BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF AtPAP25, A NOVEL CELL WALL-LOCALIZED PURPLE ACID PHOSPHATASE ISOZYME UPREGULATED BY PHOSPHATE-STARVED *ARABIDOPSIS THALIANA*

by

HERNAN A. DEL VECCHIO

A thesis submitted to the Department of Biology in conformity with the requirements for the degree of Master of Science

Queen’s University
Kingston, Ontario - Canada
August, 2012

Copyright © Hernan A. Del Vecchio, 2012
ABSTRACT

Upregulation of intracellular and secreted acid phosphatases (APases) is a universal response of orthophosphate-starved (-Pi) plants. APases hydrolyze Pi from a broad spectrum of phosphomonoesters at an acidic pH. Plant APases belong to a relatively large multigene family whose specific functions in Pi metabolism are poorly understood. This study focuses on the identification and characterization of cell wall (CW) localized purple acid APases (PAPs) upregulated by -Pi Arabidopsis thaliana. Three glycosylated PAP isozymes secreted into the CW of -Pi Arabidopsis suspension cells were purified and identified by peptide mass fingerprinting using mass spectrometry (MALDI-TOF MS) and N-terminal microsequencing as AtPAP12 (At2g27190; subunit size 60-kDa), AtPAP25 (At4g36350; subunit size 55-kDa) and AtPAP26 (At5g34850; subunit size 55-kDa). Both AtPAP12 and AtPAP26 were previously shown to be upregulated and secreted by –Pi Arabidopsis to scavenge Pi from extracellular organic-P. However, the novel AtPAP25 has never been suggested to be involved in the plant Pi-starvation response. Biochemical characterization of AtPAP25 revealed a monomeric 55 kDa protein. Similar to other PAPs it was purple-in-solution and insensitive to tartrate. Glycoprofiling via LC MS/MS revealed highly complex NXS/T glycosylation motifs at Asn172, Asn367 and Asn424. I hypothesize that these motifs play a role in AtPAP25 targeting and function. Kinetic characterization revealed a broad pH optimum centered at 5.6 and inhibition of activity by several common APase inhibitors. AtPAP25 exhibited broad substrate selectivity, low V_{max}, and a K_{m} (phosphoenolpyruvate) value of 0.52 mM. Immunoblot and semi-quantitative RT-PCR transcript analysis indicated that AtPAP25 is exclusively synthesized under –Pi conditions. Deduced amino acid sequences were compared using multiple sequence alignment and phylogenetic analysis. Growth of atpap25 T-DNA insertion mutant knockout seedlings was completely arrested when transferred to a soluble Pi deficient organic-P containing soil mix, pointing to a potential regulatory function of AtPAP25 during nutritional Pi stress. Overall, this research is helping to shed light on the functional importance of specific PAP isozymes in facilitating plant acclimation to nutritional Pi
deficiency. This is important because there is an urgent need to engineer Pi-efficient transgenic crops to minimize the huge input of expensive, non-renewable, and polluting Pi fertilizers in agriculture.
CO-AUTHORSHIP

With the exception of the literature review and general discussion, all sections were co-authored with Dr. Plaxton. N-terminal amino acid sequencing was performed at the Centre for Applied Genomics at The Hospital for Sick Kids (Toronto ON, Canada). Collaborators in Chapter 2 include Dr. Yi-Min She and his director Terry Cyr, who provided the mass spectrometry data for the glycosylation analysis of AtPAP25, which I analyzed. Peptide Mass fingerprinting via MALDI-TOF MS was performed by Hernan Del Vecchio with the guidance of Dr. David McLeod at the Protein Discovery Function Facility in the Biomedical and Molecular Biosciences Department at Queen’s University. All other research was conducted by Hernan Del Vecchio in the Plaxton lab.
AKNOWLEDGEMENTS

First and foremost, I would like to Thank God for his mercy, for protecting and guiding me. Without Him I would never have been here. Because You are the King and the Lord and You gave me my life. Thank You for every new day full of opportunities and blessings.

To my family! For your unconditional love. Not only for giving me the freedom to dream big, but also for helping me make those dreams come true. Mum and Dad, your support, your encouragement, your lives inspire and teach me. Thank you for your understanding and for letting me fly away when I thought it was the right time. The freedom you gave me has been the engine of all this adventure. To my Sister, thank you for your eternal support and your desire to see me grow day after day. You are the example of the so many things I want to do in life, your conviction of service and initiative to help others uninterestedly are your most precious treasures. You are the purest human I’ve ever met. My aunt, uncle and my grandfather, you are the guardians of my well-being. Thank you so much for always caring about my life and my future, and for helping me in every way. It is a fact that I would never have dared to achieve any of this without you. I love you all!

I would like to thank Dr. Bill Plaxton for the enormous opportunity he gave me when he accepted to have me as part of his lab personnel. Thanks Bill for all your guidance, for the knowledge and also the challenging but personally enriching learning experience.

My dearest friends of all!!! To all my “domestic” and my “international” friends. Very especially to Ricardo, Marisela, Christopher, Federico and David. You guys have been my inspiration when I was up, and my coziest refuge when I was down. Thanks so much for your patience, for your understanding, for your determination to make me push a bit harder every day, because you always want the best for me. David, just a few words for you now. Buddy, you are my brother and much more. Thanks for making me reconsider so many matters in life, and for teaching me how to deal with the world the way it is, but especial thanks for making me believe that the essential is invisible to the eye. The power of words, the price of values, the love for hard work, the thrill of chasing dreams, just a few things we share which have taught me to be a better person. Your success, determination and ambition are true inspirational sources for me; your humility the best example to follow. Endless thanks for your constancy, your support and the adventures together. Cheers for all the ones yet to come!!!

Past and present lab members! Thank you all guys for your guidance, your patience to teach me new stuff and for being there for all the fun we always had in and out the lab. Especial thanks to Whitney, my PAP-mate!! Thanks a lot for all the hours shared, all the goods and bads and for making my time at the lab way funnier!!! It wouldn’t have been the same with you, “Pump”. Also thanks a lot to Kyle from the Snedden Lab for his eternal patience with my million questions, and for his commitment with good humour!

Finally I would like to thank Dr. Wayne Snedden (and all his lab crew) and Dr. Kenton Ko for their helpful advice and support as committee members throughout my Masters.
TABLE OF CONTENTS

ABSTRACT........................................................................................................................................... i
CO-AUTHORSHIP ............................................................................................................................... iii
ACKNOWLEDGEMENTS ...................................................................................................................... iv
TABLE OF CONTENTS .......................................................................................................................... v
LIST OF TABLES ...................................................................................................................................... vii
LIST OF FIGURES .................................................................................................................................... viii
LIST OF ABBREVIATIONS ................................................................................................................... ix

Chapter 1 – GENERAL INTRODUCTION AND LITERATURE REVIEW .............................................. 1
  PLANT PHOSPHATE NUTRITION ........................................................................................................ 1
  PLANT PHOSPHATE STARVATION RESPONSE ............................................................................... 2
    Morphological adaptations of Pi-deprived plants ........................................................................... 3
    Mycotrophic versus non-mycotrophic plants ............................................................................... 3
    Transcriptional and post-transcriptional responses to Pi starvation ......................................... 4
    Biochemical adaptations of Pi-starved plants ............................................................................. 6
  PLANT ACID PHOSPHATASES ........................................................................................................ 9
    PURPLE ACID PHOSPHATASES ................................................................................................. 11
      Phosphate starvation inducible purple acid phosphatases ......................................................... 13
    ARABIDOPSIS THALIANA PURPLE ACID PHOSPHATASES .................................................. 14
      Phosphate starvation inducible Arabidopsis purple acid phosphatases .................................. 16
  DYNAMIC PLANT CELL WALLS ....................................................................................................... 18
  THESIS OBJECTIVES ......................................................................................................................... 21

Chapter 2 – Biochemical and molecular characterization of AtPAP25, a novel cell wall-localized purple acid phosphatase isozyme upregulated by phosphate-starved Arabidopsis thaliana ......................................................................................... 23
  INTRODUCTION ................................................................................................................................. 24
  MATERIALS AND METHODS .......................................................................................................... 27
    Plant material ................................................................................................................................. 27
    Enzyme Assays .............................................................................................................................. 28
    Acid phosphatase assay B ............................................................................................................. 28
    Kinetic analysis and protein assays ............................................................................................. 29
    Buffers used during AtPAP purification ....................................................................................... 29
    AtPAP extraction and purification from cell walls of Arabidopsis suspension cells ............... 29
    Estimation of native molecular mass by gel filtration chromatography ..................................... 31
    AtPAP25 antibody production ....................................................................................................... 31
    Protein electrophoresis and immunoblotting .............................................................................. 31
    Mass Spectrometry identification and aminoacid sequencing .................................................... 32
    Glycosylation analysis of AtPAP25 using Mass Spectrometry .................................................... 33
    RNA-isolation and semi-quantitative RT-PCR ........................................................................... 34
    atpap25 T-DNA insertional knockout mutant line isolation ..................................................... 34
    Bioinformatic analysis .................................................................................................................. 35
RESULTS AND DISCUSSION ................................................................. 35
Influence of Pi starvation on cell wall APase activity and immunoreactive PAP polypeptides of Arabidopsis suspension cell cultures ................................................................. 35
Purification, identification and physical characterization of cell wall PAPs from Pi-starved Arabidopsis suspension cells ................................................................. 36
AtPAP25 complex glycosylation motifs and its potential role in protein function and activity ........................................................................................................... 37
AtPAP25 kinetic properties ........................................................................ 38
AtPAP25 substrate specificity .................................................................... 39
AtPAP25 is only synthesized during Pi stress ............................................ 39
Bioinformatic analysis ............................................................................. 41
Identification and characterization of an atpap25 knockout mutant .......... 41
CONCLUDING REMARKS .................................................................. 42

Chapter 3 – GENERAL DISCUSSION .................................................. 55
OVERVIEW .......................................................................................... 55
FUTURE DIRECTIONS ........................................................................ 58
REFERENCES .................................................................................... 61
SUMMARY ........................................................................................... 69
APPENDIXES ..................................................................................... 70
LIST OF TABLES

Table 1. PAP purification from cell wall extracts of –Pi Arabidopsis suspension cell culture .................. 46

Table 2. Influence of various compounds on the activity of purified AtPAP25 ................................. 47

Table 3. Substrate specificity of purified AtPAP25 ........................................................................... 47
LIST OF FIGURES

Figure 1.1. A model suggesting various adaptive metabolic processes that are believed to help plants acclimate to nutritional Pi deficiency

Figure 2.1. Up-regulation of cell wall APase activity in Arabidopsis suspension cells becoming Pi deficient

Figure 2.2. Separation of cell wall-localized PAPs isoforms from CaCl2-extracted +Pi and -Pi Arabidopsis suspension cells via SO3- FractoGel cation exchange chromatography

Figure 2.3. SDS–PAGE and immunoblot analysis of purified cell wall PAPs from -Pi Arabidopsis

Figure 2.4. Immunoblot analysis of Butyl Sepharose purified cell wall extracts of Arabidopsis suspension cells

Figure 2.5. Semi-quantitative RT-PCR analysis of AtPAP25 gene expression in suspension cell cultures and shoots and roots of Arabidopsis seedlings grown under +Pi and -Pi conditions

Figure 2.6. Bioinformatic analysis of AtPAP25 with Arabidopsis paralogs and orthologs

Figure 2.7. Influence of Pi deprivation on phenotype of soil-grown Col-0 and atpap25 loss of function Arabidopsis seedlings
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>APase</td>
<td>acid phosphatase</td>
</tr>
<tr>
<td>AtPAP</td>
<td><em>Arabidopsis thaliana</em> purple acid phosphatase</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Linear Alignment Search Tool</td>
</tr>
<tr>
<td>CBB</td>
<td>Coomassie brilliant blue</td>
</tr>
<tr>
<td>CCF</td>
<td>cell culture filtrates</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CHCA</td>
<td>α-cyano-4-hidroxcinnamic acid</td>
</tr>
<tr>
<td>Col-0</td>
<td><em>Arabidopsis thaliana</em> ecotype Columbia, accession 0</td>
</tr>
<tr>
<td>Con-A</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EC</td>
<td>enzyme commission</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>gDNA</td>
<td>genomic DNA</td>
</tr>
<tr>
<td>GI</td>
<td>genome index</td>
</tr>
<tr>
<td>HMW</td>
<td>high molecular weight</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<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>LMW</td>
<td>low molecular weight</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix Assisted Laser Desorption Ionization – Time of Flight</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
</tbody>
</table>
miRNA       micro RNA
MOWSE       molecular weight search
$M_r$        molecular mass
MS           Mass Spectrometry
NADH        nicotinamide adenine dinucleotide (reduced form)
NMR          nuclear magnetic resonance
PAGE         polyacrylamide gel electrophoresis
PAP          purple acid phosphatase
PAP26-CW1-CW2 cell wall-localized isoforms of AtPAP26
PAP26-V      vacuolar isoform of AtPAP26
PEP          phosphoenolpyruvate
PEPC         phosphoenolpyruvate carboxylase
Pi           phosphate
+Pi and –Pi  Pi sufficient and Pi deficient
$p$NPP       para-nitrophenyl phosphate
PPi          pyrophosphate
PSI          phosphate starvation inducible
PSR          phosphate starvation response
PVDF         poly(vinylidene difluoride)
RNase        ribonuclease
rpm          revolutions per minute
RSA          root structure architecture
T-DNA        transferred DNA
TF           transcription factor
U            unit
$V_{\text{max}}$ maximum velocity
Chapter 1. General Introduction and Literature Review

PLANT PHOSPHATE NUTRITION

Orthophosphate (Pi) is an essential macronutrient for plant growth and development. It is contained in the structure of important biomolecules such as ATP, nucleic acids, phospholipids, and sugar-phosphates. Pi takes part in many fundamental processes in plant life, including photosynthesis, respiration, biosynthesis and signal transduction. Despite its diverse range of usage, 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 (Vance et al. 2003; Plaxton, 2004; Fang et al. 2009). Therefore crop productivity is directly limited by soil Pi availability. In order to maximize the yield of most crops, modern agricultural techniques have relied upon the application of extensive amounts of Pi-fertilizers. Although this has succeeded in increasing crop productivity, it has also posed many problems. Less than 20% of the estimated 120 million metric tons of Pi-fertilizer currently applied worldwide each year is absorbed by crops, whereas Pi-runoff from fertilized fields into nearby water bodies results in environmentally destructive processes such as aquatic eutrophication (Vance et al. 2003; Plaxton & Tran, 2011). Pi-fertilizers mostly rely on extraction from non-renewable rock–Pi reserves, derived from fossilized bone deposits. These deposits are expected to be depleted by the end of this century. Furthermore, 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 poses an interesting dilemma in the face of the world population explosion (Vance et al. 2003). 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.
Pi is one of the least accessible macronutrients in soil, as it forms insoluble calcium salts in alkaline soils, or complexes with iron and aluminum oxides in acidic soils, thus being inaccessible for root uptake (Fang et al. 2009; Abel et al. 2002; Raghothama & Karthikeyan, 2005; Yuan & Liu, 2008). Moreover, plants can only assimilate Pi in its fully oxidized state, $\text{H}_2\text{PO}_4^-$ or $\text{HPO}_4^{2-}$. The combination of these factors supports the fact that even when Pi is abundant in soil, it still remains highly unusable. On the other hand, up to 80% of soil Pi reserves exist as organic-Pi, mainly in the form of Pi-esters derived from decomposing biomatter (Vance et al. 2003; Fang et al. 2009; Yuan & Liu 2008; Xiao et al. 2006; Richardson et al. 2000). There are therefore two primary challenges plants have to face 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 (Vance et al. 2003; Fang et al. 2009; Yuan & Liu, 2008; Plaxton & Tran, 2011). Secondly, roots possess the ability to mobilize Pi from the soil organic-Pi pools via the secretion of hydrolytic enzymes that free esterified-Pi (Vance et al. 2003; Fang et al. 2009; Yuan & Liu, 2008; Duff et al. 1994; Tran et al. 2010b). 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 (Richardson et al. 2001; Wang et al. 2009).

