional mutant lacking AtPAP26 transcripts and immunoreactive AtPAP26 polypeptides exhibited: (i) 9- and 5-fold lower shoot and root APase activity, respectively, which did not change in response to Pi starvation, (ii) a 40% reduction in secreted APase activity during Pi deprivation, (iii) 35 and 50% reductions in free and total Pi concentration, respectively, in shoots of -Pi.
plants, and (iv) impaired shoot and root development when subjected to Pi deficiency. By contrast, no deleterious influence of AtPAP26 loss of function was apparent in +Pi plants. The results establish a firm role for AtPAP26 in the acclimation of Arabidopsis to Pi deficiency. The identification and functional characterization of secreted proteins upregulated by –Pi Arabidopsis is relevant to applied efforts to engineer Pi-efficient transgenic plants, needed to minimize the input of expensive, unsustainable, and polluting Pi fertilizers in crop production.
Co-Authorship

With the exception of the literature review and general discussion, all chapters were co-authored with Dr. Plaxton. N-terminal amino acid sequencing was performed at the Protein and Peptide Sequencing Facility of the Biotechnology Research Institute (Montreal QC, Canada). Oligonucleotide sequencing was done at the Centre for Applied Genomics at The Hospital for Sick Kids (Toronto ON, Canada). Cloning of the AtPAP26-mCherry construct was performed by Dr. Joonho Park. Transient expression and fluorescence microscopy of AtPAP26-mCherry was performed in collaboration with Drs. Naomi Marty and Rob Mullen from the University of Guelph. All other research was conducted by Hue Tran in the Plaxton lab. Collaborators in Chapter 3 include Weiqiang Qian, who assisted with the purification and kinetic characterization of the three PAPs, Brenden Hurley, who performed the semi-quantitative RT-PCR shown in Fig. 3.5, and Dr. Yimin-She who provided the mass spectrometry data which I analyzed. Chapter 4 is co-authored by Brenden Hurley, a former MSc student with WC Plaxton, who helped with the isolation and genotypic screen of a homozygous atpap26 mutant, and performed the phenotypic analysis of plate grown atpap26 knockout mutants (Figs. 4.1C, 4.2, and 4.4).
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<tbody>
<tr>
<td>2-DE</td>
<td>two dimensional polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>A</td>
<td>absorbance</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>
</tr>
<tr>
<td>bis-pNPP</td>
<td><em>bis</em>-para-nitrophenylphosphate</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Linear Alignment Sequencing Tool</td>
</tr>
<tr>
<td>CBB</td>
<td>Coomassie Brilliant Blue</td>
</tr>
<tr>
<td>CCF</td>
<td>cell culture filtrate</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CHCA</td>
<td><em>α</em>-cyano-4-hydroxycinnamic acid</td>
</tr>
<tr>
<td>Col-0</td>
<td><em>Arabidopsis thaliana</em> ecotype Columbia</td>
</tr>
<tr>
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<td>Concanavalin A</td>
</tr>
<tr>
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<td>digoxigenin</td>
</tr>
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<td>DPDS</td>
<td>2,2'-dipyridyl disulfide</td>
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<tr>
<td>DSA</td>
<td><em>Datura stramonium</em> agglutinin</td>
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<td>EC</td>
<td>enzyme commission</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>fast protein liquid chromatography</td>
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<td>genomic DNA</td>
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<td>genome index</td>
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<td><em>Galanthus nivalis</em> agglutinin</td>
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<tr>
<td>HMW</td>
<td>high molecular weight</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IPG</td>
<td>immobilized pH gradient</td>
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<tr>
<td>$K_{av}$</td>
<td>partition coefficient</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>MAA</td>
<td><em>Maackia amurensis</em> agglutinin</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>matrix assisted laser desorption ionization- time of flight</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
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<td>MOWSE</td>
<td>molecular weight search</td>
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<tr>
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<td>messenger RNA</td>
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<tr>
<td>$M_r$</td>
<td>molecular mass</td>
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<td>MS</td>
<td>mass spectrometry</td>
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<tr>
<td>MWCO</td>
<td>molecular weight cut off</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAP</td>
<td>purple acid phosphatase</td>
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<td>PAP26-S1 or S2</td>
<td>secreted isoforms of PAP26</td>
</tr>
<tr>
<td>PAP26-V</td>
<td>vacuolar isoform of PAP26</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
</tr>
<tr>
<td>$pI$</td>
<td>isoelectric point</td>
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<tr>
<td>Pi</td>
<td>orthophosphate</td>
</tr>
<tr>
<td>+Pi and -Pi</td>
<td>phosphate sufficient and phosphate deficient respectively</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PNA</td>
<td>peanut agglutinin</td>
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<td>pNPP</td>
<td>para-nitrophenylphosphate</td>
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<tr>
<td>PPI</td>
<td>pyrophosphate</td>
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<tr>
<td>PQ</td>
<td>paraquat</td>
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<tr>
<td>PSI</td>
<td>phosphate starvation inducible</td>
</tr>
<tr>
<td>PSR</td>
<td>phosphate starvation response</td>
</tr>
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<td>PTM</td>
<td>post-translational modification</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
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<tr>
<td>RGP</td>
<td>reversibly glycosylated polypeptide</td>
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<td>ribonucleic acid</td>
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<tr>
<td>RNase</td>
<td>ribonuclease</td>
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<tr>
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<td>reactive oxygen species</td>
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<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SNA</td>
<td><em>Sambucus nigra</em> agglutinin</td>
</tr>
<tr>
<td>SCF</td>
<td>seedling culture filtrate</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>maximum velocity</td>
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Chapter 1. General Introduction and Literature Review

PLANT PHOSPHATE NUTRITION

Orthophosphate (Pi) is an essential macronutrient for plant growth and development. It is a key structural constituent of important biomolecules such as ATP, nucleic acids, phospholipids, and sugar-phosphates. Pi is involved in many fundamental processes in plant life, including photosynthesis, respiration, biosynthesis, membrane construction and signal transduction. However, the soluble Pi concentration of many soils ranges from about 1-10 µM, far lower than the intracellular Pi concentrations (5-20 mM) required for optimal plant growth [1-3]. Plant growth and crop productivity is directly limited by soil Pi availability. Maximizing the yield of most modern crop varieties therefore relies on the extensive application of Pi-fertilizers. While the use of Pi-fertilizers has increased crop productivity, it has also posed many problems. Of the estimated 40 million metric tons of Pi-fertilizer currently applied worldwide each year, less than 20% is absorbed by crops, whereas Pi-runoff from fertilized fields into nearby waterways results in environmentally destructive processes such as aquatic eutrophication [1]. Pi-fertilizers mostly rely on extraction from non-renewable rock–Pi reserves, derived from fossilized bone deposits. The application of Pi-fertilizers is quite expensive, particularly in developing countries, and the problem is exacerbated in tropical and sub-tropical regions containing acidic soils, which greatly reduce the availability of Pi to the plants. The projected depletion of global rock–Pi reserves within the next 80 years raises an interesting dilemma in the face of the world population explosion [1]. In order to ensure agricultural sustainability and a reduction in Pi-fertilizer overuse, plant and soil scientists must address the need to bioengineer Pi-efficient transgenic crops. The design of effective biotechnological strategies to enhance crop Pi acquisition necessitates our
detailed understanding of Pi-starvation inducible gene expression and the complex biochemical adaptations of Pi-deficient plants.

In soil, plants can only assimilate Pi in its fully oxidized state, $\text{H}_2\text{PO}_4^-$ or $\text{HPO}_4^{2-}$. Though Pi is abundant in soil, it is one of the least accessible macronutrients, as it readily forms insoluble calcium salts in alkaline soils, or complexes with iron and aluminium oxides in acidic soils, rendering it inaccessible for root uptake [3-6]. Up to 80% of soil Pi reserves exist as organic-Pi, mainly in the form of Pi-esters derived from decomposing biomatter [1,3,6-8]. Plants therefore face two primary challenges in acquiring Pi from their environment. The first is the solubility of free Pi, which plants can improve by root secretion of large amounts of organic acids into the rhizosphere that saturate soil anion exchange capacity [1,3,6]. Secondly, roots possess the ability to mobilize Pi from the soil organic-Pi pools via the secretion of hydrolytic enzymes that free esterified-Pi [1-3,6,9]. Factors that contribute to the accumulation and turnover of different forms of inorganic- and organic-Pi in the soil are complex and controlled by various processes. However, which soil organic-Pi pools are accessible to roots remains unclear, as most plants appear to be unable to utilize phytate (myo-inositol hexaphosphate), an abundant organic-Pi component of certain soils, as a source of nutritional Pi [10,11].

**THE PLANT PHOSPHATE STARVATION RESPONSE**

Plants have evolved the ability within species-dependent limits to acclimate for extended periods of Pi deficiency by eliciting a complex array of morphological, physiological and biochemical adaptations, collectively known as the Pi-starvation response (PSR). The PSR arises in part from the coordinated induction of hundreds of Pi-starvation inducible (PSI) genes that reprioritize internal Pi use and maximize external
Pi acquisition [3,5,12,13]. The evolution of elaborate strategies to enhance the acquisition and use of Pi from the environment allows many plants to effectively acclimate to periods of Pi stress.

*Morphological adaptations of Pi-deprived plants*

Enhancing the root’s surface area thereby allowing greater Pi absorption underlies the morphological adaptations of Pi-deficient (-Pi) plants that include: (i) increasing the root:shoot growth ratio, (ii) alterations in root architecture and diameter, (iii) a shift from primary to lateral root growth, which promotes the exploration of topsoil for available Pi, and (iv) increased root hair growth and density [1,3,6]. For example, -Pi Arabidopsis seedlings have elongated root hairs and a five-fold increase in root hair density accounting for up to 70% of the total root absorptive area [6,14]. An analogous adaptive strategy of most -Pi plant species is the formation of symbiotic associations between their roots and beneficial mycorrhizal fungi.

*Mycotrophic versus non-mycotrophic plants*

Pi acquisition by mycotrophic plants is significantly enhanced by the presence of arbuscular mycorrhizae formed between soil-inhabiting fungi of the order Glomales and roots of about 90% of plant species (excluding Cheonpodiaeae, Cruciferae, Cyperaceae, Junaceae, and Proteaceae families) [15]. This symbiosis develops in roots when the fungus colonizes the apoplast and cells of the cortex, accessing photosynthate (sucrose) supplied by the host plant. Formation of mycorrhizal associations increases the effective length and surface area of roots, enabling the plant to exploit a larger volume of soil, thereby increasing the uptake of Pi and some micronutrients. However,
disruption of beneficial mycorrhizal associations due to Pi-fertilization and soil tilling has been an undesirable consequence of modern agriculture.

The dependency of plant species on mycorrhizal infection and the extent of host infection is dependent on plant Pi status and the availability of soil Pi. Although normally regarded as a beneficial symbiont, in situations where there is no nutritional benefit for the host plants in terms of Pi uptake, arbuscular mycorrhizal fungi can be detrimental to plant growth by the consumption of host carbon [16]. It is notable that many non-mycotrophic plants such as buckwheat (*Fagopyrum esculentum*), white lupin (*Lupinus albus*), and harsh hakea (*Hakea prostrata*) are notorious for their ability to thrive on -Pi soils. This reflects the view that relative to mycotrophic species, the non-mycotrophs have evolved mechanisms that allow more efficient acclimation to low Pi conditions [17]. Non-mycotrophic members of the Proteaceae family such as white lupin form proteoid roots when cultivated under -Pi conditions. Proteoid roots are clusters of short lateral roots that can absorb Pi at a faster rate than non-proteoid roots, thus enhancing Pi uptake [1]. 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.

*Transcriptional and post-transcriptional responses to Pi starvation*

Many elements of the PSR are controlled at the transcriptional level, and -Pi plants extensively remodel their transcriptome and proteome in ways that coordinate the requisite metabolic and morphological adaptations. A large collection of microarray data regarding plant, particularly Arabidopsis, transcriptional responses to -Pi has shed light on the molecular identity and regulation underlying many classical biochemical and
physiological adaptations to -Pi [6,13,18-21]. PSI gene expression is highly coordinated in a temporal and tissue specific manner [6]. The marked reduction in cytoplasmic Pi pools that accompanies prolonged Pi starvation is met by a highly specific response that differs in roots and shoots of Arabidopsis [18,20]. Despite the induction of 600-1800 genes across all tissues, there appears to be only an approximate 25% overlap between those specifically induced in the root and shoot, implying strong tissue-specific adaptations to -Pi. Transcriptional repression also plays a critical role in determining the PSR, with 250-700 genes having decreased transcript accumulation in response to -Pi. Again tissue specificity plays a key role in this response with only a 5-10% overlap in repressed gene expression between roots and shoots [18-20]. Also, transcriptional responses to Pi starvation may occur in distinct stages. The so-called ‘early’ genes are those that are induced rapidly by -Pi and often represent genes involved in general stress response, rather than being Pi starvation-specific. Conversely, ‘late’ genes are suggested to be more tightly related to the morphological, metabolic, or physiological responses to Pi starvation [18-20].

Post-transcriptional mechanisms also play a critical role in the control of PSI gene expression and activity. This is reflected by proteomic profiling of –Pi corn and rice demonstrating that transcript abundance of various genes is not always indicative of protein accumulation during Pi stress [22-24]. One of the best characterized examples of post-transcriptional mechanisms in the Arabidopsis PSR is the regulatory pathway defined by the microRNA 399 (miR399), PHO2, and the transcription factor PHR1 [6,25]. During Pi sufficiency, the PHO2 gene expresses an E2 ubiquitin conjugase, which exerts its regulatory effects by decreasing the expression of critical PSI genes [26]. Upon Pi starvation, PHR1 is induced and activates expression of miR399 which leads to the targeted destruction of the E2 ubiquitin conjugase, and consequent accumulation of its
targets [25]. However, miR399 regulatory effects appear to be mediated by the PSI non-coding RNA At4 via ‘target mimicry.’ Combined, the use of miR399 and the riboregulator At4 allow fine control of PHO2 activity. This suggests that two layers of post-transcriptional regulation influence the expression of a subset of PSI genes [27]. The targets of E2 ubiquitin conjugase or miR399 that are regulated by inhibition of translation rather than mRNA cleavage are currently unknown. Future work must focus on these downstream post-transcriptional mechanisms as they may define novel PSR genes and new targets for biotechnological engineering of Pi efficient crops.

Reversible phosphorylation and differential glycosylation are also emerging as important post-translational modifications for the control and/or subcellular targeting of diverse enzymes upregulated by -Pi plants. Upregulation of phosphoenolpyruvate (PEP) carboxylase is an archetypical metabolic adaptation of -Pi plants (Fig. 1.1) [1,3,28-32]. The simultaneous induction and in vivo phosphorylation-activation of the PEP carboxylase isozyme AtPPC1 contributes to the metabolic adaptations of -Pi Arabidopsis [30]. Interestingly, the most responsive PSI Arabidopsis transcripts include those encoding both PEP carboxylase protein kinase isozymes (AtPPCK1 and AtPPCK2) [18,19,30]. Similarly, corn cultivars resistant to Pi starvation upregulate phosphoprotein phosphatase type-2A catalytic subunits [24], whereas alterations to in vivo protein phosphorylation patterns were documented in Brassica napus suspension cells responding to -Pi [33]. A challenging goal for future research will be to document the functional consequences of reversible protein phosphorylation in the signaling and metabolic pathways involved in plant acclimation to nutritional Pi stress.

Biochemical adaptations of Pi-starved plants
Plants utilize a host of biochemical adaptations as part of their PSR. These include anthocyanin accumulation in leaves, and the induction of high-affinity Pi-transporters and alternative bypass enzymes to Pi- and the adenylate-dependent reactions of glycolysis and mitochondrial respiration (Fig. 1.1). Anthocyanin accumulation has frequently been observed in shoots of -Pi plants and is believed to protect chloroplasts against photoinhibition [2,3,6]. The upregulation of high-affinity Pi transporters of the plasma membrane is another important component of the plant PSR (Fig. 1.1) [3,5]. These transporters are induced when the external Pi concentration is low, and actively assimilate Pi against a steep concentration gradient, as the soil Pi concentration can be 10,000-fold lower than that of root cells [5]. High-affinity Pi transporters of Arabidopsis belong to the nine member PHT1 family and consist of Pi/H+ symporters with 12 transmembrane domains [34]. While all nine members are responsive to -Pi, each appears to have a certain degree of tissue specific expression, with some expressed in epidermal and root hair cells while others are expressed in root stelar cells [35]. Arabidopsis Pht1;4 and Pht1;1 have been shown to be the two major Pi transporters involved in Pi uptake, as other members of the PHT1 family are believed to function in Pi transport in various tissues [35, 36]. Consistent with this, knockout mutants of Pht1;4 or Pht1;1 show decreased Pi acquisition during -Pi [36], while knockout of Pht1;1 (pho1) also results in failure to accumulate Pi in shoots during Pi sufficiency due to its role in xylem Pi loading [37].

The induction and expression of PSI genes and proteins is associated with the cellular Pi status. An early phase response to Pi starvation is the dramatic decrease in intracellular Pi concentrations. Cytoplasmic Pi homeostasis is maintained at the expense of Pi distribution from the vacuole, which stores up to 95% of the cell’s total Pi during Pi sufficiency [2]. The decrease in cytoplasmic Pi during Pi stress is correlated with a
significant reduction in adenylate levels, indicating that reorganization of cellular metabolism to conserve limited pools of adenylates and Pi is another important biochemical adaptation of -Pi plants. This is accomplished by altering the organization of glycolysis, mitochondrial respiration, and tonoplast H⁺ pumps allowing adenylate and Pi-dependent reactions to be bypassed during -Pi [2,29]. Several of these bypasses facilitate respiration and vacuolar pH maintenance during extended Pi stress by using pyrophosphate (PPI) in performing cellular work, while simultaneously conserving ATP and recycling Pi (Fig. 1.1). Glycolytic bypass enzymes such as PPI-dependent phosphofructokinase and PEP carboxylase also promote intracellular Pi recycling during -Pi, as Pi is a byproduct of their reactions [29]. The PEP carboxylase catalyzed bypass of cytosolic pyruvate kinase, coupled with the enhanced activity of malate dehydrogenase and citrate synthase, results in the synthesis of organic acids from glycolytic metabolites (Fig. 1.1). This is critical for the anaplerotic replenishment of tricarboxylic cycle intermediates, as well as the root excretion of organic acids, a common response to -Pi [1,3,29,31,32]. Organic acid excretion is believed to aid in chelating metal cations (e.g., Al³⁺, Ca²⁺, Fe²⁺) that immobilize Pi thus increasing Pi solubility [1].
Fig. 1.1. A model suggesting various adaptive metabolic processes (indicated by asterisks) that are believed to help plants acclimate to nutritional Pi deficiency. Alternative pathways of cytosolic glycolysis, mitochondrial electron transport, and tonoplast H⁺-pumping facilitate respiration and vacuolar pH maintenance by Pi-starved plant cells because they negate the dependence on adenylates and Pi, the levels of which become markedly depressed during severe Pi starvation. Large quantities of organic acids produced by PEP carboxylase (PEPC), malate dehydrogenase (MDH), and citrate synthase (CS) are excreted by roots to increase the availability of mineral bound Pi, by solubilizing Ca-, Fe- and Al-phosphates (denoted Met–Pi). During Pi stress vacuolar purple acid phosphatases (PAP-V) is believed to recycle Pi from non-essential intracellular Pi-esters. Similarly, secreted purple acid phosphatases (PAP-S) likely function to scavenge Pi from extracellular Pi-monoester and nucleic acid fragment pools for its eventual uptake by Pi-starvation inducible high-affinity Pi transporters of the plasma membrane. The following abbreviations are used: ME, malic enzyme, PK, pyruvate kinase, H⁺-PPiase, H⁺ pyrophosphatase, 2':3'-cNMP, cyclic nucleotide monophosphate, 3'-NMP, nucleotide monophosphate.
Plants also increase the efficiency of Pi use during -Pi via upregulation of PSI-hydrolases that scavenge Pi from non-essential Pi-esters. Classical PSI-hydrolases include non-specific phospholipases, ribonucleases, and acid phosphatases (APases) [2,3,5,9,38,39]. Phospholipase induction is accompanied by the replacement of membrane phospholipids with amphipathic sulfolipids (in the thylakoid membranes) and galactolipids (in both thylakoid and extraplastidic membranes) [3,40,41]. Phospholipids are a dynamic and indispensable P-reserve during Pi starvation [42]. Increased phospholipid degradation during Pi starvation is consistent with microarray results. Genes encoding phospholipases are upregulated and those involved in phospholipid biosynthesis are downregulated during Pi stress [18-21]. Knockout of phospholipase activity or the downstream synthases required for membrane lipid remodeling results in the impaired development of -Pi Arabidopsis seedlings [40,43].

Roots and suspension cell cultures of -Pi plants also induce secreted ribonucleases, phosphodiesterases, and APases which participate in systematic Pi mobilization from soil localized organic-Pi, including nucleic acids (Fig. 1.1) [2-4]. Arabidopsis ribonuclease-1 (RNS1) transcript and secreted protein levels are highly induced in response to Pi stress [39] and Arabidopsis plants cultivated on nucleic acids as their sole source of exogenous Pi grow as well as Pi-fertilized control plants [12,38].

**PLANT ACID PHOSPHATASES**

APases (E.C. 3.1.3.2), which are ubiquitous in plant and non-plant species, catalyze the hydrolysis of Pi from a broad and overlapping range of Pi-monoesters with an acidic pH optimum. Eukaryotic APases exist as a wide variety of tissue- and/or cellular compartment-specific isozymes that vary in their physical and kinetic properties. The majority of plant APases display non-specific substrate selectivity and function in the
production, transport, and recycling of Pi [9]. Plant APases also display considerable variation in terms of native and subunit Ms, metal inhibition or activation, subcellular localization, cofactor requirements, pH optima, substrate specificity, and expression determinants [9]. The APase reaction product, Pi, serves as a potent competitive inhibitor of most plant APases, as do the following oxyanions: molybdate, vanadate, ascorbate, arsenate, tungstate, and tartrate [9]. Product inhibition by Pi suggests it serves as a physiological feedback inhibitor in plant tissues.

The Arabidopsis genome encodes over 50 different APases including 10 vegetative storage protein type APases, four phosphatidic acid APases, and one histidine APase [44]. The unexpected diversity of vascular plant APases poses a fascinating biological question in its own right. This is complicated by the fact that not all APases appear to function as metabolic enzymes. For example, a vegetative storage protein accumulates up to almost 40% of total soluble protein in leaves of depodded soybean plants, but contributes less than 1% to extractable APase activity, while a single point mutation can increase its APase activity by up to 20-fold [45].

The upregulation of intracellular and secreted APase activity has long been recognized as a universal biochemical response to plant Pi deprivation [9]. Intracellular APases are ubiquitous within plant tissues, and their pH optima (pH 5-6) suggest they are localized to the acidic vacuole [9]. During Pi stress, the large decrease in vacuolar Pi levels relieves the APases from Pi inhibition, allowing the vacuolar APases to remobilize and recycle Pi from expendable intracellular Pi-monoesters and anhydrides. This is accompanied by marked reductions in cytoplasmic Pi-metabolites during extended Pi stress [9]. Extracellular APases belong to a group of PSI-hydrolases secreted by -Pi plants. In the rhizosphere, they hydrolyze Pi from external organic-Pi sources [3,12]. 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 [46-50]. The genetic and functional redundancy of plant APases necessitates greater study, with particular focus on the largest group, the purple acid phosphatases (PAPs).

**PURPLE ACID PHOSPHATASES**

PAPs received their colourful nomenclature owing to their distinctive purple or pink colour in solution. This results from a charge transfer transition at about 560 nm from the metal-coordinating tyrosine to the metal ligand Fe(III) [51]. PAPs belong to a metallophosphoesterase superfamily that includes phosphoprotein phosphatases and exonucleases. Also, unlike other plant APases, PAPs are insensitive to tartrate inhibition [51]. All contain five blocks of conserved metal ligating residues, although the location, number and identity of the residues differ between the family groups [44,51]. 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 are highly conserved amongst bacterial, mammalian and plant PAPs and form dimetallic active sites [44,51]. Despite conservation of metal ligating residues, mammalian PAPs contain a Fe(III)-Fe(II) active site whereas plant PAPs typically contain a Fe(III)-X(II) active site where X is either Zn$^{2+}$ or Mn$^{2+}$ [51,52]. The availability of these metals in mammalian or plant cells implies that divalent metal cation specificity may provide a form of functional PAP specialization [52].

The structure of PAP catalytic sites and domains are also highly conserved [51,52]. Bacterial, mammalian and plant PAPs all contain catalytic domains that consist of two sandwiched β-α-β-α-β motifs, with almost perfect alignment and order of the conserved metal ligating residues [44,52]. Despite the conservation of catalytic domains,
mammalian and plant PAPs differ in their oligomeric structure [51,52]. Mammalian PAPs exist as 35 kDa monomers consisting solely of a catalytic domain. Although mammalian-like low molecular weight PAPs exist in plants [53,54], plants also possess high molecular weight (HMW) oligomeric PAPs composed of 50-60 kDa subunits that consist of an N-terminal non-catalytic domain fused to a C-terminal catalytic domain which is structurally related to monomeric low molecular weight PAPs [51,52]. Although most HMW PAPs appear to exist as homodimers [50,51,55-58], several HMW PAPs secreted by -Pi plant cells also appear to exist as a monomer (Table 1.1) [47]. Dimeric HMW PAPs form either through disulfide bridges or via non-covalent interactions [51,55,57]. It is not yet understood how oligomeric structure impacts PAP function, or even why plant PAPs exist in two different oligomeric states.

