PURPLE ACID PHOSPHATASE 12: A TOOL TO STUDY THE PHOSPHATE STARVATION RESPONSE IN *Arabidopsis thaliana*

A Dissertation

by

KETAN PATEL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2006

Major Subject: Biology

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Approved by:

Chair of Committee, Thomas D. McKnight
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ABSTRACT

Purple Acid Phosphatase 12: A Tool to Study the Phosphate Starvation Response in *Arabidopsis thaliana*. (December 2006)

Ketan Patel, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Thomas D. McKnight

Phosphorus is an essential element for plant growth and development. Due to its low availability, solubility and mobility, phosphate is often the limiting macronutrient for crops and other plants. Plants have evolved several responses to phosphate deficiency. However, very little is known about the molecular basis of these responses.

Here, I study the expression of *PAP12*, its role in the phosphate starvation response and the interaction of its promoter with nuclear factors. Analysis of a *PAP12* T-DNA insertion line (*pap12-1*) revealed *PAP12* is responsible for the majority of the acid phosphatase activity detected by the standard in-gel assay. RNA gel blots showed that *PAP12* was induced only by P_i deficiency, and not by general nutrient stress. *PAP12* expression, at the RNA and protein level, reflected endogenous phosphate levels in two mutants with altered phosphate accumulation. In the *pho1* mutant, *PAP12* expression and activity were up-regulated with respect to wild-type plants, and in the *pho2* mutant, *PAP12* expression and activity were reduced. Analysis of the *PAP12* promoter using promoter-*GUS* fusions revealed expression in leaves, roots, flowers,

hydathodes, root tips, and pollen grains. This broad pattern of expression suggests that *PAP12* functions throughout the plant in response to low phosphate concentrations.

The results showed *PAP12* does not play a major role in phosphate remobilization, acquisition or in helping plants cope with low phosphate environments. Instead, the major phenotype associated with *PAP12* deficiency was a significant delay in flowering in the low-phosphate *pho1* background and a slight acceleration of flowering in the high-phosphate *pho2* background over-expressing *PAP12*. These results suggest that *PAP12* may have a role in linking phosphate status with the transition to flowering.

Finally, I used promoter deletion and DNA-protein interaction assay to understand *PAP12* expression upon phosphate starvation. A 35-bp region of the *PAP12* promoter was identified as an important phosphate regulatory *cis*-element required for induction during phosphate starvation. We isolated a 23.5 kDa nuclear factor, which binds to this 35-bp region of the *PAP12* promoter in a phosphate-dependent manner.

The work presented here will add to our knowledge about the molecular processes that regulate phosphate nutrition.

DEDICATION

To my mom, dad and brother for their support and patience.

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Background

9,000,000,000 - the amount of people expected by 2050 according to a report from 2004 Revision of the official United Nations population estimates and projections (www.unpopulation.org). Feeding a population this large seems like a daunting task. It can be accomplished, but at a cost. In the 1960s, it was thought that food production would not keep pace with the growing population. This crisis was averted through the extensive use of fertilizers, in addition to germplasm improvement and expanded use of irrigation (Waggoner, 1994). One consequence of this approach is that we will need additional nitrogen and phosphorus fertilizers in the future. This will result in a rapid depletion of phosphate sources, a nonrenewable resource. To meet the challenge of increasing food supplies without further damaging the environment or using all of our non-renewable resource, we will need to better understand is that of nutrient acquisition and uptake in plants.

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This dissertation follows the style and format of The Plant Cell.

Plants may there be developed through breeding programs or genetic engineering that are more efficient at acquiring nutrients from natural soils, thus reducing the need to applying additional fertilizers. Only then can we begin to solve the problem of food production for a growing population without harming the environment (Vance et al., 2003).

Importance of Phosphate and Limits of Its Availability

Phosphorous, in the form of phosphate (P_i), is an important nutrient for all organisms. It is a key structural component of nucleic acids, ATP, phospholipids and it plays a regulatory role in protein regulation and signal transduction cascades. Therefore, all organisms require phosphate for proper growth and development. Animals usually obtain sufficient quantities from their diet. Plants, however, have to acquire phosphate from the environment where it is mainly taken up in the diprotic form, $H_2PO_4^-$ or orthophosphate (Tate, 1985). In most soils, phosphorus is second only to nitrogen as the most limiting element for plant growth (Vance et al., 2000). Phosphate mineralization, association with cations and organic compounds to create insoluble complexes, lowers the concentration of phosphate available for plant growth (Misson et al., 2005).

Limitation of Phosphate Availability

Even though the total phosphate content in soil is 500-1000 parts per million, most is in a form that is not available for uptake by plants (Kelling and Schulte, 1992). Total phosphate is comprised of two pools, an organic and an inorganic (insoluble and

soluble). The organic pool consists of dead and decaying plant and animal matter, which is not readily available for uptake. The inorganic pool, containing orthophosphate which can be taken up by plants, is also not readily available, because a majority of the phosphate is bound to cations, such as Al^{+3}, Fe^{+3} and Ca^{+2} forming insoluble complexes. This leaves a very small fraction of soluble phosphate, as low as 1% of the total phosphate in the soil available for plant nutrition (Raghothama, 1999; Vance et al., 2003).