THE PLANT PHOSPHATE STARVATION RESPONSE

All plants have evolved the ability to acclimate within species-dependent limits to extended periods of Pi deprivation. This involves a complex array of morphological, physiological and biochemical adaptations, collectively known as Pi-starvation response or PSR. This response is a consequence of the coordinated expression of hundreds of Pi-starvation inducible (PSI) genes, that reprioritize internal Pi use and maximize external Pi acquisition (Vance et al. 2003; Raghothama & Karthikeyan, 2005; Ticconi & Abel 2004; Hammond et al. 2004; Plaxton & Tran, 2011). The successful acclimation strategies displayed by
numerous species is based on the evolution of sophisticated adaptations to enhance acquisition and efficient use of Pi.

*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 (Vance et al. 2003; Fang et al. 2009; Yuan & Liu, 2008). 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 (Yuan & Liu, 2008; Ma et al. 2009). 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*

Symbiotic relationships between plant and soil-inhabiting fungi of the order Glomales can significantly enhance Pi acquisition of about 90% of plant species that form arbuscular mycorrhizae (excluding Cheonpodiaceae, Cruciferae, Cyperaceae, Junaceae, and Proteaceae families) (Bolan, 1991). This symbiosis develops in roots when the fungus colonizes the apoplast and cells of the cortex, accessing photosynthate (sucrose) supplied by the host plant. Plants can exploit a larger volume of soil, by the formation of mycorrhizal associations, as this implies an increased effective length and surface area of roots, thereby increasing the uptake of Pi and some micronutrients. However, Pi-fertilization and soil tilling has been an undesirable consequence of modern agriculture, disrupting beneficial mycorrhizal associations.

Although mostly beneficial, in situations where there is no nutritional benefit for the host plants in terms of Pi uptake, arbuscular mycorrhizal fungi can be detrimental to plants by the consumption of host carbon (Richardson et al. 2009). 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 (Murley
et al. 1998). 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

It is possible to relate many classical biochemical and physiological adaptation of –Pi plants to
transcriptional responses. A large collection of microarray data regarding plant, particularly Arabidopsis,
transcriptional changes induced by -Pi has shed light on the molecular identity and regulation of these
adaptations (Yuan & Liu, 2008; Hammond et al. 2004; Misson et al. 2005; Morcuende et al. 2007; Wu et
al. 2003; Muller et al. 2007). PSI gene expression is highly coordinated in a temporal and tissue specific
manner (Yuan & Liu, 2008). 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 (Misson
et al. 2005; Wu et al. 2003). Despite the induction of 600-1800 genes across all tissues, there is a strong
tissue-specific adaptation to –Pi with only an approximate 25% overlap between those specifically
induced in the root and shoot. As many as 700 genes appear to decrease transcript accumulation in
response to –Pi, suggesting that transcriptional repression also plays a critical role in determining the
PSR. Again tissue specificity plays a key role in this response with only a 5-10% overlap in repressed
gene expression between roots and shoots (Misson et al. 2005; Morcuende et al. 2007; Wu et al. 2003).
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 (Misson et al. 2005;
Morcuende et al. 2007; Wu et al. 2003).

Post-transcriptional mechanisms also play a critical role in the control of PSI gene expression and
activity. Proteomic profiling of –Pi Arabidopsis, and rice demonstrates that transcript abundance is not
always indicative of protein accumulation during Pi stress (Veljanovski et al. 2006; Fukuda et al. 2007; Li et al. 2008; Tran and Plaxton, 2008; Yoshimoto et al. 2007). 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 PHRI (Yuan & Liu 2008; Bari et al. 2006). During Pi sufficiency, the PHO2 gene expresses an E2 ubiquitin conjugase, which exerts its regulatory effects by decreasing the expression of critical PSI genes (Chiou et al. 2006). Upon Pi starvation, PHRI is induced and activates expression of miR399 which leads to the targeted destruction of the E2 ubiquitin conjugase, and consequent accumulation of its targets (Bari et al. 2006). 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 ribo-regulator 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 (Franco-Zorrilla et al. 2007). 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.

Post-translational modifications such as reversible phosphorylation and differential glycosylation are also important 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) (Vance et al. 2003; Fang et al. 2009; Duff et al. 1989; Plaxton & Podesta, 2006; Gregory et al. 2009; Uhde-Stone et al. 2003; Johnson et al. 1996). Interestingly, the most responsive PSI Arabidopsis transcripts include those encoding both PEP carboxylase protein kinase isozymes (AtPPCK1 and AtPPCK2) (Misson et al. 2005; Morcuende et al. 2007; Gregory et al. 2009). The simultaneous induction and in vivo phosphorylation-activation of the PEP carboxylase isozyme AtPPC1 contributes to the metabolic adaptations of -Pi Arabidopsis (Gregory et al. 2009). Similarly, corn cultivars resistant to Pi starvation upregulate phosphoprotein phosphatase type-2A catalytic subunits (Li et al. 2008), whereas alterations to in vivo protein phosphorylation patterns were documented in –Pi Brassica napus suspension cells (Carswell et al. 1997). 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 show different though well-defined biochemical adaptations that directly relate to 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). Photoinhibition protection of chloroplasts is believed to be the main purpose of the high accumulation of anthocyanins in –Pi plant shoots (Fang *et al.* 2009; Yuan & Liu, 2008; Plaxton & Tran, 2011). The upregulation of high-affinity Pi transporters of the plasma membrane is another important component of the plant PSR (Fig. 1.1; Fang *et al.* 2009; Raghothama & Karthikeyan, 2005). They actively assimilate Pi against a steep concentration gradient, where the soil Pi concentration can be 10,000-fold lower than that of root cells (Raghothama & Karthikeyan, 2005), supporting the observation that transporters are markedly induced when the external Pi concentration is low. High-affinity Pi transporters of Arabidopsis belong to the nine member PHT1 family and consist of Pi/H⁺ symporters with 12 transmembrane domains (Gonzalez *et al.* 2005). 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 (Mudge *et al.* 2003). 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 (Mudge *et al.* 2003; Shin *et al.* 2004). Consistent with this, knockout mutants of *Pht1;4* or *Pht1;1* show decreased Pi acquisition during Pi deficiency (Shin *et al.* 2004), 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 (Stefanovic *et al.* 2007).

The vacuole stores up to 95% of the cell’s total Pi during Pi sufficiency, and cytoplasmic Pi homeostasis is maintained at the expense of Pi distribution from this organelle during the early phase response to Pi starvation characterized by the dramatic decrease in intracellular Pi concentrations.
(Plaxton, 2004). The decrease in cytoplasmic Pi during long-term Pi stress is correlated with a significant reduction in levels of adenylate and other P-metabolites, 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 (Plaxton & Podesta, 2006; Plaxton & Tran, 2011). Several of these bypasses facilitate respiration or 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 deprivation, as Pi is a byproduct of their reactions (Plaxton & Podesta, 2006). The PEP carboxylase catalyzed bypass of cytosolic pyruvate kinase, coupled with the enhanced activity of malate dehydrogenase and citrate synthase also 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 of –Pi plants (Vance et al. 2003; Fang et al. 2009; Plaxton & Podesta, 2006; Uhde-Stone et al. 2003; Johnson et al. 1996). Organic acid excretion is believed to aid in chelating metal cations (e.g., Al\(^{3+}\), Ca\(^{2+}\), Fe\(^{2/3+}\)) that immobilize Pi thus increasing Pi solubility (Vance et al. 2003).
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 PSI 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. Adapted from: Plaxton & Tran (2011). Plant Physiology, 156: 1006-1015.
Plants also increase the efficiency of Pi use during Pi-deficiency 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) (Duff et al. 1994; Bariola et al. 1999; Abel et al. 2000; Fang et al. 2009; Raghoothama & Karthikeyan, 2005; Plaxton & Tran, 2011). 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) (Fang et al. 2009; Kobashayi et al. 2009; Okazaki et al. 2009). Phospholipids are a dynamic and indispensable P-reserve during Pi starvation (Tjellstrom et al. 2008). 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 (Wu et al. 2003; Misson et al. 2005; Morcuende et al. 2007; Muller et al. 2007). Knockout of phospholipase activity or the downstream synthases required for membrane lipid remodeling results in the impaired development of -Pi Arabidopsis seedlings (Kobayashi et al. 2009; Gaude et al. 2008).

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) (Fang et al. 2009; Abel et al. 2002; Plaxton & Tran, 2011; Robinson et al. 2012). Arabidopsis ribonuclease-1 (RNS1) transcript and secreted protein levels are highly induced in response to Pi stress (Bariola et al. 1999) and Arabidopsis plants cultivated on nucleic acids as their sole source of exogenous Pi grow as well as Pi-fertilized control plants (Ticconi & Abel 2004; Abel et al. 2000; Robinson et al. 2012).

PLANT ACID PHOSPHATASES

APases (E.C. 3.1.3.2) - 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 isozymes vary in their physical and kinetic properties and have been found to exist in a wide variety of tissue-
and/or cellular compartment-specific manner. They are mainly involved in the production, transport, and recycling of Pi and display non-specific substrate selectivity as a common feature (Tran et al. 2010b). Plant APases also display considerable variation in terms of native and subunit Mr, metal inhibition or activation, subcellular localization, substrate specificity and cofactor requirements, pH optima, and expression determinants (Duff et al. 1994). Pi, a product of the APase reaction, serves as a potent competitive inhibitor of many plant APases, as do the following oxanions: molybdate, vanadate, ascorbate, arsenate, tungstate, and tartrate (Duff et al. 1994). Product inhibition by Pi suggests it serves as a physiological feedback inhibitor in plant tissues.

There are over 50 different APases genes encoded by the Arabidopsis genome, including 10 vegetative storage protein type APases, four phosphatidic acid APases, and one histidine APase (Li et al. 2002). The great diversity of vascular plant APases poses a fascinating biological question in its own right. Furthermore, it becomes even more intricate as not all APases appear to function as metabolic enzymes. Such is the case of a vegetative storage protein which seems to accumulate up to almost 40% of total soluble protein in leaves of depodded soybean plants, contributes less than 1% to extractable APase activity, and a single point mutation can increase its APase activity by up to 20-fold (Leelapon et al. 2004).

As part of a universal biochemical response to plant Pi deprivation, upregulation of intracellular and secreted APase (including cell wall-localized) activity has been known for a long time (Duff et al. 1994; Tran et al. 2010). Intracellular APases have been found in virtually all plant tissues, and are a well-known marker of the cell vacuole (Duff et al. 1994; Veljanovski et al. 2006; Tran et al. 2010a). The large decrease in vacuolar Pi levels that accompanies prolonged Pi stress, relieves the APases from Pi inhibition, allowing the vacuolar APases to remobilize and recycle Pi from expendable intracellular Pi-monoesters and anhydrides. Simultaneously, there are marked reductions in cytoplasmic Pi-metabolites during extended Pi starvation (Tran et al. 2010a; Plaxton & Tran, 2011). Extracellular APases belong to a group of PSI-hydrolases secreted by -Pi plants. In the rhizosphere, they hydrolyze Pi from external organic-Pi sources (Fang et al. 2009; Ticconi & Abel, 2004; Robinson et al. 2012). Induction of
intracellular and secreted APase activity has been correlated with exclusive APase synthesis in several -Pi plants, including *Brassica nigra*, tomato, and Arabidopsis suspension cells and seedlings (Duff *et al.* 1991; Bozzo *et al.* 2002; Bozzo *et al.* 2004; Bozzo *et al.* 2006; Veljanovski *et al.* 2006; Olczak *et al.* 2003; Tran *et al.* 2010a) In order to shed light on the high genetic and functional redundancy of plant APases deeper insights and more detailed studies are needed. However, it is interesting to focus the attention on the greater and most diverse group of plant APases, purple acid phosphatases (PAPs).

**PURPLE ACID PHOSPHATASES**

PAPs owe their name to the distinctive purple or pink colour in solution. This comes from a charge transfer transition at about 560 nm from the metal-coordinating tyrosine to the metal ligand Fe(III) (Olczak *et al.* 2003). PAPs are part of a metallophosphoesterase superfamily that includes phosphoprotein phosphatases and exonucleases. Also, unlike other plant APases and as a common feature of all members of this family, PAPs are insensitive to tartrate inhibition (Olczak *et al.* 2003). All contain five blocks of conserved metal ligating residues, although the location, number and identity of the residues differ between the family groups (Li *et al.* 2002; Olczak *et al.* 2003). Members of the PAP family contain seven metal ligating residues: **DXG-GDXXY-GNH(D/E)-VXXX-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 (Li *et al.* 2002; Olczak *et al.* 2003). However, 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$^{2+}$ (Olczak *et al.* 2003; Klabunde *et al.* 1995). The availability of these metals in mammalian or plant cells implies that divalent metal cation specificity may provide a form of functional PAP specialization (Klabunde *et al.* 1995).

The structure of PAP catalytic sites and domains are also highly conserved (Olczak *et al.* 2003, Klabunde *et al.* 1995). Bacterial, mammalian and plant PAPs all contain catalytic domains that consist of two sandwiched $\beta$-$\alpha$-$\beta$-$\alpha$-$\beta$ motifs, with almost perfect alignment and order of the conserved metal ligating residues (Li *et al.* 2002; Klabunde *et al.* 1995). Nevertheless, there is variation regarding the
oligomeric structure of PAPs between mammalian and plant isozymes (Olczak et al. 2003; Klabunde et al. 1995). Mammalian PAPs exist as 35 kDa monomers consisting solely of a catalytic domain. Although mammalian-like low molecular weight PAPs exist in plants (del Pozo et al. 1999; Liang et al. 2010); 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 (Olczak et al. 2003; Klabunde et al. 1995). Although most HMW PAPs appear to exist as homodimers (Veljanovski et al. 2006; Olczak et al. 2003; Lebansky et al. 1992; Duff et al. 1989; Olczak & Watorek, 2003; Gellatly et al. 1994; Tran et al. 2010a), several HMW PAPs secreted by -Pi plant cells also appear to exist as a monomer (Bozzo et al. 2002; Tran et al. 2010a). Disulfide bridges or non-covalent interactions are responsible for the formation of dimeric HMW PAPs (Olczak et al. 2003; Lebansky et al. 1992; Olczak & Watorek 2003; Tran et al. 2010b). It has not been elucidated to date the effect caused by oligomeric status on PAP function, or even why plant PAPs are found in two different oligomeric states.

As APases, most PAPs are non-specific phosphatases that catalyze the hydrolysis of Pi from a wide range of Pi-esters (Olczak et al. 2003; Tran et al. 2010). However, there is also proof that they may be somehow involved in certain oxidative mechanisms. For example, mammalian PAPs present 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 (Klabunde et al. 1995). In a similar way some plant PAPs have been shown to have alkaline peroxidase activity that remains unaffected when treated with APase inhibitors (Bozzo et al. 2002, 2004; Veljanovski et al. 2006; del Pozo et al. 1999). In addition to this, overexpression of a soybean PAP, GmPAP3, increased tolerance to oxidative damage imposed during salinity stress (Francisca et al. 2008). Mammalian PAPs also function as phosphotyrosyl phosphatases, implying a role in signal transduction (Schenk et al. 2000). A parallel plant example is represented by a tobacco cell wall PAP that was demonstrated to be active against phosphotyrosylated peptides (Kaida et al. 2008). Phosphotyrosine activity as well as other phosphoamino acids have been shown to be substrates for a variety of PAPs from other plant sources (Veljanovski et al. 2006; Gellatly et
al. 1994; Bozzo et al. 2004). Interestingly, transgenic expression of the tobacco cell wall PAP resulted in altered cell wall composition, implying an *in vivo* regulatory role (Kaida et al. 2009).

**Phosphate starvation inducible purple acid phosphatases**

Pi starvation induces temporal and tissue specific expression of PAPs and the concomitant down-regulation of other PAPs (Misson et al. 2005; Wu et al. 2003; Li et al. 2002; Bozzo et al. 2006; Haran et al. 2000; Zimmerman et al. 2004; Tran et al. 2010b). The transcription factors PHR1, WRKY75, and ZAT6 have been implicated in the control of PSI PAP expression (Devaiah et al. 2007), while other studies have revealed PSI PAPs that are controlled by post-transcriptional mechanisms (Veljanovski et al. 2006). 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 (Veljanovski et al. 2006; Bozzo et al. 2004; Muller et al. 2004; Tran et al. 2010b). 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.