Most PAPs are classified as non-specific APases that catalyze the hydrolysis of Pi from a broad spectrum of Pi-esters [44,51]. However, mammalian PAPs expressed in macrophages and spleen cells after phagocytosis likely play a role in the generation of reactive oxygen species via a Fenton reaction involving the Fe(II) of the active site [52]. Similarly, several plant PAPs exhibit alkaline peroxidase activity that is unaffected by APase inhibitors [47,48,50,53] and overexpression of a soybean PAP, GmPAP3, increased tolerance to oxidative damage imposed during salinity stress [59]. Mammalian PAPs also function as phosphotyrosyl phosphatases, implying a role in signal transduction [60]. Similarly, a PAP from tobacco cell walls was demonstrated to be active against phosphotyrosylated peptides [61]. A variety of PAPs from other plant sources have significant activity with phosphotyrosine or other phosphoamino acids as substrates [50,58,62]. Interestingly, transgenic expression of the tobacco cell wall PAP resulted in altered cell wall composition, implying an in vivo regulatory role [63].
Phosphate starvation inducible purple acid phosphatases

Many studies have focused on the role that plant PAPs might play in Pi scavenging and recycling from Pi-esters and anhydrides during nutritional Pi deficiency. Pi starvation induces temporal and tissue specific expression of PAPs and the concomitant down-regulation of other PAPs [18,20,44,49,64,65]. The transcription factors PHR1, WRKY75, and ZAT6 have been implicated in the control of PSI PAP expression [66-68], while other studies have revealed PSI PAPs that are controlled by post-transcriptional mechanisms (Table 1.1) [50]. In contrast, Pi re-supply to -Pi plants quickly represses PSI PAPs genes while inducing specific proteases that appear to target intracellular and secreted PSI PAPs [50,62,69]. Identification and characterization of PSI PAPs is required to define the molecular mechanisms underlying this archetypical plant response to Pi starvation, as well as to identify suitable targets for improving crop Pi acquisition.

A variety of intracellular and secreted APase isozymes that are upregulated following Pi stress in plants were demonstrated to be PAPs. These have been biochemically characterized from several species including tomato [47,48], lupin [57,70], bean [54], and Arabidopsis [50,53]. White lupin secretes copious amounts of APase activity from its proteoid roots when cultivated under -Pi conditions [70]. The transcript and corresponding protein levels of the secreted APases that are responsible for this activity are highly induced upon -Pi [70]. The protein exists as a glycosylated homodimer composed of 70 kDa subunits, and although not directly determined to be a PAP, it shares high amino acid sequence identity (63 %) with AtPAP12 [70]. This similarity extends to its promoter region, which can direct enhanced gene expression under -Pi. A secreted APase isozyme of yellow lupin (Lupinus luteus) roots is orthologous to AtPAP26 (Table 1.1) [57]. As with AtPAP26, this lupin AtPAP26 ortholog is constitutively
transcribed regardless of Pi nutritional status, but has enhanced secretion when plants were cultivated under Pi starvation.

Three APases purified to homogeneity from -Pi tomato suspension cell cultures were markedly upregulated in response to Pi deficiency. They are bona fide PAPs as they have: (i) a pink color in solution, (ii) amino acid sequence similarity to putative or previously characterized plant PAPs, (iii) insensitivity to tartrate, and (iv) APase activity [47,48]. All three are glycoproteins having broad substrate specificity with acidic pH-activity optima. Two of these PSI PAPs are secreted into the rhizosphere as 84 and 57 kDa monomers; the third was a novel heterodimer of 142 kDa composed of an equivalent ratio of 63 and 57 kDa subunits [47], and is most likely localized to the cell vacuole [48]. The biochemical characterization of the intra- and extracellular PSI tomato PAP isozymes indicates their probable physiological role in Pi scavenging and Pi recycling by -Pi tomato. These plant PSI proteins are subject to both temporal and tissue-specific synthesis in -Pi plants [49]. Decreased cytoplasmic Pi, a consequence of prolonged Pi starvation, is met by a highly specific response that involves the differential synthesis of PSI tomato PAP isozymes. These results corroborated aforementioned Arabidopsis transcriptomic studies suggesting that some PSI genes have temporal as well as tissue-specific expression [13,18,20,71].

Most recently, a PAP upregulated by –Pi bean plants (Phaseolus vulgaris) was characterized and identified as PvPAP3 by mass spectrometry [54]. This 34 kDa 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. PvPAP3 was highly induced in both leaves and roots of Pi deficient bean plants. The transient expression of 35S:PvPAP3-GFP constructs in onion epidermal cells indicated that it is secreted into the apoplast. PvPAP3 was most
active with ATP as a substrate, suggesting that it may function in the adaptation of common bean to Pi stress through the use of extracellular ATP as a Pi source from the environment [54].

**ARABIDOPSIS THALIANA PURPLE ACID PHOSPHATASES**

The Arabidopsis PAP (AtPAP) family is encoded by 29 genes, although only 28 appear to be actively transcribed [44,72]. The complexity and variation of plant PAP expression and regulation is demonstrated by the numerous Arabidopsis PAPs, and suggests that other plant species probably possess many PAP isozymes. The AtPAPs are classified into three distinct phylogenetic groups according to their deduced amino acid sequences (Fig. 1.2) [44]. 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 [72]. Although some AtPAP transcripts accumulate in response to stress [44,53,64], cell specific expression patterns of AtPAPs remain elusive. There is also a lack of information regarding the subcellular location of most AtPAPs. Plant PAPs have been localized in mitochondria (GmPAP3) [59], the cell vacuole (AtPAP26) [73], the cell wall (AtPAP10) [74], and the secretome (AtPAP10 and AtPAP12) [64,75]. This is curious given that all PAPs characterized to date contain transit peptides and are glycosylated implying that they are all targeted to the early secretory system where glycosylation occurs.

The Arabidopsis PAPs characterized to date have absorption maxima ranging from 500-520 nm. They display non-specific substrate specificity and are capable of catalyzing sufficient hydrolysis of ATP, para-nitrophenyl phosphate (pNPP), PEP, and
phytic acid (phytate) [11,50,53,64,72,76,77]. However, many of these PAPs exhibit preferences for certain substrates (Table 1.1). Those characterized to date have subunit sizes of various molecular weights, and differences in their structural organization are reflected in their substrate preference.
Fig. 1.2. A classification scheme for Arabidopsis purple acid phosphatases 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 purple acid phosphatases (AtPAP3, AtPAP7–AtPAP13, AtPAP17, AtPAP18) derived from cDNA analysis. The main groups (groups I, II, and III) are further divided to yield the eight subgroups (second column). The bootstrap values for the three main groups are boxed, whereas those for the eight subgroups are indicated by arrows. The predicted molecular masses of the deduced polypeptides are listed in the third column. Fig. modified from Li et al. [44].
Although the molecular and biochemical properties of a variety of plant PAPs have been well documented, their precise physiological functions have not been resolved [3,51]. To date, only AtPAP15 and AtPAP23 have been functionally characterized in transgenic Arabidopsis [72,76]. AtPAP15 is the only member of the AtPAP family that has thus far been shown to possess significant phytase activity (Table 1.1) [76,77]. Phytate is a major component of the soil organic-Pi complement, as well as the primary Pi storage reserve in seeds and animal feedstuffs. AtPAP15 is thought to be involved in ascorbate synthesis via production of myo-inositol [77]. Lower phytase activity was exhibited in extracts of AtPAP15 T-DNA knockout mutants, but unaltered activity was observed with the generic APase substrate pNPP relative to wild-type controls [77]. AtPAP15 also appears to play an important role in mobilizing Pi from phytate reserves during seed or pollen germination [76]. Constitutive overexpression of AtPAP15 containing a carrot 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 [11], confirming the phytase activity of AtPAP15.

In contrast to AtPAP15, AtPAP23 is a non-specific APase that is predominantly expressed in flower apical meristems, but becomes restricted to petals and anther filaments in fully developed flowers [72]. Despite its highly specific expression pattern and the demonstrated APase activity of its protein product, AtPAP23 knockout and overexpression lines had unaltered extractable APase activity and were indistinguishable from wild-type plants in the development of flower or other organs [72]. Although alterations in AtPAP23 expression levels did not result in any obvious phenotype, the Fe and Mn content of the overexpressed lines was significantly elevated relative to wild type plants. Thus, apart from its role as a non-specific APase, AtPAP23
possibly functions in Fe and Mn homeostasis in flower development and metabolism (Table 1.1) [72].

_Posphate starvation inducible Arabidopsis purple acid phosphatases_

In contrast to the wealth of AtPAP genomic and transcript expression data, comparatively little information is available on the specific AtPAP isozyme(s) that contribute to intra- versus extracellular Pi scavenging by -Pi Arabidopsis. AtPAP17 is one of the few PSI PAPs that has been purified and characterized from -Pi Arabidopsis seedlings [53]. AtPAP17 exists as a low molecular weight (34 kDa) monomeric PAP and is transcriptionally induced in roots and leaves of -Pi Arabidopsis (Table 1.1) [53]. The AtPAP17 promoter contains a binding site for AtPHR1, a transcription factor involved in the Arabidopsis PSR; thus, the upregulation of AtPAP17 during Pi stress appears to be mainly controlled at the transcriptional level [53]. AtPAP17 transcripts also accumulate in response to oxidative or salt stress, and AtPAP17 is a bifunctional enzyme that has both APase and alkaline peroxidase activity. This suggests that AtPAP17 could be involved in the metabolism of reactive oxygen species during general stress, rather than playing significant Pi recycling or scavenging roles in -Pi Arabidopsis [53]. The presence of an N-terminal signal peptide suggests that AtPAP17 may be linked to the cell wall or plasma membrane [53]. The determination of the subcellular localization of AtPAP17, as well as the phenotypic impact that modifying its expression has on transgenic Arabidopsis will help to fully establish its function(s) during Pi deprivation. Two PAPs that play significant roles in the PSR of Arabidopsis are AtPAP12 and AtPAP26, both of which will be discussed in detail in the following chapters.
Table 1.1 Properties and proposed roles of functionally and/or biochemically characterized Arabidopsis PAPs.

<table>
<thead>
<tr>
<th>AtPAP Designation</th>
<th>AGI code</th>
<th>Upregulated by Pi Starvation?</th>
<th>Subcellular Localization</th>
<th>Best Substrate</th>
<th>Physical Properties</th>
<th>Proposed Function(s)</th>
<th>Reference(s)</th>
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<tr>
<td></td>
<td>Transcript</td>
<td>Protein</td>
<td></td>
<td></td>
<td>Native $M_r$ (kDa)</td>
<td>Subunit $M_r$ (kDa)</td>
<td>$A_{max}$ (nm)</td>
</tr>
<tr>
<td>AtPAP12</td>
<td>At2g27190</td>
<td>Yes</td>
<td>secretome</td>
<td>PEP</td>
<td>130</td>
<td>60</td>
<td>520</td>
</tr>
<tr>
<td>AtPAP15</td>
<td>At3g07130</td>
<td>No</td>
<td>n.d.*</td>
<td>phytic acid</td>
<td>58</td>
<td>60</td>
<td>n.d.</td>
</tr>
<tr>
<td>AtPAP17</td>
<td>At3g17790</td>
<td>Yes</td>
<td>n.d.</td>
<td>pNPP**</td>
<td>n.d</td>
<td>34</td>
<td>n.d.</td>
</tr>
<tr>
<td>AtPAP23</td>
<td>At4g13700</td>
<td>No</td>
<td>n.d.</td>
<td>ATP</td>
<td>n.d</td>
<td>52</td>
<td>n.d.</td>
</tr>
<tr>
<td>AtPAP26 -V</td>
<td>At5g34850</td>
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<td>cell vacuole</td>
<td>PEP</td>
<td>100</td>
<td>55</td>
<td>520</td>
</tr>
<tr>
<td>AtPAP26 – S1</td>
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<td>No</td>
<td>secretome</td>
<td>PEP</td>
<td>65</td>
<td>55</td>
<td>500</td>
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<td>65</td>
<td>55</td>
<td>n.d.</td>
</tr>
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</table>

*n.d., not determined

**pNPP, para-nitrophenyl-phosphate
RESEARCH OBJECTIVES

The objectives of the thesis project were to analyze the effect of Pi deficiency on the secretome of Arabidopsis suspension cell culture. It is hypothesized that acclimation of Arabidopsis to nutritional Pi deprivation involves extensive remodeling of its secreted proteome including upregulation of specific PAP isozymes. Protein profiling using 2D-PAGE and MS has allowed for the characterization of global changes in the Arabidopsis secretome upon Pi starvation. The identification of proteins that are upregulated in response to Pi stress was performed by MS and these results identified potential targets for future research on the plant PSR. Arabidopsis suspension cell cultures were chosen since a relatively large biomass of cells cultured under a well defined nutritional regime (and their surrounding liquid media containing secreted proteins) can be obtained over a relatively short period of time. The availability of the complete Arabidopsis genomic sequence, together with a wide assortment of Arabidopsis genomic resources (including high throughput transcript profiling and a large collection of T-DNA insertion mutants) has made this non-mycotropic species an excellent model organism for studying the plant Pi starvation response.

A biochemical approach was taken to identify and characterize specific secreted APase isozymes involved in extracellular Pi scavenging by -Pi Arabidopsis. This involved the purification and characterization of secreted PAPs, coupled with bioinformatic analysis of peptide mass fingerprint and N-terminal amino acid sequence data obtained with the purified native PAPs. The successful isolation and identification of secreted PSI Arabidopsis PAPs will not only result in the identification of the corresponding Arabidopsis genes, but will also allow for the eventual overexpression of secreted APases in transgenic plants as a possible strategy to improve Pi acquisition.
Although the physical and kinetic properties and expression of several plant PAPs have been described, their physiological functions have not yet been resolved. Functional genomics was used to determine if a specific PAP isozyme (AtPAP26), that is dual targeted to the vacuole and secretome during Pi stress, plays a role in the Pi metabolism of Arabidopsis during nutritional Pi deprivation. This involved the molecular, biochemical, and phenotypic characterization of a homozygous T-DNA insertional mutant of the AtPAP26 in Arabidopsis. T-DNA insertional mutants can be obtained from a variety of T-DNA databases if the corresponding genes of the APase isozymes are known.

REFERENCES


Chapter 2. Proteomic analysis of alterations in the secretome of *Arabidopsis thaliana* suspension cells subjected to nutritional phosphate deficiency


ABSTRACT

A proteomic approach was applied to compare the secretome (culture filtrate proteome) of phosphate-sufficient (+Pi) and Pi-deficient (-Pi) *Arabidopsis thaliana* suspension cell cultures. Secretomes harvested from the +Pi and –Pi cells yielded dissimilar 2-DE maps. PMF via MALDI-TOF MS resulted in the identification of 50 protein spots representing 37 discrete proteins having unique gene identities. A total of 24 Pi-starvation responsive proteins were identified, with 18 of these being upregulated and 6 down-regulated. Secreted proteins upregulated by the –Pi cells included a ribonuclease involved in Pi scavenging from extracellular nucleic acids, as well as enzymes of cell wall modification, proteolysis, pathogen responses, and ROS metabolism. Enzyme activity assays and immunoblotting demonstrated that a pair of purple acid phosphatase isoforms having subunit *M*ₐₜₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖₖ₆
INTRODUCTION

Phosphate (Pi) is a crucial but limiting macronutrient for plant growth and metabolism. Agricultural Pi deficiency is alleviated by the massive application of Pi fertilizers. However, Pi assimilation by fertilized crops is inefficient and unsustainable, as the majority of applied Pi becomes insoluble in the soil or runoffs into and pollutes nearby surface waters. The projected depletion of non-renewable global rock-Pi reserves by the year 2100 [1] has prompted plant scientists to develop strategies and molecular tools for engineering Pi-efficient crops. This goal necessitates our thorough understanding of the intricate molecular and biochemical adaptations of Pi-deprived (-Pi) plants.

Plants respond to Pi starvation by increasing their root growth at the expense of shoot growth, and by forming lateral roots or root clusters, thereby increasing the surface area for absorption of limiting soil Pi [1]. -Pi plants also upregulate alternative bypass enzymes to Pi- and adenylate-dependent glycolytic and respiratory electron transport reactions, and scavenge and conserve Pi by replacing membrane phospholipids with amphipathic galacto- and sulfonyl lipids [2-5]. In addition, secreted ribonuclease (RNS), phosphodiesterase, and acid phosphatase (APase) cooperate in the catabolism of soil-localized nucleic acids to mobilize Pi [3, 4, 6, 7], which is made available for root uptake by Pi-starvation inducible (PSI) high-affinity Pi transporters [8]. -Pi plants also upregulate phosphoenolpyruvate (PEP) carboxylase which can result in the root excretion of large quantities of malic and citric acids [1, 2]. This acidifies the rhizosphere and contributes to the solubilization and assimilation of mineral Pi from the environment.

Important insights into plant Pi starvation responses have been acquired through extensive transcriptomic analyses that have revealed hundreds of Pi-starvation inducible (PSI) genes encoding proteins that are believed to help –Pi plants reprioritize internal Pi
use and maximize Pi acquisition from the soil [8-18]. However, these results must be balanced by the fact that transcript abundance does not necessarily reflect cognate protein levels [2, 19-21], and that transcript profiling provides no information about either the subcellular location of gene products, or PTMs that may be essential for their function, transport and activation. A proteomic study of –Pi rice seedlings documented numerous alterations in root protein expression that had not been revealed in earlier transcriptomic studies [20]. Similarly, AtPAP26 encodes a vacuolar purple APase that is markedly upregulated by –Pi Arabidopsis thaliana [21]. However, AtPAP26 transcripts are constitutively expressed in Pi-sufficient (+Pi) and –Pi Arabidopsis indicating that transcription exerts little influence on AtPAP26 polypeptide levels relative to translational and/or proteolytic controls [21]. Therefore, it is crucial that transcriptomic studies are integrated with proteomic, enzymological, and metabolomic analyses so that the full suite of the metabolic/biochemical adaptations of –Pi plants can be fully understood.

Owing to the availability of genomic resources we recently initiated biochemical and molecular studies of -Pi Arabidopsis suspension cells and seedlings [21]. Although suspension cells cultures provide a robust model for assessing the biochemical adaptations of –Pi plants [5, 7, 21-25], few reports describe the use of cell cultures for investigating Arabidopsis Pi starvation responses. In this study we employ 2-DE, MS, and immunoblotting to identify secretome alterations due to Pi-deprivation of Arabidopsis suspension cells.

**MATERIALS AND METHODS**

*Plant material and cell viability assay*

Heterotrophic Arabidopsis thaliana (cv. Landsberg erecta) suspension cells were cultured at 25°C in the dark as previously described [21]. For secretome analysis, 50 mL
of a 7-d +Pi culture was used to inoculate 450 mL of fresh media containing 0 or 5 mM K$_2$HPO$_4$. Cells were harvested after 7-d by filtration, frozen in liquid N$_2$, and stored at -80°C. Cell culture filtrates (CCFs) were processed as described below. Double staining with fluorescein diacetate and propidium iodide was used to respectively discriminate between living (stained green) and dead cells (stained red) via fluorescence microscopy using an Axio Imager Z1 Fluorescence Microscope (Carl Zeiss) as previously described [25].

**Protein extraction and secretome preparation for 2-DE**

Quick-frozen cells (1 g) were ground to a powder under liquid N$_2$ and homogenized (1:2 w/v) in ice-cold buffer (50 mM Na-acetate, pH 5.6, 1 mM DTT, 1 mM PMSF, 1% insoluble PVP), and clarified by centrifugation at 14 000 x g and 4°C for 10 min. The corresponding CCF (~1 L obtained from replicate 500 mL cultures) was rapidly concentrated to about 100 mL with a Pellicon and further concentrated using an Amicon Ultra-15 (10 kDa cut-off each) (Millipore Canada Inc.) until the final protein concentration was ≥1 mg/mL.

**Enzyme, protein, and phosphate assays**

All enzymes were assayed at 25°C by continuously monitoring NADH oxidation at 340 nm using a Gilford 260 recording spectrophotometer. APase assay conditions were 50 mM Na-acetate, pH 5.6, 5 mM PEP, 10 mM MgCl$_2$, 0.2 mM NADH, and 3 units of rabbit muscle lactate dehydrogenase in a final volume of 1 mL. Aldolase and PEP carboxylase activities were determined using coupled assays [26, 27]. One unit of activity is defined as the amount of enzyme resulting in the use of 1 µmol of substrate/min. Protein concentrations were determined with a CBB G-250 dye-binding
method using bovine \( \gamma \)-globulin as the standard, whereas CCF PI levels were
determined at 660 nm as previously described [7].

**Immunoblotting**

SDS-PAGE mini-gels were prepared and electroblotted onto PVDF membranes as previously described [7] and probed using IgGs described in the relevant Fig. legends. Immunoreactive polypeptides were visualized using a horseradish peroxidase-conjugated secondary antibody and ECL detection (ECL Plus, GE Healthcare). Immunoblots probed with anti-(AtPAP12 or AtPAP26)-immune serum were pretreated with sodium-\( m \)-periodate [7] to oxidize antigenic glycosylated side chains of glycoproteins. All immunoblots were performed in triplicate with representative results shown in the various Figs.

**2-DE and image analysis**

All 2-DE equipment and reagents were from GE Healthcare. Concentrated CCF proteins were precipitated with a 2D Clean-UP Kit. Following centrifugation, pellets were solubilized in 50 \( \mu \)L of rehydration buffer (6 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer (pH 4-7), and 50 mM DTT), and proteins quantified using the 2D Quant Kit. IEF was conducted at 20°C using IPG strips (pH 4-7 L, 7 cm) and an Ettan IPGphor II system. Protein (50 \( \mu \)g in 125 \( \mu \)L) was loaded into the IEF tray and passive rehydration carried out overnight under a layer of mineral oil. IEF was performed as follows: 250 V for 15 min, and 1000 V for 6 h, and 8000 V for a total of 40 000 Vh. Focused strips were equilibrated for 15 min in 50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, and 1% DTT, followed by incubation for 15 min in the same buffer without DTT, but containing 2.5% iodoacetamide. Equilibrated strips were placed on 12% SDS-
gels (10 cm X 10.5 cm X 1 mm) and sealed with a 3% agarose solution. SDS-PAGE was
performed with a MiniVE system at 5 mA for 30 min and then 30 mA until the tracking
dye reached the bottom of the gel. Gels were stained with CBB R-250. For each
condition analyzed, 3 replicate gels were prepared from 3 independent samples. Gels
were scanned using a desktop scanner (Epson perfection 600) and image analysis
performed with PDQuest software (version 7.0; Bio-Rad). After background subtraction
and spot detection, spots were matched and normalized using the method of total
density in gel image. The statistical significance of quantitative data was determined
using the Student’s *t*-test (*n* = 3, *p*<0.05), and the obviously different protein spots
defined as *p*<0.05 and change in amount at least 2-fold.

**In-gel digestion, MS, and database searching**

Protein-staining spots were excised from 2-DE gels, diced into ~1 mm pieces,
and destained in 50 mM ammonium bicarbonate in 50% ACN. Proteins were reduced
with 10 mM DTT in 100 mM ammonium bicarbonate and alkylated in 100 mM
ammonium bicarbonate containing 55 mM iodoacetamide. Samples were dehydrated
using 100% ACN and digested with 10 µL of 20 ng/µL of trypsin (sequencing-grade,
Promega) in 50 mM ammonium bicarbonate at 37°C for 5 h. Peptides were gel extracted
with a 1% formic acid/2% ACN solution, followed by two extractions with 50% ACN. All
three extractions were pooled and evaporated to ~10 µL using a Speedvac.

PMF by MALDI-TOF MS was performed using a Voyager DE-PRO MALDI-TOF
mass spectrometer (Applied Biosystems). Each pooled tryptic digest (0.5 µL) was mixed
with an equal volume of CHCA matrix (Sigma) (5 mg/mL in 70% ACN, 0.1% TFA, and 10
mM diammonium citrate) and spotted onto the MALDI target. Spectra were acquired in
positive ion reflector mode under 17000 kV accelerating voltage and a mass range of
800-3500 Da. Internal calibration was performed using trypsin autolysis fragments at $m/z$
842.5100, 2211.1046, and 2807.3000. LC-MS/MS was performed as previously
described [28]. Briefly, digests were injected onto an LC Packings C18 PepMap 100
column (75 µm X 150 mm, particle size 3 µm, pore size 100 Å), equilibrated with 0.1%
formic acid using a Waters CapLC XE chromatography system connected to a Waters
QTOF Global mass spectrometer. The column was developed at 0.2 µL/min with a 5-
80% ACN gradient over 35 min. Data acquisition and analyses were performed using
Waters MassLynx 4.0 software.

Protein identification was performed by searching in the National Center for
Biotechnology Information nonredundant database (NCBI_nr) using both MASCOT
(http://www.matrixscience.com) and MS-FIT (http://www.prospector.ucsf.edu/). The
following parameters were used for the database searches: 50 ppm mass tolerance, one
allowed trypsin miscleavage, carbamidomethylation of Cys (fixed modification), and Met
oxidation (variable modification). For a positive identification, the identified protein must
rank as the top hit in both search programs, match at least 4 peptides, cover ≥10% of
the total sequence, and generate a MOWSE score greater than the significant threshold,
at the $p<0.05$ level (MOWSE score >60 using MASCOT and ≥10³ with MS-FIT).