A common method used to address limiting amounts of phosphate in soils is to apply soluble phosphate in the form of phosphate fertilizers. This results only in a temporary increase of soluble P_i available to plants, because most of the P_i soon becomes immobile due to absorption. Additionally, some of the applied phosphate can runoff and pollute nearby surface waters, which over a long period of time can lead to eutrophication (Abelson, 1999).

Plant Responses to P_i-Limitation

P_i concentrations in soil rarely exceed 10μM, while the internal P_i concentration is 1-10 mM (Rausch and Bucher, 2002; Bieleski, 1973). In addition, rate of P_i uptake by plants is faster than the rate of diffusion of P_i in soil, thus creating a P_i-depletion zone around the root (Schachtman et al., 1998; Marschner, 1995).

Deficiency symptoms of plants grown under low phosphate include increased root growth, reduced shoot growth, stimulation of root hair elongation, stunted growth,

delayed maturity, dark green leaves and accumulation of anthocyanins (Raghothama, 1999).

Despite the chronically P_i limited environment, plants still survive; in-part because plants have evolved numerous mechanisms for phosphate acquisition and use under nutrient limiting conditions. The strategies can be classified under two major categories, conservation of use and enhanced acquisition or uptake (Vance et al., 2001; Raghothama, 1999). Mechanisms that conserve the use of phosphate involve decreased growth rate, remobilization of internal P_i and use of alternative pathways for photosynthesis and respiration (Theodorou and Plaxton, 1993; Duff et al., 1989; Raghothama, 1999).

Enhanced acquisition or uptake mechanisms include morphological, physiological, biochemical and molecular changes (Raghothama, 1999). Morphological changes include an increase in root:shoot ratio, increase in root hair growth and elongation, proteoid root formation and increased association with mycorrhizal fungi (Lynch, 1995; Harrison, 1998). Physiological responses include secretion of organic acids and other chelators, mobilization of P_i from the vacuole as well as increased translocation of phosphate within the plant (Abel et al., 2002; Schachtman et al., 1998). Pathways for respiration, carbon metabolism, photosynthesis, and nitrogen fixation are altered (Plaxton, 2004). Biochemical responses include increase production of phosphatases, RNAses, organic acids, changes in protein phosphorylation, decrease in photosynthetic rate and activation of alternative respiration pathway, which "bypass" phosphate requiring pathways (Raghothama, 1999). Molecular changes include but are

not limited to changes in gene expression. Genes, such as RNAses, acid phosphatases, and phosphate transporters are induced upon phosphate starvation (Bariola et al., 1994; Muchhal et al., 1996; Raghothama, 1999).

Plant Acid Phosphatases

Acid phosphatases (APs) have been assumed to play an important role in phosphate acquisition in plants. They exist in both intracellular (IAP) and extracellular, secreted (SAP) forms and have pH optima under acidic (acid phosphatases) conditions. They hydrolyze phosphate esters releasing inorganic phosphate as a reaction product. One group of acid phosphatases, the purple acid phosphatases (PAPs), are of considerable interest and have been studied intensely. Purple acid phosphatases are distinguished from other acid phosphatases by their purple color in solution, which is due to the presence of a binuclear iron center and arises from tyrosine-to-iron charge transfer transition (Strater et al., 1995). PAPs are present in both plants and animals. In animals they play a role in iron transport, bone resorption, antigen presentation and redox reactions (Buhi et al., 1982; Hayman et al., 1996; Hayman et al., 2000). In plants, their role is still unknown, although several roles have been proposed. Two groups of PAPs exist in plants: small and large plant purple acid phosphatases. Small plant PAPs are 35 kilodaltons monomeric proteins and are closely related to their mammalian and bacterial homologs. Large PAPs are homodimeric proteins with subunits bound by a disulfide bridge. The monomeric subunits of large PAPs are 55 kilodaltons (Olczak et al., 2003; Strater et al., 1995). Several physiological roles have been proposed for these large

PAPs: acquiring inorganic phosphate under phosphate-deficient conditions, scavenging P_i from extracellular phosphate-esters during P_i deprivation; remobilizing of phosphate from metabolically less active tissue to developing or growing tissue (Smith et al., 1990). In *Arabidopsis thaliana*, there are 29 predicted PAPs, differing in their expression patterns. To date only *PAP11* and *PAP12* are known to be induced by phosphate starvation (Li et al., 2002; Zhu et al., 2005; this work).

Phosphate Starvation Response

A few transcription factors have been identified that are implicated in regulating phosphate responsive genes. A conserved MYB transcription factor in *Arabidopsis*, *PHR1*, involved in phosphate starvation signaling, was shown to act downstream in the P_i starvation signaling pathway (Rubio et al., 2001). The P_i-responsive expression of *AtIPS1*, *At4* and *RNS1* genes is impaired in the *phr1* mutant. PHR1 binding sites are also found in the upstream regions of phosphate starvation-responsive genes (Rubio et al., 2001). In rice, a transcription factor, *OsPF1*, with a basic helix-loop-helix domain is involved in tolerance to phosphate starvation (Yi et al., 2005).