The upregulation of several intracellular and secreted APase isozymes has been well documented. Such APases have been found to be PAPs and have as well been biochemically characterized from a number of species including tomato (Bozzo et al. 2002, 2004), lupin (Olczak & Watorek 2003; Miller et al. 2001), bean (Liang et al. 2010), and Arabidopsis (Veljanovski et al. 2006; del Pozo et al. 1999; Tran et al. 2010a). White lupin secretes copious amounts of APase activity, accompanied by high levels of the corresponding transcript and protein expression when subjected to –Pi conditions (Miller et al. 2001). The glycoprotein exists as a 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 (Miller et al. 2001), even covering its promoter region. A secreted APase isozyme of yellow lupin (*Lupinus luteus*) roots is orthologous to AtPAP26 (Olczak & Watorek 2003).

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 (Bozzo et al. 2002, 2004). 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 (Bozzo et al. 2002), and is most likely localized to the cell vacuole (Bozzo et al. 2004). 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 (Bozzo et al. 2006). 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 (Hammond et al. 2004; Misson et al. 2005; Wu et al. 2003; Hammond et al. 2003).

Most recently, a PAP upregulated by –Pi bean plants (Phaseolus vulgaris) was characterized and identified as PvPAP3 by mass spectrometry (Liang et al. 2010). 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 (Liang et al. 2010).

**ARABIDOPSIS THALIANA PURPLE ACID PHOSPHATASES**

The Arabidopsis PAP (AtPAP) family is encoded by 29 genes, although only 28 appear to be actively transcribed (Li et al. 2002; Zhu et al. 2005). The high number of Arabidopsis PAPs directly correlates with the complexity and variation of plant PAP expression and regulation, and suggests that other plant
species probably possess many PAP isozymes, as it has recently been shown for rice (Zhang et al. 2010). AtPAPs classification follows the deduced amino acid sequences, and allows for the distribution into three distinct phylogenetic groups (Li et al. 2002). Groups I and II represent oligomeric HMW AtPAPs, with group I consisting of PAPs of slightly smaller monomer size than group II. Group III includes the monomeric low molecular weight mammalian-like AtPAPs. Transcript profiling of the AtPAP family revealed that seven members are predominantly expressed in flower tissue, although most AtPAPs are found throughout all tissues (Zhu et al. 2005). AtPAP transcripts expression patterns remain elusive to date, despite a small group of examples where they accumulate in response to a given stress (Li et al. 2002; Olczak & Watorek 2003; Haran et al. 2000; Tran et al. 2010a). There is also a lack of information regarding the subcellular location of most AtPAPs. Plant PAPs have been localized in mitochondria (GmPAP3) (Francisca et al. 2008), the cell vacuole (AtPAP26) (Carter et al. 2004; Veljanovski et al. 2006; Hurley et al. 2010), the cell wall (AtPAP10) (Chivasa et al. 2002; Wang et al. 2011), and the secretome (AtPAP12 and AtPAP26) (Haran et al. 2000, Kaffarnik et al. 2009; Tran et al. 2010a). 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), and PEP (Wang et al. 2009; Veljanovski et al. 2006; Olczak & Watorek 2003; Haran et al. 2000; Zhu et al. 2005; Kuang et al. 2009; Zhang et al. 2008; Tran et al. 2010a). However, many of these PAPs exhibit preferences for certain substrates. Those characterized to date have subunit sizes of various molecular weights, and differences in their structural organization are reflected in their substrate selectivity.

Although the molecular and biochemical properties of a variety of plant PAPs have been well documented, their precise physiological functions have not been resolved (Fang et al. 2009; Olczak et al. 2003). To date, AtPAP15, AtPAP23, AtPAP26 (vacuolar), AtPAP26 and AtPAP12 (secreted) and AtPAP10 (cell wall) have been functionally characterized in transgenic Arabidopsis (Zhu et al. 2005;
Kuang et al. 2009; Hurley et al. 2010; Tran et al. 2010a; Wang et al. 2011; Sun et al. 2012). AtPAP15 is the only member of the AtPAP family that has thus far been shown to possess significant phytase activity (Kuang et al. 2009; Zhang et al. 2008). 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 (Zhang et al. 2008). 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 (Kuang et al. 2009). AtPAP15 also appears to play an important role in mobilizing Pi from phytate reserves during seed or pollen germination (Kuang et al. 2009). 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), 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 (Zhu et al. 2005). 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 (Zhu et al. 2005). 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 (Zhu et al. 2005).

Phosphate 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 (del Pozo et al. 1999). AtPAP17 exists as a low molecular weight (34 kDa) monomeric PAP and is transcriptionally induced in roots and leaves of -Pi Arabidopsis (del Pozo et al. 1999). 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 (del Pozo et al. 1999). AtPAP17 transcripts also accumulate during leaf senescence and 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 (del Pozo et al. 1999). The presence of an N-terminal signal peptide suggests that AtPAP17 may be targeted to the cell wall or vacuole (del Pozo et al. 1999). 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. Upregulation of AtPAP12 and AtPAP26 during Pi deprivation has been shown in a variety of studies using different approaches. AtPAP26 represents the main intracellular PAP upregulated by –Pi Arabidopsis and is targeted to the vacuole (Veljanovski et al. 2006). The absence of expression of AtPAP26-V in an atpap26 T-DNA insertional mutant resulted in the impairment of growth under –Pi conditions, proving that it is essential for Pi stress acclimation in Arabidopsis (Hurley et al. 2010). A different AtPAP26 isoform together with an AtPAP12 was actively upregulated and secreted into the culture media for both Arabidopsis suspension cell cultures and liquid-grown seedlings in –Pi conditions (Tran et al. 2010a). Purification and biochemical characterization of both AtPAP26 isozymes and AtPAP12 showed an overlap in substrate specificity and kinetic properties pointing to a common function as Pi scavengers and recyclers during Pi-stress periods. To this respect, the secreted AtPAP26 and AtPAP12 proved to be key players in scavenging Pi from organic phosphate compounds allowing plants to better acclimate to Pi deprivation (Robinson et al. 2012). Mutant lines analysis showed an arrested growth phenotype of atpap26/atpap12 double mutant seedlings transplanted into soluble-Pi free soil,
supporting the previous observations on Pi acquisition from organic phosphate sources (Robinson et al. 2012).

**DYNAMIC PLANT CELL WALLS**

A supportive cellulosic wall surrounds plant cells, providing shape and structure, as well as an environment for extracellular interactions. The cell wall is an extremely dynamic compartment with roles in cell growth and development, signaling, defense, communication, and stress responses. The cell wall is composed mainly of carbohydrates, which account for about 90% of the dry weight and constitute a framework of cellulose microfibrils embedded in a matrix of hemicellulose and pectins (Bayer et al. 2006) while proteins contribute less than 10% to the cell wall mass. Nevertheless, this may represent several hundred different proteins that are crucial for sustaining and regulating both physical and biological functions of the plant extracellular matrix (Bayer et al. 2006).

During the last few decades it has become evident that the cell wall is a dynamic organization that is essential for cell division, enlargement, differentiation, as well as for responding to biotic and abiotic stress (Jamet et al. 2006). Although the complexity and abundance of cell wall proteins have initially been evaluated following a genetic (transcriptomic) approach, the main caveat to this still lies on the lack of information regarding alternative splicing occurrence or post-translational modifications. Undoubtedly, proteomic approaches have succeeded in doing so, as they not only give a larger vision of the proteins present in a particular organ at a given stage of development, but also deal with some of these issues. More recently there have been detailed proteomic studies of the Arabidopsis cell wall (Chivasa et al. 2002; Rose et al. 2004; Lee et al. 2004; Borderies et al., 2003; Feiz et al. 2006; Bayer et al. 2006). These new perspectives on the study of the cell wall have included the identification of cell wall protein families and diverse post-translational modifications exerted over them, answering many interrogatives but also creating many more. One specific concern is the relatively accurate estimation of the number of proteins present in the cell wall. The annotation of the Arabidopsis genome shows that approximately 17% of the genome (i.e. 5000 genes) encodes proteins with a predicted signal peptide that targets them to the
secretory pathway. If cell wall proteins recently identified in proteomic studies and the multiple forms of proteins produced by alternative splicing and post-translational modifications are taken into account, an estimate would be between 1000 and 2000 different proteins (Jamet et al. 2006).

The isolation, purification and identification of cell wall proteins constitute a great challenge from a methodological point of view. Despite the concomitant low-abundance associated problem of many proteins (Hunter et al. 2002) the structural nature of the cell wall presents difficulties itself. Cell wall proteins are embedded in an insoluble polysaccharide matrix and interact with other cell wall components, making their extraction a delicate task. The available cell wall proteomes include weakly bound proteins (Jamet et al. 2006). Weakly bound cell wall proteins are extracted from purified cell walls with salts or chelating agents, as yet, there is no efficient procedure to release cell wall proteins that are strongly bound to the extracellular matrix. All mentioned caveats are also combined with the difficulties in separation using classical proteomic techniques as 2D-PAGE where extreme hydrophobic and basic proteins from isolated cell walls are not well resolved (Bayer et al. 2006). Various technical approaches have recently been explored for extraction and identification of the Arabidopsis cell wall proteome. The use of ionic solutions to elute loosely-bound cell wall proteins from tissues and suspension cells is an important component of effective extraction procedures for both cell cultures and seedlings (Bayer et al. 2006; Feiz et al. 2006).

Application of current and new methods will allow for the successful isolation of cell wall proteins, and further research concerning the influence of Pi-nutrition on cell wall proteins. Pi starvation emerges as one of the most important nutritional deficiency syndromes affecting worldwide crop production, and thus investigation of the PSR response in cell walls is of great importance.

The complexity of the cell wall and the intricate mechanisms of protein secretion into extracellular spaces poses a complex challenge for studying cell wall proteins. Suspension cell cultures represent a simplified biological system able to offer a more accessible insight to work with cell wall proteins. Besides, investigating the biochemical properties of suspension cell cultures has proved to be a
key tool to understand diverse processes occurring in Arabidopsis biochemical adaptations to environmental stresses (Veljanovski et al. 2006; Tran et al. 2010a).

The distinction between secreted proteins that become localized to the cell wall (and interact with cell wall components) and cytosolic proteins has been well defined, with little overlap between both proteomes. This constitutes strong evidence that extracted cell wall proteins are actual proteins targeted and localized in such compartment, and do not represent artifacts of intracellular contamination during cell wall protein extraction (Chivasa et al. 2002). On the other hand, filtration and concentration of proteins being actively secreted outside of the cell into the surrounding media by suspension cell cultures constitute “cell culture filtrates” (CCF) (Tran et al. 2010a). Purification and identification of CCF proteome shows distinctive differences with those coming from cell wall extracts (Chivasa et al. 2002; Slabas et al. 2004), supporting the idea of a tight regulation throughout the secretion process, targeting different proteins to the secretome while others remain embedded into the cell wall. Interestingly, unexpected proteins have been found in Arabidopsis cell wall proteome (Slabas et al. 2004). Their presence within the cell wall has been explained through analysis of their N- or C-terminal consensus amino acid motifs which target different isozymes to the secretory pathway, although previously reported to function in other cell compartments (e.g. citrate synthase) (Slabas et al. 2004). Simultaneously, non-classical secretory pathways are also thought to exist for proteins that are secreted despite of lacking a secretory signal peptide (Slabas et al. 2004).

Cell wall-localized PAPs contribution to PSR has received little attention and has only recently been the focus of research (Wang et al. 2011). The structural as well as metabolic importance of the cell wall makes this compartment a key target for the search of PAPs’ presence and their roles during PSR. A successful approach to the production of Pi-efficient crops can only rely on a broad view of PAP activity during Pi stress, and this necessarily includes the study of diverse cell compartments, including the cell wall.
THESIS OBJECTIVES

The objective of this thesis is to test the hypothesis that PSI PAPs occur in cell wall of \(-\)Pi Arabidopsis and if so, to purify, identify and characterize them. Arabidopsis suspension cell cultures were used for initial studies since a relatively large biomass of cells cultured under a well-defined nutritional regime 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-mycotrophic species an excellent model organism for studying the plant Pi starvation response. Furthermore, it is hypothesized that acclimation of Arabidopsis to nutritional Pi deprivation involves extensive remodeling of its proteome including upregulation of specific PAP isozymes that might be playing specific roles in a diverse number of subcellular locations, including the extracellular matrix.

A biochemical approach will initially be taken to identify and characterize specific cell wall-localized APase isozymes involved in Pi scavenging and remobilization by \(-\)Pi Arabidopsis. This will involve the purification and characterization of cell wall-extracted 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 cell wall PSI Arabidopsis PAPs will not only result in pinpointing 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 intracellular and secreted plant PAPs have been described, very few have focused on the identification and characterization of specifically cell wall-localized PAPs and their physiological contributions to the global PSR. A functional genomic approach will be used to determine if the strictly cell wall-localized PAP isozymes play a role in Pi metabolism of Arabidopsis during nutritional Pi deprivation. This will include the molecular and biochemical characterization of the suspension cell cultured purified AtPAPs, and also the phenotypic analysis of a homozygous AtPAP T-DNA insertional mutant in Arabidopsis. T-DNA insertional mutants
can be obtained from a variety of T-DNA databases if corresponding genes that encode the APase isozymes are known.
Chapter 2.

Biochemical and molecular characterization of AtPAP25, a novel cell wall-localized purple acid phosphatase isozyme upregulated by phosphate-starved *Arabidopsis thaliana*
INTRODUCTION

Acid phosphatases (APases; EC 3.1.3.2) catalyze the hydrolysis of Pi from a broad range of P-monoesters and anhydrides with an acidic pH optimum (Tran et al. 2010b). 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 (Tran et al. 2010b), a common abiotic stress that frequently limits plant growth in natural ecosystems. During Pi stress, intracellular APases likely remobilize and recycle Pi from expendable P-monoesters and anhydrides. This upregulation is accompanied by a marked reduction in cytoplasmic P-metabolites during extended Pi stress (Plaxton & Tran, 2011). 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; Tran et al. 2010b). For example, the combined action of secreted ribonucleases, phosphoesterases and APases allows Pi-starved (-Pi) tomato and Arabidopsis thaliana plants to efficiently scavenge extracellular nucleic acids as their sole source of nutritional Pi (Nurnberger et al. 1990; Bosse & Kock, 1998; Abel et al. 2000; Ticconi & Abel, 2004; Robinson et al. 2012).

Purple APases (PAPs) represent the largest class of PSI, non-specific plant APases, and are characterized by their pink or purple colour in solution (owing to a bimetallic active centre), and insensitivity to L-tartrate inhibition (Tran et al. 2010b). 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; Tran et al. 2010b). Genome annotation of Arabidopsis identified 29 putative PAP genes, several of which are transcriptionally induced during Pi deprivation (del Pozo et al. 1999; Haran et al. 2000; Li et al. 2002). These and subsequent studies (Zhu et al. 2005; Veljanovski et al. 2006; Tran & Plaxton, 2008; Zhang et al. 2008; Tran et al. 2010; Wang et al. 2011)
demonstrated the complexity and variation of \textit{AtPAP1-29} expression and regulation. A major contribution to the information available on the principal AtPAP isozyme(s) that mediate intra- versus extracellular Pi scavenging by -Pi Arabidopsis has been recently made by Veljanovski \textit{et al.} (2006), Hurley \textit{et al.} (2010), Tran \textit{et al.} (2010a) and Robinson \textit{et al.} (2012) among others. In order to understand the role of PAPs in the Pi starvation response (PSR), we have taken an initial biochemical approach that involves: (1) purification and characterization of Arabidopsis PAPs upregulated during Pi stress, and (2) bioinformatic analysis of peptide mass fingerprint and/or N-terminal amino acid sequence data obtained with the purified native PAPs. Suspension cell cultures have been invaluable in this regard because a relatively large biomass of Arabidopsis cells and their surrounding liquid media containing secreted proteins can be obtained over a relatively short period (Veljanovski \textit{et al.} 2006; Tran \& Plaxton, 2008; Gregory \textit{et al.} 2009, Tran \textit{et al.} 2010a). The principal intracellular (vacuolar) PAP upregulated by -Pi Arabidopsis was fully purified and identified as AtPAP26 (Veljanovski \textit{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 \textit{et al.} 2006). Molecular and phenotypic analyses of an \textit{atpap26} T-DNA insertional loss of function mutant 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 (Hurley \textit{et al.} 2010). Tran \textit{et al.} (2010a) subsequently described the dual targeted nature of AtPAP26 isoforms present intracellularly and in the secretome of Arabidopsis. Studies of secreted PAP isozymes upregulated by –Pi Arabidopsis also showed AtPAP12 to be a significant contributor to extracellular Pi scavenging. Secreted AtPAP12 and AtPAP26 account for about 75\% of the total APase activity secreted by roots of Arabidopsis seedlings grown in –Pi conditions as well as on a variety of organic-P sources (Robinson \textit{et al.} 2012). Studies performed on an \textit{atpap12/atpap26} double knockout mutant showed that these two PAPs also make a major (~90\%) contribution to PSI shoot cell wall APase activity (Robinson \textit{et al.} 2012). It is crucial to note that 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 non-renewable Pi fertilizers for maximum agronomic benefit (Richardson et al. 2009).