**RNA extraction and RT-PCR**

Total RNA was extracted from +Pi and -Pi cells using an RNeasy kit (Qiagen)
and semi-quantitative RT-PCR performed as described [27] using gene specific primers
listed (Appendix 1). On column DNase treatment was incorporated to eliminate genomic
DNA. RNA samples were assessed for purity by their $A_{260}/A_{280}$ ratio and integrity by
resolving 1 µg of total RNA on a 1.2% (w/v) denaturing agarose gel. Normalization of
RNA for the purpose of equal starting material during RT was done for each sample by
density measurement of 28S RNA bands from the above gel (scanned using ImageJ software from the National Institute of Health, USA).

RESULTS AND DISCUSSION

Optimization of +Pi and -Pi Arabidopsis cell cultures, and influence of Pi starvation on cell growth and viability

When subcultured in the presence of 5 mM Pi, the CCF of 7-d Arabidopsis cells contained 1.7 ±0.3 mM Pi (n = 3, ± SEM), indicating that the cells were still completely +Pi. By contrast, the 1.25 mM Pi of conventional Murashige-Skoog media [29] was inadequate for maintaining +Pi batch-cultured Arabidopsis suspension cells; 7-d following their subculture into this media: (i) Pi was undetectable in the CCF, and (ii) biomass accumulation was reduced by at least 25% relative to cells subcultured for 7-d in 5 mM Pi. The inability of conventional Murashige-Skoog media to maintain plant (including Arabidopsis) suspension cells fully +Pi for at least one week in batch culture has been well documented [5, 7, 21, 23, 24]. All subsequent studies were conducted using optimized 7-d -Pi and +Pi Arabidopsis suspension cells subcultured into media containing 0 and 5 mM Pi, respectively.

The –Pi cells attained about 50% of the fresh weight of the corresponding +Pi cells (about 25 and 50 g of –Pi and +Pi cells were respectively obtained/500 mL culture). Growth inhibition of the –Pi cells was correlated with depletion of CCF Pi to undetectable levels within 1-d following subculture of 7-d +Pi cells into –Pi media. Cellular viability quantification using fluorescein diacetate/propidium iodide double staining and fluorescence microscopy revealed that >95% of the 7-d-old –Pi cells were still alive (Appendix 2). Similarly, the vast majority (>90%) of Brassica napus suspension cells remained viable for at least 21-d following their subculture into –Pi media (although
negligible growth occurred beyond 9-d), at which point they began to enter into programmed cell death [25].

To assess possible secretome contamination by soluble intracellular proteins, immunoblots of a clarified cell extract, and concentrated +Pi and –Pi secretomes were probed with antibodies raised against the cytoplasmic marker enzymes PEP carboxylase and cytosolic aldolase. Immunoreactive polypeptides comigrating with the respective purified antigens were only observed on immunoblots of cell lysates (Fig. 2.1). Similarly, PEP carboxylase and aldolase activities were readily assayed in cell lysates, but were not detected in the concentrated CCFs (Fig. 2.1). The combined results (Figs. 2.1 and Appendix 2) indicate that proteins localized in the +Pi or –Pi secretome were actively secreted, and not artifacts due to cell lysis.

Influence of Pi-starvation on secreted acid phosphatase activity and immunoreactive polypeptides

A ubiquitous biochemical marker of plant Pi stress is the upregulation of intracellular and secreted APases [1, 3, 4, 7, 21, 23, 24]. Extractable (intracellular) and secreted (concentrated CCF) APase activities of the -Pi cells used in the current study were respectively about 4- and 6-fold greater than that of the corresponding +Pi cells (Fig. 2.2A and results not shown). Similarly, –Pi tomato suspension cells exhibited 8-fold greater APase activity in their CCF relative to +Pi cultures [7, 23]. Time-course studies revealed that the activity of both intracellular and secreted APases of the –Pi Arabidopsis cells maximized 6- to 7-d following their subculture into –Pi media [21] (results not shown). This was paralleled by the appearance of 65- and 55-kDa immunoreactive polypeptides on immunoblots of –Pi but not +Pi secretomes respectively probed with rabbit antibodies raised against the Arabidopsis purple APases, AtPAP12.
and AtPAP26 (Fig. 2.2B). Our current efforts include the purification, molecular/biochemical characterization, and functional analysis of secreted AtPAP12 and AtPAP26 isoforms (subunit $M_r = 65$- and 55-kDa, respectively) from the CCF of the -Pi Arabidopsis cells (H. Tran, W. Qian, D. Wang, and W. Plaxton, unpublished data). Secreted APases are believed to play a pivotal role in root Pi-scavenging from soil-localized Pi-esters [1, 3, 4, 7, 23, 24].

Protein separation by 2-DE and identification by MS

Initially, pH 3–10 IPG strips were employed for IEF of concentrated CCF proteins prior to second dimension SDS-PAGE. As all proteins focused within the 4–7 pH range (results not shown), subsequent 2-DE was conducted using pH 4-7 IPG strips (Fig. 2.3). 2-DE of the +Pi and –Pi secretomes yielded reproducible protein patterns between the three sets of independent culture repetitions. Although the respective patterns were relatively distinct, an obvious overlap of some spots was observed (Fig. 2.3). About 110 CBB-staining spots were detected in total following 2-DE of the +Pi and –Pi secretomes. PMF via MALDI-TOF MS was performed on 64 spots, including all 46 spots whose abundance changed by $\geq 2$-fold following Pi-stress, and resulted in 50 protein assignments representing 37 different proteins having unique gene identities (Table 2.1). LC-MS/MS was performed on four spots (#10, 13, 15, and 44; Fig. 2.3) and confirmed identifications obtained using PMF (Appendix 3). Thirteen different proteins identified following 2-DE of the –Pi secretome appeared to be absent in the +Pi secretome, whereas levels of 5 different proteins of the +Pi secretome were upregulated by $\geq 2$-fold in response to Pi stress (Fig. 2.3, Table 2.1). By contrast, 13 of the identified proteins were secreted at similar levels irrespective of Pi nutritional status, whereas 6 proteins present in the +Pi secretome appeared to be down-regulated by $\geq 2$-fold in response to
Pi stress (Fig. 2.3, Table 2.1). The discrepancy between the experimental and theoretical values observed for some proteins (Table 2.1) might be explained by proteolytic degradation of polypeptides, PTM events, or variability arising from alternate splicing of mRNAs. Similar levels of discrepancy between the predicted and experimental $M_r$ and $pI$ of proteins identified by MS have been noted in previous studies [30, 31].

Although 2-DE coupled with MS is a powerful tool for investigating stress-induced proteome modifications, a disadvantage of this technique is that key protein players of low abundance may be masked by more plentiful proteins. For example, although APases were secreted in response to Pi stress (Fig. 2.2), no APase was identified following MS analysis of all CBB-250 staining spots that were upregulated in the –Pi secretome. This is likely due to the low concentration of APase polypeptides in the –Pi secretome, and corroborates studies demonstrating that secreted APases of –Pi plants exhibit relatively high specific activities (>300 units/mg) [7, 24].

Some of the proteins identified in the secretome of Arabidopsis suspension cells lack predicted signal peptides

About 40% of proteins identified from the +Pi and –Pi secretomes lack putative N-terminal secretory signal peptides (Table 2.1). Several of the same proteins including dehydroascorbate reductase 1, phosphoglycerate kinase, and enolase have been localized to the Arabidopsis cell wall [30, 32-35]. As discussed by Slabas and co-workers [33], the results imply that certain cytoplasmic proteins without obvious targeting signals are secreted to perform specific extracellular functions.

Proteins secreted by –Pi Arabidopsis perform diverse functions
Accumulation of the glycolytic enzymes phosphoglycerate mutase and enolase in the CCF of the –Pi cells implies that they are multifunctional proteins. This is not unreasonable since various extracellular animal proteins with functions unrelated to glycolysis were eventually discovered to be encoded by glycolytic enzyme genes [36]. For example, enolase is a structural protein of the mammalian eye lens and in yeast is a heat shock protein that may confer thermotolerance [36]. Enolase was also upregulated in response to cold stress in Arabidopsis, anoxia, cold, and heat shock in *Echinochloa phyllopogon*, and Pi-deprivation in maize [31, 36]. Apart from its glycolytic role, enolase likely functions as a general stress protein that protects cellular components at the structural level [36].

The role of the PSI secreted ribonuclease 1 (RNS1) in Pi scavenging from extracellular nucleic acids has been well documented in –Pi Arabidopsis and tomato [4, 6]. RNS1 hydrolyzes extracellular RNA into mononucleotides, thereby allowing secreted APases to cleave Pi from resultant Pi-monoesters for its uptake by PSI high-affinity Pi transporters of the plasmalemma. RNS1 was the most abundant protein in the CCF of –Pi Arabidopsis cells (Fig. 2.3B, spots #43-45). As is the case with several of the other identified proteins, RNS1 may be subject to PTM since multiple spots of similar *M*<sub>r</sub> were detected following 2-DE (Fig. 2.3).

Cell wall modifying enzymes located in the CCF included xyloglucan endotransglycosylase 6 (XTR6), several glycosyl hydrolases, as well as an expansin, and polygalacturonase (Table 2.1). Many of these proteins contain predicted N-terminal targeting sequences and several have been localized to the Arabidopsis cell wall [30, 32, 33, 35, 37]. Those specific to the Pi starvation response included β-fructofuranoside 5, a monocopper oxidase-like protein, a reversibly glycosylated polypeptide (RGP), and an XTR6 isoform (spot #36) (Table 2.1). Three spots corresponding to a monocopper
oxidase-like protein were also upregulated in the –Pi secretome (Fig. 2.3B, Table 2.1). Although little is known about its physiological role(s), the monocopper oxidase-like protein encoded by At5g51480 is believed to exist as a GPI-anchored protein of the Arabidopsis plasma membrane [38]. GPI-anchored proteins targeted to the plant cell surface may function in extracellular matrix remodeling and/or signaling. Since the phosphatidylinositol moeity of their GPI anchor is susceptible to cleavage by specific phospholipases, GPI-anchored proteins exist in both soluble and membrane-associated forms [38].

RGPs are plant-specific proteins that catalyze self-glycosylation reactions [39, 40]. These proteins are highly conserved, with five RGP genes identified in Arabidopsis. Because RGP1-RGP3 are so closely related (95% amino acid sequence identity) our PMF data was unable to discriminate as to which RGP isozyme occurred in the -Pi secretome (Fig. 2.3B, Table 2.1). RGP self-glycosylation promotes RGP complex formation and association with the Golgi [40]. In Arabidopsis, the maximal concentration of RGP1 transcripts and protein occurred in roots and suspension cell cultures where it was suggested to function in cell wall modification [39].

XTR6 forms part of a large gene family of Arabidopsis xyloglucan endotransglucosylases/hydrolyases [41]. XTRs possess xyloglucan endohydrolase and endotransglucosylase activities which respectively catalyze the hydrolysis of xyloglucan polymers and transfer of the newly generated reducing ends to adjacent xyloglucan chains. A specific XTR was upregulated in the CCF of Arabidopsis suspension cells treated with fungal elicitors [42], indicating a role in pathogen defense. Similarly, an XTR6 isoform was upregulated in the –Pi secretome (spot #36, Fig. 2.3B; Table 2.1).

The majority of proteins accumulating in the –Pi secretome function in cellular defense and/or ROS detoxification (Table 2.1). Proteomic studies of –Pi maize and rice
roots also demonstrated the upregulation of many antioxidant enzymes [20, 31], and corroborate transcriptomic studies documenting numerous PSI Arabidopsis genes involved in cell defense and oxidative stress amelioration [10-13]. Secreted antioxidant enzymes identified in the current study included glutathione transferase, dehydroascorbate reductase, and Fe and Mn superoxide dismutase 1 (Table 2.1).

Numerous extracellular peroxidase isozymes exist in plants and likely function in pathogen responses and oxidative stress resistance. Peroxidases promote the oxidative cross-linking of cell wall polymers thereby forming a physical barrier that may hinder pathogen penetration. Several peroxidases accumulated in the CCF of Arabidopsis suspension cells treated with fungal elicitors [42], and peroxidases also occur in the cell wall [32, 35, 37]. Although two peroxidases (At5g64120 and At5g19880) were transcriptionally induced in –Pi Arabidopsis seedlings [11, 12], the peroxidase encoded by At5g64120 was down-regulated in the –Pi secretome (Table 2.1). By contrast, peroxidase 17 and 53 (respectively encoded by At2g22420 and At5g06720) accumulated in the CCF of the –Pi cells, suggesting that the –Pi cells experienced oxidative stress.

Secreted proteases are involved in maturation of enzymes, signaling, protein turnover, and pathogen defense [35, 42], and may also contribute to secretome remodeling according to nutritional Pi status [22]. Of the five proteases we identified in the Arabidopsis secretome, three were upregulated in response to Pi starvation, including two leucine aminopeptidases and a serine carboxypeptidase (Table 2.1). A serine carboxypeptidase was also secreted by Arabidopsis cultures treated with fungal elicitors [42]. Interestingly, transcript profiling has identified a PSI subtilisin-like serine protease in Arabidopsis, despite the Arabidopsis genome encoding 56 subtilisin-like proteases [11]. Our understanding of these proteins is limited, although they have been
implicated in protein turnover, response to pathogen infection, and control of plant development. It is notable that within 48 h of resupply of 2.5 mM Pi to -Pi tomato suspension cells, CCF APase activity and immunoreactive polypeptides corresponding to a pair of secreted PSI purple APase isozymes disappeared [22]. Their disappearance was correlated with the de novo synthesis and secretion of a pair of CCF-localized serine proteases. Biotechnological strategies for engineering Pi-efficient crops should consider the possibility that PSI protein overexpression in transgenic plants may be enhanced by modified protease expression and/or the design of protease-resistant PSI proteins.

Semi-quantitative RT-PCR analysis of selected transcripts

Recent studies of plant Pi-starvation responses have focused on identifying genes displaying enhanced transcription during Pi stress [8-18]. However, alterations in transcript abundance do not necessarily translate into a correlated change in protein amount or enzymatic activity, or vice versa [2, 19-21]. Indeed, only 2 (XTR6 and RNS1) of the 18 identified proteins that accumulated in the –Pi secretome (Table 2.1) have been documented to be transcriptionally induced in –Pi Arabidopsis [6, 11]. This was further investigated by semi-quantitative RT-PCR analysis of selected transcripts from the +Pi and –Pi Arabidopsis cells (Fig. 2.4). RNS1, AtPAP17, and AtPAP26 were employed as positive controls since their mRNAs have been documented to either markedly increase (AtPAP17, RNS1) or remain invariant (AtPAP26) when Arabidopsis is subjected to Pi deprivation [6, 21]. The RNS1, XTR6, and cyclase transcripts correlated well with relative levels of the corresponding polypeptides in the +Pi and –Pi secretomes (Figs. 2.3 and 2.4, Table 1). In contrast, monocopper oxidase-like protein, dehydroascorbate reductase-1, enolase, phosphoglycerate mutase, polygalacturonase,
**serine carboxypeptidase-50** and **leucine aminopeptidase-1** transcripts were expressed at similar levels irrespective of nutritional Pi status, whereas the corresponding polypeptides were only detected in the +Pi (polygalacturonase) or –Pi (remaining enzymes) secretomes. Similarly, the purple APase AtPAP26 was markedly upregulated by –Pi Arabidopsis, but AtPAP26 transcripts were constitutively expressed (Fig. 2.4) [21]. *In silico* analysis of Arabidopsis microarray data using Genevestigator (www.genevestigator.ethz.ch/) confirmed that: (i) **RNS1** (At2g02990) and **AtPAP17** (At3g17790) are significantly induced in –Pi tissues, whereas (ii) all other genes that we analyzed via RT-PCR (Fig. 2.4) have generally high basal transcript levels and are constitutively expressed in all tissues examined to date. Thus, transcriptional control appears to exert less impact on levels of certain intracellular and secreted proteins upregulated in –Pi Arabidopsis, relative to translational and post-translational controls that influence protein synthesis and degradation.

**CONCLUDING REMARKS**

We have employed 2-DE and MS to identify differentially expressed proteins in the secretome of Arabidopsis suspension cells subjected to nutritional Pi deficiency. Our results identified 50 protein spots, ~50% of which were significantly upregulated in response to Pi stress. These included the Pi scavenging RNS1, as well as various defense and detoxifying enzymes that may counteract the increased susceptibility of –Pi Arabidopsis to pathogen attack or oxidative stress. The latter hypothesis is corroborated by the widespread horticultural observation that plants become less vulnerable to pathogen infection when subjected to Pi stress [11]. Enzyme activity assays and immunoblots indicated that the +Pi and –Pi secretomes were free of contaminating cytoplasmic proteins, and that a pair of purple APase isozymes were also secreted by
the --Pi cells. Purple APases secreted by roots of --Pi plants are believed to play a key function in scavenging Pi from extracellular Pi-esters, known to comprise up to 80% of total soil P [4, 7, 23].

Adaptive mechanisms that plant cells employ to acclimate to Pi limitation are complicated as the secreted proteins identified in low Pi tolerance are involved in multiple functional categories. A significant challenge will be to assess the specific role of each of these proteins in the Arabidopsis Pi stress response. Future studies using knockout lines should prove invaluable in this regard. Our study also highlights the importance of complementing transcriptomics with proteomics, as the combined datasets provides a more robust depiction of how alterations in gene expression may be linked to adaptive changes in the metabolism of --Pi plants.

REFERENCES


Table 2.1. Functional characterization of proteins identified following 2-DE of the CCF secretome from 7-d old +Pi and -Pi Arabidopsis suspension cells. A unique number was assigned to spots identified as the same protein and migrating to the same coordinate upon 2-DE, while different spot numbers were assigned to spots identified as the same protein and migrating to different coordinates (Fig. 2.3). Protein spots reproducibly detected only under –Pi or +Pi conditions are indicated by (+ +) and (– –), respectively, whereas proteins up-regulated or down-regulated by at least two-fold in the CCF of –Pi cells are indicated by (+) and (–), respectively; (0) corresponds to proteins exhibiting similar expression under +Pi or –Pi conditions. Genes containing a coding sequence for a putative transit peptide (as predicted by SignalP, version 3.0) are indicated with a diamond ◊.

<table>
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<th>pI / M_r (kDa)</th>
<th>Response to Pi stress</th>
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<td>5.6 / 52.6</td>
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<td>At5g51480 ♯</td>
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**Defense/detoxifying**

| Glutathione transferase 8, At1g78380 | 81 (47) | 7 | 28 | 5.8 / 27 | 5.8 / 25.7 | ++ |
| Dehydroascorbate reductase 1, At1g19570 | 87 (46) | 7 | 35 | 5.6 / 27 | 5.6 / 23.7 | ++ |
| Fe superoxide dismutase 1, At4g25100 | 70 (49) | 4 | 19 | 6.5 / 26 | 6.1 / 23.8 | 0 |
| Mn superoxide dismutase 1, At3g10920 | 81 (50) | 5 | 29 | 6.3 / 26 | 8.5 / 25.4 | + |
| Glutathione reductase, At3g54660 | 65 (21) | 7 | 17 | 6.4 / 55 | 8.0 / 60.8 | 0 |
| NADPH-dependent thioredoxin reductase 2, At2g17420 ♯ | 96 (33) | 7 | 27 | 6.3 / 36 | 6.3 / 40.0 | + |
| Peroxidase, At5g64120 ♯ | 135 (6) | 10 | 37 | ≥7.0 / 70 | 8.6 / 34.9 | – |
| Peroxidase 17, At2g22420 ♯ | 97 (27) | 10 | 31 | 5.1 / 41 | 5.1 / 36.7 | + |
| Peroxidase 53, At5g06720 ♯ | 78 (11) | 5 | 26 | 4.7 / 50 | 4.7 / 35.0 | + |
| Peroxidase 58, At5g19880 ♯ | 77 (26) | 4 | 18 | 5.0 / 41 | 5.1 / 35.4 | 0 |
Table 2.1. continued

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<td>5.4 / 49.2</td>
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<td>94 (41)</td>
<td>9</td>
<td>33</td>
<td>6.1 / 34</td>
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</table>

<sup>a</sup>MOWSE score: statistical probability of a positive identification of predicted proteins calculated by MASCOT (http://www.matrixscience.com) with 50 ppm mass tolerance and one permissible missed cleavage (MOWSE > 60). All identifications were corroborated using MS-FIT (http://www.prospector.ucsf.edu/) (MOWSE > 10<sup>3</sup>) as outlined in the Materials and methods.

<sup>b</sup>Calculated using the EXPASY pI / M<sub>r</sub> tool (http://www.expasy.org/tools/pi_tool.html).

<sup>c</sup>Spots identified by both PMF and LC MS/MS (Appendix 3).

<sup>d</sup>PMF via MALDI-TOF MS matched corresponding peptides from RGP 1-3, and did not allow for discrimination between these three very closely related isoymes.
Fig. 2.1. Immunological detection of PEP carboxylase and cytosolic aldolase in clarified Arabidopsis suspension cell extracts. Concentrated CCF proteins from +Pi and –Pi cells (CCF, 30 µg each), a clarified cell extract from +Pi cells (CE, 5 µg) and homogenous castor seed PEP carboxylase (PEPC) and cytosolic aldolase (ALD, 10 ng each) [26, 27] were resolved by SDS-PAGE and electroblotted onto PVDF membranes. Immunoblots were probed with a 500-fold dilution of affinity-purified rabbit anti-(castor seed PEP carboxylase or cytosolic aldolase)-IgG [26, 27]. Immunoreactive polypeptides were detected using a HRP-conjugated secondary antibody and ECL detection. Corresponding PEP carboxylase and aldolase activities appear below the respective lanes.
Fig. 2.2. APase activities (A) and immunological detection of secreted purple APase isozymes (B) in CCFs of +Pi versus –Pi Arabidopsis suspension cells. CCFs were harvested and concentrated as described in Materials and Methods, and (A) assayed for APase activity and (B) subjected to immunoblots analysis using anti-(AtPAP12 or AtPAP26)-IgGs. (A) All APase activities represent the means ±SEM of n = 3 separate flasks. (B) Concentrated CCF proteins from the +Pi and –Pi cells (5 µg/lane), as well as homogenous AtPAP26 (20 ng) [21] were resolved by SDS-PAGE and electroblotted onto PVDF membranes as previously described [7]. Immunoblots were probed with a 5000-fold dilution rabbit anti-(AtPAP12 or AtPAP26) immune serum as indicated. Immunoreactive polypeptides were detected using a HRP-conjugated secondary antibody and ECL detection. Lanes labeled “+Pi” and “–Pi” denote CCF proteins from +Pi and –Pi cultures, respectively. The migration of various M₆ standards, in kDa, is indicated.
Fig. 2.3. 2-DE comparison of the secretomes of +Pi (A) versus –Pi (B) Arabidopsis suspension cells. Concentrated CCF proteins (50 µg each) were resolved via 2-DE using pH 4-7 IPG strips in the first dimension and 12% SDS gels in the second dimension. Gels were stained with CBB R-250. Image analysis of scanned gels was performed using PDQuest software (version 7.0). Spots identified by LC-MS/MS and/or MALDI-TOF MS are numbered and correspond to those listed in Table 2.1.
Fig. 2.4. Semi-quantitative RT-PCR analysis of gene expression in +Pi and -Pi Arabidopsis suspension cells. Levels of mRNA were analyzed by RT-PCR using primers specific for the following genes: **monocopper oxidase-like protein** (*Cu oxidase*; At5g51480), **dehydroascorbate reductase 1** (DHAR-1; At1g19570), **enolase** (At2g36530), **phosphoglycerate mutase** (PGM; At3g08590), **leucine aminopeptidase 1** (Leu amino-1; At2g24200), **serine carboxypeptidase 50** (Ser carboxy-50; At1g15000), **RNS1** (At2g02990), **XTR6** (At4g25810), **cyclase** (At4g34180), **polygalacturonase** (polygal; At3g16850), **AtPAP17** (At3g16850), and **AtPAP26** (At5g34850). **Actin 2** (At3g18780) and **ubiquitin** (At5g25760) were used as reference controls to ensure equal template loading. All PCR products were taken at cycle numbers determined to be nonsaturating. The amount of cDNA template for each primer pairs is indicated in parentheses and was based on linear range testing for non-saturating condition. Control RT-PCR reactions lacking RT did not show any bands. The *Cu oxidase*, DHAR1, enolase, PGM, Leu amino-1, Ser carboxy-50, RNS1, XTR6, cyclase, polygal, AtPAP17, and AtPAP26 primers amplified as 233, 221, 153, 229, 163, 220, 153, 206, 151, 169, 469 and 610-bp fragments of the 5' region of their respective cDNAs, as expected.
Chapter 3. Biochemical and molecular characterization of AtPAP12 and AtPAP26: the predominant purple acid phosphatase isozymes secreted by phosphate-starved Arabidopsis thaliana

Submitted to Plant Cell and Environment Feb. 19, 2010

ABSTRACT

Plant purple acid phosphatases (PAPs) belong to a large multigene family whose specific function in Pi metabolism is poorly understood. Two PAP isozymes secreted by Pi-deficient (-Pi) Arabidopsis thaliana cell cultures and seedlings were purified and characterized from culture filtrates of -Pi suspension cells. They correspond to an AtPAP12 (At2g27190) homodimer and AtPAP26 (At5g34850) monomer composed of glycosylated 60- and 55-kDa subunits, respectively. Each PAP exhibited broad pH-activity profiles centered at about pH 5.6, and overlapping but non-identical substrate specificities. Concanavalin-A chromatography resolved a pair of secreted AtPAP26 glycoforms that differentially cross-reacted with Galanthus nivalis agglutinin. AtPAP26 is dual-targeted during Pi stress since it is also the principal intracellular (vacuolar) PAP upregulated by -Pi Arabidopsis. Our results indicate that differential glycosylation influences the subcellular targeting and substrate selectivity of AtPAP26. Immunoblotting correlated the marked increase in secreted APase activity of –Pi Arabidopsis with the appearance of immunoreactive AtPAP12 and AtPAP26 polypeptides. Semiquantitative RT-PCR of seedling mRNAs corroborated earlier reports that: (i) transcriptional controls exert little influence on AtPAP26 upregulation during Pi stress, whereas (ii) Pi-starvation inducible AtPAP12 transcripts correlate well with relative levels of secreted AtPAP12
polypeptides. We hypothesize that AtPAP12 and AtPAP26 facilitate Pi scavenging from soil-localized organophosphates during nutritional Pi deprivation.