We do not know much about the phosphate sensing and signaling pathways in plants, but several mutants have been isolated that may shed some light. The *Arabidopsis* mutant *pho1*, is deficient in xylem loading, accumulating only 5% as much free phosphate and 24 to 44% of total phosphate relative to wild-type plants (Poirier et al., 1991). The *pho1* mutant defines a novel class of proteins involved in ion transport in plants (Hamburger et al., 2002). There are 11 members of the *PHO1* gene family in

Arabidopsis having broad patterns of expression in leaves, roots, flowers, and stem (Wang et al., 2004).

The *pho2* mutant in *Arabidopsis* accumulates up to 3-fold more total P_i in leaves than wild-type seedlings (Delhaize and Randall, 1995). This mutant has a 2 fold greater P_i uptake rate than wild-type plants under P_i-sufficient conditions (Dong et al., 1998). Map-based cloning recently identified *PHO2* (At2g33770), as an unusual E2 conjugase gene that is targeted by a microRNA, miR399 (Bari et al., 2006; Aung et al., 2006).

The *phosphate underproducing* mutants (*pup*) from *Arabidopsis* have reduced staining for acid phosphatase activity in roots of plants grown under low-phosphate concentrations (Trull and Deikman, 1998; Tomscha et al., 2004). These mutants might help determine the role of acid phosphatases in plant response to phosphorous starvation, although none of the *pup* genes have been cloned, yet.

The *pdr2* mutant has disrupted local P_i sensing and may help understand the mechanism that monitors environmental P_i status and regulate cell cycle progression. The *pdr2* mutation does not affect P_i-responsive gene expression but inhibits in a P_i -dependent manner cell division in primary root tips and subsequent root growth (Ticconi et al., 2004). This mutant has been mapped but the gene has not been identified.

The *At4* mutant has an altered internal allocation of P_i. The *At4* gene contains multiple short open reading frames whose expression is induced by P_i-starvation (Shin et al., 2006).

Although several P_i-responsive genes and transcription factors have been identified, we still do not know how the molecular mechanism that regulates P_i

homeostasis in plants. The above mutants should help us to understand how plants sense and respond to phosphate.

This dissertation will focus on understanding transcriptional regulation of *PAP12*, a P_i-responsive gene from *Arabidopsis thaliana*. The approach we have taken is to establish that *PAP12* is a phosphate-repressible gene and study its regulation at the molecular level. This will allow us to engineer or design crops to be more phosphate efficient, develop more precise methods to monitor crop phosphate status, and give us insight as to how plants sense phosphate and respond to phosphate starvation. This could ultimately lead to a reduction in the agricultural requirement of P_i, while maintaining environmental quality, and minimize the use of non-renewable resources. Efficient use of P_i will be important as a potential phosphate crisis looms for agriculture in the twenty-first century (Abelson, 1999).

CHAPTER II

CHARACTERIZATION OF *PAP12* EXPRESSION IN *Arabidopsis*

thaliana

Introduction

Despite decades of research using forward and reverse genetic approaches, we have few details of how gene expression is regulated in response to P_i limitation. Most phosphate responsive mutants are defective in structural or downstream genes. Others are defective in phosphate accumulation, such as *pho1* which is deficient in the accumulation of P_i (Poirier et al., 1991) and *pho2*, which accumulate too much P_i (Delhaize and Randal, 1995). Phosphate-starvation response mutants include, *psr* (Chen et al.,2000), *phosphatase-underproducer 1*, (*pup1)* (Trull and Deikman, 1998; Tomscha et al., 2004), *At4*, a gene predicted to contain multiple short open-reading frames (ORFs), whose expression is strongly induced by P_i-starvation and is involved in internal allocation of P_i (Shin et al., 2006), and *phosphate starvation response 1* (*phr1*) (Rubio et al., 2001). The PHR1 protein contains a MYB domain and a coiled-coil domain, and mutants show reduced P_i-inducibility of some P_i-starvation induced genes (Rubio et al., 2001). Thus, there have been few regulatory genes (transcription factors, proteins involved in signaling or sensing phosphate, kinases or phosphatases involved in modulating the phosphate starvation response (PSR) or other proteins involved in the PSR signal transduction pathway) identified using a forward genetics approach. This lack of success may be because plants have a highly redundant system that senses and regulates

the amount of phosphate in the plant, leading to a tight P_i homeostasis. Mutations in these regulatory genes may be lethal, as having too much or too little phosphate can result in toxicity or poor to no growth. Limited insight into the phosphate starvation response may be gained by a forward genetics approach.

We use a reverse genetics approach in order to understand and identify components involved in the phosphate starvation response in *Arabidopsis thaliana*. We identified a phosphate-starvation induced gene, *Purple Acid Phosphatase 12* (*PAP12*) At2g27190, proposed to function in scavenging P_i from the environment under conditions of P_i-deficiency. To identify its role in the PSR, we obtained a *pap12*-null mutant and looked for phenotypes under low P_i growth conditions. A phosphate responsive element (PRE), important for expression during phosphate starvation, was identified in the *PAP12* promoter using an approach that combined *PAP12* promoter deletions analysis and EMSA. We also isolated and attempted to identify the *PAP12* phosphate responsive element binding protein (PAP12 PREBP) using a avidin-biotin affinity purification approach.