The aim of the current study was to identify and characterize cell wall PAPs upregulated by –Pi Arabidopsis. The cell wall is a dynamic and biochemically complex structure that plays essential roles throughout the life of a plant including growth, development, response to environmental stresses and interactions with pathogens and symbionts. Polysaccharides comprise about 90% of the cell wall, a framework of cellulose microfibrils embedded in a matrix of hemicelluloses and pectins. Cell wall proteins represent about 10% of the cell wall mass (Rose & Lee, 2010), and comprise hundreds of different proteins with various functions including: (i) cell wall modifying enzymes, (ii) structural proteins, and (iii) “defense proteins” synthesized in response to biotic and abiotic stress (Rose & Lee, 2010). The complexity and importance of the cell wall is comprised by the outstanding number of genes that are suspected to play a role in its biogenesis, assembly and modification (Carpita et al. 2001). The estimated number of genes participating in cell wall genesis and remodeling exceeds 2000, meaning around 15% of the Arabidopsis genome is dedicated to cell wall synthesis and maintenance (Carpita et al. 2001). Previous proteomic research has shown the existence of PAPs (e.g. AtPAP10, AtPAP25 and AtPAP26) in the Arabidopsis cell wall, but unfortunately lacked any attempt to biochemically characterize them (Bayer et al. 2006; Chivasa et al. 2002; Kaida et al. 2008; Irshad et al. 2008). Our approach allowed us to specifically target the cell wall proteome for purification and identification of PAPs from –Pi in Arabidopsis suspension cell cultures. Accordingly, AtPAP12 and AtPAP26 were strongly upregulated, and a novel AtPAP25 was exclusively synthesized in cell walls of –Pi Arabidopsis. Analysis of an atpap25 T-DNA insertion mutant indicated that AtPAP25 makes an important contribution to the ability of Arabidopsis seedlings to acclimate to suboptimal Pi nutrition.
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 8 separate 2.6 L 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. Seedling growth for RNA isolation was performed as follows. Wild-type Arabidopsis seeds (Columbia ecotype, Col-0) were surface sterilized as previously described (Veljanovski *et al*. 2006). Approximately 5 mg of seeds were placed in Magenta boxes containing 50 mL of 0.5x 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 medium was replaced with fresh media containing 0 or 1.5 mM Pi, and at 14 d, seedlings were harvested by filtration in a Buchner funnel, snap-frozen in liquid N$_2$ and stored at -80 °C for later analyses.

For mutant isolation and routine plant growth, Arabidopsis (Col-0 ecotype) seeds were sown in a standard soil mixture (Sunshine Aggregate Plus Mix 1; SunGro, Vancouver, Canada) and stratified at 4 °C for 3 d. Plants were cultivated in growth chambers at 23 °C (16/8 h photoperiod at 100 µmol m$^{-2}$ s$^{-1}$ PAR) and fertilized biweekly by subirrigation with 0.25x Hoagland’s media. To assess the influence of Pi deprivation on soil-grown plants, seeds of Col-0 and an *atpap25* T-DNA insertion mutant lines were stratified at 4 °C for 2 d, then placed in 24-well tissue culture plates containing 0.5 mL of 0.5x Murashige and Skoog media, pH 5.7, 1% (w/v) sucrose, and 0.2 mM Pi, and cultivated at 24 °C under continuous illumination (100 µmol m$^{-2}$ s$^{-1}$) on an orbital shaker set at 80 rpm. After 7 d seedlings were transplanted into a 75% to 85% sphagnum peat moss/perlite soil mix lacking all nutrients (Sunshine Mix 2; SunGro). Plants were fertilized biweekly with 0.25x Hoagland’s media (pH 6.0) containing either 0 or 2 mM KH$_2$PO$_4$. Whenever Pi was eliminated, it was replaced by 2 mM KH$_2$SO$_4$ and 0.5 mM MES. For growth
on agar-solidified nutrient media, stratified seeds were placed on horizontal or vertically oriented 0.8% (w/v) agar (Micropropagation Type I Agar from Caisson Labs, Cat. #A038) plates containing 0.5x MS media and 1% (w/v) sucrose supplemented with 50 µM or 1.5 mM KH$_2$PO$_4$.

**Enzyme 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 (assay A), 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 SpectramaxPlus 250 Microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Standard APase assay conditions were 50 mM sodium acetate, pH 5.6, 5 mM PEP, 10 mM MgCl$_2$, 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.

**Phosphatase assay B**

Acid-washed microtitre plates were used for all kinetic studies. For substrates other than PEP, the Pi released by the APase reaction was quantified. Between 1 and 10 mU of APase (determined using assay A) was incubated in a 96-well microtitre plate in a final volume of 40 µL. Reaction mixtures contained 50 mM sodium acetate (pH 5.6), 10 mM MgCl$_2$ and an alternative substrate (5 mM unless otherwise stated). Assays were initiated by the addition of substrate, allowed to progress at 25 °C for 6 min and terminated by the addition of 200 µL of a solution prepared daily by mixing four parts of 10% (w/v) ascorbate with one part of 10 mM ammonium molybdate in 15 mM zinc acetate (pH 5.0). Samples were incubated for 25 min at 40 °C and the $A_{660}$ determined using the SpectromaxPlus Microplate spectrophotometer. Controls
were run to check background levels of Pi present at each substrate concentration tested. To calculate activities, a standard curve over the range 1–133 nmol of Pi was constructed for each set of assays.

**Kinetic analyses and protein assays**

All kinetic parameters are the means of at least three separate experiments and are reproducible to within ±10% of the mean value. Apparent \( K_m \) (PEP) and \( I_{50} \) (Pi) values were performed using assay A and calculated using a computer kinetics program (Brooks, 1992). Substrate selectivity studies were performed by quantifying the Pi released by the APase reaction as described above. Protein concentrations were determined using a Coomassie Blue G-250 dye binding method with bovine \( \gamma \)-globulin as the standard.

**Buffers used during AtPAP purification**

Buffer A contained 50 mM sodium acetate (pH 5.7), 1 mM DTT and 35% (saturation) \((\text{NH}_4)_2\text{SO}_4\). Buffer B contained 50 mM sodium acetate (pH 5.7), 1 mM DTT and 10% (v/v) ethylene glycol. Buffer C contained 50 mM sodium acetate (pH 4.7) and 1 mM DTT. Buffer D contained 25 mM sodium acetate (pH 5.6), 50 mM KCl, 1.5 mM MgCl\(_2\), 0.2 mM CaCl\(_2\), 0.2 mM MnCl\(_2\), 1mM DTT and 10% (v/v) glycerol. Buffer E contained 25 mM MES (pH 5.6), 30% (saturation) \((\text{NH}_4)_2\text{SO}_4\) and 1 mM DTT. Buffer F contained 25 mM MES (pH 5.6) and 1 mM DTT, whereas Buffer G contained 25 mM MES (pH 5.6), 1 mM DTT and 15% (v/v) ethylene glycol.

**AtPAP extraction and purification from cell walls of Arabidopsis suspension cells**

All procedures were performed at room temperature (23 °C) unless otherwise noted. For extraction of cell wall proteins, intact +Pi or -Pi Arabidopsis cells were treated as previously described (Lebansky et al. 1992). Cells (200 g) were incubated overnight at 4 °C in 200 mL of 5 mM HEPES-\( \text{NaOH} \) (pH 7.8) containing 0.5 M CaCl\(_2\) with constant agitation. Extracts were clarified by filtration through Whatman #1 paper, and brought to 35% (saturation) \((\text{NH}_4)_2\text{SO}_4\). APase activity was absorbed onto a column (1.6 x
12.5 cm; 25 mL of resin) of Butyl Sepharose 4 Fast Flow (GE Healthcare, Montréal, Canada) that had been pre-equilibrated in buffer A and connected to an ÄKTA FPLC system (GE Healthcare). The resin was washed with 150 mL of buffer A, and APase activity was eluted at 2 mL min$^{-1}$ of Buffer B, in two consecutive steps. The majority (~80%) of APase activity eluted during the first step gradient that consisted of 50 mL of 60% Buffer B and 40% Buffer A. A second step gradient of 30 mL of 100% Buffer B completed the elution of APase activity. Pooled APase activity fractions (around 60 mL) were concentrated to 6 mL using Amicon Ultra-15 ultrafiltration devices (30 kDa cut-off). Concentrated Butyl Sepharose APase peak activity fractions were dialyzed overnight at 4 °C against two 2 L changes of buffer C. The sample was clarified by centrifugation, and APase activity absorbed onto a Fractogel EMD SO$_3^-$ cation exchange column (1.5 x 1.7 cm, 3 mL of resin) at 1 mL min$^{-1}$, 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 (60 mL) of 0–1 M KCl in buffer C (2 mL fraction$^{-1}$). APase activity resolved as three distinct peaks (AtPAP12, AtPAP25 and AtPAP26, see below) at approximately 160, 360 and 1000 mM KCl, respectively (Fig. 2.2a). Peak activity fractions were separately pooled and concentrated as above to 2 mL. Samples were desalted into buffer D and applied at 0.5 mL min$^{-1}$ onto a column (1 x 1.3 cm, 1.3 mL of resin) of concanavalin A (Con-A) Sepharose pre-equilibrated with buffer D. AtPAP26 was resolved as two distinct peaks of APase activity using Con-A, as one peak did not bind (denoted as AtPAP26-CW1), whereas the other peak (AtPAP26-CW2) bound and was eluted using 20 mL of a linear 0–500 mM methyl-α-D-mannopyranoside gradient in buffer E (1 mL fraction$^{-1}$). Pooled peak fractions from the Con-A column were brought to 30% (saturation) (NH$_4$)$_2$SO$_4$ and applied at 0.5 mL min$^{-1}$ onto a Phenyl Superose HR 5/5 column (GE Healthcare) pre-equilibrated with buffer E. APase activity was eluted using 20 mL of a 0–100% linear gradient of buffer F (100–0% buffer E) (0.75 mL fraction$^{-1}$). Pooled peak fractions were concentrated as above to about 250 μL, divided into 25 μL aliquots, frozen in liquid N$_2$ and stored at -80 °C. APase activity was stable for at least 4 months when stored at -80 °C.
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 5.6), 100 mM KCl, 1.5 mM MgCl₂, 1 mM DTT and 10% (v/v) glycerol. Native Mr's were calculated from a plot of $K_{av}$ (partition coefficient) against log molecular mass using the following protein standards: ferritin (440 kDa), catalase (232 kDa), albumin (67 kDa), chymotrypsinogen (25 kDa), and ribonuclease (13.6 kDa).

AtPAP25 Antibody Production

Purified AtPAP25 (200 µg) was dialyzed overnight against Pi-buffered saline, filtered through a 0.2-µm membrane, and emulsified (1 mL total volume) in Titer Max Gold adjuvant (Uptima, Interchim Research, Cedex, France). After collection of preimmune serum, the APase was injected subcutaneously into a 2-kg New Zealand rabbit. A secondary injection (100 µg) was administered after 28 d. At 7 d after the final injection, blood was collected by cardiac puncture. After incubation overnight at 4 °C, the clotted cells were removed by centrifugation at 1,000g for 10 min. The antiserum was adjusted to contain 0.04% (w/v) NaN₃, frozen in liquid N₂ and stored at -80 °C.

Protein electrophoresis and immunoblotting

SDS–PAGE, subunit Mr 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 (Bozzo et al. 2002; Veljanovski et al. 2006). Anti-(recombinant AtPAP12) immune serum (anti-AtPAP12) was a kind gift of Prof. Thomas McKnight (Texas A&M University), whereas anti-(native AtPAP26-V) immune serum (anti-AtPAP26) was obtained as previously described (Veljanovski et al. 2006). Prior to probing immunoblots with anti-AtPAP25 or anti-AtPAP26, carbohydrate epitopes were abolished by a Na-m-periodate oxidation procedure as described previously (Bozzo et al. 2002). All immunoblot results were replicated at least three times with representative results shown in the figures.
Mass spectrometry identification and aminoacid sequencing

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). Excised gel bands were distained, dehydrated, reduced and alkylated. Digestion was performed using 10 ng of sequencing grade trypsin (Calbiochem, San Diego, CA, USA). Protein identification was performed by searching against the National Center for Biotechnology (NCBI) non-redundant database (NCBInr, released 22 October 2010, containing 9,251,875 protein sequences) using both MASCOT (version 2.3.0, Matrix Science, Boston, MA, USA) and MS-Fit (version 5.3; ProteinProspector, University of California, San Francisco, CA, USA) programs. These searches allowed one missed cleavage of trypsin digestion and the fixed modification of cysteine carbamidomethylation. Deamidation of asparagines 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 ppm. 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 ≥ 100 with MS-FIT).

For N-terminal amino acid sequencing, purified AtPAPs were subjected to SDS-PAGE gel and electroblotted onto a BioRad Sequi-Blot PVDF membrane using 10 mM \( \text{N-cyclohexyl-3-aminopropanesulfonic acid (CAPS)} \) and 10% (v/v) methanol buffer (pH 11). Membranes were then stained with a 0.1% (w/v) Coomassie Brilliant Blue R-250 for 1 h. Membranes were subsequently destained in 40% (v/v) methanol, containing 10% (v/v) acetic acid until blue-stained protein bands appeared on a clear background. Dried membranes were sent to the Advanced Protein Technology Centre (The Hospital for Sick Kids, Toronto, ON, Canada) for N-terminal microsequencing.
Glycosylation analysis of AtPAP25 using Mass Spectrometry

Following reduction with 10 mM DTT (56 °C, 1 h) and alkylation by 55 mM iodoacetamide (room temperature, 1 h), AtPAP25 was dialyzed against 10 mM NH₄HCO₃, and dried using SpeedVac concentrator (Savant, Fisher Scientific, Nepean ON, Canada). Sequential enzymatic digestions were then performed on 20 µg of the protein using sequencing grade bovine trypsin (100 ng, Roche Diagnostics GmbH, Indianapolis, IN) followed by proteinase K (200 ng, Promega, Madison WI, USA) digestion for 4 h. The digests were subsequently diluted by 0.2% formic acid and analyzed by online nanoAcquity ultra-performance liquid chromatography (UPLC, Waters, Milford, MA) coupled with linear ion-trap Fourier transform ion cyclotron resonance (LTQ-FIT ICR, Thermo Fisher, San Jose, CA, USA) MS as previously described (Dalziel et al., 2012). Briefly, the peptides were trapped by a RP Symmetry C18 column (180 µm i.d. x 20 mm length, 5 µm) at 5 µl min⁻¹, and subsequently separated on a C18 analytical column (100 µm i.d. x 100 mm, 1.7 µm, BEH 130) at 400 nl min⁻¹. Peptides were eluted using a mobile phase consisting of solvent A (0.1% FA) and solvent B (acetonitrile/0.1% FA). NanoUPLC separation was achieved by a linear gradient from 5 % to 45%, and then 85% of solvent B during 65 min. Automated data dependent acquisition was employed to obtain FT-MS survey scan (resolution 100,000) at mass range of m/z 300-2000, and MS/MS measurements of the top eight most intense precursor ions at multiply charged states of 2+, 3+, and 4+. Dynamic exclusion was enabled for a period of 180 s.