**INTRODUCTION**

Acid phosphatases (APases; EC 3.1.3.2) catalyze the hydrolysis of Pi from a broad range of Pi-monoesters and anhydrides with an acidic pH optimum (Duff *et al.* 1994). Eukaryotic APases exist as a wide variety of tissue- and/or cellular compartment-specific isozymes. They function in the production, transport, and recycling of Pi, which is crucial for cellular metabolism and bioenergetics. The induction of intracellular and secreted APases appears to be a universal plant response to nutritional Pi-deprivation (Duff *et al.* 1994), a common abiotic stress that frequently limits plant growth in natural ecosystems. During Pi stress, intracellular APases likely remobilize and recycle Pi from expendable Pi-monoesters and anhydrides. This is accompanied by a marked reduction in cytoplasmic P-metabolites during extended Pi stress (Duff *et al.* 1994). Extracellular APases belong to a group of Pi-starvation inducible (PSI) phosphohydrolases secreted by roots and cell cultures of –Pi plants to hydrolyze Pi from external organophosphates, the predominant form of P in soil solutions which can comprise up to 80% of total soil P (Ticconi & Abel 2004; Richardson *et al.* 2009). For example, the combined action of secreted ribonucleases, phosphoesterases, and APases allows Pi-starved (–Pi) tomato and Arabidopsis plants to efficiently scavenge extracellular nucleic acids as their sole source of nutritional Pi (Bosse & Kock 1998; Abel *et al.* 2000; Ticconi & Abel 2004).

Purple APases (PAPs) represent a specific class of PSI, non-specific plant APases, and are characterized by their pink or purple color in solution (due to a bimetallic active center), and insensitivity to L-tartrate inhibition. PAPs have been studied in a wide variety of bacterial, animal, and plant species. Conserved sequence motifs
containing the metal ligating residues are common to animal and plant PAPs (Li et al. 2002). Genome annotation of Arabidopsis thaliana identified 29 PAP genes, several of which are transcriptionally induced during Pi-deprivation (del Pozo et al. 1999; Haran et al. 2000; Li et al. 2002). This and subsequent studies (Zhu et al. 2005; Veljanovski et al. 2006; Tran & Plaxton 2008; Zhang et al. 2008) demonstrated the complexity and variation of AtPAP1-29 expression and regulation. Nevertheless, little information is available on the principal AtPAP isozyme(s) that contribute to intra- versus extracellular Pi scavenging by –Pi Arabidopsis. In order to help solve this conundrum we have taken an initial biochemical approach that involves: (i) purification and characterization of Arabidopsis APases upregulated during Pi stress, coupled with (ii) bioinformatic analysis of peptide mass fingerprint and/or N-terminal amino acid sequence data obtained with the purified native APases. Suspension cell cultures have been invaluable in this regard since a relatively large biomass of Arabidopsis cells and their surrounding liquid media containing secreted proteins can be obtained over a relatively short period (Veljanovski et al. 2006; Tran & Plaxton 2008; Gregory et al. 2009). The principal intracellular (vacuolar) APase upregulated by –Pi Arabidopsis was fully purified and identified as AtPAP26 (Veljanovski et al. 2006). A pronounced decrease in intracellular free Pi levels was correlated with the accumulation of vacuolar AtPAP26 (AtPAP26-V) polypeptides and concomitant marked increase in intracellular APase activity of –Pi Arabidopsis suspension cells and seedlings (Veljanovski et al. 2006). Our recent molecular and phenotypic analyses of an atpap26 T-DNA insertional mutant lacking AtPAP26 transcripts and immunoreactive AtPAP26 polypeptides confirmed that AtPAP26 is the main contributor to intracellular APase activity, and that it makes an important contribution to the Pi metabolism of –Pi Arabidopsis (see Chapter 4, Hurley et al. 2010).
Genomic and proteomic approaches using Arabidopsis have led to the identification and characterization of genes that drive efficient use of Pi by vascular plants. Factors that influence Pi availability, mobility, and subsequent uptake and use by plants are of great interest to plant and soil scientists hoping to engineer Pi-efficient crops needed to optimize inputs of unsustainable and nonrenewable Pi fertilizers for maximum agronomic benefit (Richardson et al. 2009). Although the extracellular APases secreted by roots are of particular importance for the mineralization of soil organophosphates, there have been no reports describing the unequivocal identification of secreted APase(s) of –Pi Arabidopsis. Coello (2002) documented a marked increase in secreted APase activity of –Pi Arabidopsis seedlings and suggested that the protein responsible for this activity was encoded by AtPAP12. Unfortunately, amino acid sequencing or peptide mass fingerprinting of the 63 and 52 kDa polypeptides that co-purified with the secreted APase activity of the –Pi Arabidopsis seedlings was not performed to support this hypothesis (Coello 2002). However, when Arabidopsis was transformed with a construct encoding the AtPAP12 promoter region and putative transit peptide fused to a green fluorescent protein gene, the transformants secreted small amounts of the fusion protein into the rhizosphere when subjected to nutritional Pi deficiency (Haran et al. 2000). Similarly, an AtPAP12 ortholog (LaSAP1) that is secreted into the rhizosphere by proteoid roots of –Pi white lupin (Lupinus albus) has been purified, characterized, and cloned (Miller et al. 2001). Enhanced expression of both the enzyme protein and its mRNA was relatively specific to proteoid roots of –Pi lupin (Miller et al. 2001). Furthermore, our proteomic study of the influence of Pi stress on the Arabidopsis secretome reported a pronounced increase in the secreted APase activity of –Pi suspension cells relative to Pi-sufficient (+Pi) cells (see Chapter 2, Tran & Plaxton 2008). Maximal secreted APase activity was achieved about one week following
A subculture of the Arabidopsis cells into –Pi liquid media, and corresponded with the appearance of approximate 60- and 55-kDa immunoreactive polypeptides on immunoblots of –Pi secretomes probed with antibodies raised against AtPAP12 and AtPAP26, respectively (Tran & Plaxton 2008). The overall aims of the current study were to identify and characterize the biochemical and molecular features of the predominant secreted APase(s) upregulated by -Pi Arabidopsis.

**MATERIALS AND METHODS**

*Plant material*

Heterotrophic Arabidopsis (*Arabidopsis thaliana*, cv. Landsberg erecta) suspension cells were maintained at 21 °C in the dark as previously described (Veljanovski et al. 2006). For large scale –Pi subculture, 50 mL aliquots of 7-d-old +Pi (5 mM K$_2$HPO$_4$) cultures were used to inoculate 10 separate Fernbach flasks that each contained 450 mL of fresh media (Murashige & Skoog 1962) lacking Pi. Cells were harvested after 7 d by filtration, frozen in liquid N$_2$, and stored at -80°C, whereas the CCF (5 L) was processed as described below.

Wild-type Arabidopsis seeds (Columbia ecotype) were surface sterilized as described (Veljanovski et al. 2006). Approximately 100 seeds were placed in Magenta boxes containing 50 mL of 0.5 X Murashige and Skoog media, pH 5.7, 1% (w/v) sucrose and 0.2 mM Pi. Seeds were stratified for 3 d at 4 °C and placed on an orbital shaker (80 rpm) at 24 °C under continuous illumination (80 µmol m$^{-2}$ s$^{-1}$). After 7 d, the media was replaced with fresh media containing 3 mM Pi (+Pi) or 0 mM Pi (–Pi) and at 14 d, the media (henceforth referred to as ‘seedling culture filtrate’ or SCF) from both groups was filtered through 0.2 µm membranes and concentrated using Amicon Ultra-15 centrifugal filter units (30-kDa cutoff).
Enzyme assays, kinetic analyses, and protein assays

All enzyme assays were linear with respect to time and concentration of enzyme assayed. One unit (U) of activity is defined as the amount resulting in the utilization of 1 µmol min⁻¹ of substrate at 25 °C. Aldolase and phosphoenolpyruvate (PEP) carboxylase activities were determined using coupled spectrophotometric assays (Tran & Plaxton 2008; Gregory et al. 2009). For routine measurements of APase activity, the hydrolysis of PEP to pyruvate was coupled to the lactate dehydrogenase reaction and assayed at 25 °C by monitoring the oxidation of NADH at 340 nm using a Spectramax plus Microplate spectrophotometer (Molecular Devices). Standard APase assay conditions were 50 mM sodium acetate, pH 5.6, 5 mM PEP, 10 mM MgCl₂, 0.2 mM NADH, and 3 units of desalted rabbit muscle lactate dehydrogenase in a final volume of 0.2 ml. All assays were initiated by the addition of enzyme preparation and corrected for background NADH oxidation by omitting PEP from the reaction mixture. Apparent $K_m$(PEP) values were determined using this assay as described (Veljanovski et al. 2006). All kinetic parameters are the means of at least three separate experiments and are reproducible to within ±10% of the mean value. Substrate selectivity studies were performed by quantifying the Pi released by the APase reaction as previously described (Bozzo, Raghothama & Plaxton 2002; Veljanovski et al. 2006). Controls were run for background amounts of Pi present at each substrate concentration tested. To calculate activities, a standard curve over the range of 1-133 nmol Pi was constructed for each set of assays. Protein concentrations were determined using a Coomassie Blue G-250 dye binding method (Veljanovski et al. 2006) with bovine γ-globulin as the standard.

Buffers used during AtPAP purification
Buffer A contained 50 mM sodium acetate (pH 5.8), 1 mM DTT, and 35% (saturation) (NH₄)₂SO₄. Buffer B contained 50 mM sodium acetate (pH 5.8), 1 mM DTT, and 10% (v/v) ethylene glycol. Buffer C contained 50 mM sodium acetate (pH 5.0), 1 mM EDTA, and 1 mM DTT. Buffer D contained 25 mM MES (pH 6.0) and 1 mM DTT. Buffer E contained 25 mM sodium acetate (pH 5.8), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM CaCl₂, 0.2 mM MnCl₂, 1 mM DTT, and 10% (v/v) glycerol. Buffer F contained 25 mM MES (pH 5.8), 30% (saturation) (NH₄)₂SO₄, and 1 mM DTT, whereas Buffer G contained 25 mM MES (pH 5.8), 1 mM DTT, and 15% (v/v) ethylene glycol.

**AtPAP purification**

All procedures were performed at room temperature (25 °C) unless otherwise noted. AtPAP26-V was purified from the –Pi cells as previously described (Veljanovski et al. 2006). For isolation of secreted PAPs, freshly harvested, dilute CCF (5 L) from the –Pi cells was rapidly concentrated to about 1 L using a Pellicon ultrafiltration device (30-kDa cutoff), immediately brought to 35% (saturation) (NH₄)₂SO₄, and APase activity absorbed batchwise onto 55 mL of Butyl Sepharose 4 Fast Flow that had been pre-equilibrated in Buffer A. The resin was washed with 250 mL of Buffer A using a sintered glass funnel and APase activity eluted with 125 mL of Buffer B at about 2 ml min⁻¹. Pooled APase activity was concentrated to 5 mL using Amicon Ultra-15 ultrafiltration devices (30-kDa cutoff), frozen in liquid N₂, and stored at -80°C. Aforementioned procedures were repeated four separate times prior to initiating large scale APase purification via column chromatography using an ÄKTA FPLC system (GE Healthcare). Concentrated Butyl Sepharose APase peak activity fractions were thawed, pooled (total volume = 20 ml) and dialyzed overnight against two 2 L changes of Buffer C. The sample was clarified by centrifugation and absorbed at 1 ml min⁻¹ onto a S-Sepharose
cation-exchange column (1.5 X 11 cm) that had been pre-equilibrated with Buffer C. The column was washed with Buffer C until the $A_{280}$ decreased to baseline, and developed with a linear gradient (170 ml) of 0-500 mM KCl in Buffer C (5 ml fraction$^{-1}$). APase activity resolved as two peaks (AtPAP26 and AtPAP12) at approximately 160 and 360 mM KCl, respectively (Fig. 1a). Peak activity fractions were pooled and concentrated separately as above to 2 ml. Both samples were desalted into buffer D and applied separately at 0.5 ml min$^{-1}$ onto a column (1.6 X 4 cm) of Phosphocellulose P-11 (Whatman), which was hydrated and precycled as described by the manufacturer and pre-equilibrated with Buffer D. The column was washed with Buffer D until the $A_{280}$ decreased to baseline, and eluted with 5 mM PEP in Buffer D. Fractions (6 ml) containing APase activity were pooled, concentrated as above, and absorbed at 0.5 ml min$^{-1}$ onto a column (1 X 1.3 cm) of Concanavalin-A (Con-A) Sepharose pre-equilibrated with Buffer E. AtPAP26 was resolved as two distinct peaks of APase activity using Con-A, as one peak did not bind (denoted as AtPAP26-S1), whereas the other peak (AtPAP26-S2) bound and was eluted using 20 mL of a linear 0-500 mM methyl-α-D-mannopyranoside gradient in Buffer E (1 ml fraction$^{-1}$) (Fig. 1b). Pooled peak fractions from the Con-A column were concentrated as above to ≤0.5 ml, and for AtPAP12 and AtPAP26-S2, brought to 30% (saturation) $(NH_4)_2SO_4$ and applied at 0.5 ml min$^{-1}$ onto a Phenyl Superose HR 5/5 column (GE Healthcare) pre-equilibrated with Buffer F. APase activity was eluted using 20 mL of a 0-100% linear gradient of Buffer G (100%-0% Buffer F) (0.5 ml fraction$^{-1}$). Pooled peak fractions were concentrated as above to about 50-μl, mixed with an equal volume of stabilization buffer (100 mM sodium acetate, pH 5.0, 10 mM MgCl$_2$, 50% (v/v) glycerol), divided into 10 μL aliquots, frozen in liquid N$_2$ and stored at -80°C. APase activity was stable for at least four months when stored at -80 °C in stabilization buffer.
Estimation of native molecular mass by gel filtration chromatography

This was performed by FPLC at 0.2 ml min\(^{-1}\) on a calibrated Superose 12 HR 10/30 column, equilibrated with 25 mM sodium acetate (pH 5.8), 100 mM KCl, 1.5 mM MgCl\(_2\), 1 mM DTT, and 10% (v/v) glycerol. Native \(M_r\) was calculated from a plot of \(K_{av}\) (partition coefficient) against log \(M_r\) using the following protein standards: ferritin (440-kDa), catalase (232-kDa), albumin (67-kDa), chymotrypsinogen (25-kDa), and ribonuclease (13.6-kDa).

Protein electrophoresis and immunoblotting

SDS-PAGE, subunit \(M_r\) estimation via SDS-PAGE, immunoblotting onto poly(vinylidene) difluoride (PVDF) membranes, and visualization of antigenic polypeptides using an alkaline-phosphatase-tagged secondary antibody and chromogenic detection were conducted as previously described (Veljanovski et al. 2006). Anti-(recombinant AtPAP12) immune serum was a kind gift of Prof. Thomas McKnight, Texas A & M University, whereas anti-(native AtPAP26-V) immune serum was obtained as previously described (Veljanovski et al. 2006). Immunoblot analysis using the monoclonal antibody YZ1/2.23 (kindly provided by Prof. Michael McManus, Massey University) was performed as described (McManus et al. 1988). All immunoblot results were replicated at least three times with representative results shown in the figures. Glycoprotein staining was performed using Pro-Q Emerald 488 fluorescent stain (Molecular Probes), and stained gels were scanned using a Typhoon 8600 fluorescence imager (GE Healthcare). Further characterization of the glycan chains of purified AtPAPs was performed using a Digoxigenin (DIG) Glycan Differentiation Kit (Roche).

Amino acid sequencing and mass spectrometry
N-terminal microsequencing was performed by automated Edman degradation at
the Protein and Peptide Sequencing Facility of the Biotechnology Research Institute
(Montreal QC, Canada). Peptide mass fingerprinting by matrix-assisted laser desorption
ionization quadrupole time-of-flight (MALDI-TOF) mass spectrometry was performed as
described (Gregory et al. 2009). Briefly, excised gel bands were destained, dehydrated,
reduced and alkylated. Digestion was performed using 10 ng of sequencing grade
trypsin (Calbiochem). Protein identification was performed by searching against the
NCBI (National Center for Biotechnology) non-redundant database (NCBInr, released
July 4, 2009, containing 9,251,875 protein sequences) using both MASCOT (version
2.1, Matrix Science, UK) and MS-Fit (version 5.3, ProteinProspector, UCSF) programs.
These searches allowed one missed cleavage of trypsin digestion and the fixed
modification of cysteine carbamidomethylation. Deamidation of asparagine and
 glutamine to aspartic acid and glutamic acid; and N-terminal pyroglutamation and
methionine oxidation were selected as variable modifications. The mass tolerance
between calculated and observed masses used for database searches was considered
within the range of ±50 p.p.m. For a positive identification, the identified protein must
rank as the top hit in both search programs, match at least four peptides, cover ≥20% of
the total sequence and generate a MOWSE score greater than the significant threshold
at the p<0.05 level (MOWSE score >60 using MASCOT and ≥10^3 with MS-FIT).

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. Normalization of
RNA for RT was performed for each sample by density measurement of 28S ribosomal
RNA bands from the above gel (scanned using ImageJ software from the National Institutes for Health, USA). RNA (5 μg) was reverse transcribed with Superscript III (Invitrogen) and non-competitive RT–PCR performed as previously described (Tran & Plaxton 2008; Gregory et al. 2009). Gene-specific primers used to amplify AtPAP26, AtPPCK, and AtACT2 were previously described (Gregory et al. 2009). Transcripts for AtPAP12 were amplified using the primers AtPAP12LP: 5’-CACGTTCTTCGTCTCGGATT-3’ and AtPAP12RP: 5’-CCCTTGCGTTACATGAACCT-3’. The amount of input cDNA necessary for non-saturating amplification for each primer pair was established by performing PCR using 0.05-1.2 ng of total RNA during first-strand cDNA synthesis.

Bioinformatic analysis

Similarity searches were performed using the BLAST program (www.ncbi.nlm.nih.gov). Multiple sequence alignment of AtPAP12 and AtPAP26 was performed with ClustalX (ver. 1.81). Signal peptide prediction was performed using SignalP. Phylogenetic analysis and a neighbor-joining tree were generated using the ‘One Click’ mode of the Phylogeny.fr program (www.phylogeny.fr) with the following settings: MUSCLE 3.7 for multiple alignment, Gblocks 0.91b for alignment refinement, PhyML 3.0 aLRT for phylogeny, and TreeDyn 198.3 for Tree rendering. NCBI accession numbers for deduced amino acid sequences of various AtPAP12 and AtPAP26 orthologs are listed in Appendix 9.

RESULTS AND DISCUSSION

Influence of Pi starvation on secreted APase activity and immunoreactive PAP polypeptides of Arabidopsis cell cultures and seedlings
Secreted APase specific activity was about six- and two-fold greater in the -Pi suspension cell CCF and seedling SCF, respectively, relative to corresponding +Pi controls (Fig. 3.2a). This was correlated with the absence of detectable extracellular Pi, and the appearance of 60- and 55-kDa immunoreactive polypeptides on immunoblots of –Pi CCF and SCF proteins respectively probed with rabbit antibodies raised against native AtPAP26-V and recombinant AtPAP12 (Fig. 3.2b). Earlier work (Tran & Plaxton 2008) demonstrated that secreted CCF proteins from 7-d-old +Pi or –Pi Arabidopsis suspension cells were free of contaminating cytoplasmic marker enzymes. Similarly, immunoreactive cytosolic PEP carboxylase and aldolase polypeptides comigrating with the respective purified antigens were observed on immunoblots of the seedling extracts, but were absent on immunoblots of the corresponding concentrated SCF proteins (Appendix 4). This was corroborated by the presence of significant PEP carboxylase and aldolase activities in seedling extracts, but not concentrated SCFs. These results indicate that: (i) proteins localized in the +Pi or –Pi SCF were actively secreted and not artifacts due to cell lysis, and (ii) the –Pi seedlings were indeed Pi stressed, as PEP carboxylase, whose parallel induction and in vivo phosphorylation-activation in -Pi Arabidopsis was recently documented (Gregory et al. 2009), was upregulated by about two-fold under the –Pi conditions (Appendix 4).

Purification and identification of APases secreted by –Pi Arabidopsis suspension cells

Concentration and enrichment of APases secreted by the –Pi cells was facilitated by Pellicon ultrafiltration of CCFs, followed by batchwise Butyl Sepharose chromatography (Table 3.1). The latter procedure eliminated gelatinous material that otherwise interfered with stabilization and efficient storage of secreted APase activity. Two peaks of APase activity were resolved during S-Sepharose cation-exchange FPLC
of the concentrated Butyl Sepharose fractions (Fig. 3.1a), and were subsequently identified as AtPAP12 and AtPAP26 (see below). Both were further purified via Phosphocellulose and Con-A affinity chromatographies (Table 3.1). Pooled AtPAP26 peak activity fractions from the Phosphocellulose column unexpectedly resolved as two isoforms (AtPAP26-S1 and -S2) during Con-A chromatography (Fig. 3.1b). AtPAP26-S1 failed to bind to the Con-A resin, whereas AtPAP26-S2 (and AtPAP12) was bound and eluted following application of the methyl-α-D-mannopyranoside gradient. The proportion of total AtPAP26-S1 to -S2 activities that eluted from the Con-A column was about 3:1. The possibility that AtPAP26-S1’s elution in the Con-A flow-through fractions was due to column overloading was excluded because subsequent application of this sample on a second, freshly prepared Con-A column resulted in the quantitative recovery of AtPAP26-S1 in the resultant unbound fractions (results not shown). As shown in Table 3.1, AtPAP12, AtPAP26-S1, and AtPAP26-S2 were purified to final PEP-hydrolyzing specific activities of 109, 1710, and 650 U mg⁻¹, respectively. These values are in the range reported for a variety of homogeneous plant PAPs, including AtPAP26-V (421 U mg⁻¹), and secreted PAPs from –Pi tomato (222 and 370 U mg⁻¹) (Bozzo et al. 2002). All three final preparations exhibited a pink color in solution. The physical basis for this color was apparent from the visible absorption spectra of AtPAP12 and AtPAP26-S1, which demonstrated that they are PAPs since they absorbed maximally in the middle of the visible region, peaking at about 520 and 500 nm, respectively (Appendix 5). An absorption spectrum for AtPAP26-S2 could not be obtained owing to its low protein concentration (Table 3.1).

The three purified PAP isoforms were subjected to N-terminal microsequencing (Fig. 3.3a), as well as peptide mass fingerprinting via MALDI-TOF MS (Appendix 6). Comparison with databank sequences demonstrated that they corresponded to
AtPAP12 (At2g27190), along with a pair of AtPAP26 isoforms (At5g34850). The former result corroborates that of Haran and coworkers (2000) who transformed Arabidopsis with an *AtPAP12-green fluorescent protein* construct, and demonstrated secretion of the resulting fusion protein into the surrounding growth media during Pi deprivation. The N-terminal sequences obtained for AtPAP26-S1 and –S2 were identical to that obtained for the native AtPAP26-V of –Pi Arabidopsis suspension cells (Fig. 3.3a) (Veljanovski *et al.* 2006), indicating a common signal peptide cleavage site for all three isoforms. Specific glycosyl hydrolase, carboxypeptidase, aminopeptidase, and enolase isozymes are also dual-targeted to both the vacuole and secretome of Arabidopsis cells (Carter *et al.* 2004; Shimaoka *et al.* 2004; Tran & Plaxton 2008). These proteins may be under the direction of C-terminal signaling regions, or might be differentially targeted by post-translational modifications (e.g., glycosylation).

**Physical and immunological properties**

Native molecular masses of AtPAP12 and AtPAP26-S1/-S2 were estimated to be 130 ±5 kDa and 60 ±5 kDa (means ± SEM, n = 3 for each), respectively, as determined by analytical gel filtration FPLC. When the final AtPAP12 and AtPAP26-S1/-S2 preparations were denatured and subjected to SDS-PAGE, single Coomassie Blue-staining polypeptides of 60- and 55-kDa were respectively observed that cross-reacted with anti-(recombinant AtPAP12) immune serum (Fig. 3.4a and c). By contrast, anti-(native AtPAP26-V) immune serum specifically cross-reacted with AtPAP26-V, -S1, and –S2 (Fig. 3.4b). AtPAP12, and AtPAP26-S1 and –S2 were detected with the glycoprotein stain Pro-Q Emerald (Appendix 7). These results indicate that native AtPAP12 exists as a glycosylated homodimer, whereas AtPAP26-S1 and –S2 are monomeric glycoproteins. Under reducing and non-reducing conditions (with and without
100 mM DTT in the sample buffer, respectively) there was no change in the mobility of the AtPAP26-S1 or –S2 polypeptides during SDS-PAGE (results not shown). However, under non-reducing conditions AtPAP12 migrated at about 140-kDa (Appendix 8). These results corroborate a previous report demonstrating that plant AtPAP12 orthologs contain a pair of conserved cysteine residues at approximately position 120 and 370 that form a disulfide bridge between the two subunits (Olczak & Watorek 2003) (also see Appendix 8). As AtPAP26 orthologs lack the C-terminal cysteine residue at position 370, they were predicted to be unable to form a disulfide bridge between their subunits (Olczak & Watorek 2003).