Here, we characterize the expression of *PAP12* in *Arabidopsis* in response to P_i-starvation and establish that it is induced by phosphate starvation. We also examine *PAP12* expression in two mutants, *pho1* and *pho2*. To determine its role in the phosphate starvation response, we identified a T-DNA insertion line from Sygenta's collection of T-DNA lines. A *pho1 pap12-1* double mutant was made to study the effects of the loss of PAP12 activity in a low-phosphate environment.

Materials and Methods

Accession Numbers

Arabidopsis Genome Initiative locus identifier for *PAP12* is At2g27190.

Arabidopsis Seed Sterilization

Arabidopsis thaliana seeds were first rinsed in 70% ethanol then sterilized in 50% bleach, 0.1% Triton X-100 solution for 7 minutes on a rotating platform. The seeds were then rinsed 3 times with sterile MQ water and plated on MS media with 1% sucrose (Murashige and Skoog, 1962).

Arabidopsis Growth Conditions

Five to seven seeds were added to a 125 ml-flask containing liquid 1xMS Media (Caisson Laboratories, Inc., catalog #: MSP009) with 1% sucrose. Seeds were allowed to grow for 7 days with continuous shaking at 110 RPM under 24 hour light at 22°C. Seven day old seedlings were then used for nutrient starvation experiments. Seedlings were transferred to media lacking phosphate, potassium or iron for different time periods and extracted for RNA.

Plant Media

Plants were grown in Murashige and Skoog (MS) media purchased from (Caisson Laboratories, Inc., catalog #: MSP009). P_i-deficient media was purchased from (Caisson Laboratories, Inc., catalog #: MSP011). MS salts were used to make potassium-deficient media, with the following substitutions: KNO_3, KH_2PO_4, and KI were omitted and replaced with $NaNO_3$, NaH_2PO_4 and NaI respectively. MS salts were used to make iron-deficient media, with the following change: Fe[EDTA] complex was omitted.

Soil-grown Plants

Plants were grown in growth chambers using RediEarth soil under conditions of 24 hour light/0 hour dark (152 umol m^{-2} s^{-1}), at 22°C.

PAP12 cDNA Isolation

Partial protein sequence from a soybean PAP (LeBansky et al., 1992) was used to screen a collection of *Arabidopsis* ESTs. Oligonucleotide probes from a PAP EST was used to screen a lambda ZAP cDNA library. The cDNA was then used to screen a lambda ZAP *Arabidopsis* genomic DNA library (ecotype *Landsberg erecta* kindly donated by Dr. Terry Thomas). Multiple clones of *PAP12* were isolated, both genomic and cDNA; DNA sequence of the longest *PAP12* cDNA (*PAP1*) was deposited into Genbank with accession number of U48448.

RNA Manipulation

RNA extraction was performed using Tri reagent (Sigma) according to the manufacturer's instruction and separated on 1.2% (w/v) formaldehyde agarose gels. RNA blots were transferred and hybridized to Hybond-N+ nylon membrane (Amersham Pharmacia) according to manufacturer's instruction. The *PAP12* probe, random primer-labeled, was a 1.6 kb fragment consisting of the 5' and 3' UTR and the entire coding region. A probe for an *Arabidopsis* tubulin gene (Marks et al., 1987) was used as a loading control. Blots were prehybridized in HybII buffer (1% BSA, 1mM EDTA, 0.5M NaHPO$_4$, 7% SDS) and hybridized overnight at 65 degrees C. Blots were washed first in 2x SSPE, followed by 3 washes in 2x SSPE/0.1%SDS, and then exposed on film or on phosphoimager plate.

Acid Phosphatase Activity Gels

Plant material was extracted using GUS extraction buffer (50 mM sodium phosphate buffer, pH 7.0; 10 mM ß- mercaptoethanol; 10 mM Na$_2$EDTA; 0.1% sodium lauryl sarcosine; 0.1% Triton X- 100). Tissue was ground to a fine powder using liquid nitrogen. Protein quantification was performed using the Bio-Rad protein assay according to manufacturer's instructions (BioRad, Catalog 500-0006). Equal amounts of protein were loaded onto discontinuous native PAGE, 5% (w/v) stacking gel, 10% (w/v) resolving gel. Gels were run at constant 20 mA at 25°C. Gels were rinsed twice in 5mM NaCl, 100mM sodium acetate (pH 5.2). Gels were developed overnight at 25°C in

development solution (5mM NaCl, 100mM sodium acetate pH 5.2, .02% α-Naphthyl

Acid Phosphate (Sigma, N-700), 0.02% Fast Garnet GBC Salt (Sigma, F-8761).

P_i and Total Phosphate Assay

Total phosphate and free phosphate (P_i) were measured according to Ames (1966).

Isolation of PAP12 T-DNA Line

An *in silico*-based method was used to identify a T-DNA insertion line from the SAIL

collection created by Syngenta. To isolate a line with a T-DNA insertion in the *PAP12*

gene, a PCR-based method was used. The flanking sequences of the T-DNA insertion in

the *PAP12* gene were confirmed by genomic PCR with primers as follows: a T-DNA left

border primer, LB3 (5'-TAGCATCTGAATTTCATAACCAATCTCG -3'); and two

PAP12 gene specific primers; 5' primer for *PAP12*, (5'-

CAACATCAACCTTGTATTTGACAAACC-3'); a 3' primer for *PAP12*, (5'-

CCAGACGGTTCTGGTTCTTTATCCC-3').