Protein identification was performed using an in-house Mascot Server (version 2.3.0, Matrix Science, London, UK), and the raw data were searched against the National Center for Biotechnology Information (NCBIInr) database for viridiplantae (green plants). The parameter settings allowed trypsin digestion for maximum 2 missed cleavage sites, and a fixed peptide modification of cystein carbamidomethylation. Deamidation of asparagine and glutamine, methionine oxidation, phosphorylation of serine, threonine and tyrosine were considered as variable modifications. Mass tolerances were set up to 10 ppm for the FT MS ions and 1 Da for ion trap MS/MS fragment ions. Glycosylation structures were
elucidated by manual interpretation of MS/MS spectra of glycopeptides as well as the accurate masses from high-resolution FT-MS scans.

**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% (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 of Health, Bethesda, MD, USA). RNA (5 µg) was reverse transcribed with Superscript III (Invitrogen, Carlsbad, CA, USA), and non-competitive RT-PCR performed as previously described (Gennidakis *et al.* 2007; Tran & Plaxton 2008; Gregory *et al.* 2009). Gene-specific primers used to amplify *AtPAP12, AtPAP26* and *AtPPCK* were previously described (Gregory *et al.* 2009, Tran *et al.* 2010a). Transcripts for *AtPAP25* were amplified using the primers 5’-TTACGCAGATGACCATTCCAA-3’ (forward, $T_m$: 58.4 °C) and 5’-AGGAATGGACGTGACCAGAC- 3’ (reverse, $T_m$: 62.4 °C). 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.

**atpap25 knockout mutant isolation and analysis**

*atpap25* T-DNA insertion mutants seeds (GK-004D10-014797) were obtained from The European Arabidopsis Stock Centre (NASC). Initial screening was performed by growing the plants on 0.8% (w/v) +Pi agar plates containing 0.5x Murashige and Skoog media, 1% (w/v) sucrose, and 5 µg mL$^{-1}$ sulfadiazine and selecting for those with resistance to the antibiotic. Resistant plants were subjected to a second round of selection with sulfadiazine, and the selected ones were transferred to soil for self-pollination and propagation. gDNA was extracted from leaves and PCR-screened for homozygous mutants using T-DNA left-border and gene specific primers (5’-CCCTAAGCATGACCCTAGCC-3’ forward, $T_m$: 62 °C; 5’-CCTTTGGACGTATGAACCTAGCC-3’ reverse, $T_m$: 63.1 °C; 5’-
GAAGGTGGCTCCTACAAATGC-3’ T-DNA forward, Tm: 61 °C) (Appendix 6). All PCR products were sequenced for verification (Centre for Applied Genomics, The Hospital for Sick Kids, Toronto, ON, Canada).

Bioinformatic analysis

Similarity searches were performed using the BLAST program (http://www.ncbi.nlm.nih.gov). Multiple sequence alignment of AtPAP12, AtPAP25 and AtPAP26 was performed with ClustalX (version 1.81). Phylogenetic tree construction and analysis was done using Geneious (Version 5.6.4; http://www.geneious.com). Signal peptide prediction was performed using SignalP software (http://www.cbs.dtu.dk/services/SignalP).

RESULTS AND DISCUSSION

Influence of Pi starvation on cell wall APase activity and immunoreactive PAP polypeptides of Arabidopsis suspension cell cultures

When Arabidopsis suspension cells were subcultured in the presence of 5 mM Pi and harvested 7 d later, the cell culture filtrates (CCF) contained 1.8 ± 0.1 mM Pi (n = 3 separate flasks, ±SEM), indicating that the cells were still +Pi. However, extracellular Pi was undetectable when parallel –Pi cells were cultivated for 7 d in liquid media containing 0 mM Pi (Fig. 2.1a). This was correlated with a significant increase in cell wall APase specific activity which was up to 4-fold greater in cell wall extracts from –Pi cells relative to +Pi controls. At day 6, -Pi cells were resupplied with 5 mM Pi and cell wall APase activity rapidly decreased to +Pi levels over the next several days (Fig. 2.1b). Parallel immunoblot analyses of cell wall extracts with anti-AtPAP12 were of poor quality owing to the elevated salt concentration in the samples. In order to eliminate this problem while simultaneously enriching the extracts in APase activity, Butyl Sepharose FPLC was performed. The resulting samples showed an increased specific APase activity in
-Pi cells (5.11 ± 0.09 U mg⁻¹) with respect to +Pi cells (1.26 ± 0.03 U mg⁻¹). This was correlated with the upregulation of 60 and 55 kDa immunoreactive polypeptides on immunoblots of -Pi cell wall samples probed with anti-AtPAP12, which respectively comigrated with native AtPAP12 and AtPAP26 purified from the CCF of the –Pi suspension cells (Fig. 2.1c) (Tran et al. 2010a).

Earlier work demonstrated that secreted CCF proteins from the +Pi or –Pi Arabidopsis suspension cells were free of contaminating cytoplasmic marker enzymes (Tran & Plaxton, 2008). Similarly, immunoreactive cytosolic PEP carboxylase and aldolase polypeptides were absent on immunoblots of the Butyl Sepharose enriched cell wall extracts, but present on immunoblots of corresponding clarified homogenates of the +Pi or –Pi cells that had previously been treated with CaCl₂ for extracting cell wall proteins. This was corroborated by presence of significant PEP carboxylase and aldolase activities in the clarified cell homogenates, but not in the corresponding cell wall extracts (Appendix 1).

**Purification, identification and physical characterization of cell wall PAPs from Pi-starved Arabidopsis suspension cells**

Three peaks of APase activity were resolved during SO₃⁻-cation exchange FPLC of the concentrated Butyl Sepharose fraction obtained from cell wall extracts of –Pi cells (Fig. 2.2a), and were subsequently identified as AtPAP12, AtPAP25 and AtPAP26 (see below). As was observed during purification of secreted AtPAP26 from CCF of the –Pi cells (Tran et al. 2010a) pooled AtPAP26 activity fractions from the SO₃⁻-cation exchange column resolved as two glycoforms (AtPAP26-CW1 and AtPAP26-CW2) during Con-A chromatography. AtPAP26-CW1 failed to bind to the Con-A column, whereas AtPAP26-CW2 (and AtPAP12 and AtPAP25) was bound and eluted following the application of the methyl-α-D-mannopyranoside gradient. The proportion of total AtPAP26-CW1 to AtPAP26-CW2 activities that eluted from the Con-A column was about 2:1. The possibility that the elution of AtPAP26-CW1 in the Con-A flow-through fractions was caused by 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-CW1 in the resultant unbound fractions. As shown in Table 1 AtPAP12, AtPAP25
and AtPAP26-CW1 and AtPAP26-CW2 were purified to final PEP hydrolyzing specific activities of 66, 5.5, 252 and 108 U mg\(^{-1}\), respectively. Visual inspection of concentrated final preparations of AtPAP12, AtPAP26-CW1, and AtPAP26-CW2 showed a pink color in solution, while AtPAP25 was purple. All four purified PAPs were subjected to peptide mass fingerprinting via MALDI-TOF MS. Comparison with databank sequences demonstrated that they corresponded to AtPAP12 (At2g27190), AtPAP25 (At4g36350) and two glycoforms of AtPAP26 (At5g34850) (Appendix 2).

When the final preparations were denatured and subjected to SDS-PAGE, protein-staining polypeptides of 60 (AtPAP12) and 55 kDa (AtPAP25 and AtPAP26-CW1/-CW2) were observed (Fig. 2.3a), which cross-reacted with rabbit antibodies raised against recombinant AtPAP12. By contrast, anti-AtPAP25 and anti-AtPAP26 were relatively monospecific for their respective antigens (Fig. 2.3b). Protein-stained SDS-PAGE gels of the purified final preparations also revealed that AtPAP12 and AtPAP26-CW2 were highly purified whereas AtPAP25 and AtPAP26-CW1 were purified to near homogeneity. The native molecular mass of AtPAP12 was estimated to be 120 ± 5 kDa, whereas that of AtPAP25, AtPAP26-CW1, or AtPAP26-CW2 was approximately 55 ± 5 kDa (means ± SEM, \(n = 3\) determinations) as determined by analytical gel filtration FPLC. These results suggest that AtPAP12 exists as a homodimer, while AtPAP25 and AtPAP26-CW1/-CW2 are monomers. This corroborates results found previously for AtPAP12 and AtPAP26 found in other cell compartments (Veljanovski et al. 2006; Tran et al. 2010a).

**AtPAP25 complex glycosylation motifs and its potential role in protein function and activity**

Plant N-linked glycans are usually composed of two structures of high-mannose type, and complex-type glycans arising from the trimming of mannose residues. The N-linked glycopeptides of purified AtPAP25 were characterized by high-resolution UPLC separation and LTQ-FT MS/MS analysis of the glycosylated peptides generated by either specific trypsin digestion or nonspecific proteinase K cleavage. Three putative N-linked glycosylation sites of AtPAP25 (Asn172, Asn367 and Asn424 which each possess the
consensus NXS/T glycosylation motifs), were each determined to contain two compositions of xylomannosides GlcNAc(Fuc)GlcNAc(Xyl)Man$_3$ and GlcNAc(Fuc)GlcNAc(Xyl)Man$_3$GlcNAc (Appendix 5), but no high-mannose-type glycan. The glycosylation site of Asn424 was further modified by the addition of terminal galactose residue to form an extended side chain of GlcNAc(Fuc)GlcNAc(Xyl)Man$_3$GlcNAcGal. The relative abundance of the three glycan chains at this site is shown in Appendix 5. MS/MS results also confirmed the absence of any phosphopeptides in AtPAP25, which contradicts a phosphoproteomic study of phosphotyrosylated proteins of Arabidopsis cell cultures which reported AtPAP25 to be phosphorylated at Tyr423 (Sugiyama et al. 2008) (Appendix 8).

**AtPAP25 Kinetic properties**

AtPAP25 exhibited a broad pH activity profile with a maximum at 5.6 (Appendix 3). All subsequent studies were performed at pH 5.6. Hyperbolic PEP saturation kinetics were observed with a $K_m$ (PEP) value of $0.52 \pm 0.03$ mM (mean $\pm$ SEM of $n = 4$ determinations). This value is somewhat lower than the $K_m$ (PEP) values reported for intracellular AtPAP26-V (0.8 mM, Veljanovski et al. 2006) or secreted tomato PAPs (1.4 and 2.1 mM, Bozzo et al. 2002), although not as low as values reported for secreted AtPAP26-S1 and AtPAP26-S2 (0.14 and 0.18 mM respectively) and AtPAP12 (0.06 mM) purified from CCF of the –Pi cells (Tran et al. 2010a). Interestingly, AtPAP25 showed a low specific activity ($V_{max}$) (5.5 U mg$^{-1}$) with PEP. It is possible that *in vitro* assay conditions (e.g. oligomerization state, cofactors, protein-protein interactions) were responsible for the low specific activity, however this result also suggests this AtPAP might be involved in a regulatory pathway rather than a Pi scavenging one (see below).

AtPAP25 activity was unaffected by the addition of 5 mM of Mg$^{2+}$, Co$^{2+}$, Mn$^{2+}$, or EDTA; or 10 mM glycolate, glutamate, tartrate, aspartate, asparagine, succinate, or isocitrate. Similar to other PAPs (Duff et al. 1989; Bozzo et al. 2002, 2004; Veljanovski et al. 2006; Tran et al. 2010a) AtPAP25 was potently inhibited by molybdate, Zn$^{2+}$, Fe$^{2+}$ and Cu$^{2+}$ (Table 2). As with all plant PAPs, AtPAP25 was not
inhibited by 5 mM L-tartrate. However, AtPAP25 exhibited potent inhibition by Pi as reflected by its extremely low I$_{50}$-Pi value of 50 µM (determined with approx. K$_{m}$ [PEP] of 0.5 mM).

**AtPAP25 Substrate specificity**

A wide range of phosphorylated compounds were assayed as potential substrates for AtPAP25 (Table 3). This PAP showed a broad specificity cleaving Pi from phenyl-P, 5-Br-4-Cl-3-indolyl phosphate (BCIP), p-NPP, and β-naphthyl-P. By contrast, AtPAP25 failed to use 5 mg mL$^{-1}$ of the egg yolk storage phosphoprotein phosvitin or 5 mM phytate as substrates. The latter result demonstrates that, unlike AtPAP15 (Zhang et al. 2008; Kuang et al. 2009), AtPAP25 is not a phytase. These results are comparable to those observed in intracellular as well as secreted AtPAPs (Veljanovski et al. 2006; Tran et al. 2010a).

Interestingly, AtPAP25 exhibited modest activity with O-phospho-L-threonine (P-Thr) and O-phospho-L-tyrosine (P-Tyr), but showed no activity on O-phospho-L-serine (P-Ser) (Table 3). Kaida et al. (2008, 2010) have hypothesized that a tobacco cell wall PAP orthologous to AtPAP10 functions as a phosphoprotein phosphatase to participate in the control of cellulose and callose synthesis. The apparent exclusive synthesis of AtPAP25 under –Pi conditions as well as its specific targeting to the cell wall might suggest a potentially similar role.

**AtPAP25 is only synthesized during Pi stress**

Immunoblot analysis of Butyl Sepharose enriched +Pi and –Pi cell wall extracts using the anti-AtPAP25 antibodies indicated that unlike AtPAP12 and AtPAP26 (Fig. 2.1c), AtPAP25 was only expressed under –Pi conditions (Fig. 2.4).

Comparison of APase activity elution profiles during SO$_3^-$-cation exchange FPLC of cell wall extracts from the –Pi versus +Pi cells also indicates that AtPAP12 and AtPAP26 were markedly upregulated whereas AtPAP25 was de novo synthesized in response to Pi deprivation (Fig. 2.2a,b). AtPAP12 and AtPAP26 have previously been shown to be markedly upregulated in suspension cell and
seedling root secretome of –Pi Arabidopsis (Tran et al. 2010a). Furthermore, analysis of an atpap12/atpap26 double mutant demonstrated that they (i) play a pivotal role in extracellular Pi scavenging from exogenous organic Pi compounds and (ii) are also markedly upregulated in cell walls of –Pi Arabidopsis shoots (Robinson et al. 2012). By contrast, although AtPAP25 was previously identified in two independent studies of cell wall proteome from Arabidopsis suspension cells (Chivasa et al. 2002; Borderies et al. 2003), the current results are the first to document its apparent involvement with the Arabidopsis Pi-starvation response. It is likely that Chivasa et al. (2002) and Borderies et al. (2003) were unknowingly studying –Pi Arabidopsis suspension cells as they were cultivating them for one week in regular Murashige and Skoog media (contains 1.25 mM Pi) which has been shown to be insufficient for maintaining Arabidopsis suspension cells fully +Pi during one week in batch culture (Veljanovski et al. 2006; Tran & Plaxton, 2008). This is why our +Pi suspension cells are cultivated in media that initially contains 5 mM Pi.

In order to evaluate the possible regulation of AtPAP25 at the transcriptional level, semi-quantitative RT-PCR was performed with +Pi and –Pi Arabidopsis seedlings and suspension cell cultures (Fig. 2.5). Results showed AtPAP25 transcripts were only detected in –Pi Arabidopsis, which supports previous trends of AtPAP25 protein expression (Figs. 2.2 and 2.4). A wide variety of transcript profiling studies have examined Arabidopsis genes expression patterns under Pi stress or other conditions, (Raghothama & Karthikeyan, 2005; Uhde-Stone et al. 2003; Wu et al. 2003; Hammond et al. 2003; Misson et al. 2005; Morcuende et al. 2007; Muller et al. 2007; Tian et al. 2007; Wasaki et al. 2006; Hernandez et al. 2007; Hammond et al. 2004; Zhu et al. 2005). Flower specific-expression of AtPAP25 has been demonstrated in +Pi Arabidopsis, with AtPAP25 transcripts being particularly abundant in stamens (Wellmer et al. 2004; Zhu et al. 2005). Although alterations in transcript abundance do not necessarily translate into a correlated change in protein amount or enzyme activity (or vice versa), these results indicate possible expression of AtPAP25 in Arabidopsis flowers.
Bioinformatic analysis

The AtPAP family is encoded by 29 genes that have been classified into three distinct groups by clustering analysis of their deduced amino acid sequences (Li et al. 2002). Group Ia-1 is comprised of AtPAP5, AtPAP6, AtPAP11, AtPAP19 and AtPAP25. All of the deduced sequences contain conserved domains involved in coordinating the bimetal nuclear centre characteristic of the PAP active site. AtPAP25 shares a 60% and a 55% sequence identity with AtPAP12 and AtPAP26 respectively, whereas its identity with AtPAP25 orthologs from other plants range from 56 to 68% (Appendix 4). The high degree of sequence identity across dicots and monocots implies an important and conserved function for AtPAP25 orthologs in vascular plants.