**Bioinformatics analysis**

Phylogenetic analysis of AtPAP12 and AtPAP26 orthologs revealed their clear grouping into distinct monophyletic groups corresponding to two plant PAP subfamilies (Fig. 3.3b) (Olczak & Watorek 2003). All of the deduced sequences contain conserved domains involved in coordinating the dimetal nuclear center characteristic of the PAP active site (Appendix 8 and results not shown). AtPAP12 and AtPAP26 share 57% sequence identity, whereas their respective identities with orthologs from other plants ranges from 71-88% (Appendix 9). The high degree of sequence identity across dicots and monocots implies an important and conserved function for AtPAP12 and AtPAP26 orthologs in vascular plants.

*In silico* analysis of the deduced AtPAP12 and AtPAP26 sequences predicted that the mature proteins have *M*_s of 50- and 51-kDa, respectively (Appendix 6). The 10- and 4-kDa discrepancies with the respective subunit *M*_s of the purified native AtPAP12 and AtPAP26-S1/-S2 as estimated by SDS-PAGE (Fig. 3.4a) can be explained by the addition of glycan groups. Signal P predicted that the N-terminus of the deduced
AtPAP12 and AtPAP26 polypeptides contain a 28- and 22-amino acid signal peptide, respectively (Fig. 3.3a). By contrast, the N-terminal sequence of the mature AtPAP12 and AtPAP26-S1/S2 polypeptides begins at position 38 and 31, respectively, implying actual signal peptide lengths of 37 and 30 amino acids (Fig. 3.3a). Interestingly, the signal peptides of AtPAP12, AtPAP26-V, -S1, and –S2, and AtPAP26 orthologs purified from tomato, soybean, and onion plants (Lebansky, Mcknight & Griffing 1992; Shinano et al. 2001; Bozzo et al. 2004a; Veljanovski et al. 2006) are all processed at exactly the same site, beginning after an invariant R residue (indicated in bold font in Fig. 3.3a).

When N-terminal amino acid sequences of the AtPAP26 orthologs were aligned, there are highly conserved amino acid residues at the -6, -5, and -3 positions (T/S, S, and Y/F) with respect to the R cleavage site (Fig. 3.3a). These residues may be required for efficient signal peptide cleavage by a signal peptidase ortholog that processes the various precursors into mature PAP polypeptides. AtPAP26 and AtPAP12 were also predicted to possess three and four N-linked glycosylation sites, respectively, which follow the consensus motif N-X-S/T, where X is any amino acid except proline (Appendix 8).

**Post-transcriptional control of AtPAP26 expression by Pi nutrition**

Results of Fig. 3.5 substantiate previous studies of Arabidopsis suspension cells documenting the marked upregulation of AtPAP26 in response to Pi deprivation, without concomitant changes in AtPAP26 transcript abundance (Veljanovski et al. 2006; Tran & Plaxton 2008). AtPAP12 and AtPPCK1 were employed as positive controls as their mRNAs are significantly induced when Arabidopsis seedlings are subjected to Pi deprivation (Haran et al. 2000; Gregory et al. 2009). Transcript profiling of the Arabidopsis PAP family has confirmed that basal levels of AtPAP26 transcripts are
relatively abundant and are constitutively expressed in all tissues (Zhu et al. 2005). Recent proteomic studies documented a variety of intracellular and secreted proteins that are also controlled post-transcriptionally mainly at the level of protein accumulation in plants responding to changes in environmental Pi availability (Fukuda et al. 2007; Li et al. 2008; Tran & Plaxton 2008). Several reports have further emphasized the involvement of post-transcriptional processes in PSI gene regulation, particularly those played by the microRNA miR399 and E2-ubiquitin conjugase AtUBC24 (pho2) (Chiou et al. 2006), with transcriptional control of miR399 mediated by the transcription factor PHR1 (Bari et al. 2006). Plant microRNAs have been shown to act by translational inhibition (Brodersen et al. 2008). In addition: (i) splice variants of AtPAP10 preferentially associate with ribosomes during Pi starvation (Li et al. 2002), (ii) reversible phosphorylation has been demonstrated to be an important post-translational enzyme modification involved in certain PSI responses (Gregory et al. 2009), whereas (iii) turnover of extracellular PSI tomato PAPs appears to be mediated by serine proteases that are induced and secreted upon Pi-resupply to –Pi cells (Bozzo, Singh & Plaxton 2004b). It stands to reason that the regulation of AtPAP26 abundance by nutritional Pi status could be controlled by any of these processes without concomitant changes in AtPAP26 transcripts. In contrast to AtPAP26, the upregulation of PAPs such as AtPAP12 and AtPAP17 during Pi stress appears to be mainly controlled at the transcriptional level (Fig. 3.5) (del Pozo et al. 1999; Haran et al. 2000; Li et al. 2002; Tran & Plaxton 2008).

Evidence that AtPAP26-S1 and –S2 are glycoforms

Glycosylation is an important post-translational modification that can influence enzyme localization, stability, and/or kinetic properties (Varki 1993; Rudd et al. 1994).
Secreted plant enzymes typically contain highly heterogeneous glycan pools. Although all characterized plant PAPs are glycosylated (Bozzo et al. 2002; Olczak & Watorek 2002; Bozzo et al. 2004a; Veljanovski et al. 2006; Olczak & Olczak 2007), few details of their glycan moieties are available. Of the identified N-linked glycan residues of plant PAPs, the majority consist of complex type oligosaccharides containing xylosylated and/or fucosylated residues, whereas the remainder are of the high-mannose type (Olczak & Watorek 1998). Plant PAPs with 55-kDa subunit sizes typically contain between 3-5 N-glycosylation sites and approximately 10% sugar content (Olczak & Watorek 1998). Our preliminary results indicated that AtPAP26-S1 and –S2 are glycoforms since they both were readily detected with Pro-Q Emerald (Appendix 7), but differed in their ability to bind to Con-A Sepharose (Fig. 3.1b). This was corroborated with a glycan differentiation kit that employs multiple DIG-conjugated lectins as probes that target different glycan groups (Appendix 10). None of the purified AtPAPs were detected by DIG-MAA, -SNA, -DSA, or –PNA (Appendix 7), indicating the absence of these lectin-specific oligosaccharide modifications. However, AtPAP26-S2 bound Galanthus nivalis agglutinin (GNA) (Fig. 3.6a), as well as Con-A Sepharose (Fig. 3.1b) indicating the presence of terminal mannose residues. By contrast, neither AtPAP26-S1 nor AtPAP26-V (or AtPAP12) bound DIG-GNA (Fig. 3.6a), although AtPAP26-V (and AtPAP12) did bind to Con-A Sepharose (Veljanovski et al. 2006). This suggests that important differences exist in the glycan moieties of the AtPAP26-S1, AtPAP26-2, and AtPAP26-V glycoforms.

Additional insights into the glycan groups bound to each AtPAP isoform were provided by immunoblot experiments using the monoclonal antibody YZ1/2.23 which is specific for xylose and/or fucose containing N-linked oligosaccharides (McManus et al. 1988). All three AtPAP26 glycoforms (as well as AtPAP12) were readily detected with
this antibody (Fig. 3.6b), indicating the presence of xylose and/or fucose residue(s) bound to their core oligosaccharide. Deglycosylation experiments with the endoglycosidase Peptide-N-Glycosidase F (PNGase F) were also attempted (Fig. 3.6c). PNGase F hydrolyzes high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins that lack core α1-3-linked fucose residues (Tretter et al. 1991). The inability of PNGase F to deglycosylate the AtPAP26s (Fig. 3.6c) coupled with the immunoblot results obtained using the xylose/fucose specific antibody (Fig. 3.6b) suggests that the three AtPAP26 glycoforms contain fucose bound to their core. However, PNGase F partially deglycosylated AtPAP12 (Fig. 3.6c), implying that a glycan group of AtPAP12 contains a xylose rather than fucose residue bound to its chitobiose core (Tretter et al. 1991). Parallel control experiments established that PNGase F efficiently deglycosylated the mammalian glycoproteins RNase-B and carboxypeptidase Y under identical incubation conditions (results not shown).

As the conserved C-terminal N-glycosylation site appears to be most important for plant PAP expression, stabilization, and secretion (Olczak & Olczak 2007), it would be interesting to establish if this site is involved in dual targeting of AtPAP26 to the vacuole versus secretome of –Pi Arabidopsis. Calreticulin glycoforms from the plant Liriodendron tulipifera have been localized to either the endoplasmic reticulum or golgi apparatus, depending on the specific glycan group bound at its N-linked glycosylation site (Navazio et al. 2002). Similarly, glycodelin is a human protein that exists as a number of tissue-specific glycoforms involved in diverse functions such as contraception and immunosuppression (Lapid & Sharon 2006). Glycodelin’s oligosaccharides vary significantly from one tissue to another and have an effect on its own secretion and role in cell communication (Lapid & Sharon 2006). As discussed below, differential glycosylation also appears to influence the substrate specificities of AtPAP26-S1 and –
S2. This may be due to steric hindrance of substrate binding to the AtPAP26s. The varying sizes and dynamics of different glycans can result in substantial shielding of functionally important protein domains, modulate the interactions of glycoconjugates with other molecules, and influence the rate of protein conformational changes (Rudd et al. 1994).

**Kinetic properties**

Although the purified secreted AtPAPs all exhibited wide pH-APase activity profiles, with a maximum centered at about pH 5.6, AtPAP12 exhibited a somewhat broader profile with significant activity detected in the pH 7.0 to 8.0 range (Appendix 11). All subsequent APase kinetic studies were performed at pH 5.6. Hyperbolic PEP saturation kinetics were observed with $K_m$(PEP) values of 138, 178, and 59 µM obtained for AtPAP26-S1, AtPAP26-S2, and AtPAP12, respectively. These $K_m$(PEP) values are markedly lower than those reported for AtPAP26-V (800 µM) (Veljanovski et al. 2006) or secreted PAPs of -Pi tomato (1.4 and 2.1 mM) (Bozzo et al. 2002), but are similar to those of intracellular PAPs isolated from -Pi Brassica nigra and tomato suspension cells (50 - 200 µM) (Duff, Lefebvre & Plaxton 1989; Bozzo et al. 2004a).

AtPAP12, AtPAP26-S1, and AtPAP26-S2 were activated from about 30 to 60% by Mg$^{2+}$ (Appendix 12), but there was no effect on their activity when the reaction mixture (lacking Mg$^{2+}$) contained 5 mM EDTA. The most notable inhibitors were Zn$^{2+}$, molybdate, vanadate, and fluoride (Appendix 12). Similar findings have been reported for other plant APases (Duff et al. 1989; Duff et al. 1994; Bozzo et al. 2002; Bozzo et al. 2004a; Veljanovski et al. 2006). As with all PAPs, the APase activity of AtPAP12, AtPAP26-S1, and AtPAP26-S2 was insensitive to 5 mM L-tartrate.
APase activity of each PAP was examined with a variety of phosphorylated compounds, tested at a concentration of 5 mM. All three PAPs exhibited non-specific substrate selectivities, although AtPAP26-S2 exhibited a broader range of substrate utilization than AtPAP26-S1 (Table 3.2). For example, AtPAP26-S2 (and AtPAP12) readily employed phenyl-P as a substrate, whereas AtPAP26-S1 exhibited little activity towards this substrate (Table 3.2). By contrast, the purified PAPs showed no activity with phytic acid or bis-(p-nitrophenyl phosphate) (5 mM each), or 5 mg mL\(^{-1}\) of phosvitin (a storage phosphoprotein). A significant component of soil organic-Pi may exist as phytate, or phytic acid (Richardson, Hadobas & Hayes 2000). AtPAP12, AtPAP26-S1, and AtPAP26-S2 are not phytases as they were unable to hydrolyze Pi from phytic acid. Phytase activity has been reported to constitute less than 0.8% of the total acid phosphomonoesterase activity of Arabidopsis root extracts, and wild-type Arabidopsis is unable to acquire P from exogenous phytate owing to the absence of extracellular phytase activity (Richardson et al. 2000). AtPAP15 is the only member of the AtPAP family that has thus far been shown to possess both APase and phytase activity (Zhang et al. 2008; Kuang et al. 2009). AtPAP15 appears to play an important role in mobilizing Pi from phytate reserves during seed or pollen germination (Kuang et al. 2009). Since its expression is unresponsive to Pi deprivation and does not occur in root hair or epidermal cells, AtPAP15 does not appear to function in extracellular Pi scavenging (Kuang et al. 2009). However, constitutive overexpression of AtPAP15 containing a carrot 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). By contrast, transgenic Arabidopsis (and other plants) overexpressing secreted phytases showed no advantage in their
growth or Pi nutrition compared with wild-type controls when cultivated in a variety of agricultural soils (Richardson et al. 2009). This indicates that the bulk of organic-Pi present in soil is not an effective substrate for transgenic plants that secrete phytase.

CONCLUDING REMARKS

This is the first report detailing the purification, identification, and biochemical and molecular characterization of secreted PAPs of Arabidopsis. Accumulation of the same PAP isozymes purified from the CCF of –Pi suspension cells was apparent in the SCF of –Pi seedlings (Fig. 3.2). The collective results indicate that AtPAP12 and AtPAP26 are the predominant secreted PAPs of –Pi Arabidopsis. Their overlapping but non-identical substrate selectivities and pH-activity profiles (Table 3.2, Appendix 11), and relatively high specific APase activities (Table 3.1) are consistent with the hypothesis that their combined activities helps –Pi Arabidopsis to efficiently scavenge Pi from a wide range of extracellular Pi-esters over a broad pH range. However, in contrast to AtPAP12, AtPAP26’s enhanced synthesis during Pi stress is under post-transcriptional control (Fig. 3.5). This highlights the need to integrate transcript profiling studies with parallel biochemical and proteomic analyses of plant stress responses, as the combined datasets will provide a more robust depiction of how alterations in gene expression may be linked to adaptive changes in plant metabolism. This is especially pertinent for our understanding of plant Pi starvation responses in which many recent studies have focused on identifying genes whose transcripts differentially accumulate during Pi stress (Hammond et al. 2003; Wu et al. 2003; Hammond, Broadley & White 2004; Misson et al. 2005; Hernandez et al. 2007; Morcuende et al. 2007; Müller et al. 2007), despite accumulating evidence that transcript abundance does not always predict intra- or extracellular proteome remodeling that ensue nutrient deprivation (Fukuda et al.
Furthermore, transcript profiling provides no information about either the subcellular location of gene products, or post-translational modifications that may be essential for their function, transport, or activation. Constitutive expression of transcripts encoding key Pi-metabolizing enzymes such as AtPAP26 is hypothesized to help ‘prime the system’, thereby accelerating their rate of biosynthesis upon exposure to suboptimal environmental Pi levels. Although our results provide insights into the adaptive biochemical mechanisms that -Pi Arabidopsis employs to scavenge Pi from extracellular organophosphates, a major challenge will be to understand the signaling pathways involved in AtPAP12 and AtPAP26 upregulation and secretion during Pi stress.

Results of our current and earlier (Veljanovski et al. 2006) studies led to the surprising conclusion that AtPAP26 is targeted to both the cell vacuole and secretome during Pi stress. This has been corroborated by the absence of 55-kDa immunoreactive AtPAP26 polypeptides on immunoblots of secretomes (SCFs) of a homozygous atpap26 T-DNA insertion mutant cultivated under –Pi conditions (correlated with a 40% decrease in secreted APase activity) (Hurley et al. 2010). Heterogeneity at the conserved C-terminal glycosylation site could potentially account for the differential targeting of AtPAP26 during Pi stress (Olczak & Watorek 2003). It is also notable that secreted AtPAP26 of –Pi Arabidopsis exists as a pair of distinct glycoforms. Future research involving the use of mass spectrometry will help to confirm the glycosylation sites of the AtPAP26-V, -S1, and –S2 glycoforms, as well as to characterize the oligosaccharides attached at each site. The use of glycosylation to control enzyme turnover, localization, association with binding partners, and activity may transcend PAPs and Pi starvation. The roles which both the individual protein and its cellular environment play in determining glycosylation site occupancy and glycan processing may allow cells to
generate an assortment of glycoforms, resulting in a single glycosylated enzyme having a range of dynamic and functional activities (Varki 1993; Rudd et al. 1994).

A thorough understanding of the functions of AtPAP12 and AtPAP26 orthologs in the Pi stress response of vascular plants will be achieved by combining enzyme biochemistry with functional genomics. Studies using Arabidopsis AtPAP12 and AtPAP26 knockout and overexpression lines are currently in progress. These studies are relevant to applied efforts to engineer Pi-efficient transgenic plants, needed to minimize the input of expensive, unsustainable, and polluting Pi fertilizers in crop production.

REFERENCES


Table 3.1. Purification of secreted PAPs originating from 19-L of culture media of 7-day old –Pi Arabidopsis suspension cells.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific Activity</th>
<th>Purification -fold</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>units</td>
<td>mg</td>
<td>Units mg⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Culture Filtrate</td>
<td>19000</td>
<td>5244</td>
<td>1660</td>
<td>3</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Pellicon concentration</td>
<td>5323</td>
<td>3981</td>
<td>1143</td>
<td>3</td>
<td>1</td>
<td>76</td>
</tr>
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<td>Batchwise Butyl Sepharose</td>
<td>20ᵃ</td>
<td>2508</td>
<td>178</td>
<td>14</td>
<td>4.6</td>
<td>48</td>
</tr>
<tr>
<td>S-Sepharose FPLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>AtPAP26</td>
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<td>1934</td>
<td>40</td>
<td>48</td>
<td>16</td>
<td>37</td>
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<td>360</td>
<td>32</td>
<td>11</td>
<td>3.7</td>
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<tr>
<td>Phosphocellulose</td>
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<td></td>
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<td>AtPAP26</td>
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<td>960</td>
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<td>738</td>
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<td>17</td>
<td>5.7</td>
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<td>Concanavalin-A Sepharose</td>
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<td>AtPAP26-S1</td>
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<td>684</td>
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<td>1710</td>
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<td>Phenyl Superose FPLC</td>
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<td>0.06</td>
<td>650</td>
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<td>AtPAP12</td>
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<td>2.2</td>
<td>109</td>
<td>36</td>
<td>5</td>
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</table>

ᵃConcentrated pooled fractions.
Table 3.2. Substrate specificities of the purified secreted AtPAPs. APase activity was determined with 5 mM of each compound using the spectrophotometric Pi assay described in the Materials and Methods. Activity is expressed relative to the rate of Pi hydrolysis from 5 mM PEP, set at 100%. All values represent means of $n = 3$ separate determinations and are reproducible to within ±10% of the mean value.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>AtPAP12</th>
<th>AtPAP26-S1</th>
<th>AtPAP26-S2</th>
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<tr>
<td>PEP</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>β-naphthyl-P</td>
<td>116</td>
<td>115</td>
<td>118</td>
</tr>
<tr>
<td>para-nitrophenyl-P</td>
<td>101</td>
<td>94</td>
<td>83</td>
</tr>
<tr>
<td>Phenyl-P</td>
<td>98</td>
<td>14</td>
<td>96</td>
</tr>
<tr>
<td>NaPPi</td>
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<tr>
<td>ADP</td>
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<td>ATP</td>
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<td>52</td>
</tr>
<tr>
<td>P-tyrosine</td>
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<td>30</td>
<td>71</td>
</tr>
<tr>
<td>6-P-gluconate</td>
<td>67</td>
<td>57</td>
<td>69</td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>40</td>
<td>54</td>
<td>63</td>
</tr>
<tr>
<td>3-P-glycerate</td>
<td>38</td>
<td>48</td>
<td>67</td>
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<tr>
<td>Glycerol-3-P</td>
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<td>α-naphthyl-P</td>
<td>43</td>
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<td>53</td>
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<td>Ribose-3-P</td>
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<tr>
<td>Ribose-5-P</td>
<td>9</td>
<td>25</td>
<td>50</td>
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<tr>
<td>P-threonine</td>
<td>29</td>
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<td>GTP</td>
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<tr>
<td>P-serine</td>
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<td>26</td>
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<tr>
<td>Fructose-6-P</td>
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<td>32</td>
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<tr>
<td>dAMP</td>
<td>12</td>
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<tr>
<td>Glucose-1-P</td>
<td>6</td>
<td>9</td>
<td>6</td>
</tr>
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</table>
Fig. 3.1. Separation of secreted PAP isoforms from cell culture filtrates of –Pi Arabidopsis suspension cells via S-Sepharose cation-exchange (A) and Con-A Sepharose affinity (B) chromatographies. (A,B) APase activity and $A_{280}$ values are indicated with a dotted and solid line, respectively. (A) The S-Sepharose column was developed with a linear KCl gradient (0-0.5 M). (B) AtPAP26-S1 does not bind to Con-A and was located in the flow-through fractions whereas AtPAP26-S2 bound to Con-A and was eluted with a linear α-methyl-D-mannoside gradient (0-0.5 M).
Fig. 3.2. Induction of secreted APase activity and immunological PAP detection in -Pi Arabidopsis suspension cell and seedling culture filtrates. (A) Arabidopsis suspension cells and seedlings were cultivated under +Pi and –Pi conditions as described in the Materials and Methods, and the cell and seedling culture filtrates (CCF and SCF, respectively) assayed for APase activity and Pi concentration. All values represent the means ±SEM of n = 3 biological replicates and are reproducible to within ±10% of the mean value. (B) Concentrated secreted proteins from +Pi and –Pi Arabidopsis (25 µg per lane) were resolved by SDS-PAGE, electrophoretically transferred onto PVDF, and probed with anti-(AtPAP12 or AtPAP26-V) immune serum. Lanes labeled “+Pi” and “-Pi” denote secreted proteins from +Pi and –Pi cells or seedlings, respectively. Homogeneous AtPAP26-V (25 ng) (Veljanovski et al. 2006) and AtPAP12 (50 ng; see Table 3.1) serve as positive controls.
Fig. 3.3. Bioinformatic analysis of AtPAP12 and AtPAP26 with orthologs from other plants. Alignment of their deduced N-terminal sequences (A) and phylogenetic analysis (B) were performed using ClustalX. (A) N-terminal sequences obtained by automated Edman degradation of secreted AtPAP12, AtPAP26-S1, and AtPAP26-S2 purified from the CCF of -Pi Arabidopsis suspension cells are shown in bold font. The N-terminal sequences obtained for the 55-kDa subunit of AtPAP26-V (Veljanovski et al. 2006), the 57-kDa subunit of PAPs from tomato (Lycopersicon) and soybean (Glycine) (Lebansky et al. 1992; Bozzo et al. 2004a), the 52-kDa subunit of an onion (Allium) APase (Shinano et al. 2001), and the 70–kDa subunit of an APase secreted by proteoid roots of -Pi lupin (Lupinus) (Miller et al. 2001) are enclosed with rectangles. Identical amino acids are denoted by an asterisk. Arrows indicate the predicted transit peptide cleavage site (using Signal P). Only bootstrap probability values of ≥60% (over 100 replicates) are indicated at the branching points. The scale bar indicates 0.1 substitutions per site.
Fig. 3.4. SDS-PAGE and immunoblot analysis of purified secreted PAPs from -Pi Arabidopsis suspension cells. (A) Purified PAPs were electrophoresed on 10% SDS gels and stained with Coomassie Blue R-250 (CBB-250). The amount of protein loaded is as follows: AtPAP26-V and AtPAP12, 2.5 µg each; AtPAP26-S1 and –S2, 4 and 1.5 µg, respectively. (B,C) Purified AtPAPs (50 ng each) were subjected to SDS-PAGE, electroblotted onto PVDF, and immunoblotting performed with anti-(AtPAP12 or AtPAP26-V) immune serum as indicated. Antigenic polypeptides were visualized using an alkaline-phosphatase–linked secondary antibody and chromogenic detection (Veljanovski et al. 2006). \( M_w \) denotes various non-prestained (A) or prestained (B,C) \( M_r \) standard.
Fig. 3.5. Semi-quantitative RT-PCR analysis of AtPAP12 and AtPAP26 gene expression in shoots and roots of Pi-sufficient (+Pi) and Pi-starved (-Pi) Arabidopsis seedlings. The seedlings were cultivated under +Pi and –Pi conditions as described in the Materials and Methods. Levels of mRNA were analyzed by semi-quantitative RT-PCR using gene specific primers for AtPAP12, AtPAP26, AtPPCK1, and AtACT2. AtPAP12 and AtPPCK1 were used as positive controls (Haran et al. 2000; Li et al. 2002; Tran & Plaxton 2008; Gregory et al. 2009), whereas AtACT2 was used as a reference to ensure equal template loading. All PCR products were taken at cycle numbers determined to be nonsaturating. Template concentrations needed to achieve non-saturating conditions for primer pairs as tested for roots of –Pi seedlings are indicated in parentheses. Control RT–PCR reactions lacking reverse transcriptase did not show any bands. The AtPAP12, AtPAP26, AtPPCK1, and AtACT2 transcripts amplified as 244, 610, 627, and 220 bp fragments of the 5’ region of their respective cDNAs, as expected.