Western Blot Using αPAP12

Antibodies were generated in New Zealand White rabbits from recombinant PAP12

protein expressed in *E.coli*. Equal amounts of proteins were loaded onto SDS-PAGE

gels and blotted to PVDF membrane (GE Osmonics Labstore, Minnetonka, MN).

Detection of PAP12 protein was carried out using a 1:3000 dilution of the αPAP12

primary antibody and a 1:3000 dilution of alkaline phosphatase-linked anti-rabbit

secondary antibody. Proteins were blotted onto PVDF membrane using CAPS buffer

(10mM CAPS (pH 11.0), 10% methanol) at 350 mA for 1.5 hours. Blots were then

incubated in blocking buffer (2% BSA in TBS, 100 mM Tris-HCl, 150 mM NaCl, pH

7.5) for 3 hours at room temperature, followed by an over night incubation with primary

antibody in blocking buffer. Blots were washed for a total of 5 times with TBS for 5

minutes each at room temperature. Secondary antibody was added and allowed to

incubate for 1 hour at room temperature followed by 5 washes in TBS. Blots were

developed using the Bio-Rad AP Conjugate Substrate Kit (Bio-Rad Laboratories, Inc.,)

according to manufacturer's instruction.

Results

Identification of PAP12

PAP12 was identified by screening an *Arabidopsis* flower cDNA library. The *PAP12*

cDNA was then used to screen an *Arabidopsis* genomic library. We sequenced the gene

and deposited it in the NCBI database (accession # U48448), where it is identified by its

original name *PAP1*.

PAP12 Gene Structure and Proposed Function

The *PAP12* (At2g27190) gene contains 7 exons separated by 6 introns. The cDNA

contain an ORF of 470 amino acids. The protein has a molecular weight of 54,163

daltons, and contains a putative signal sequence at the N-terminus but no other known targeting signals, indicating that it is most likely an extracellular protein.

It is 87% similar to *Ipomoea batatas* PAP (gi|4210712|emb|CAA07280.1|), 84% to *Nicotiana tabacum* PAP (gi|27597231|dbj|BAC55156.1|), 85% to *Medicago truncatula* PAP (gi|60459337|gb|AAX20028.1|), 81% to *Oryza sativa* PAP (japonica cultivar-group) (gi|34910020|ref|NP_916357.1|), 83% to *Glycine max* PAP (gi|6635439|gb|AAF19820.1|), and 82% to *Phaseolus vulgaris* PAP (gi|2344871|emb|CAA04644.1|). Thus *PAP12* has homologs present in monocots and dicots.

PAP12 contains a metallo-phosphoesterase domain and the complete set of residues involved in the ligation of the dimetal nuclear center found in other PAPs (Li, 2002). PAP12, belonging to the high molecular weight type PAPs, is a homodimer held together by a disulfide linkage.

PAP12 Expression is Induced Upon Phosphate Starvation

Previous groups have reported an increase in acid phosphatase activity upon P_i starvation in several species (tomato, soybean, potato). To test whether this is true of *PAP12*, we looked at *PAP12* expression in plants grown on P_i-rich and P_i-deficient media. We also looked at *PAP12* expression in the *pho1* and *pho2* mutant.

In order to use *PAP12* as a tool to understand the PSR in *Arabidopsis*, we needed to determine whether *PAP12* was specifically responsive to phosphate or induced by general nutrient starvation. To distinguish among these possibilities *PAP12*, we

starved *Arabidopsis* seedling for P_i and two other macronutrients, Fe and K. Seedlings

were grown in P_i-rich media for 5 days and then transferred to P_i-deficient media for 6,

12, 24, 48, and 72 hours. Plants starved for 72 hours were transferred to media with P_i

for 24 hours to see if *PAP12* is repressed by phosphate in addition to being induced by

the absence of phosphate, i.e. phosphate repressed. Northern blots containing RNA from

these samples were probed with cDNA for *PAP12* and *tubulin*. Seedling grown in media

with P_i showed *PAP12* transcripts were present at low levels, but were induced to high

levels upon P_i-starvation. *PAP12* transcript levels were repressed in seedlings that were

shifted to media with phosphate for 24 hours after being starved for 72 hours in media

without phosphate. Seedlings starved 24 hours for K or Fe showed little change in

PAP12 transcript levels compared to seedlings starved for phosphate for 24 hours

(Figure 1).

PAP12 Expression in Arabidopsis Wild-type, pho1 and pho2 Mutants

To determine in what tissues *PAP12* is expressed, a northern blot was performed using

RNA isolated from soil grown roots, leaves, stems, siliques, and flowers, and hybridized

with the *PAP12* cDNA. *PAP12* was present in all tissues examined, with highest

transcript in flowers (Figure 2). This is consistent with the results obtained by Li et al.