In silico analysis of the deduced AtPAP25 sequence predicted that the mature protein has molecular mass of 53 kDa (Appendix 2). The 2 kDa discrepancy with the respective subunit Mr of the purified native AtPAP25 as estimated by SDS–PAGE (Fig. 2.3a) can be explained by the addition of glycan groups. Signal P predicted that the N-terminus of the deduced AtPAP25 polypeptide contain a 21-amino acid signal peptide (Fig. 2.6a). By contrast, the N-terminal sequence of the mature AtPAP25 polypeptide begins at position 30 implying actual signal peptide lengths of 29 amino acids (Fig. 2.6a). Interestingly, the signal peptides of cell wall targeted AtPAP12, AtPAP25, and AtPAP26-CW1, as well as AtPAP26-V, along with AtPAP12 and AtPAP26-S1/-S2 upregulated in the –Pi Arabidopsis secretome (Veljanovski et al. 2006; Tran et al. 2010a) are all processed at exactly the same site, beginning after an invariant R residue (indicated with a box in Fig. 2.6a). This implies that a common signal peptidase isozyme may cleave the transit peptides of the various secreted and/or vacuolar AtPAP isozymes during their subcellular targeting in –Pi Arabidopsis.

Identification and characterization of an atpap25 mutant

41
To further assess the potential role that AtPAP25 plays in planta, an atpap25 T-DNA insertion line was obtained and screened as described in Materials and Methods. Confirmation of AtPAP25 gene disruption in the atpap25 mutant was confirmed by PCR of gDNA using T-DNA insertion left border and AtPAP25-gene specific primers (Appendix 6).

Growth of atpap25 in an organic-Pi containing soil that lacks any soluble Pi (Hurley et al. 2010; Robinson et al. 2012) showed a strong phenotype compared to control, Col-0 plants (Fig. 2.7). The atpap25 seedlings suffered a complete arrest in growth, and showed a dark purple color indicating their extreme inability to cope with Pi stress. By contrast, +Pi atpap25 plants showed no obvious phenotype relative to +Pi Col-0 plants (Fig. 2.7). AtPAP25 immunoreactive polypeptides were not observed on anti-AtPAP25 immunoblots of cell wall or total protein extracts of shoots or roots of –Pi or +Pi Col-0 seedlings. Immunoblots of +Pi and –Pi 21-d-old Col-0 Arabidopsis flower extracts also showed no immunoreactive 55-kDa AtPAP25 polypeptides. Additionally, root surface APase staining using BCIP and β-naphthyl-P did not show any obvious difference between atpap25 and Col-0 14-d-old seedlings grown in –Pi agar solidified media (Appendix 7). The recurrent inability to document AtPAP25 protein expression in planta, pointed to the hypothesis of AtPAP25 having a signaling role such as a phosphoprotein phosphatase. Experiments conducted to date are insufficient to corroborate such a hypothesis, however future research is intended to tackle this important point, allowing to determine AtPAP25 role in vivo. Furthermore, immunoblots of atpap12/26 cell wall preparations of –Pi seedlings lacked detectable AtPAP25 polypeptides using chromogenic or chemiluminescent detection.

**CONCLUDING REMARKS**

To the best of our knowledge, this is the first report detailing the purification, identification and biochemical and molecular characterization of cell wall-localized PAPs of -Pi Arabidopsis. Results indicate that AtPAP12, AtPAP25, and AtPAP26 are the predominant PAP isozymes localized in the extracellular matrix of -Pi Arabidopsis suspension cells. Identification and characterization of the novel
AtPAP25 was the main focus of the present study. Two independent proteomic studies identified AtPAP25 as part of the cell wall proteome of Arabidopsis suspension cell cultures (Chivasa et al. 2002; Borderies et al. 2003), and it was also reported to be phosphorylated at Tyr423 following the Arabidopsis phosphoproteome study by Sugiyama et al. (2008). AtPAP25 transcripts appear to only be synthesized during Pi starvation, suggesting AtPAP25 possesses a different regulation of expression in suspension cell cultures and seedlings (Fig. 2.5), unlike other extracellular PSI PAPs such as AtPAP26 (Veljanovski et al. 2006; Tran et al. 2010a).

The existence of AtPAP25 as a cell wall PAP of –Pi Arabidopsis suspension cells may imply this enzyme acts as a Pi scavenger/remobilizer due to its highly specific localization and its apparent unique –Pi-exclusive synthesis nature. Moreover, preliminary studies conducted on an atpap25 T-DNA insertion mutant established that AtPAP25 is required for efficient acclimation of Arabidopsis to nutritional Pi deprivation (Fig. 2.7). However, in spite of every attempt to find AtPAP25 protein expression in planta, results were not successful. Root surface APase activity staining of –Pi seedlings using BCIP or β-naphthyl-P as substrates also showed no differences between Col-0 and atpap25 plants. Additionally, the purified AtPAP25 showed a very low APase specific activity (V_max) (Table 1) and potent inhibition by Pi. Taken together, these results suggest that AtPAP25 may have a signaling role and not an extracellular Pi scavenging/remobilizing function as has been established for other secreted PAPs of –Pi Arabidopsis such as AtPAP10, AtPAP12 and AtPAP26 (Robinson et al. 2012; Wang et al. 2011).

MS/MS analysis of purified AtPAP25 revealed a complex array of glycan motifs, present in three Asn residues possessing the conserved NXS/T glycosylation motif sequence. 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 (Rademacher et al. 1988; Varki, 1993; Bond et al. 2011). AtPAP25’s restricted cell wall localization may be the result of glycosylation-driven targeting, and its proposed signaling role might
also rely on this, adding more complexity to its \textit{in vivo} function. A deeper understanding of the effects that glycosylation exerts on AtPAP25 such as aiding in binding with partners or potential substrates may help explain the impaired development of the –Pi \textit{atpap25} knockout seedlings.

Finally, it is premature to outlay a metabolic mechanism for AtPAP25 action, especially as it might involve highly complex and intricate pathways and many other participating enzymes. However, it is possible to speculate that its signaling role might be directly or indirectly associated to cell wall-component synthesis or remodeling leading to impaired cell wall structure. An altered arrangement of cell wall scaffold might in turn be detrimental for appropriate secretion and/or function of dedicated Pi scavenging/recycling enzymes thus supporting the observations on –Pi soil-grown mutant lines (Fig. 7). Additional experiments using \textit{atpap25} knockout and overexpression lines will help determine the exact role exerted by this PAP. They will also contribute to a better mutant characterization analysis by combining the work done by Robinson \textit{et al.} (2012) with the \textit{atpap12/atpap26} double knockout line, creating a triple \textit{atpap12/atpap25/atpap26} knockout line. Complete physiological assessment will be a crucial asset in order to determine the final contribution of all three PAPs to the PSR of Arabidopsis cultivated under a variety of different conditions, including exogenous Pi sources. Overall, these studies are relevant to current efforts to engineer Pi-efficient transgenic plants, as there is an ultimate need to minimize the input of expensive, unsustainable and polluting Pi fertilizers in crop production.
### Table 1. Purification of cell wall PAPs from 400 g of 7-d-old –Pi Arabidopsis suspension cells

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (mL)</th>
<th>Total Activity (Units)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (Units mg(^{-1}))</th>
<th>Purification (-fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarified CaCl(_2) Extract</td>
<td>400</td>
<td>1600</td>
<td>2145</td>
<td>0.75</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Butyl Sepharose</td>
<td>11</td>
<td>820</td>
<td>164</td>
<td>5</td>
<td>7</td>
<td>51</td>
</tr>
<tr>
<td>SO(_3)^– - Fracto Gel FPLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pool 2 (AtPAP12)</td>
<td>1.8(^a)</td>
<td>40</td>
<td>11</td>
<td>3.6</td>
<td>5</td>
<td>2.5</td>
</tr>
<tr>
<td>Pool 3 (AtPAP25)</td>
<td>1.8(^a)</td>
<td>261</td>
<td>18</td>
<td>14.5</td>
<td>19</td>
<td>16.3</td>
</tr>
<tr>
<td>Pool 1 (AtPAP26)</td>
<td>1.8(^a)</td>
<td>520</td>
<td>28.1</td>
<td>18.6</td>
<td>25</td>
<td>32.5</td>
</tr>
<tr>
<td>Con-A Sepharose FPLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtPAP12</td>
<td>7(^a)</td>
<td>84</td>
<td>6.7</td>
<td>12.5</td>
<td>17</td>
<td>5.25</td>
</tr>
<tr>
<td>AtPAP25</td>
<td>9(^a)</td>
<td>17.2</td>
<td>3</td>
<td>5.7</td>
<td>7.6</td>
<td>1.1</td>
</tr>
<tr>
<td>AtPAP26-CW1</td>
<td>13(^a)</td>
<td>168</td>
<td>7.8</td>
<td>22</td>
<td>30</td>
<td>10.5</td>
</tr>
<tr>
<td>AtPAP26-CW2</td>
<td>8(^a)</td>
<td>82.4</td>
<td>3.6</td>
<td>23</td>
<td>31</td>
<td>5.2</td>
</tr>
<tr>
<td>Phenyl Superose FPLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtPAP12</td>
<td>0.5(^a)</td>
<td>40</td>
<td>0.61</td>
<td>66</td>
<td>87</td>
<td>2.5</td>
</tr>
<tr>
<td>AtPAP25</td>
<td>0.3(^a)</td>
<td>1.6</td>
<td>0.29</td>
<td>5.5</td>
<td>7.4</td>
<td>0.1</td>
</tr>
<tr>
<td>AtPAP26-CW1</td>
<td>0.25(^a)</td>
<td>116</td>
<td>0.46</td>
<td>252</td>
<td>336</td>
<td>7.25</td>
</tr>
<tr>
<td>AtPAP26-CW2</td>
<td>0.25(^a)</td>
<td>52</td>
<td>0.5</td>
<td>108</td>
<td>144</td>
<td>3.3</td>
</tr>
</tbody>
</table>

\(^a\) Concentrated pooled fractions
Table 2. Effect of metal ions and other substances on the activity of AtPAP25
APase activity was measured with the addition of 5 mM of each listed compound to the reaction mixture of assay A as described in Materials and Methods. Activity is expressed relative to the rate of Pi hydrolysis from 5 mM PEP (in the absence of any compound), set at 100%. All values represent means of at least $n = 3$ separate determinations and are reproducible to within ±10% of mean value.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Relative Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaF</td>
<td>52</td>
</tr>
<tr>
<td>Vanadate</td>
<td>6</td>
</tr>
<tr>
<td>Arsenate</td>
<td>3</td>
</tr>
<tr>
<td>Molybdate</td>
<td>2</td>
</tr>
<tr>
<td>ZnCl$_2$</td>
<td>0</td>
</tr>
<tr>
<td>FeCl$_2$</td>
<td>0</td>
</tr>
<tr>
<td>CuSO$_4$</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3. Substrate specificity of AtPAP25. APase activity was determined with 5 mM of each compound using assay B as described in 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 at least $n = 3$ separate determinations and are reproducible to within ±10% of the mean value.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-naphthyl-P</td>
<td>111</td>
</tr>
<tr>
<td>PEP</td>
<td>100</td>
</tr>
<tr>
<td>BCIP</td>
<td>91</td>
</tr>
<tr>
<td>pNPP</td>
<td>82</td>
</tr>
<tr>
<td>ATP</td>
<td>64</td>
</tr>
<tr>
<td>Phenyl-P</td>
<td>56</td>
</tr>
<tr>
<td>NaPPi</td>
<td>48</td>
</tr>
<tr>
<td>AMP</td>
<td>41</td>
</tr>
<tr>
<td>P-Thr</td>
<td>33</td>
</tr>
<tr>
<td>Glc-6-P</td>
<td>32</td>
</tr>
<tr>
<td>6-P-Gluconate</td>
<td>30</td>
</tr>
<tr>
<td>Glc-1-P</td>
<td>14</td>
</tr>
<tr>
<td>P-Tyr</td>
<td>11</td>
</tr>
<tr>
<td>α-naphthyl-P</td>
<td>9</td>
</tr>
</tbody>
</table>
Figure 2.1. Upregulation of cell wall APase in Arabidopsis suspension cells becoming Pi deficient. Time course of +Pi and -Pi suspension cell cultures. At 0 d, 50 mL aliquots of cells cultured for 7 d in 5 mM Pi were subcultured into 450 mL of fresh Murashige and Skoog media containing 5 mM -Pi or 0 mM +Pi. Time courses for CCF Pi (A), and APase activity (B) of -Pi and +Pi cells were determined. All values represent means±SE of n = 3 separate flasks. Where invisible, the error bars are too small to be seen. (C) Pooled Butyl Sepharose extracts from +Pi versus -Pi cell wall extracts (1 μg per lane) were subjected to SDS-PAGE, electroblotted onto PVDF membranes and immunoblotted with anti-AtPAP12. Antigenic polypeptides were visualized using an alkaline phosphatase-linked secondary antibody and chromogenic detection (Veljanovski et al. 2006). AtPAP12 and AtPAP26 (50 ng/lane) purified from CCF of -Pi cells (Tran et al. 2010a) served as positive controls.
Figure 2.2. Separation of APase isoforms from pooled Butyl Sepharose peak APase activity fractions of cell wall extracts of −Pi (A) versus +Pi (B) Arabidopsis suspension cells via SO₃⁻-FractoGel cation-exchange FPLC. APase activity and A₂₈₀ values are indicated with a dotted and solid line, respectively. The column was developed with a linear 0-1 M KCl gradient (represented by the dashed line).
Figure 2.3. SDS–PAGE and immunoblot analysis of purified cell wall 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 order of protein loaded is as follows: AtPAP26-CW1 and -CW2, AtPAP12 and AtPAP25 (5 μg each). MW denotes several non-pre-stained molecular mass standards. B, immunoblot analysis of purified PAPs was performed following 10% SDS-PAGE using anti-(AtPAP12, AtPAP25, or AtPAP26) as indicated. For anti-(AtPAP12 and AtPAP26) blots, 250 ng of each sample were loaded, while for anti-(AtPAP25) blots 100 ng of AtPAP12 CW, 200 ng of AtPAP26-CW1, 300 ng of AtPAP26-CW2, and 55 ng of AtPAP25 were loaded. Antigenic polypeptides were visualized using an alkaline phosphatase-linked secondary antibody and chromogenic detection (Veljanovski et al. 2006).
Figure 2.4. Pooled Butyl Sepharose peak APase activity fractions from +Pi versus –Pi cell wall extracts (1 μg per lane) were subjected to SDS-PAGE and immunoblotted with anti-AtPAP25. Antigenic polypeptides were visualized using an alkaline phosphatase-linked secondary antibody and chromogenic detection (Veljanovski et al. 2006). Purified secreted AtPAP12 and AtPAP25 purified from cell walls of –Pi suspension cells (50 ng each) served as positive controls.