<table>
<thead>
<tr>
<th></th>
<th>Shoot +Pi</th>
<th>Shoot -Pi</th>
<th>Root +Pi</th>
<th>Root -Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtPAP12</td>
<td>(0.4 ng)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtPAP26</td>
<td></td>
<td>(0.6 ng)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtPPCK1</td>
<td></td>
<td></td>
<td>(0.17 ng)</td>
<td></td>
</tr>
<tr>
<td>AtACT2</td>
<td></td>
<td></td>
<td>(0.05 ng)</td>
<td></td>
</tr>
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</table>
Fig. 3.6. Characterization of glycan chains of secreted PAP isoforms purified from CCF of –Pi Arabidopsis suspension cells. (A,B) PAPs were subjected to SDS-PAGE, electrobotted onto PVDF and probed with (A) 1 µg mL⁻¹ of DIG-GNA (1 µg per lane), followed by immunological DIG detection using anti-DIG-IgG, or (B) the monoclonal antibody YZ1/2.23 (3 ng per lane) which specifically cross-reacts with xylose or fucose residues linked to the chitobiose core of plant glycoproteins (McManus et al. 1988). (C) Deglycosylation of purified AtPAPs (50 µg each) was attempted with PNGase F under denaturing conditions using the N-Glycosidase F Protein Deglycosylation Kit (Roche) according to the manufacturer’s instructions (overnight incubation at 37 °C). PNGase F cleaves between the innermost N-acetylglucosamine and asparagine residues of many N-linked glycoproteins (Chu 1986).
Chapter 4. The dual-targeted purple acid phosphatase isozyme

AtPAP26 is essential for efficient acclimation of Arabidopsis thaliana to nutritional phosphate deprivation

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¹ these authors contributed equally to the article

ABSTRACT

Induction of intracellular and secreted acid phosphatases (APases) is a widespread response of Pi-starved (-Pi) plants. APases catalyze Pi hydrolysis from a broad range of phosphomonoesters at an acidic pH. The largest class of non-specific plant APases is the purple APases (PAPs). Although the physical and kinetic properties, subcellular location, and expression of several plant PAPs have been described, their physiological functions have not been resolved. Recent biochemical studies indicated that AtPAP26, one of 29 PAPs encoded by the Arabidopsis thaliana genome, is the predominant intracellular APase, as well as a major secreted APase isozyme upregulated by -Pi Arabidopsis. A T-DNA atpap26 insertional mutant lacking AtPAP26 transcripts and 55-kDa immunoreactive AtPAP26 polypeptides exhibited: (i) 9- and 5-fold lower shoot and root APase activity, respectively, which did not change in response to Pi starvation, (ii) a 40% decrease in secreted APase activity during Pi deprivation, (iii) 35 and 50% reductions in free and total Pi concentration, respectively, as well as 5-fold higher anthocyanin levels in shoots of soil grown –Pi plants, and (iv) impaired shoot and root development when subjected on Pi deficiency. By contrast, no deleterious influence
of AtPAP26 loss of function was apparent under Pi replete conditions, or during nitrogen or potassium-limited growth, or oxidative stress. Transient expression of 35S:AtPAP26-mCherry in Arabidopsis suspension cells verified that AtPAP26 is targeted to the cell vacuole. Our results indicate that AtPAP26 is a principal contributor to Pi-stress inducible APase activity, and that it makes an important contribution to Pi-recycling and scavenging in –Pi Arabidopsis.

INTRODUCTION

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

A well documented component of the plant Pi stress response is the upregulation of intracellular and secreted acid phosphatases (APase; E.C. 3.1.3.2) which catalyze the hydrolysis of Pi from various Pi-monesters and anhydrides in the acidic pH range (Duff, et al., 1994). APase induction by –Pi plants has been correlated with de novo APase synthesis in several species, including Arabidopsis thaliana, tomato, and lupin
The probable function of intracellular APases is to recycle Pi from expendable intracellular organophosphate pools. This is accompanied by a marked reduction in cytoplasmic P-metabolites during extended Pi stress (Duff, et al., 1994; Bozzo and Plaxton, 2008). Secreted APases belong to a group of PSI phosphohydrolases believed to mobilize Pi from the external organophosphates that are prevalent in many soils (Vance, et al., 2003; Bozzo and Plaxton, 2008). Molecular analyses of PSI transcripts and proteins have hinted at complex control of plant APase gene expression. Pi deprivation induces temporal and tissue specific expression of PSI APase isozymes (Bozzo, et al., 2006; Haran, et al., 2000; Wu, et al., 2003; Zimmerman, et al., 2004) and the concomitant down-regulation of other APases (Misson, et al., 2005). The transcription factors PHR1, WRKY75, and ZAT6 have been implicated in the control of Arabidopsis PSI APases (Rubio, et al., 2001; Devaiah, et al., 2007a; Devaiah, et al., 2007b), while post-transcriptional mechanisms appear to be essential for the upregulation of the purple APase (PAP) AtPAP26 during Pi stress (Veljanovski, et al., 2006; Tran and Plaxton, 2008; Tran, et al., 2010). In contrast, Pi re-supply to –Pi plants rapidly represses PSI APase genes (Veljanovski, et al., 2006; del Pozo, et al., 1999; Müller, et al., 2004) while inducing proteases that target PSI secreted APases (Bozzo, et al., 2004). Further characterization of PSI APases is required to define the molecular mechanisms underlying this archetypical plant response to Pi starvation, as well as to identify additional targets for biotechnological improvement of crop Pi acquisition.

A variety of intracellular and secreted APases upregulated by –Pi plants have been characterized as PAPs, which represent a specific APase class characterized by a bimetallic active site that endows them with a pink or purple colour in solution (Veljanovski, et al., 2006; Bozzo, et al., 2006; Tran, et al., 2010; del Pozo, et al., 1999;
Bozzo, et al., 2002; Bozzo, et al., 2004). Although mammals contain two PAP-encoding genes, vascular plant PAPs belong to a large multigene family. Li and coworkers (Li, et al., 2002; Flanagan, et al., 2006) classified 29 putative PAP genes in Arabidopsis, several of which appear to respond to Pi deficiency. Apart from functioning as non-specific APases, several PSI plant PAPs (including AtPAP17 and AtPAP26) exhibit alkaline peroxidase activity indicating their potential alternative role in the metabolism of reactive oxygen species (Veljanovski, et al., 2006; del Pozo, et al., 1999; Bozzo, et al., 2002; Bozzo, et al., 2004). Moreover, AtPAP17 was induced by oxidative stress (del Pozo, et al., 1999) and ectopic expression of a soybean mitochondrial PAP (GmPAP3) conferred increased resistance to oxidative stress in transgenic Arabidopsis (Francisca Li, et al., 2008). It is therefore important to determine the physiological roles of the various plant PAPs and which of their dual enzymatic activities are functional in –Pi plants.

The aim of the present study was to build upon biochemical analyses indicating that AtPAP26 is the predominant intracellular (vacuolar), as well as a major secreted APase isozyme upregulated by –Pi Arabidopsis (Veljanovski, et al., 2006; Tran and Plaxton, 2008; Tran, et al., 2010). In particular, we sought to test the hypothesis that AtPAP26 plays a pivotal role in the Pi metabolism of Arabidopsis during Pi stress. This was done by taking advantage of the publicly available T-DNA tagged insertional mutagenized populations of Arabidopsis (Alonso, et al., 2003). We identified and characterized a null atpap26 allele that abrogated AtPAP26 expression. This was correlated with the elimination of PSI intracellular APase activity, as well as a significant reduction in secreted APase activity during Pi deprivation. The atpap26 mutant demonstrated impaired development and altered free and total Pi levels relative when
exposed to Pi deficiency. Our results establish a firm role for AtPAP26 in facilitating the acclimation of Arabidopsis to suboptimal Pi nutrition.

MATERIALS AND METHODS

Plant Material and Growth Conditions

For mutant isolation and routine plant growth Arabidopsis (Arabidopsis thaliana, Col-0 ecotype) seeds were sown in a standard soil mixture (Sunshine Aggregate Plus Mix 4; SunGro, Vancouver, BC) and stratified at 4 °C for 2 d. Plants were cultivated in growth chambers at 22 °C and 100 μmol m⁻² s⁻¹ photosynthetically active radiation (PAR) with a 16-h-light/8-h-dark photoperiod, and fertilized biweekly by subirrigation with 0.25x Hoagland’s media. No additional watering was required. To assess the influence of Pi deprivation on the development of soil-grown seedlings, 7 d old seedlings were transplanted into a 55% to 65% sphagnum peat moss/perlite soil mix lacking all nutrients (Sunshine Mix 2; SunGro, Vancouver, BC) at a density of 4 seedlings per pot. Plants were fertilized as above with 0.25x Hoagland’s media containing either 2 or 0 mM Pi. At 3 weeks post-germination entire rosettes were harvested and either dried for 48 h at 65 °C, or snap frozen in liquid N₂, and stored at -80 °C for later analysis. Whenever Pi was reduced or eliminated from the growth medium, Pi salts were replaced with appropriate sulfate salts, so that the conjugate cation remained constant.

For liquid culture, approximately 100 seeds were surface sterilized and stratified for 2 d at 4 °C, then placed in 250 mL Magenta boxes containing 50 mL of 0.5x Murashige-Skoog media, pH 5.7, 1% (w/v) sucrose and 0.2 mM Pi, and cultivated at 24 °C under continuous 100 μmol m⁻² s⁻¹ PAR on an orbital shaker set at 80 rpm. After 7 d the seedlings were transferred into fresh media containing 1.5 or 0 mM Pi for an additional 7 d. The 14 d old seedlings were blotted dry, and snap frozen in liquid N₂ and
stored at -80 °C, whereas growth media containing secreted proteins was passed through a 0.45 μM syringe filter and concentrated over 250-fold with an Amicon Ultra-15 ultrafiltration device (30,000 Mₐ cutoff; Millipore) at 4 °C.

**Mutant Isolation**

A potential *atpap26* (Salk_152821) mutant line was identified from the Salk T-DNA lines (Alonso, et al., 2003) via analyses of the SiGnAL database (http://signal.salk.edu/cgi-bin/tdnaexpress). Seeds were obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University. Homozygous mutant plants were isolated by PCR screening of gDNA from the T4 generation using primers mapped on Fig. 4.1A and described in Appendix 13. All PCR products were sequenced for verification (Génome Québec, Montréal, Canada and the Centre for Applied Genomics at The Hospital for Sick Kids, Toronto, Canada). Southern blotting was performed on *atpap26* gDNA to determine the T-DNA insert number. Genomic DNA was isolated according to (Zhang and Zeevaart, 1999) except that isopropanol-precipitated nucleic acids were pelleted by centrifugation at 15,000 g for 10 min, followed by 2 washes with 70% (v/v) ethanol; DNA pellets were dissolved in 10 mM Tris-HCl (pH 8.0). Gel blot analysis was performed as described (Quan, et al., 2007). The 795 bp neomycin phosphotransferase coding sequence (*NPTII* CDS) was used to generate a digoxigenin (DIG) labelled probe using Roche’s dig labelling kit. Southern blot hybridization was performed at 70 °C for 18 h using hybridization solution (250 mM NaPi, pH 7.2, 1 mM EDTA, 20% (w/v) SDS, 0.5% (w/v) milk powder) with dig-labelled *NPTII* CDS probes followed by three 20 min washes at 60 °C in 20 mM NaPi (pH 7.2) containing 1 mM EDTA and 1% (w/v) SDS. The blot was incubated for 90 min in 1% (w/v) milk powder and probed for 30 min with 1:5000 anti-DIG-IgG conjugated to alkaline phosphatase.
(Roche) followed by two 15 min washes in detection wash solution. Bands were visualized by chemiluminescence using CDP-Star (Roche).

**RNA Isolation and Semi-quantitative RT–PCR**

Total RNA was extracted and purified as described previously (Veljanovski, et al., 2006). RNA samples were assessed for purity via their $A_{260}/A_{280}$ ratio and integrity by resolving 1 µg of total RNA on a 1.2% (w/v) denaturing agarose gel. Normalization of RNA for RT was performed for each sample by density measurement of 28S ribosomal RNA bands from the above gel (scanned using ImageJ software from the National Institutes for Health, U.S.A.). RNA (5 µg) was reverse transcribed with Superscript III (Invitrogen) and non-competitive RT–PCR was performed as described in Gennidakis et al. (Gennidakis, et al., 2007). Gene specific primers used to amplify *AtPPCK1*, *AtPAP17*, *AtPAP26*, and *AtACT2* were previously described (Veljanovski, et al., 2006; Gregory, et al., 2009). Transcripts for *AtPAP12* were amplified using gene specific primers (Appendix 13); all PCR products were sequenced for verification. Conditions were optimized for all semi-quantitative RT-PCR reactions to ensure linearity of response for comparison between samples.

**Protein Extraction**

Tissues were homogenized (1:2; w/v) in ice-cold extraction buffer composed of 20 mM sodium acetate (pH 5.6), 1 mM EDTA, 1 mM DTT, 1 mM 2,2′ dipyridyl disulfide, 1 mM phenylmethlysulfonyl fluoride, 5 mM thiourea, and 1% (w/v) insoluble polyvinyl (polypyrrolidone). Homogenates were centrifuged at 4 °C and 14,000g for 5 min, and the supernatants reserved as clarified extract.
APase Assays and Determination of Protein Concentration

APase activity was routinely measured by coupling the hydrolysis of PEP to pyruvate to the lactate dehydrogenase reaction and continuously monitoring NADH oxidation at 340 nm using a Molecular Devices Spectromax Plus Microplate spectrophotometer and the following optimized assay conditions: 50 mM Na-acetate (pH 5.6), 5 mM PEP, 10 mM MgCl₂, 0.2 mM NADH, and 3 units of desalted rabbit muscle lactate dehydrogenase in a final volume of 0.2 mL. Assays were corrected for background NADH oxidation by omitting PEP from the reaction mixture. APase assays were also carried out in an assay mix containing 50 mM sodium acetate (pH 5.6), 5 mM ρNPP, and 10 mM MgCl₂ by measuring the formation of para-nitrophenol at 405 nm (ε = 18,000 M⁻¹cm⁻¹). All APase assays were linear with respect to time and concentration of enzyme assayed. One unit of activity is defined as the amount of enzyme resulting in the hydrolysis of 1 µmol of substrate·min⁻¹ at 25 °C. Protein concentrations were determined using a modified Bradford assay (Bozzo, et al., 2002) with bovine γ-globulin as the protein standard.

Protein Electrophoresis and Immunoblotting

SDS-PAGE, immunoblotting onto poly(vinylidene difluoride) membranes (Immobilon transfer; 0.45 µm pore size; Millipore Canada) and visualization of antigenic polypeptides using an alkaline-phosphatase-tagged secondary antibody were conducted as previously described (Veljanovski, et al., 2006; Tran and Plaxton, 2008). Densitometric analysis of immunoblots was performed using an LKB Ultroscan XL laser densitometer and GELSCAN software (Version 2.1; Pharmacia LKB Biotech). Derived A₆₆₀ values were linear with respect to the amount of the immunoblotted extract. All immunoblot results were replicated a minimum of three times, with representative results
shown in the various figures. Non-denaturing PAGE was carried out using 7% separating gels (Gennidakis, et al., 2007). In-gel APase activity staining was performed by equilibrating the gels in 100 mM Na-acetate (pH 5.6) containing 10 mM MgCl₂ for 30 min, and then incubating in equilibration buffer containing 1 mg·mL⁻¹ Fast Garnet GBC and 0.03% (w/v) β-naphthyl-P.

Quantification of Total and Soluble Pi

Total Pi determinations were carried out using dried leaf tissue that had been flamed to ash by heating at 500 °C for 3 h. The ash was dissolved in 30% (v/v) HCl containing 10% (v/v) HNO₃, and centrifuged at 14,000g for 10 min. The supernatant was diluted 50-fold in water and its Pi concentration quantified as previously described (Bozzo, et al., 2006). Diluted sample (800 µL) was mixed with 200 µL of a Pi assay reagent and incubated at 45 °C for 20 min. Pi assay reagent consisted of four parts of freshly prepared 10% [w/v] ascorbic acid plus one part 10 mM ammonium molybdate containing 15 mM zinc acetate (pH 5.0). Total Pi content was measured at A₆₆₀ using appropriate Pi standards and is expressed as µmol Pi/mg dry weight. Soluble Pi was determined by extracting frozen tissues (1:2, w/v) with 0.1 M HCl. Samples were centrifuged at 14,000g and supernatants assayed for Pi as described above. For available soil Pi, 0.3 g of soil was suspended in 10 mL of deionized water and the eluate removed for free Pi determination. To determine the total amount of Pi in the soil, 0.1g was converted to ash as described above prior to Pi determination.

Determination of Anthocyanin Concentration

Frozen leaf material was transferred to extraction buffer (18% [v/v] 1-propanol containing 1% [v/v] concentrated HCl), boiled for 3 min, and centrifuged at 14,000g for
10 min. The optical density was measured at $A_{532}$ and $A_{653}$. Subtraction of 0.24 $A_{653}$ compensated for the small overlap in $A_{532}$ by the chlorophylls (Schmidt and Mohr, 1981). Anthocyanin concentration was determined using the corrected absorbance and the molar extinction coefficient ($\varepsilon$) of 38,000 L mol$^{-1}$ cm$^{-1}$ for anthocyanin.

Subcellular Localization of AtPAP26-mCherry Fusion Protein

An AtPAP26 cDNA clone (U11049) was obtained from ABRC, and amplified using PCR and appropriate oligonucleotide primers (Appendix 13). The resulting PCR fragment, containing the entire open reading frame of AtPAP26, was inserted into EcoRI to XmaI of plasmid pSAT4A-mCherry-N1 (ABRC) to yield pSAT4A-AtPAP26-mCherry. The plasmid, p35S-NTPP-GFP, encoding the Arabidopsis basic chitinase N-terminal signal sequence, followed by the 16 amino-acid-long sweet potato sporamin N-terminal protopeptide (NTPP) fused to the green fluorescent protein (GFP) was a gift from Christopher Trobacher and John Greenwood (University of Guelph, Canada) and was constructed in the following manner. First, the plasmid p35S-mGFP5-HDEL (provided by Jim Haseloff, Univ. of Cambridge, UK) containing the 35S cauliflower mosaic virus promoter driving the expression of a fusion protein consisting of the Arabidopsis basic chitinase signal sequence fused to GFP and a C-terminal HDEL ER retrieval sequence (Haseloff, et al., 1997) was modified (using the Quikchange PCR-based site-directed mutagenesis kit [Stratagene, La Jolla, CA] and appropriate oligonucleotide primers [Appendix 13]) to introduce an XhoI site following the basic chinase signal sequence. Thereafter, p35S-mGFP5-HDEL was modified further (via site-directed mutagenesis) to replace the codon encoding the histidine at the –4 position of the –HDEL ER retrieval motif with a stop codon, yielding p35S-mGFP5-NXS. Finally, annealed (complementary) oligonucleotides encoding the 16 amino-acid long sporamin NTPP containing lytic
vacuolar sorting information (Matsuoka and Nakamura, 1991; Koide, et al., 1997), along with XhoI compatible ends, were ligated directly into XhoI-digested p35S-mGFP5-NXS, yielding p35S-NTTP-GFP.

Culturing of Arabidopsis (var Landsberg erecta) suspension cells in standard Murashige-Skoog media (contains 1.25 mM Pi), and co-transient-transformations of 4 d old cells with 10 µg of each plasmid using a biolistic particle delivery system 1000/HE (Bio-Rad, Mississauga, ON, Canada) were performed as previously described (Lingard, et al., 2008). Bombarded cells were incubated for 8 h to permit expression and sorting of the expressed proteins, and then fixed in formaldehyde. Epifluorescent images were acquired using a Zeiss Axioskope 2 MOT epifluorescence microscope (Carl Zeiss Inc., Thornwood, NY, USA) with a Zeiss 63x Plan Apochromat oil-immersion objective. Image capture was performed using a Retiga 1300 charge-coupled device camera (Qimaging, Burnaby, BC, Canada) and Northern Eclipse 5.0 software (Empix Imaging Inc., Mississauga, ON, Canada). Figure compositions were generated using Adobe Photoshop CS (Adobe Systems Inc., Toronto, ON, Canada).

Statistics

All values are presented as means ±SE. Data were analyzed using the one tailed Student's t-test, and deemed significant if $P < 0.01$.

RESULTS AND DISCUSSION

Identification and Validation of an atpap26 Mutant Allele

To assess the contribution of AtPAP26 to intracellular and secreted APase activity during Pi deficiency, as well as its impact on the phenotype of +Pi versus –Pi
Arabidopsis, a T-DNA insertion line was identified in the Salk collection (Salk_152821) (Alonso, et al., 2003). The T-DNA insert was located in the seventh intron of the *AtPAP26* gene (locus At5g34850) (Fig. 4.1A) and this position was verified by PCR screening of genomic DNA (gDNA) using an *AtPAP26* gene specific primer and a T-DNA left border primer (Fig. 4.1B). Homozygosity of the T-DNA mutant was confirmed by PCR of gDNA using *AtPAP26*-specific primers (Fig. 4.1B). T-DNA insert number was assessed by Southern blotting of gDNA that had been digested with *Eco*RI, *Hind*III, or *Sac*I. Each restriction enzyme cleaves a single digestion site within the integrated *pBIN*-p*ROK2* vector. All digestions yielded a single strongly hybridizing band (Fig. 4.1C), indicating a single T-DNA insertion site.

The impact of the T-DNA insertion on *AtPAP26* expression was investigated by performing RT-PCR using primer pairs to amplify cDNA sequences flanking the second exon of *AtPAP26* (Fig. 4.1A). *AtPAP26* transcripts were observed in shoot and root mRNA isolated from wild-type Col-0, but not *atpap26* mutant plants (Fig. 4.2A). *AtPAP12*, *AtPAP17*, and *AtPPCK1* (encodes PEP carboxylase protein kinase 1) were employed as positive controls as their transcript levels are markedly increased in -Pi Arabidopsis (Tran and Plaxton, 2008; Haran, et al., 2000; Tran, et al., 2010; del Pozo, et al., 1999; Li, et al., 2002; Gregory, et al., 2009). All of these Pi-responsive control transcripts showed a similar induction in –Pi *atpap26* mutant and wild-type Col-0 seedlings (Fig. 4.2A), indicating that the mutant is unimpaired in Pi starvation signaling.

Consistent with previous studies (Veljanovski, et al., 2006; Tran and Plaxton, 2008; Tran, et al., 2010), the amount of 55-kDa *AtPAP26* immunoreactive polypeptides were about 2-fold greater in root or shoot extracts from the –Pi relative to +Pi Col-0 seedlings (Fig. 4.2B), and this was paralleled by the pronounced accumulation of secreted *AtPAP26* polypeptides in the media of –Pi Col-0 seedlings (Fig. 4.3). By
contrast, immunoblotting of clarified shoot or root extracts, or secretome proteins of the +Pi or -Pi atpap26 mutant failed to reveal any immunoreactive polypeptides (Figs. 4.2B and 4.3), whereas the upregulation and secretion of 60-kDa AtPAP12 polypeptides during Pi stress was unaffected (Fig. 4.3). Therefore, atpap26 defines a null allele of AtPAP26, with abrogated expression of AtPAP26 transcript and protein. Results of Fig. 4.2A corroborate previous studies of Arabidopsis suspension cells and seedlings documenting the upregulation of intracellular and secreted AtPAP26 polypeptides in response to Pi starvation, without concomitant changes in AtPAP26 transcript abundance (Veljanovski, et al., 2006; Tran and Plaxton, 2008; Tran, et al., 2010). Recent proteomic studies have observed a variety of intracellular and secreted proteins that are also controlled post-transcriptionally at the level of protein accumulation in Arabidopsis, maize, and rice plants responding to changes in environmental Pi availability (Tran and Plaxton, 2008; Fukuda, et al., 2007; Li, et al., 2008). In contrast to AtPAP26, the upregulation of Arabidopsis PAPs such as AtPAP12 and AtPAP17 during Pi deprivation appears to be mainly controlled at the transcriptional level (Tran and Plaxton, 2008; Haran, et al., 2000; Tran, et al., 2010; del Pozo, et al., 1999; Li, et al., 2002) (Fig. 4.2A). As AtPAP12 is secreted by -Pi Arabidopsis suspension cells and seedlings (along with AtPAP26) it is expected to play an extracellular Pi-scavenging role (Fig. 4.3B) (Tran and Plaxton, 2008; Tran, et al., 2010). AtPAP17 (formerly known as ACP5) transcripts also accumulate in response to oxidative or salt stress, similar to GmPAP3 (del Pozo, et al., 1999; Francisca Li, et al., 2008). AtPAP17 may thus function to detoxify reactive oxygen species during general stress rather than play a significant Pi remobilization and scavenging role in –Pi Arabidopsis.
AtPAP26 is the Predominant Intracellular and a Major Secreted APase Isozyme Upregulated by -Pi Arabidopsis

Pi deprivation of Col-0 seedlings resulted in a 2- to 3-fold increases in shoot, root, and secreted APase activities (Figs. 4.2C and 4.3), as previously reported (Veljanovski, et al., 2006; Tran and Plaxton, 2008; Tran, et al., 2010; Zakhleniuk, et al., 2001). However, -Pi atpap26 mutants exhibited 9- and 5-fold lower shoot and root APase activities, respectively (Fig. 4.2C), as well as a 40% reduction in secreted APase activity relative to Col-0 (Fig. 4.3). This decrease in secreted APase activity of the atpap26 mutant corresponds with the disappearance of an APase activity staining band that comigrated with homogenous AtPAP26 (Fig. 4.3C). It is notable that no increase in intracellular APase activity was detected in response to Pi starvation of the atpap26 mutant (Fig. 4.2C). APase assays employing 5 mM para-nitrophenol-P (pNPP) rather than 5 mM PEP as the substrate were also performed with shoot and root extracts of -Pi Col-0 and atpap26 seedlings. Consistent with the PEP-based assays (Fig. 4.2C), extracts from -Pi shoots and roots of atpap26 seedlings respectively exhibited about 4-fold and 6-fold lower pNPP-hydrolyzing activity relative to Col-0 controls (shoots = 20 ±1 and 82 ±44 nmol pNPP hydrolyzed/min/mg protein, respectively; roots = 35.3 ±3 and 202 ±33 nmol pNPP hydrolyzed/min/mg protein, respectively; means ±SE of n = 4 biological replicates).