(2002). *PAP12* was expressed in a phosphate dependent manner in both *pho1* and *pho2*

mutants. *PAP12* transcript levels are increased in *pho1* leaves, which have low amount

of phosphate. To test whether this was due to low phosphate levels and not a defect in

signaling, we analyzed *PAP12* expression in *pho1* plants grown in P_i-rich and P_i-

deficient media. *PAP12* expression was low in *pho1* plants grown in phosphate and high

in plants grown in media without phosphate (Figure 3). The expression of *PAP12* in the

pho1 mutant can be down regulated by supplying exogenous phosphate. In *pho2* leaves,

which contain 2.5 times higher levels of phosphate than wild type, *PAP12* transcripts

were low (Figure 3). Therefore *PAP12* expression was similar whether P_i levels are

controlled exogenously by growth in P_i-rich or P_i-poor media or endogenously

controlled in *pho1* and *pho2* mutants.
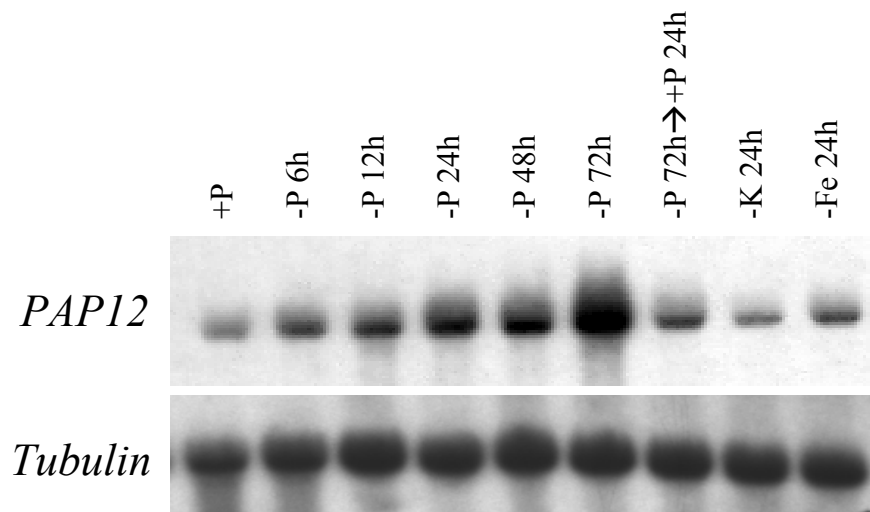
Figure 1. *PAP12* expression in plants starved for P, K and Fe.

Arabidopsis plants were grown in MS media with phosphate, transferred 5 days after germination to media without phosphate, potassium or iron for the selected time points. Total RNA was extracted from these plants, and a northern blot was performed with 10 µg of RNA per lane. The blot was hybridized with a *PAP12* probe and a *Tubulin* probe was used to ensure equal loading of RNA.
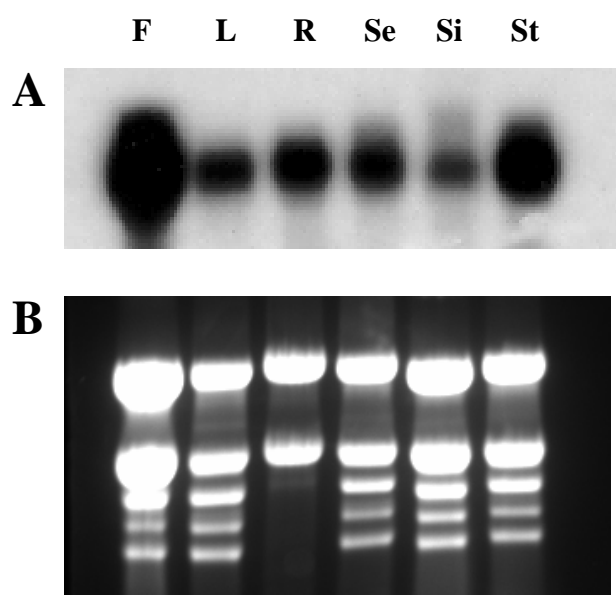
Figure 2. *PAP12* expression in tissues of soil grown *Arabidopsis* plants.

(A) RNA was isolated from the indicated tissues: flower (F), leaf (L), root (R), seedlings (Se), silique (Si), and stem (St). 10 µg of total RNA was loaded per lane for RNA blot analysis, which was hybridized with a *PAP12 probe*.
(B) Ethidium bromide-stained RNA image.
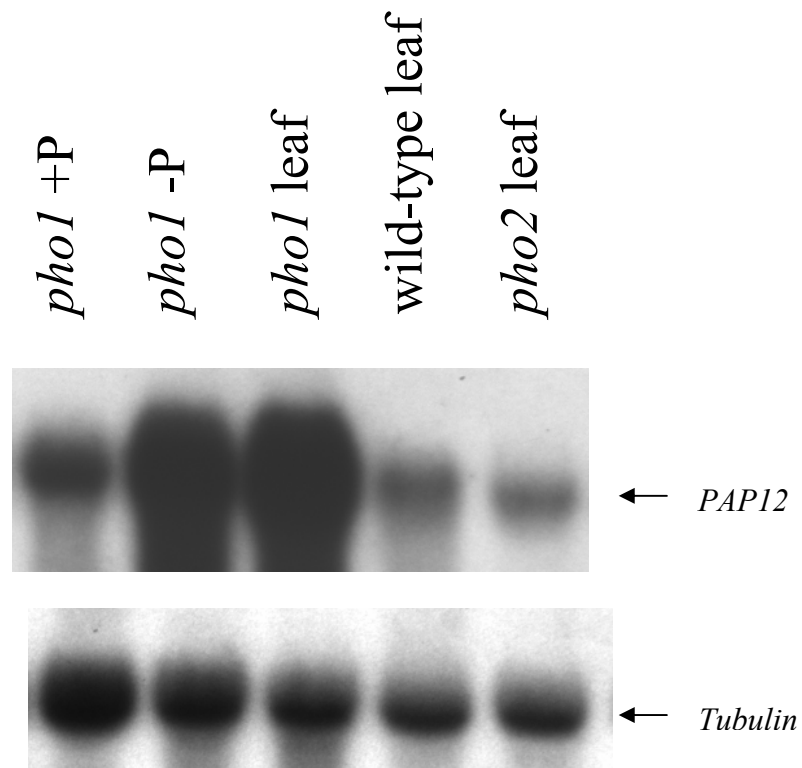
Figure 3. *PAP12* expression in wild-type, *pho1*, and *pho2* mutants.