Figure 2.5. Semi-quantitative RT-PCR analysis of AtPAP25 gene expression in (A) suspension cell cultures and (B) shoots and roots of Arabidopsis seedlings grown under Pi-sufficient (+Pi) and Pi-starved (-Pi) conditions. The suspension cell cultures as well as seedlings were cultivated as described in Materials and Methods. Levels of mRNA were analyzed by semi-quantitative RT-PCR using gene specific primers for AtPAP12, AtPAP25, AtPAP26, AtPPCK1 and Actin. AtPAP12 and AtPPCK1 were used as positive controls for PSI genes (Haran et al. 2000; Li et al. 2002; Tran & Plaxton, 2008; Gregory et al. 2009), whereas Actin was used as a reference to ensure equal template loading. All PCR products were taken at cycle numbers determined to be non-saturating (30 cycles). 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, AtPAP25, AtPAP26, AtPPCK1, and Actin transcripts amplified as 244, 610, 627, 483 and 220 bp fragments of the 5’ region of their respective cDNAs, as expected.
Figure 2.6. Bioinformatic analysis of AtPAP25 with Arabidopsis paralogs (A) and orthologs from other plants (B). Alignment of their deduced amino acid sequences was performed using ClustalX. A, N-terminal sequences obtained by automated Edman degradation of AtPAP12, AtPAP25 and AtPAP26 purified from cell wall of –Pi Arabidopsis suspension cell cultures. Their predicted signal peptide cleavage sites (using SignalP) is indicated by the arrow. However, N-terminal analysis shows that indeed AtPAP12, AtPAP25 and AtPAP26 have their transit peptide cleavage site located immediately after a conserved Arg residue (enclosed in a vertical rectangle). Identical amino acids are denoted by an asterisk. AtPAP25 amino acid sequences obtained through tandem MS/MS analysis of tryptic peptides are underlined. Complex glycosylation motifs N-linked to Asn172, Asn367, and Asn424, are boxed. B, AtPAP25 orthologs phylogenetic analysis. Only bootstrap probability values $\geq 60\%$ (more than 100 replicates) are indicated at the branching points. The scale bar represents 0.4 substitutions per site. Phylogenetic analysis was performed using Geneious (Version 5.6.4).
Figure 2.7. Influence of Pi deprivation on appearance of soil-grown Col-0 and atpap25 mutant Arabidopsis seedlings. Seedlings were grown for 7-d in a 24-well microtitre plate (1 seedling per well) containing 0.5 mL of 0.5x MS media, 1% (w/v) sucrose, and 0.2 mM Pi, then transplanted into a Pi-deficient organic-P containing soil mix (Hurley et al. 2010) and grown for additional 14-d. Fertilization occurred biweekly with 0.25x Hoagland’s media containing 0 or 2 mM Pi (-Pi and +Pi, respectively); white scale bars = 1 cm; white dashed scale bar = 0.6 cm. Inset shows same atpap25 seedling with a clear background for improving contrast. These results are representative of a minimum of 3 independent trials with at least 3 plants per trial.
Chapter 3. General Discussion

OVERVIEW

Plants owe their general nutritional condition to the diversity of components present in the soil they grow in, a concomitant feature of their sessile nature. However, this has allowed them to develop an intricate and dynamic set of physiological and biochemical strategies to compensate for the scarcity of certain nutrients. Although environmentally limiting, Pi plays key roles in major pathways needed for plant growth and development. Pi is involved in many different cell processes and metabolic pathways, is a constituent of nucleic acids as well as one of the most important post-translational modification enzymatic control mechanism. The marked Pi impoverishment of many soils around the world poses a great challenge for optimal crop production. Amongst the widespread responses of plants to low Pi conditions is the upregulation of Pi scavenging and remobilizing enzymes, such as PAPs. PAP induction is a common trend among many plant species subjected to Pi starvation, and these enzymes have been found to play diverse roles in certain species (Veljanovski et al. 2006; Tran et al. 2010; Hurley et al. 2010; Bozzo et al. 2002, 2004; Zhang et al. 2008; Wang et al. 2011). Indeed, PAPs have been localized to specific cell compartments and tissues as varied as the vacuole (Veljanovski et al. 2006), secretome (Tran et al. 2010a), mitochondria (Francisca et al. 2008), and roots (Wang et al. 2011). Large scale proteomic approaches have identified PAPs in the cell wall of a few species (Bayer et al. 2006; Chivasa et al. 2002, Kaida et al. 2008, 2010). With the exception of a few complete studies, most of the work conducted to date refers only to the identification of cell wall-localized PAPs. The present thesis has focused on the search, purification, identification and biochemical and molecular characterization of PAP members specifically targeted to the cell wall of –Pi Arabidopsis. The approach followed in the present study allowed for the extraction of proteins present in the extracellular matrix of –Pi cell cultures and subsequently led to the discovery of three PSI PAPs being the most abundantly expressed in this compartment. FPLC derived protein peaks showing APase activity were identified as AtPAP12, AtPAP25, and AtPAP26. These results confirmed the dual targeted nature of AtPAP12, while AtPAP26
seems to be expressed in the vacuole, cell wall and secreted (Veljanovski et al. 2006; Tran et al. 2010a; Robinson et al. 2012). In accordance with cell wall proteomics literature AtPAP25 appears to be only localized in the cell wall compartment (Chivasa et al. 2002; Borderies et al. 2003).

This thesis focused on the characterization of the newly found AtPAP25 and its relationship with the biochemical adaptations of –Pi Arabidopsis. Purification as well as immunoblotting analyses indicated that AtPAP25 was exclusively synthesized by the –Pi suspension cells only under –Pi conditions. Semi-quantitative RT-PCR also showed that AtPAP25 is transcriptionally induced during Pi starvation. In contrast to AtPAP25, the other two cell wall PAP isozymes (AtPAP12 and AtPAP26) were upregulated under –Pi conditions, and their transcripts were Pi-starvation induced and constitutively expressed, respectively. Both these results are in agreement with previous studies (Hurley et al. 2010; Tran et al. 2010a; Veljanovski et al. 2006; Robinson et al. 2012).

Assessing AtPAP25’s potential Pi scavenging and recycling role in –Pi Arabidopsis was the main objective of the present thesis. However, biochemical characterization of purified, native AtPAP25, was also essential. Confirming its PAP nature, the purified AtPAP25 showed a dark purple color in solution and insensitivity to tartrate inhibition. In addition, there was a broad and unspecific range of phosphomonoesters that served as substrates for this enzyme. Among the most potent inhibitors were Zn$^{2+}$, NaF, molybdate, vanadate, and arsenate, commonly reported APase inhibitors. Hyperbolic substrate saturation kinetics were observed with a $K_m$ (PEP) value of 0.52 mM. Surprisingly, the $V_{max}$ of purified AtPAP25 (~5 units mg$^{-1}$) was orders of magnitude lower than values obtained for purified AtPAP12 and AtPAP26 of the –Pi Arabidopsis cell wall or secretome (Table 1; Tran et al. 2010a). This result, coupled with AtPAP25’s potent Pi inhibition ($I_{50} = 50 \mu$M) may – unlike its mentioned counterparts and contrary to our first expectations – point to a potential function as a signaling PAP, rather than as a Pi scavenging enzyme.
The characterization of AtPAP25 from cell walls of –Pi Arabidopsis suspension cells expanded our knowledge of the PAP family members involved in Pi metabolism during Pi starvation periods. However, to deepen the understanding of AtPAP25 role in vivo a complementary initiative was taken. An atpap25 T-DNA insertion mutant line was obtained and analyzed for a Pi-starvation related phenotype. –Pi soil-grown atpap25 mutants presented a severe phenotype characterized by a failure to develop and a dark purple color, both common symptoms of severe Pi stress (Hurley et al. 2010; Tran et al. 2010a, Plaxton & Tran, 2011). By contrast, the atpap25 cultivated under +Pi conditions showed no significant phenotypic differences compared to Col-0. The existence of such a strong phenotype in –Pi soil argues in favor of a significant role for AtPAP25 in planta. Other physiological studies involving root surface APase activity staining failed to show any significant difference between Col-0 and atpap25 plants (Appendix 7). Immunoblotting of cell-wall specific as well as total protein extracts of +Pi and –Pi Col-0 or atpap12/atpap26 double knockout seedlings was unable to detect immunoreactive 55 kDa AtPAP25 polypeptides in any tissue. Previous studies by Kaida and coworkers (2008, 2009, 2010) has indicated the possible involvement of NtPAP12 in remodeling the cell wall of Nicotiana tabacum suspension cell cultures. NtPAP12 appears to dephosphorylate two polysaccharide remodeling enzymes, and thus affects cell wall reorganization and synthesis through the degradation of xyloglucan and cello-oligosaccharides. AtPAP25 might be playing a similar role, and in doing so it might compromise growth and development in atpap25 plants grown in soil with no available soluble Pi. Furthermore, following the irrefutable presence of this protein in the cell wall of Arabidopsis suspension cell cultures, the strong phenotype of –Pi atpap25 plants and the relationship of its expression to –Pi conditions, it is likely that this PAP is somehow playing an important role in vivo as regulator of a Pi-signaling related process. This might explain its low abundance and low V\text{max}. Additional research is currently being done on the atpap25 plants in order to shed more light on the actual role carried out by AtPAP25 in –Pi Arabidopsis.
FUTURE DIRECTIONS

One of the most interesting features about AtPAP25 is its apparent specific localization in the cell wall but not secretome of Arabidopsis suspension cells (Chivasa et al. 2002; Borderies et al. 2003; Tran et al. 2010a). As stated above the combination of a relatively high expression of this protein in –Pi Arabidopsis suspension cell cultures and the marked phenotype observed in –Pi soil-grown atpap25 knockout lines both argue in favor of an important role associated with Pi metabolism. In this regard further assessment of the knockout lines would be extremely helpful in order to complete the physiological analysis of the mutant, including biomass and total Pi contents of hydroponic and soil grown +Pi versus -Pi seedlings. Anthocyanin content analysis would be another way to metabolically confirm the biological changes induced by Pi starvation and the impact on atpap25 mutant lines lacking AtPAP25 compared to Col-0. Also, a microarray analysis of –Pi versus +Pi atpap25 plants could be very informative in order to survey what other genes present altered expression patterns.

Previous work conducted at our and other labs have explored the world of secreted or cell wall-localized PAP members whose main role relies on Pi scavenging and remobilization (Bozzo et al. 2002, 2004; Hurley et al. 2010; Tran et al. 2010a; Wang et al. 2011; Robinson et al. 2012). The upregulation of AtPAP12 and AtPAP26 in the secretome of –Pi Arabidopsis suspension cell cultures and seedlings was the first evidence of the extracellular Pi scavenging role of these closely related PAP isozymes in the Arabidopsis PSR (Tran et al. 2010a). More recently, individual studies carried out in both atpap26 and atpap12 single knockout lines and double knockout lines atpap12/atpap26, have confirmed the ability of these enzymes to scavenge Pi from a diverse and broad group of exogenous organic Pi compounds (Robinson et al. 2012). Confronting our negative attempts to find AtPAP25 in planta with the impaired growth of atpap25 mutant lines on organic-P containing soil lacking soluble Pi, implies an altered PAP-driven Pi scavenging machinery. As mentioned before, this in turn, points to AtPAP25 as a potential regulator and/or intermediary in the regulation of other Pi-scavenging and remobilizing enzymes, possible due to its putative protein phosphatase activity. It would be helpful to overexpress AtPAP25 in atpap25
lines and observe the recovery of a phenotype comparable –Pi grown WT plants. On the other hand, the low abundance of AtPAP25 either in total or cell wall-specific protein extracts of Arabidopsis seedlings urges for another mechanisms of detection, including interaction analyses. To this respect, a more ambitious and probably long term goal would be yeast two hybrid screening searching or co-immunopurification of cell wall extracts with anti-AtPAP25, looking for AtPAP25 interactors. This would constitute an asset to help create a better picture of the underlying biochemical processes taking place and their phenotypic/functional implications.

In order to further analyze AtPAP25 contribution to Pi metabolism, the construction of triple knockout lines atpap12/atpap25/atpap26 would be interesting. The contribution to the total APase activity as well as the immunological presence of such PAP members in +Pi and -Pi plants have already been documented for atpap12/atpap26 double knockouts as well as for individual mutant atpap12 and atpap26 lines (Hurley et al. 2010; Robinson et al. 2012). It would also be extremely interesting to see what the phenotype of this triple mutant is when grown in solid or liquid Murashige and Skoog +Pi and -Pi media as well as in soil with and without Pi fertilization. It would be particularly attractive to assess the survival rate of the triple mutant in –Pi soil, given the already strong phenotype and arrested development shown by atpap12/atpap26 (Robinson et al. 2012) and atpap25 seedlings (Fig. 2.7). Similarly, an overexpressor AtPAP25 mutant line is expected to be constructed and tested under a broad range of different growing conditions, including the presence and absence of soluble Pi or organic-P sources. This approach will be useful to compensate for the low abundance of AtPAP25 and unravel its expression in planta within any particular tissue.

Confocal laser scanning microscopy imaging of transient and/or stably overexpressed AtPAP25-fluorescent protein fusions in Arabidopsis suspension cells and seedlings would also be informative. This would unequivocally confirm AtPAP25 cell wall localization and would provide evidence of the target tissues for its expression in planta. Additionally, targeting AtPAP25 localization in vivo will improve our understanding of its biological function. It would be interesting to deepen the study of AtPAP25 putative
role as a phosphoprotein phosphatase and a signaling Pi status protein. Proteomic studies might be useful for preliminary screening and identification of potential AtPAP25 \textit{in vivo} phosphoprotein substrates. Confirming AtPAP25 targets and their localization would be of great impact, since it would shed light on how that might relate to such a strong phenotype observed in \textit{atpap25} plants grown in soil lacking any soluble Pi. A recent study by Kaida \textit{et al.} (2010) proved this approach to be successful with tobacco NtPAP12. NtPAP12 is potentially involved in the control of cellulose and callose synthesis in the cell wall. AtPAP25 might also appear to be involved in similar pathways during Pi stress, although future studies remain to determine its actual \textit{in vivo} role and thus fully explain the results of the current thesis.
REFERENCES


64


SUMMARY

To the best of my knowledge this thesis represents the first study of the influence of nutritional Pi deprivation on cell wall proteins of the model plant *Arabidopsis thaliana*.

1. Pi starvations of Arabidopsis suspension cells was correlated with increased APase activity of cell wall extracts, as well as the pronounced upregulation of 60- and 55-kDa immunoreactive polypeptides on immunoblots probed with anti-AtPAP12.

2. Four APase isoforms of the –Pi cell wall extracts were purified to near homogeneity activity and identified as AtPAP12, AtPAP25, and AtPAP26-CW1/-CW2.

3. AtPAP12 appears to be dual-targeted during Pi deprivation, being upregulated in the secretome as well as cell walls of –Pi Arabidopsis, whereas AtPAP26 exhibits a triple-targeted nature in –Pi Arabidopsis, having been localized to the cell vacuole, secretome, and cell wall. By contrast, AtPAP25 appears to be strictly cell wall-localized and although previously identified in this compartment, this study constitutes the first to document AtPAP25’s biochemical and molecular properties or apparent involvement in the Arabidopsis PSR.

4. Like most PAPs, AtPAP25 shows broad substrate selectivity, being able to cleave Pi from a wide range of phosphomonoesters. It exhibited maximum activity at pH 5.6 and a $K_m$ (PEP) value of 0.52 mM. AtPAP25 was potently inhibited by Pi ($I_{50} = 50$ µM) and has a relatively low $V_{max}$ activity (5.5 U mg$^{-1}$) supporting a potential signaling role hypothesis.

5. *AtPAP25* transcript expression profiles together with AtPAP25 immunoblotting analyses are in accordance with its exclusive synthesis in response to –Pi growth conditions.

6. Complex NXS/T glycosylation motifs were mapped to be on Asn172, Asn367, and Asn424. The complete oligosaccharide structure was determined for each site. I hypothesize that distinct glycosylation may be an important post-translational modification playing a role in AtPAP25’s targeting and function.
APPENDIXES

Appendix 1

Immunological detection of PEP carboxylase and cytosolic aldolase in clarified Arabidopsis suspension cell extracts.

APase activity present in CaCl$_2$-extracted cell wall fractions of +Pi vs. –Pi cells was enriched via Butyl Sepharose FPLC (10 µg per lane for both +Pi and –Pi butyl). Immunoblotting was also performed with clarified cell extracts from the same -Pi CaCl$_2$-treated cells (CE, 5 µg) and homogeneous castor seed PEP carboxylase (PEPC) and cytosolic aldolase (ALD) (50 ng each) (Uhrig et al. 2008; Hodgson & Plaxton, 1998). Immunoblots were probed with a 1000-fold dilution of affinity-purified rabbit anti-(castor seed PEP carboxylase or cytosolic aldolase)-IgG (Uhrig et al. 2008; Hodgson & Plaxton, 1998). Immunoreactive polypeptides were detected using an alkaline phosphatase-conjugated secondary antibody and chromogenic detection. Corresponding PEP carboxylase and aldolase specific activities appear below the respective lanes.
Appendix 2

TABLE S1. MALDI-TOF MS analyses of tryptic peptides derived from native AtPAP isoforms isolated from –Pi Arabidopsis cell wall extracts.