Clarified extracts of –Pi shoots were also resolved by non-denaturing PAGE and subjected to in-gel APase activity staining using β-naphthyl-P as the substrate, and parallel immunoblotting with anti-AtPAP26 immune serum was performed (Fig. 4.2D). Shoot extracts of –Pi Col-0 yielded several APase activity-staining bands on non-denaturing gels, in agreement with previous results (Tomscha, et al., 2004). However, an abundant high molecular mass APase activity staining band that strongly cross-
reacted with anti-AtPAP26 immune serum was absent in the *atpap26* mutant (Fig. 4.2D). The collective results of Figs. 4.2 and 4.3 support our earlier biochemical studies (Veljanovski, et al., 2006; Tran, et al., 2010), and unequivocally demonstrate that AtPAP26 is a principal contributor of intracellular and secreted APase activity of –Pi Arabidopsis. Similar results were obtained by Tomscha and co-workers (Tomscha, et al., 2004) who characterized an Arabidopsis phosphatase-underproducer (*pup3*) ethylmethane sulfonate mutant that exhibited about 40% lower APase activity in shoot and root extracts. Although immunoblotting using anti-(recombinant AtPAP12) immune serum led them to conclude that AtPAP12 was one of the AtPAP isozymes defective in *pup3*, AtPAP26 was also implicated since the *pup3* mutation mapped to a 2.7 Mb sequence of chromosome 5 within the Arabidopsis genome that encompasses *AtPAP26* (Tomscha, et al., 2004). That *pup3* may have been defective in AtPAP26 rather than AtPAP12 is supported by the observations that: (i) *AtPAP12* transcript levels were unaffected in *pup3* (Tomscha, et al., 2004), whereas (ii) the same anti-(recombinant AtPAP12) immune serum employed by Tomscha et al. (Tomscha, et al., 2004) effectively cross-reacts with both AtPAP12 and AtPAP26 (Fig. 4.3) (Tran, et al., 2010).

By contrast, no detectable influence on total APase activity was obtained with several other AtPAP isozyme loss-of-function mutants. For example, no alteration in extractable APase activity was reported in *atpap23* T-DNA mutants (Zhu, et al., 2005). Similarly, extracts of *atpap15* T-DNA mutants contained 6-fold lower phytase activity, but unaltered *pNPP* hydrolytic activity relative to wild-type controls, possibly due to AtPAP15’s specificity as a phytase (Zhang, et al., 2008) and/or its low abundance relative to AtPAP26.

*AtPAP26 is Essential for Efficient Acclimation of Arabidopsis to Pi starvation*
Shoot development of 14-d-old Arabidopsis seedlings cultivated on +Pi and –Pi agar media is depicted in Figs. 4.4A and 4.4B. No differences were noted in the appearance or fresh weight of +Pi Col-0 versus atpap26 shoots. However, atpap26 shoot development was significantly impaired during growth on –Pi media (Fig. 4.4A), exhibiting a 30% decrease in fresh weight relative to Col-0 (Fig. 4.5B). The development of atpap26 mutants was also characterized in plants cultivated on +Pi or -Pi agar media, as well as on +Pi media deficient in nitrogen or potassium, or supplemented with 1 μM paraquat (PQ). Similar to 14-d-old plants (Fig. 4.4B), rosette fresh weight of 3-week-old –Pi atpap26 seedlings was reduced by about 25% relative to –Pi Col-0, but unaffected under +Pi conditions (Fig. 4.4C). Likewise, no phenotypic differences relative to Col-0 were apparent when +Pi atpap26 seedlings were subjected N- or K-deficiency, or PQ treatment (Fig. 4.4C and results not shown). Loss of AtPAP26 function also failed to influence the sensitivity of Arabidopsis to PQ-mediated oxidative stress, suggesting that the in vitro alkaline peroxidase activity of purified AtPAP26 (Veljanovski, et al., 2006) has little in vivo relevance during Pi stress. This is consistent with AtPAP26’s vacuolar localization (Carter, et al., 2004) in which an acidic pH of about pH 5.5 closely aligns with the enzyme’s APase pH-activity optimum of pH 5.6, but is far below AtPAP26’s peroxidase pH-activity optimum of pH 9.0 (Veljanovski, et al., 2006). It therefore appears that while AtPAP26 is indispensable for the acclimation of Arabidopsis to Pi deprivation, it is expendable in +Pi Arabidopsis, or during other macronutrient deficiencies or oxidative stress.

Root development of the atpap26 mutant was examined by cultivating seedlings on vertically orientated agar plates for 12-d. Primary roots of the atpap26 mutants were about 20% shorter than those of Col-0 during growth on the –Pi media (Fig. 4.4D, Appendix 14). However, there was no influence of the loss of AtPAP26 expression on
root structure architecture of +Pi or –Pi seedlings, or root hair number within 5 mm of the root tip (Appendix 14). Although altered root development is a common phenomenon within functional analyses of Arabidopsis PSI genes, this response can be grouped into two categories based on the function of the PSI gene in question. Disruption of genes involved in transcriptional reorganization or hormone-mediated responses to Pi starvation generally causes a shift in root structure architecture in addition to decreased primary root growth. For example, knockout of PSI transcription factors has been reported to reduce primary root growth with concomitant increases in lateral root and root hair development (Devaiah, et al., 2007a; Devaiah, et al., 2007b). Similar results have been obtained with genes that mediate signal transduction in –Pi Arabidopsis, such as phospholipase Dζ1 and Dζ2, in which a double knockout exhibited reduced phosphatidic acid levels and altered root structure architecture during Pi starvation (Li, et al., 2006). By contrast, disruption of PSI genes encoding metabolic enzymes often results in a reduction of total root growth during Pi stress without obvious changes in root structure architecture. For example, knockout of monogalactosyldiacylglycerol synthase 2 resulted in an overall 20% decrease in root growth, without influencing root structure architecture (Kobayashi, et al., 2009). The observed upregulation of three well documented PSI genes (Fig. 4.2A) and overall reduced root growth without concomitant alterations in root structure architecture are consistent with AtPAP26’s function in Pi scavenging and recycling, rather than Pi signaling, during Pi starvation.

PSI gene expression is influenced by sugar levels (Karthikeyan, et al., 2006), while exogenous sucrose may exacerbate Pi starvation through increased cell proliferation signaling (Lai, et al., 2007). Thus, plant cultivation under sterile conditions in the presence of exogenous sucrose may generate phenotypes not seen under more physiologically relevant conditions. We thus examined whether soil grown atpap26
plants displayed any phenotype. The *atpap26* mutant exhibited markedly impaired development during cultivation for 21 d under -Pi conditions on a nutrient depleted soil mixture (Fig. 4.5A), as reflected by the 25% reduction in rosette dry weight relative to Col-0 control plants (Fig. 4.5B). By contrast, 21-d-old *atpap26* plants showed a small, but significant 16% increase in rosette dry weight during their cultivation on +Pi soil. One of the most obvious symptoms of plant Pi stress is anthocyanin accumulation in shoots which is believed to protect chloroplasts against photo-inhibition (Vance, et al., 2003). Since petioles of soil-cultivated -Pi *atpap26* plants were purple in color (Fig. 4.5A) their leaf anthocyanin content was quantified. Although anthocyanins accumulated in Col-0 and *atpap26* leaves during growth on the -Pi soil, the anthocyanin level of *atpap26* leaves was about 5-fold greater than that of Col-0 (Fig. 4.5C). The markedly impaired development of -Pi *atpap26* seedlings coupled with their drastically elevated shoot anthocyanin levels suggested that the mutant was suffering from more pronounced nutritional Pi-deficiency relative to Col-0. Indeed, a 35 and 50% reduction in free and total Pi concentration, respectively, was quantified in leaves of –Pi *atpap26* plants, relative to Col-0 controls (Fig. 4.5D). This adds further evidence to support an important role for AtPAP26 in the Pi metabolism of –Pi Arabidopsis. The peat-vermiculite soil mix used in these experiments contained 13 ± 0.48 μmol total Pi/g dry weight (mean ±SE, of *n* = 3 determinations). However, all this soil’s Pi was in the form of organic-Pi, as its free Pi content was undetectable. As AtPAP26 is also secreted by Arabidopsis roots during Pi stress (Fig. 4.3) (Tran and Plaxton, 2008; Tran, et al., 2010) it is possible that decreased hydrolysis of soil-localized organic-Pi reduced the total amount of Pi made available to the *atpap26* mutant during its cultivation on the Pi-deficient soil. In contrast to –Pi conditions, the *atpap26* leaves accumulated about 25% more free and total Pi.
relative to Col-0 during their growth on Pi-fertilized soil (Fig. 4.5D), indicating that AtPAP26 may also play a role in intracellular Pi homeostasis of +Pi plants.

*Vacuolar Localization of AtPAP26-mCherry*

To determine the subcellular localization of AtPAP26, its coding region was fused with the 5’ end of a *mCherry* reporter gene and transiently expressed in Arabidopsis suspension cells under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Imaging under the confocal fluorescence microscope demonstrated that *AtPAP26-mCherry* was targeted to the vacuole (Fig. 4.6A). The vacuolar localization of the AtPAP26-mCherry fusion protein was confirmed through its co-localization with sporamin NTPP-green fluorescent protein (GFP) (Fig. 4.6B and 4.6C), a well characterized lytic vacuole marker fusion protein (Jin, et al., 2001). These results are consistent with those of Carter and coworkers (2004) who detected the protein encoded by gene locus At5g34850 (*i.e.*, AtPAP26) in the vacuolar proteome of Arabidopsis leaves.

**CONCLUDING REMARKS**

Results of the present study corroborate parallel biochemical analyses of Arabidopsis suspension cells and seedlings indicating that *AtPAP26* encodes the principal vacuolar, as well as a major secreted APase isozyme upregulated by -Pi Arabidopsis (Veljanovski, et al., 2006; Tran and Plaxton, 2008; Tran, et al., 2010). However, in contrast to PSI enzymes such as AtPAP12 and AtPAP17, AtPAP26’s enhanced synthesis during Pi stress is under post-transcriptional control (Fig. 4.3). Recent proteomic studies have confirmed that transcriptional controls exert little influence on levels of certain intracellular and secreted proteins upregulated by –Pi plants relative to translational and post-translational controls that influence protein...
synthesis and degradation (i.e., see Tran et al. 2008, 2010 and refs. therein). These results highlight the need to integrate transcript profiling with parallel biochemical and proteomic analyses of plant stress responses, as the combined datasets will provide a more robust depiction of how alterations in gene expression may be linked to adaptive changes in plant metabolism. As the predominant APase isozyme upregulated by –Pi Arabidopsis, it was not unexpected that development of atpap26 seedlings was specifically impaired during their cultivation on –Pi media (Figs. 4.4 and 4.5). Although AtPAP26 was essential for efficient acclimation of Arabidopsis to Pi starvation, it was expendable in +Pi seedlings or during exposure to other types of macronutrient deprivation, or oxidative stress (Fig. 4.4). Shoots of atpap26 mutants accumulated less free and total shoot Pi relative to Col-0 during Pi stress (Fig. 4.5). This implies that AtPAP26 functions in the recycling of internal Pi-ester pools to increase the efficiency of Pi utilization in –Pi Arabidopsis. Impaired growth of –Pi Arabidopsis resulting from disrupted expression of genes encoding PSI metabolic enzymes has previously been reported, albeit in the context of altered Pi recycling from membrane phospholipids resulting from loss of the PSI phospholipase C isoform NPC5 or monogalactosyldiacylglycerol synthase 2 (Kobayashi, et al., 2009; Gaude, et al., 2008). Similarly, loss of PSI high-affinity Pi transporters disrupted the shoot development of –Pi, but not +Pi Arabidopsis (Shin, et al., 2004). However, this is the first study showing that elimination of a single member of the AtPAP family simultaneously exerts a significant inhibitory effect on non-specific APase activity and growth of –Pi Arabidopsis.

Several studies have reported that overexpression of secreted PAPs can improve plant biomass and Pi accumulation (Xiao, et al., 2006; Hur, et al., 2007; Ma, et al., 2009; Wang, et al., 2009). However, these attempts have either focused around high specificity phytases or PAPs with extracellular roles, but unclear intracellular function.
On the other hand, AtPAP26 not only has a demonstrated vacuolar localization (Fig. 4.7), but is a dual targeted enzyme that is also secreted by –Pi Arabidopsis (Fig. 4.3) (Veljanovski, et al., 2006; Tran and Plaxton, 2008; Tran, et al., 2010). Intracellular and secreted AtPAP26 glycoforms of –Pi Arabidopsis are highly active against a wide range of Pi-ester substrates over a broad pH range (Veljanovski, et al., 2006; Tran, et al., 2010), making it an ideal candidate for overexpression studies and biotechnological strategies aimed at improving crop Pi acquisition and utilization. It will thus be of interest to examine the Pi metabolism and growth characteristics of *AtPAP26* overexpressors cultivated on unfertilized soil and/or with various Pi-esters as their sole source of exogenous P.

**REFERENCES**


Lingard MJ, Gidda SK, Bingham S, Rothstein SJ, Mullen RT, Trelease RN (2008) Arabidopsis PEROXIN11c-e, FISSION1b, and DYNAMIN-RELATED PROTEIN3A


Fig. 4.1. Confirmation of T-DNA insert location and copy number in an atpap26 T-DNA insertional mutant. (A) Schematic representation of AtPAP26 gene (At5g34850); open boxes and solid lines represent exons and introns, respectively. T-DNA insertion location is indicated by atpap26 T-DNA, while arrows represent primers used for RT-PCR and genotyping. (B) Assessment of T-DNA location and homozygosity of mutants via PCR-based screening of gDNA template isolated from +Pi seedlings. PCR products were amplified from Col-0 and atpap26 gDNA in a 30 cycle PCR reaction containing the indicated primers. M denotes a 100-bp ladder for confirmation of product size. (C) Analysis of T-DNA insert number by Southern blot analysis of atpap26 gDNA probed with NPTII. Arrows indicate the base pair length of EcoRI and HindIII digested phage λ-DNA markers labeled with digoxigenin (Roche).
Fig. 4.2 AtPAP26 is the predominant intracellular APase isozyme upregulated by –Pi Arabidopsis and whose expression is nullified in atpap26 mutant seedlings. RNA and soluble proteins were isolated from seedlings cultivated in liquid media containing 0.2 mM Pi for 7-d prior to transfer into media containing 0 (-Pi) or 1.5 mM Pi (+Pi) for an additional 7-d. (A) Levels of mRNA were analyzed by 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. Control RT–PCR reactions lacking reverse transcriptase did not show any PCR product. (B) Purified native AtPAP26 from -Pi Arabidopsis suspension cells (50 ng/lane) (Veljanovski, et al., 2006) and clarified extract proteins from shoots (2 μg/lane) and roots (4 μg/lane) of the +Pi and –Pi seedlings were resolved by SDS-PAGE and electroblotted onto a poly(vinylidene difluoride) membrane. Following oxidation of antigenic glycosyl groups with sodium-m-periodate (Laine, 1988), blots were probed with a 1000-fold dilution of anti-(native AtPAP26)-immune serum and immunoreactive polypeptides detected using an alkaline-phosphatase linked secondary antibody and chromogenic detection (Veljanovski, et al., 2006). (C) APase activity of clarified extracts represent means (±SE) of duplicate assays on n = 3 biological replicates; asterisks denote values that are significantly different from Col-0 (P < 0.05). (D) Clarified extracts from –Pi shoots of Col-0 and atpap26 were resolved by non-denaturing PAGE and subjected to in-gel APase activity staining (50 μg protein/lane) or immunoblotting with anti-(native AtPAP26)-immune serum (7 μg protein/lane). O, origin; TD, tracking dye front.
Fig. 4.3. Immunological AtPAP detection, in-gel APase activity staining, and corresponding specific APase activities of proteins secreted into the media by +Pi and –Pi Col-0 and atpap26 seedlings. Growth medium containing secreted proteins was passed through a 0.45 µM syringe filter and concentrated >250-fold with Amicon Ultra-15 ultrafiltration devices (30,000 Mₐ cutoff; Millipore) at room temperature. (A,B) Concentrated secreted proteins (15 µg/lane) as well as homogeneous AtPAP12 and AtPAP26 from –Pi Arabidopsis suspension cells (Veljanovski, et al., 2006; Tran, et al., 2010) (20 ng each) were subjected to SDS-PAGE and immunoblotting as described in the legend for Fig. 4.2, except that the immunoreactive polypeptides were visualized using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescent detection (ECL Plus, GE Healthcare). (C) Concentrated secreted proteins (30 µg protein/ lane) and homogenous AtPAP26 (2 µg) were subjected to non-denaturing PAGE and in-gel APase activity staining as described in the legend for Fig. 4.2. APase activity was assayed from the concentrated liquid culture media collected from seedlings cultivated in sterile liquid media as described in the legend for Fig. 4.2. All APase activities represent the means of duplicate determinations on n = 3 biological replicates and are reproducible to within ±15% of the mean value.
Fig. 4.4. Influence of nutrient deprivation or oxidative stress on growth of atpap26 and Col-0 seedlings. (A) Shoot fresh weight of seedlings cultivated for 14-d under continuous illumination (100 μmol·m⁻²·s⁻¹ PAR) on agar-solidified 0.5x Murashige-Skoog media containing 1% sucrose and 1.5 mM or 50 μM Pi. (B) Shoot fresh weight of seedlings cultivated on agar media containing 1.5 mM Pi for 7-d, then grown for an additional 14-d on media containing 1.5 mM or 50 μM Pi, or on +Pi media lacking nitrogen (-N) or potassium (-K), or containing 1 μM paraquat (PQ). (C) Primary root length of seedlings cultivated on vertically oriented plates for 6-, 9-, or 12-d as described in panel A. All values in A - C represent means ±SE of n = 16 seedlings from four different plates; asterisks denote values that are significantly different from Col-0 (P < 0.01).
Fig. 4.5. Effect of Pi deprivation on soil-grown atpap26 and Col-0 seedlings. (A) Seedlings were cultivated for 7-d on Pi-fertilized soil, then transplanted into a Pi-deficient soil mixture and grown for an additional 14-d; fertilization occurred biweekly with 0.25x Hoagland's media containing 2 mM Pi (+Pi) or 0 mM Pi (-Pi). (B) Rosette dry weight of seedlings cultivated as in panel A. (C-E) Anthocyanin (C), and free and total Pi concentrations (D, E) of rosette leaves of seedlings cultivated as in panel A. All values are means ±SE (n = 20 for panel A, 5 for panel C, and 10 for panels D and E); asterisks denote values that are significantly different from Col-0 (P < 0.01).
Fig. 4.6. AtPAP26-mCherry localizes to lytic vacuoles of transiently-transformed Arabidopsis suspension cells. The heterotrophic suspension cells were transiently co-transformed via biolistic bombardment with AtPAP26-mCherry and sporamin NTTP-GFP. Following bombardment, cells were incubated for 8 h to allow for gene expression and protein sorting, then fixed in formaldehyde and viewed using epifluorescence microscopy. Note that the fluorescence patterns attributable to co-expressed AtPAP26-mCherry (A) and sporamin NTTP-GFP (B) co-localize in the same cell, as evidenced by yellow color in the merged image (C). Also shown (D), is the differential interference contrast (DIC) image of the cell depicted in panels A-C. This result is representative of ≥25 cells from at least 2 independent biolistic bombardments. Bar = 10 µm.
Chapter 5. General Discussion

OVERVIEW

Plants are sessile organisms, whose growth and metabolism are influenced by the availability of soil Pi. During periods of nutritional Pi deficiency, plants initiate a PSR that includes a variety of morphological, biochemical, and molecular adaptations. Although transcriptomic approaches have been taken to decipher the mechanisms involved in the plant PSR [1-11], Chapter 2 of this thesis reports the first proteomic approach taken to survey the influence of Pi deficiency on the secretome of a –Pi plant. The secretome of +Pi versus –Pi Arabidopsis cells yielded highly dissimilar protein spot patterns on 2-DE gels, which indicates major changes occur as the secretome is tailored in response to Pi deficiency. The study provides novel insights of secreted proteins involved in the Arabidopsis PSR, as proteomics may give a better understanding of the effects of Pi stress on plants than genomics. Unlike previous microarray studies of –Pi plants, where transcript levels did not always correlate with protein content, proteomics confirms the presence of the protein and provides a direct measure of the quantity present. Over 50 different spots were analyzed using both MALDI-TOF MS and LC MS/MS, which resulted in the identification of 37 unique proteins, of which 24 were Pi responsive (18 were upregulated and six downregulated in response to -Pi, respectively). Also, these proteins were found to have many diverse functions. Results from the proteomic analysis of the –Pi secretome suggest candidate proteins that may be involved in the plant PSR, and although it will be challenging, further research will be needed to assess the specific role of each protein.

The de novo synthesis and expression of APase is a universal indicator of Pi starvation in plants [12]. Although analysis of the –Pi secretome by MS did not result in
the identification of any APases, this is likely due to the low abundance and high specific activity of the secreted APases. A 6-fold increase in APase activity correlated with the presence of 55 and 60-kDa polypeptides on immunoblots probed with antibodies against AtPAP26 and AtPAP12, respectively, in the –Pi versus the +Pi secretome, therefore it was decided that the purification and identification of the corresponding APase(s) would be undertaken.

Chapter 3 detailed the purification, identification, and biochemical and molecular characterization of secreted PAPs of Arabidopsis. Two of these PAP isoforms (AtPAP26-S1 and AtPAP26-S2) arise from the same gene product and the third secreted PAP was identified as AtPAP12, whose orthologs in other plant species have induced transcript and protein levels in response to Pi stress, suggests its involvement in the PSR. The overlapping but non-identical substrate selectivities and pH-activity profiles and high specific APase activities suggest that the combined activities of AtPAP12, AtPAP26-S1, and AtPAP26-S2 help –Pi Arabidopsis to efficiently scavenge Pi from a wide range of extracellular Pi-esters over a broad pH range. Collectively, these results indicate that AtPAP12 and AtPAP26 are the predominant secreted PAPs of –Pi Arabidopsis. Also, accumulation of the same PAP isozymes purified from the CCF of –Pi suspension cells was apparent in the SCF of –Pi seedlings, therefore validating the use of suspension cell cultures to investigate the in planta response to Pi stress.

Glycosylation is an important post-translational modification known to alter a protein’s enzymatic activity, localization, and stability. Although glycoforms of plant proteins are known, very few have been functionally characterized [13]. This is the first purification and identification of two secreted PAP26 glycoforms (AtPAP26-S1 and AtPAP26-S2) and a third PAP26 glycoform also exists in the vacuole (AtPAP26-V). Differences in the substrate selectivity of the AtPAP26 glycoforms may be due to steric
hindrance that large glycan groups impose on functionally important protein domains. A key area for future research will be to establish the role that differential glycosylation plays in the dual targeting of AtPAP26 to cell vacuole and the secretome in –Pi Arabidopsis. Differential glycosylation of AtPAP26 may allow different it to interact with various binding partners. Lectins, which specifically bind carbohydrate groups, have been shown to modify the APase activity of soybean seed APases [14]. It will be interesting to see if this is the case with the AtPAP26 glycoforms, where differences in their oligosaccharide side chains may allow one secreted glycoform to interact with a certain lectin, leading to the activation of AtPAP26.

Both AtPAP12 and AtPAP26 play important roles in the Pi stress response of plants, but the control of AtPAP12 expression is believed to be at the transcriptional level, whereas AtPAP26 appears to be under post-transcriptional control. Transcript profiling of AtPAP26 indicated the constitutive expression of this gene, regardless of nutritional Pi status. Similar results where gene expression does not correspond to the levels of protein produced, have been seen with other genes not only in Arabidopsis, but in other vascular plants [15-17]. This suggests that post-transcriptional control is much more common than originally thought, and further emphasizes the integration of both microarray and proteomic studies in order to gain a complete understanding of the plant PSR.

By combining enzyme biochemistry and functional genomics, a thorough understanding of the functions of AtPAP12 and AtPAP26 orthologs in the Pi starvation response of vascular plants can be achieved. Once the APases purified from the –Pi Arabidopsis secretome were identified as AtPAP12 and AtPAP26, their corresponding gene numbers were used to interrogate the publically available genomic resources for Arabidopsis. A T-DNA insertional mutant was found for AtPAP26 from the Salk collection
(SALK_15281). The *atpap26* mutant had abolished expression of *AtPAP26* as verified by RT-PCR. Immunoblots of the secretome of the *atpap26* mutant, as well as shoot and root extracts did not detect any immunoreactive 55 kDa polypeptides using an anti-*AtPAP26* antibody, indicating the mutant lacked *AtPAP26* protein.