pho1 seedlings were grown in media with phosphate (+P) and then transferred to media without phosphate (-P) for 48 hours. Leaf tissue from *pho1*, *pho2* and wild-type plants was harvested from 2 week old soil grown plants. RNA gel blots containing 10 μg of total RNA per lane were hybridized with a *PAP12* and *Tubulin* probe.

Identification of PAP12-1, a T-DNA Insertion Line

To determine the role of *PAP12* during the PSR, we obtained a T-DNA insertion in the

PAP12 gene (SAIL_1187_A05) in the Columbia background by searching the SAIL

(formerly GARLIC) Collection available from Syngenta Biotechnology, Inc; we have

designated this mutant as *pap12-1*. Homozygous *pap12-1* lines were selected by PCR

analysis using gene specific primers and a T-DNA specific primer. The T-DNA

insertion was localized to the third intron, resulting in a truncated mRNA in the *pap12-1*

mutant versus wild-type Columbia plants (Figure 4, Figure 5).

Phenotype, Growth and P_i Content of pap12-1

Plant growth, seed set, germination, and total and free phosphate levels were not affected

in *pap12-1* when grown on soil. Growth of *pap12-1* on various phosphate sources was

also not affected. Wild-type and *pap12-1 Arabidopsis* seeds were germinated on MS

media, where potassium phosphate was substituted with 1.25 mM glucose-1-phosphate,

ά-napthyl phosphate, glycerol-2- phosphate, o-phosphoethanolamine or hydroxyapaptite.

We did not observe any visible phenotype between the *pap12-1* and wild-type plants in

seed germination or overall plant growth on these different phosphate sources (Figure 6).

We also measured total and free phosphate in *pap12-1* and wild-type plants to see if

PAP12 was involved in redistribution or remobilization of phosphate from old leaves to
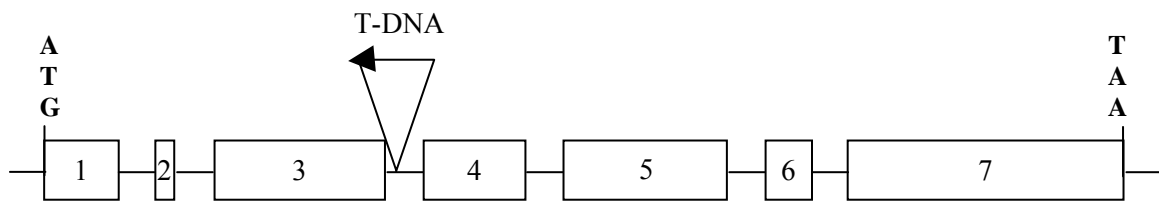
Figure 4. T-DNA insertion in *PAP12*.

The *PAP12* gene has 7 exons interrupted by 6 introns. A plant containing a T-DNA insertion in the third intron was identified from the GARLIC lines. Two gene specific primers and a T-DNA specific primer were used to genotype homozygous lines.

1   2

Figure 5. *PAP12* transcript expression in the *pap12-1* T-DNA insertion line.