<table>
<thead>
<tr>
<th>Identification</th>
<th>Gene</th>
<th>MASCOT MOWSE Score</th>
<th>MS-FIT MOWSE Score</th>
<th>Sequence Coverage</th>
<th>N° of matching Peptides</th>
<th>M_r predicted/M_r observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtPAP12</td>
<td>At2g27190</td>
<td>74</td>
<td>11331</td>
<td>20</td>
<td>7</td>
<td>54/60</td>
</tr>
<tr>
<td>AtPAP25</td>
<td>At4g36350</td>
<td>89</td>
<td>6250000</td>
<td>34</td>
<td>22</td>
<td>53/55</td>
</tr>
<tr>
<td>AtPAP26 – CW1</td>
<td>At5g34850</td>
<td>114</td>
<td>957937</td>
<td>27</td>
<td>12</td>
<td>55/55</td>
</tr>
<tr>
<td>AtPAP26 – CW2</td>
<td>At5g34850</td>
<td>111</td>
<td>380115</td>
<td>28</td>
<td>14</td>
<td>55/55</td>
</tr>
</tbody>
</table>

Appendix 3

AtPAP25 pH activity profile.

AtPAP25’s APase activity was measured as a function of assay pH. Assays were performed using assay A as described in Materials and Methods, except that they were buffered by a mixture of 25 mM sodium acetate, 25 mM MES, and 25 mM Bis-Tris propane. Activity is expressed as relative percent to the maximum (100%). All values represent the means of n = 3 independent measurements and are reproducible to within ±10% of the mean value.
### Appendix 4

Comparison of the amino acid sequence identity of AtPAP25 with AtPAP12 and AtPAP26, as well as with AtPAP25 orthologs from other vascular plant sources.

<table>
<thead>
<tr>
<th>AtPAP</th>
<th>Protein Accession&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent Identity to AtPAP25</th>
<th>Percent Similarity to AtPAP25</th>
<th>Predicted pI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Predicted Localization&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtPAP10</td>
<td>NP_179235.1</td>
<td>59</td>
<td>74</td>
<td>7.3</td>
<td>Secretory</td>
</tr>
<tr>
<td>AtPAP12</td>
<td>NP_180287.2</td>
<td>60</td>
<td>75</td>
<td>5.9</td>
<td>Secretory</td>
</tr>
<tr>
<td>AtPAP25</td>
<td>AEE86645.1</td>
<td>100</td>
<td>100</td>
<td>6.5</td>
<td>Secretory</td>
</tr>
<tr>
<td>AtPAP26</td>
<td>NP_198334.1</td>
<td>55</td>
<td>73</td>
<td>6.8</td>
<td>Secretory</td>
</tr>
</tbody>
</table>

<sup>a</sup>Given by NCBI protein database  
<sup>b</sup>Predicted by ExPASy compute pI/Mw tool  
<sup>c</sup>Predicted by TargetP program. Secretory denotes the presence of putative signal peptides for the secretory pathway.

<table>
<thead>
<tr>
<th>Plant Source</th>
<th>Protein Accession&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent identity to AtPAP25</th>
<th>Percent similarity to AtPAP25</th>
<th>Predicted Mr&lt;sub&gt;r&lt;/sub&gt; (kDa)</th>
<th>Predicted localization&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtPAP25</td>
<td>AEE86645.1</td>
<td>100</td>
<td>100</td>
<td>53.0</td>
<td>Secretory</td>
</tr>
<tr>
<td>Castor</td>
<td>XP_002534135.1</td>
<td>68</td>
<td>81</td>
<td>53.3</td>
<td>Secretory</td>
</tr>
<tr>
<td>Soybean</td>
<td>NP_001240926.1</td>
<td>63</td>
<td>77</td>
<td>53.1</td>
<td>Secretory</td>
</tr>
<tr>
<td>Clover</td>
<td>AAX20028.1</td>
<td>63</td>
<td>77</td>
<td>53.8</td>
<td>Secretory</td>
</tr>
<tr>
<td>Bean</td>
<td>CAA04644.1</td>
<td>60</td>
<td>75</td>
<td>52.9</td>
<td>Secretory</td>
</tr>
<tr>
<td>White Lupin</td>
<td>BAA97745.1</td>
<td>57</td>
<td>71</td>
<td>52.8</td>
<td>Secretory</td>
</tr>
<tr>
<td>Tobacco</td>
<td>BAC55155.1</td>
<td>61</td>
<td>78</td>
<td>54.0</td>
<td>Secretory</td>
</tr>
<tr>
<td>Potato</td>
<td>AAT37527.1</td>
<td>62</td>
<td>78</td>
<td>51.2</td>
<td>Non-Secretory</td>
</tr>
<tr>
<td>Sweet Potato</td>
<td>CAA06921.1</td>
<td>61</td>
<td>75</td>
<td>53.3</td>
<td>Secretory</td>
</tr>
<tr>
<td>Poplar</td>
<td>XP_002306126.</td>
<td>62</td>
<td>77</td>
<td>53.7</td>
<td>Secretory</td>
</tr>
<tr>
<td>Grape</td>
<td>XP_002274392.1</td>
<td>64</td>
<td>78</td>
<td>53.7</td>
<td>Secretory</td>
</tr>
<tr>
<td>Rice</td>
<td>NP_001044416.1</td>
<td>60</td>
<td>73</td>
<td>53.0</td>
<td>Secretory</td>
</tr>
<tr>
<td>Canola</td>
<td>ABV89741.1</td>
<td>59</td>
<td>73</td>
<td>54.1</td>
<td>Secretory</td>
</tr>
<tr>
<td>Cotton</td>
<td>AET86953.1</td>
<td>62</td>
<td>75</td>
<td>54.9</td>
<td>Secretory</td>
</tr>
<tr>
<td>Wheat</td>
<td>ACR23330.1</td>
<td>56</td>
<td>74</td>
<td>55.2</td>
<td>Secretory</td>
</tr>
<tr>
<td>Corn</td>
<td>NP_001147979.1</td>
<td>58</td>
<td>74</td>
<td>55.1</td>
<td>Secretory</td>
</tr>
</tbody>
</table>

<sup>a</sup>Given by NCBI protein database  
<sup>b</sup>Predicted by Target P program. Secretory denotes the presence of putative signal peptides for the secretory pathway.
Appendix 5

Identification of AtPAP25 glycopeptides by UPLC LTQ-FT LC MS/MS

Glycosylation motifs are located on Asn172, Asn367, and Asn424. The complete number of glycosyl residues. GlcNAc: N-acetyl-D-glucosamine; Man: mannose; Xyl: xylose; Fuc: fucose; Gal: galactose.

<table>
<thead>
<tr>
<th>Glycopeptide m/z (charge)</th>
<th>Meas. MH+</th>
<th>Calc. MH+</th>
<th>Ppm</th>
<th>Peptide sequence</th>
<th>Glycan composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1061.4296(2+)</td>
<td>2121.8514</td>
<td>2121.8493</td>
<td>1</td>
<td><a href="L">AtPAP25: 170-178</a>ASNETLYHY(M)</td>
<td>(GlcNAc)₃(Man)₂(Xyl)</td>
</tr>
<tr>
<td>1162.9691(2+)</td>
<td>2324.9304</td>
<td>2324.9287</td>
<td>1</td>
<td><a href="L">AtPAP25: 170-178</a>ASNETLYHY(M)</td>
<td>(GlcNAc)₂(Man)₃(Xyl)(GlcNAc)</td>
</tr>
<tr>
<td>1027.4910(3+)</td>
<td>3080.4574</td>
<td>3080.4556</td>
<td>1</td>
<td><a href="R">AtPAP25: 361-377</a>VSNIKYNITNGLSYPVK(D)</td>
<td>Fuc(GlcNAc)₃(Man)₂(Xyl)</td>
</tr>
<tr>
<td>1095.1838(3+)</td>
<td>3283.5358</td>
<td>3283.5350</td>
<td>0</td>
<td><a href="R">AtPAP25: 361-377</a>VSNIKYNITNGLSYPVK(D)</td>
<td>Fuc(GlcNAc)₂(Man)₂(Xyl)(GlcNAc)</td>
</tr>
<tr>
<td>1018.4524(2+)</td>
<td>2035.8970</td>
<td>2035.8952</td>
<td>1</td>
<td><a href="I">AtPAP25: 365-371</a>KYNITNI(T)</td>
<td>Fuc(GlcNAc)₂(Man)₂(Xyl)</td>
</tr>
<tr>
<td>1119.9914(2+)</td>
<td>2238.9750</td>
<td>2238.9746</td>
<td>0</td>
<td><a href="I">AtPAP25: 365-371</a>KYNITNI(T)</td>
<td>Fuc(GlcNAc)₂(Man)₂(Xyl)(GlcNAc)</td>
</tr>
<tr>
<td>1270.0714(2+)</td>
<td>2539.1350</td>
<td>2539.1332</td>
<td>1</td>
<td><a href="K">AtPAP25: 366-377</a>YNTNGLSYPVK(D)</td>
<td>Fuc(GlcNAc)₂(Man)₂(Xyl)</td>
</tr>
<tr>
<td>1371.6089(2+)</td>
<td>2742.2100</td>
<td>2742.2126</td>
<td>-1</td>
<td><a href="K">AtPAP25: 366-377</a>YNTNGLSYPVK(D)</td>
<td>Fuc(GlcNAc)₂(Man)₂(Xyl)(GlcNAc)</td>
</tr>
<tr>
<td>780.8189(2+)</td>
<td>1560.6300</td>
<td>1560.6270</td>
<td>2</td>
<td><a href="Y">AtPAP25: 424-426</a>NRTH(H)</td>
<td>Fuc(GlcNAc)₂(Man)₂(Xyl)</td>
</tr>
<tr>
<td>882.3588(2+)</td>
<td>1763.7098</td>
<td>1763.7064</td>
<td>2</td>
<td><a href="Y">AtPAP25: 424-426</a>NRTH(H)</td>
<td>Fuc(GlcNAc)₂(Man)₂(Xyl)(GlcNAc)</td>
</tr>
<tr>
<td>918.8911(2+)</td>
<td>1836.7744</td>
<td>1836.7744</td>
<td>0</td>
<td><a href="E">AtPAP25: 422-426</a>YNRT(H)</td>
<td>Fuc(GlcNAc)₂(Man)₂(Xyl)</td>
</tr>
<tr>
<td>1020.4311(2+)</td>
<td>2039.8544</td>
<td>2039.8538</td>
<td>0</td>
<td><a href="E">AtPAP25: 422-426</a>YNRT(H)</td>
<td>Fuc(GlcNAc)₂(Man)₂(Xyl)(GlcNAc)</td>
</tr>
<tr>
<td>983.4127(2+)</td>
<td>1965.8176</td>
<td>1965.8169</td>
<td>0</td>
<td><a href="L">AtPAP25: 421-426</a>EIYRTH(H)</td>
<td>Fuc(GlcNAc)₂(Man)₂(Xyl)</td>
</tr>
<tr>
<td>1084.9521(2+)</td>
<td>2168.8964</td>
<td>2168.8963</td>
<td>0</td>
<td><a href="L">AtPAP25: 421-426</a>EIYRTH(H)</td>
<td>Fuc(GlcNAc)₂(GlcNAc)(Man)₂(Xyl)</td>
</tr>
<tr>
<td>566.5674(3+)</td>
<td>1697.6865</td>
<td>1697.6859</td>
<td>0</td>
<td><a href="Y">AtPAP25: 424-427</a>NRTH(A)</td>
<td>Fuc(GlcNAc)₂(Man)₂(Xyl)</td>
</tr>
<tr>
<td>634.2606(3+)</td>
<td>1900.7661</td>
<td>1900.7653</td>
<td>0</td>
<td><a href="Y">AtPAP25: 424-427</a>NRTH(A)</td>
<td>Fuc(GlcNAc)₂(Man)₂(Xyl)(GlcNAc)</td>
</tr>
<tr>
<td>688.2775(3+)</td>
<td>2062.8168</td>
<td>2062.8181</td>
<td>-1</td>
<td><a href="Y">AtPAP25: 424-427</a>NRTH(A)</td>
<td>Fuc (GlcNAc)₂(Man)₂(Xyl)(GlcNAc)(Gal)</td>
</tr>
<tr>
<td>849.3476(2+)</td>
<td>1697.6879</td>
<td>1697.6859</td>
<td>1</td>
<td><a href="Y">AtPAP25: 424-427</a>NRTH(A)</td>
<td>Fuc(GlcNAc)₂(Man)₂(Xyl)</td>
</tr>
<tr>
<td>950.8878(2+)</td>
<td>1900.7678</td>
<td>1900.7653</td>
<td>1</td>
<td><a href="Y">AtPAP25: 424-427</a>NRTH(A)</td>
<td>Fuc(GlcNAc)₂(Man)₂(Xyl)(GlcNAc)</td>
</tr>
<tr>
<td>1031.9142(2+)</td>
<td>2062.8206</td>
<td>2062.8181</td>
<td>1</td>
<td><a href="Y">AtPAP25: 424-427</a>NRTH(A)</td>
<td>Fuc(GlcNAc)₂(Man)₂(Xyl)(GlcNAc)(Gal)</td>
</tr>
<tr>
<td>926.0786(3+)</td>
<td>2776.2202</td>
<td>2776.2194</td>
<td>0</td>
<td><a href="R">AtPAP25: 412-425</a>EASFHGAVLEYNRT(T)</td>
<td>Fuc(GlcNAc)₂(Man)₂(Xyl)</td>
</tr>
<tr>
<td>993.7716(3+)</td>
<td>2979.2992</td>
<td>2979.2988</td>
<td>0</td>
<td><a href="R">AtPAP25: 412-425</a>EASFHGAVLEYNRT(T)</td>
<td>Fuc(GlcNAc)₂(Man)₂(Xyl)(GlcNAc)</td>
</tr>
<tr>
<td>1047.7892(3+)</td>
<td>3141.3519</td>
<td>3141.3516</td>
<td>0</td>
<td><a href="R">AtPAP25: 412-425</a>EASFHGAVLEYNRT(T)</td>
<td>Fuc (GlcNAc)₂(Man)₂(Xyl)(GlcNAc)(Gal)</td>
</tr>
</tbody>
</table>
Appendix 6

A. Schematic representation of the AtPAP25 gene; white boxes and solid lines represent exons and introns, respectively. T-DNA insertion location is indicated by “atpap25 T-DNA”, while arrows represent primers. B. Assessment of T-DNA location and homozygosity of mutants via PCR-based screening of gDNA template isolated from leaves of +Pi soil grown seedlings. PCR products were amplified using AtPAP25-specific primers (primer pair A+B) and the overlapping region between AtPAP25 and the T-DNA insert-specific primers (primer pair B+C) from the Col-0 and atpap25 gDNA. The lack of multiple amplification products for AtPAP25 DNA in atpap25 demonstrates that the mutant is homozygous. NC: negative control.

Confirmation of T-DNA insert location in atpap25 mutant
Appendix 7

Root surface APase activity staining

Root surface APase activity staining on 14-d-old +Pi (1.5 mM Pi) and –Pi (50 µM) Arabidopsis seedlings with BCIP (A) and β-naphthyl-P (B). Seedlings were grown on agar-solidified nutrient media as described in the Materials and Methods. For BCIP staining, roots were covered with a 0.5% agar solution containing 0.01, 0.04, 0.08, or 0.16 % (w/v) BCIP. β-naphthyl-P staining was performed as described by Gilbert et al. (1999). Results are representative of a minimum of 3 independent trials with at least 3 seedlings per trial. Scale bar = 1 cm.
Confirmation of absence of phosphorylation on Tyr423 of purified AtPAP25

LC MS/MS analysis on purified AtPAP25 was unable to find the existence of phosphopeptide 412-425 containing the phosphorylated Tyr423. MS/MS spectra failed to show the 80 Da shift corresponding to the presence of the phosphate group attached to the tyrosine residue. This result confirms that AtPAP25 is not phosphorylated, and contradicts what had previously been shown by Sugiyama et al. (2008).