The *atpap26* mutants were also characterized for their biochemical and molecular phenotype. Loss of *AtPAP26* expression resulted in almost a complete loss of soluble APase activity, a 40% reduction in secreted APase activity, and a failure to upregulate intracellular APase activity in response to Pi starvation. During Pi stress, the *atpap26* mutant had impaired root and shoot development, however the *atpap26* mutant underwent normal growth during Pi sufficient conditions, oxidative stress, or exposure to nitrogen or potassium-limited growth. The knockout mutant of *AtPAP26* was also grown on Pi deficient soils containing organic-Pi as its sole source of Pi. The soil grown *atpap26* mutants exhibited decreased shoot growth (25% reduction in rosette dry weight) and a 5-fold increase in anthocyanin accumulation under Pi deficient conditions. Also, *atpap26* mutants accumulated decreased levels of both free and total Pi concentrations (35 and 50% reductions, respectively) in the shoots during Pi starvation. This implies that the growth of *atpap26* mutants was constrained both by total Pi content but also due to lower efficiency of Pi utilization. Taken together, these results suggest that *AtPAP26* is indispensable for acclimation to Pi starvation and maintenance of nominal growth rates, as *AtPAP26* plays an important role in the recycling of internal Pi-ester pools and scavenging of organic-Pi to increase the efficiency of Pi utilization in –Pi Arabidopsis.

**FUTURE DIRECTIONS**

To date, it is not clear how different AtPAP isozymes contribute to overall APase activity in the vacuole or extracellular compartment, as multiple APases have been
discovered in the vacuole, cell wall, and secretome of Arabidopsis. Both AtPAP10 [18-20] and AtPAP17 [21] have been localized to the cell wall, therefore future studies will include the identification and characterization of cell wall localized PAPs, and to determine if these PAPs are involved in the Pi stress response. Previous research on tobacco cell wall APases identified four different PAP isozymes, including one (NtPAP12) having high amino acid identity (71%) to AtPAP12 [22]. NtPAP12 was further investigated and although it shares the same subunit size and other enzymatic properties as AtPAP12, it has been suggested to play a function as a protein phosphatase in the control of cell wall biosynthesis [23, 24]. Taken together, this data indicates that PAP12 may be dual targeted to the cell wall and secretome.

Immunolocalization using fluorescently labeled AtPAP12 will help determine its subcellular localization. Plasmolysis can be used to visualize fluorescently labeled proteins in the cell wall fraction, whereas immunoblots using antibodies against the fluorescent protein can be performed to detect the fluorescent protein tag in the secretome of transformed cells. Also Brefelden A, which is commonly used to study protein transport, can be used as a control to inhibit protein secretion.

To establish the in planta function of AtPAP12 during Pi stress, we obtained an AtPAP12 T-DNA knockout line (from Dr. Thomas McKnight, Texas A& M University), which will be used for molecular, biochemical and phenotypic characterization. Preliminary data from RT-PCR experiments has shown that homozygous atpap12 mutant seedlings express no AtPAP12 transcripts nor do they secrete any 60 kDa immunoreactive AtPAP12 polypeptides. Measurement of APase activity in the secretome, root and shoot extracts of the –Pi atpap12 mutants will be performed to determine the extent of AtPAP12’s role in the Pi starvation response. Growth of atpap12 on Pi deficient and sufficient soil will be documented and any phenotype observed will
be investigated. Also, measurements of total and free Pi concentrations, as well as anthocyanin accumulation will be performed on –Pi soil grown \textit{atpap12} mutant plants. These results will be used to determine if there is a phenotype associated with the \textit{atpap12} mutant. It is hypothesized that, because AtPAP26 is the major intracellular PAP involved in acclimation of plants to Pi stress, the \textit{atpap12} mutant will be able to survive Pi deficiency by employing AtPAP26 to recycle Pi intracellularly and scavenge Pi extracellularly. As such, the \textit{atpap12} mutant plants may not exhibit a pronounced phenotype when subjected to nutritional Pi stress.

Double knockouts of \textit{AtPAP12}/\textit{AtPAP26} are currently being developed by crossing single Arabidopsis T-DNA insertional mutants and genotypically screening for a homozygous \textit{atpap12/atpap26} double knockout. The \textit{atpap12/atpap26} double knockout will be used to determine if these two family members play complementary roles in extracellular function. The characterization of \textit{atpap12/atpap26} double knockout mutant cultivated on different extracellular sources of organic-Pi (such as glycerophosphate or DNA) will help to confirm the role of these proteins in scavenging P from the rhizosphere during periods of Pi deprivation. The combined APase activities of AtPAP12 and AtPAP26 increase the number of viable substrates, allowing plants to grow on a larger variety of organic-Pi sources. Therefore, it is hypothesized that growth of the \textit{atpap12/atpap26} mutant will be severely depressed when grown on exogenous sources of organic-Pi, as these mutants would lack the ability to scavenge Pi from such molecules. Also, as we have hypothesized that AtPAP12 and AtPAP26 are the major secreted APases involved in acclimation of Arabidopsis to –Pi stress, the \textit{atpap12/atpap26} double knockout mutant will be crucial in ascertaining the importance and function of the secreted AtPAP12 and AtPAP26 in scavenging Pi from the rhizosphere during Pi starvation.
Pi applied as fertilizer is mostly unavailable to plants, thereby making Pi fertilization inefficient and dangerous to the environment. However, much of Pi applied as fertilizer may be converted into organic-Pi by soil dwelling microbes [25]. This would allow it to be degraded and scavenged by proteins such as the secreted PAPs. Thus, the over-expression of secreted AtPAP12 and AtPAP26 by roots of crop plants may be an ideal strategy for increasing the efficiency of agricultural Pi fertilizer application. Over-expression studies will be performed by characterizing the growth and Pi acquisition of the AtPAP12 and AtPAP26 over-expressors on free Pi and organic-Pi. According to our hypothesis, we would expect to see increased levels of Pi uptake in both the AtPAP12 and AtPAP26 over-expressors. The AtPAP26 over-expressor will also be used in complementation studies of atpap26 knockout mutants to see if it can rescue the mutant phenotype. The aforementioned work will greatly expand our knowledge of the function and regulation of PSI APases, and 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.

This thesis suggests that post-transcriptional control mediates changes in AtPAP26 gene expression. Characterization of post-transcriptional control of not only AtPAP26 but also PSI genes in general may provide key insights into the regulation of Pi starvation responses and provide new biotechnological targets. Previous studies of hypoxic roots of corn, heat stressed wheat, and dehydration stressed tobacco and Arabidopsis suggest post-transcriptional control of gene expression as a mechanism to regulate the appropriate abiotic stress response [26-29]. Under the applied stress, a decrease in the pool of actively translating ribosomes is accompanied by differential phosphorylation of translation initiation factors and ribosomal proteins, indicative of a
translationally incompetent state [30, 31]. Consistent with this, Pi starvation causes a broad repression of genes involved in RNA synthesis, processing, aminoacyl tRNA activation and protein synthesis while some ribosomal kinases are induced [6]. A survey of the expression and phosphorylation status of the translational machinery during Pi starvation should be performed in order to determine if the same post-transcriptional control mechanisms are in place to regulate the Pi stress response. Elucidation of mechanisms controlling \textit{AtPAP26} gene expression may shed light on general mechanisms of PSI gene regulation that are currently unknown.

\textit{AtPAP26} accumulation may also be regulated at the level of protein stability, possibly through the upregulation of a specific protease. This alternative means of regulation could be investigated using Arabidopsis seedlings that are subjected to Pi stress then resupplied with Pi. It is hypothesized that, much like tomato, the re-addition of Pi will upregulate Pi-inducible secreted proteases that are targeted to PSI extracellular APases. Secreted PSI PAPs in tomato have been shown to be selectively degraded by proteases expressed during Pi sufficiency [32]. To date, there is no information on the proteolytic machinery that mediates PSI protein turnover following Pi stress reversal due to Pi resupply. Although the role of \textit{AtPAP26} in the Pi stress response is now well documented, its means of regulation are still unknown. The involvement of secreted proteases in the turnover of secreted PAPs will be a novel discovery that may imply a means of regulation for other PSI secreted proteins, such as ribonucleases and phosphodiesterases

Our knowledge of the regulation of secreted PAPs is imperative and key to the manipulation of biotechnological strategies to enhance crop Pi acquisition. The discovery of Pi-inducible proteases that appear to target PSI APases implies that stable overexpression of extracellular PSI proteins in transgenic plants could be enhanced by
modified protease expression and/or the design of protease-resistant PSI proteins. The results of this future research will lead to a better understanding of PAP regulation and plant biochemical adaptations to Pi deprivation. This information will help industry to develop molecular tools and rational strategies for engineering crops that have a reduced requirement on expensive, polluting, and nonrenewable Pi fertilizers.

REFERENCES


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Summary

Major conclusions from Chapter 2 are:

1. +Pi and –Pi secretomes yielded dissimilar 2-DE maps. Of the more than 100 Coomassie brilliant blue stained protein spots, 50 were selected for identification by MALDI-TOF MS. This resulted in the identification of 24 –Pi responsive proteins, of which 18 were upregulated and six downregulated.

2. Secreted proteins upregulated by Pi stress perform diverse functions, ranging from Pi scavenging, cell wall modification, proteolysis, pathogen response, and ROS metabolism.

3. RT-PCR was used to assess the relationship between mRNA levels and relative amounts of selected secreted proteins. The results show poor correlation between the levels of mRNA and expressed proteins, and thus indicate that transcriptional control is but one of many factors contributing to Arabidopsis Pi starvation responses. This highlights the need for parallel biochemical and proteomics analyses of –Pi plants.

Major conclusions from Chapter 3 are:

1. This is the first work detailing the purification, identification, and characterization of secreted APases from the secretome of -Pi Arabidopsis suspension cell culture.

2. An increase in secreted APase activity corresponded with the appearance of 55 and 60 kDa immunoreactive polypeptides on immunoblots of –Pi but not +Pi secretomes of both Arabidopsis suspension cell culture filtrate and seedling culture filtrate, probed with antibodies raised against AtPAP26 and AtPAP12. Therefore, the use of Arabidopsis suspension cell cultures to investigate secreted
PAPs is validated, as the same PAPs appear to be secreted by roots of –Pi seedlings.

3. Results from RT-PCR suggest that transcriptional control exerts little influence on AtPAP26 upregulation during Pi deficiency of Arabidopsis seedlings, whereas Pi starvation inducible AtPAP12 transcripts correlate well with relative levels of secreted AtPAP12 protein.

4. All three final APase preparations exhibited a pink color in solution indicating they are bona fide PAPs. The three PAP isoforms were identified by N-terminal Edman microsequencing and peptide mass fingerprinting via MALDI-TOF MS as AtPAP12 (At2g27190, 60 kDa subunits) along with the unprecedented discovery of two differentially glycosylated AtPAP26 glycoforms (AtPAP26-S1 and AtPAP26-S2, At5g34850 gene, 55 kDa subunits).

5. All three purified PAPs have broad substrate specificities and pH activity profiles, although there are obvious kinetic differences between AtPAP26-S1 and –S2 glycoforms. It is hypothesized that differences in substrate selectivities of the three secreted PAPs allows -Pi Arabidopsis plants to efficiently scavenge Pi from extracellular Pi-esters over a broad pH range.

6. AtPAP26 is dual-targeted during Pi stress; it was previously shown to be localized to the vacuole and our current study purified AtPAP26 glycoforms from the secretome of –Pi Arabidopsis. Despite possessing identical N-terminal signal peptides, the AtPAP26 glycoforms have different substrate selectivities and the proteins are localized differently. These results suggest that glycosylation likely influences AtPAP26 substrate specificity and subcellular targeting.

7. All three PAPs purified from the –Pi Arabidopsis CCF are glycosylated, as shown by the 55 and 60 kDa immunoreactive polypeptides detected with the anti-
xylose/fucose antibody, the use of the DIG-glycan differentiation kit to recognize specific glycans in each PAP protein, the sensitivity of the AtPAPs to deglycosylation using PNGase F deglycosidase, and their ability to bind to Con-A (AtPAP12 and AtPAP26-S2).

8. This is the first work to identify both AtPAP12 and AtPAP26 as the major secreted PAPs upregulated during Pi stress. This data suggests that these secreted PAPs function to scavenge extracellular Pi.

Major conclusions from Chapter 4 are:

1. The work identified and characterized a T-DNA atpap26 mutant that lacked AtPAP26 transcripts and 55-kDa immunoreactive AtPAP26 polypeptides. This correlated with 9- and 5-fold lower shoot and root APase activity, respectively, which did not change in response to Pi starvation. Also, a 40% decrease in the secreted APase activity was observed in the atpap26 mutant during Pi starvation.

2. When subjected to Pi stress, the atpap26 mutant had impaired root and shoot development. However, the atpap26 mutant had normal growth under Pi sufficient conditions, oxidative stress, or exposure to nitrogen or potassium-limited growth.

3. atpap26 mutants grown in Pi deficient soil exhibited decreased shoot growth, as shown by the 25% reduction in rosette dry weight. The soil grown atpap26 mutants also had a 5-fold increase in anthocyanin accumulation, and decreased levels of both free and total Pi concentrations (35 and 50% reductions, respectively) in the shoots. This implies that AtPAP26 functions in the recycling of internal Pi-ester pools to increase the efficiency of Pi utilization in –Pi Arabidopsis. These results indicate that AtPAP26 is an essential contributor to Pi
stress inducible APase activity, and that it plays an important role in Pi-recycling and scavenging in –Pi Arabidopsis.
### Appendix 1

**Primers used for RT-PCR analysis of proteins found in the CCF of Arabidopsis suspension cell cultures**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Designation</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin 2</td>
<td>Actin-F</td>
<td>TCGGTGGTTCCATTCTTGCT</td>
</tr>
<tr>
<td></td>
<td>Actin-R</td>
<td>GCTTTTAAAGCCTTTTGTACCTTTGAGAG</td>
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<td>GGTGTCGATCGTTAAATATCC</td>
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<tr>
<td></td>
<td>PAP17-R</td>
<td>TCTACCAACTCTGACATCAACG</td>
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<tr>
<td>AtPAP26</td>
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<td></td>
<td>PAP26-R</td>
<td>GCTATCCCATCTCACACACACG</td>
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<td>Cyclase-F</td>
<td>TCTCACGTCAGGAATTATATCTC</td>
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<td>Cyclase-R</td>
<td>AATTTCCTGACACCAACACAC</td>
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<tr>
<td>Dehydroascorbate reductase 1</td>
<td>DHAR1-F</td>
<td>TGGCTCTGGAAATCTGTTG</td>
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<tr>
<td>Enolase</td>
<td>Enolase-F</td>
<td>GTCGGAATCATACACCTCACCACT</td>
</tr>
<tr>
<td></td>
<td>Enolase-R</td>
<td>CCTCGAACACATCGCTTATTA</td>
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<tr>
<td>Leu aminopeptidase</td>
<td>Leu amino-F</td>
<td>TGGCTCTGGAAATCTGTTG</td>
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<td></td>
<td>Leu amino-R</td>
<td>GCTATCCCATCTCACACACG</td>
</tr>
<tr>
<td>Monocopper oxidase</td>
<td>Cu oxidase-F</td>
<td>TCTCTCGCTGGTGCGTACCT</td>
</tr>
<tr>
<td></td>
<td>Cu oxidase-R</td>
<td>ACCGTCTTGCACGAGATTAC</td>
</tr>
<tr>
<td>Phosphoglycerate mutase</td>
<td>Pmutase-F</td>
<td>TCTCTCGCTGGTGAC</td>
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<tr>
<td>Polygalacturonase</td>
<td>Pmutase-R</td>
<td>GCGGTACCATGAGCTTTG</td>
</tr>
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<td>RNS1</td>
<td>RNS1-F</td>
<td>TTCACCCATTAACCATATCAATC</td>
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<td></td>
<td>RNS1-R</td>
<td>TCAGAGGAAGAGAAGCAGAGACAGAGA</td>
</tr>
<tr>
<td>Ser carboxypeptidase 50</td>
<td>Ser carboxy-F</td>
<td>CGTCTCCGTTGAGTCCTC</td>
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<tr>
<td>Ubiquitin</td>
<td>UBC-F</td>
<td>ACCGAGCTCGTTAAGAGATTG</td>
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<tr>
<td></td>
<td>UBC-R</td>
<td>CCTTTCTATTAGCATACCG</td>
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<td>GGCACCCAAAAGAAACAA</td>
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<td></td>
<td>XTR6-R</td>
<td>CCTGAGCCTGAGCTTTG</td>
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Appendix 2

Fluorescence micrographs of Arabidopsis suspension cells stained with fluorescein diacetate/propidium iodide

Cells that were cultured for 7 days in +Pi or –Pi Murashige and Skoog medium were examined under brightfield (A) and then stained with fluorescein diacetate (B; green, live cells) or propidium iodide (C; red, dead cells) prior to image capture. The proportion of live to dead cells was evaluated from the merged fluorescence images of cells (D). The images represent typical examples.
Appendix 3

Identification of protein spots from 2-DE gels of concentrated CCF proteins from Pi Arabidopsis cells using LC-MS/MS

<table>
<thead>
<tr>
<th>Spot #</th>
<th>Protein assignment</th>
<th>Gene ID</th>
<th>MOWSE score</th>
<th>Number of peptides</th>
<th>% sequence coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>Glycosyl hydrolase family 17 protein</td>
<td>At5g58090</td>
<td>417</td>
<td>14</td>
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<tr>
<td>10</td>
<td>Polygalacturonase</td>
<td>At3g16850</td>
<td>197</td>
<td>12</td>
<td>17</td>
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<tr>
<td>15</td>
<td>Enolase</td>
<td>At2g36530</td>
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<td>34</td>
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<tr>
<td>44</td>
<td>Ribonuclease 1</td>
<td>At2g02990</td>
<td>590</td>
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</table>
Appendix 4

Immunological detection of PEP carboxylase and cytosolic aldolase in clarified Arabidopsis seedling extracts

Immunological detection of PEP carboxylase (A) and cytosolic aldolase (B) in clarified Arabidopsis seedling extracts. Concentrated seedling culture filtrate (SCF) proteins from +Pi and –Pi seedlings (30 µg/lane), the corresponding seedling extract (SE) containing soluble intracellular proteins from +Pi and –Pi seedlings (5 µg/lane), and homogenous castor seed PEP carboxylase (PEPC) and cytosolic aldolase (ALD) (10 ng each) were resolved by SDS-PAGE and electroblotted onto PVDF membranes. Immunoblots were probed with affinity-purified rabbit anti-(castor seed PEPC or cytosolic aldolase)-IgG as indicated. Immunoreactive polypeptides were detected using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescent detection. Corresponding PEP carboxylase and aldolase activities appear below the respective lanes; all values represent the means ±SEM of n = 3 separate determinations and are reproducible to within ±10% of the mean value.
Appendix 5

Visible absorbance spectra of secreted APases isolated from CCF of -Pi Arabidopsis suspension cells

The spectra were obtained using a solution of 11 and 7 mg/ml of the final AtPAP12 and AtPAP26-S2 preparations, respectively.
Appendix 6

MALDI-TOF MS analyses of tryptic peptides derived from native AtPAP isoforms isolated from -Pi Arabidopsis secretome

<table>
<thead>
<tr>
<th>Identification</th>
<th>Gene</th>
<th>MASCOT Score</th>
<th>MS-FIT MOWSE Score</th>
<th>Sequence coverage</th>
<th>No. of Matching Peptides</th>
<th>Mr predicted/observed</th>
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<tbody>
<tr>
<td>AtPAP26-S1</td>
<td>At5g34850</td>
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<td>2820000</td>
<td>52</td>
<td>18</td>
<td>51/ 55</td>
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<tr>
<td>AtPAP26-S2</td>
<td>At5g34850</td>
<td>114</td>
<td>705513</td>
<td>38</td>
<td>13</td>
<td>51/ 55</td>
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<tr>
<td>AtPAP12</td>
<td>At2g27190</td>
<td>126</td>
<td>37718</td>
<td>20</td>
<td>10</td>
<td>50/ 58</td>
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</table>
Appendix 7

Glycosylation of purified AtPAP12, AtPAP26-S1 and AtPAP26-S2

Purified PAPs (0.5 µg each) were resolved via SDS-PAGE and stained with Pro-Q Emerald (A). Purified PAPs (1 µg each) were electroblotted and probed with various DIG-conjugated lectins (MAA, SNA, DSA, PNA), followed by DIG immunodetection using anti-DIG-IgG. Positive controls are indicated by +.
Appendix 8

AtPAP12 contains an intersubunit disulfide bridge

(A) Non-denaturing SDS-PAGE of purified AtPAP12, with and without the addition of 100 mM DTT to the sample buffer. (B) Deduced amino acid sequence alignment of AtPAP12 and AtPAP26 using ClustalX. Identical amino acids are indicated by asterisks. Cys residues required for the formation of disulfide bridges between AtPAP12 monomers appear in bold font (at about position 120 and 370). Putative N-linked glycosylation sites, which follow the consensus sequence N-S-S/T are underlined, whereas conserved sequence motifs containing potential metal-ligating residues are enclosed in a rectangle.
## Appendix 9

Comparison of the amino acid sequence identity of AtPAP26 and AtPAP12 with each other, as well as with AtPAP26 and AtPAP12 orthologs from other vascular plants

<table>
<thead>
<tr>
<th>Species</th>
<th>NCBI protein accession #</th>
<th>Identity to AtPAP26(^a)</th>
<th>Identity to AtPAP12(^a)</th>
<th>Predicted Size(^b)</th>
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<td>Glycine max</td>
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<td>Populus trichocarpa-2</td>
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<td>52.8</td>
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\(^a\)Determined using BLAST (http://blast.ncbi.nlm.nih.gov/)

\(^b\)Calculated using the EXPASY M\(_r\) prediction program (http://www.expasy.org/tools/pi_tool.html)
Appendix 10

Binding specificities of several plant lectins

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<th>Lectin</th>
<th>Affinity</th>
<th>Linkage</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Con A (Concanavalin A)</td>
<td>α-D-mannose</td>
<td>N-glycan</td>
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</tr>
<tr>
<td></td>
<td>α-D-glucose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GNA (Galanthus nivalis agglutinin)</td>
<td>mannose α-linked mannose</td>
<td>N-glycan</td>
<td>(2)</td>
</tr>
<tr>
<td>PNA (Peanut agglutinin)</td>
<td>galactose-β(1,3)-N-acetylgalactosamine</td>
<td>N-glycan</td>
<td>(3)</td>
</tr>
<tr>
<td>SNA (Sambucus nigra agglutinin)</td>
<td>sialic acid-α(2,6)-galactose</td>
<td>N-or O-glycan</td>
<td>(4)</td>
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<tr>
<td>DSA (Datura stramonium agglutinin)</td>
<td>galactose-β(1,4)-N-acetylglucosamine</td>
<td>N-or O-glycan</td>
<td>(3)</td>
</tr>
<tr>
<td>MAA (Maackia amurensis agglutinin)</td>
<td>sialic acid-α(2,3)-galactose</td>
<td>N-or O-glycan</td>
<td>(5)</td>
</tr>
</tbody>
</table>

REFERENCES

Appendix 11

Phosphatase activities of purified AtPAP12, AtPAP26-S1, and AtPAP26-S2 as a function of assay pH

Assays were buffered by a mixture of 25 mM sodium acetate, 25 mM MES, and 25 mM Bis-Tris-Propane and employed 5 mM PEP as a substrate. All values represent the means ±SEM of $n = 3$ separate determinations and are reproducible to within ±10% of the mean value.
Appendix 12

Effect of various substances on the activity of purified AtPAP26-S1, AtPAP26-S2, and AtPAP12

The standard APase assay was used with 5 mM PEP as the substrate but lacking added MgCl₂. Enzyme activity in the presence of each substance (5 mM) is expressed relative to the respective control determined in the absence of any additions and set at 100%. All values are the means of $n = 3$ independent determinations and are reproducible to within ±10% of the mean value.

<table>
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<th>AtPAP26-S2</th>
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<td>MnCl₂</td>
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<tr>
<td>CoCl₂</td>
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<td>Vanadate</td>
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<td>Molybdate</td>
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Appendix 13

Primers used for cloning and RT-PCR analysis of *atpap26*

<table>
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<th>Primer Designation</th>
<th>Sequence (5’→3’)</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>AtPAP26-F1</td>
<td>ATTGCTGAAAACTTAAGCGGG</td>
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</tr>
<tr>
<td>AtPAP26-R2</td>
<td>TACCAGATATCAAATGTGCAGG</td>
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<td>T-DNA-LBb1.3</td>
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<tr>
<td>AtPAP12-F</td>
<td>CACGTTCTTCGTCTCGGATT</td>
<td>Used for semi-quantitative RT-PCR analysis of AtPAP12 transcripts</td>
</tr>
<tr>
<td>AtPAP12-R</td>
<td>CCCTTGCCTTACATGAACCT</td>
<td>Used for semi-quantitative RT-PCR analysis of AtPAP12 transcripts</td>
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<tr>
<td>AtPAP26-F2</td>
<td>CGAATTCATGAATCATTTGGTGATA</td>
<td>Used for amplifying AtPAP26 cDNA clone U11049</td>
</tr>
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<td>CT-F12</td>
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<td>Complementary oligonucleotide coding for the sporamin NTPP and <em>XhoI</em> ends</td>
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Appendix 14
Comparison of root growth of Col-0 and atpap26 seedlings cultivated for 12-d under continuous illumination

Comparison of root growth of Col-0 and atpap26 seedlings cultivated for 12 days under continuous illumination (125 µmol·m⁻²·s⁻¹ PAR) on vertically oriented agar plates containing 0.5X Murashige and Skoog media, 1% (w/v) sucrose, and 1.5 mM or 50 µM Pi. (A) Plates shown are representative of four replicates, scale bar = 1 cm. (B) Root hairs within 5 mm of root tip were counted under a dissecting microscope. Values represent means ±SE of at least 20 different seedlings.

![Image showing root growth comparison](image-url)