RNA gel blot analysis using 10 µg of total RNA from *pap12-1* (lane 1) and wild-type (lane 2) leaf were hybridized with a *PAP12* probe. Plants were grown on soil for 2 weeks and leaves were extracted.
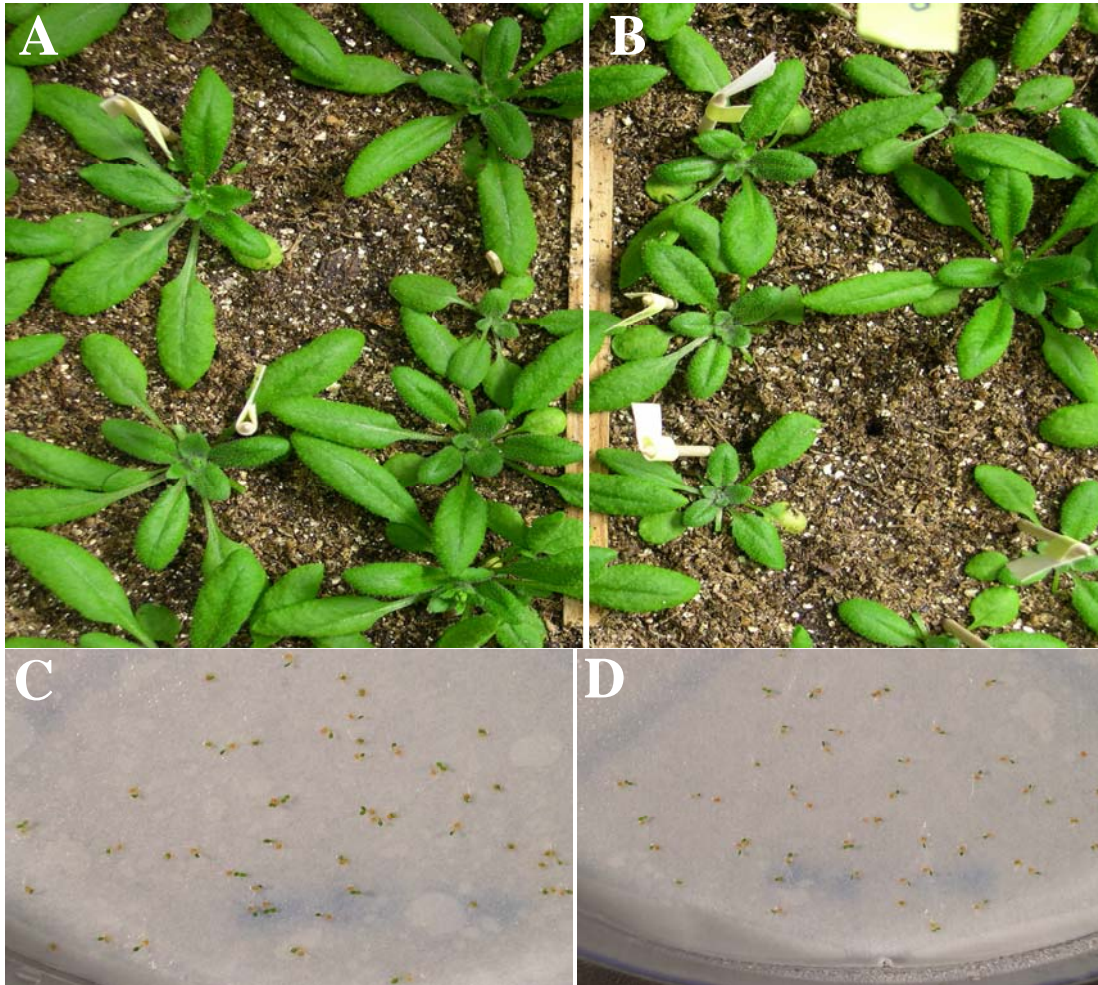
Figure 6. Germination and growth of wild-type and *pap12-1* plants on soil.

Panel A and C show wild-type *Arabidopsis* (ecotype Columbia) plants and B and D show *pap12-1* plants. Seeds were germinated on Whatman paper. For growth analysis, plants were directly germinated on soil and grown under constant light.

new growing leaves. There was no significant difference in total or free levels of phosphate in true leaves or dry seeds (Table 1).

The *pho1 pap12-1* double mutant displayed a delay in flowering time of about 2 weeks compared to that of the *pho1* single mutant (Figure 7). Total phosphate content of *pho1* and *pho1 pap12-1* was measured in leaf tissue and dry seeds. There was no significant difference in the total phosphate content between *pho1* and *pho1 pap12-1* mutants in leaves or in dry seeds (Table 1). We also did not observe any obvious difference in growth between the *pho1* and *pho1 pap12-1* mutants during the vegetative phase of growth (Figure 8).

To test the hypothesis that the role of *PAP12* may be in the activation of a stress-induced flowering pathway, we over-expressed *PAP12* in the *pho2* and wild-type backgrounds. We decided to use *pho2* and wild-type because *PAP12* expression is low in these backgrounds. We did not over-express *PAP12* in the *pho1* mutant because *PAP12* expression was already high. The prediction is that if a *PAP12* knockout has a delay in the timing of flowering then over-expression of *PAP12* may cause acceleration of flowering. We have successfully generated plants over-expressing *PAP12* in the *pho2* background but have not been able to obtain lines with increased PAP12 activity in the wild-type Col-O background. The *pho2* plants over-expressing *PAP12* flowered 4-5 days earlier than *pho2* (Figure 9).

Table 1. P_i content in wild-type and *pap12-1* plants grown in soil

Plant genotype	Total P_i leaf content nmoles/mg	Free P_i leaf content nmoles/mg
wild-type	19.19 +/- 3.87 n=12	15.55 +/- 2.95 n=12
pap12-1	21.52 +/- 4.6 n=12	17.45 +/- 3.08 n=12
pho1	5.1 +/- 2.1 n=10	
pho1 pap12-1	6.3 +/- 2.1 n=11	

Plant genotype	Total P_i seed content nmoles/mg
wild-type	269.3 +/- 13.2 n=5
pap12-1	238.75 +/- 16.4 n=5
pho1	121.89 +/- 21.1 n=5
pho1 pap12-1	111 +/- 17.7 n=5

Plants were grown on soil and leaf Pi content was measured on individual plants. Values represent mean +/- SD from 1 leaf.

pho1 pap12-1   pho1

Figure 7. Delayed flowering of *pho1* and *pho1 pap12-1* mutants.

Seeds of *pho1* and *pho1 pap12-1* mutants were germinated directly on soil and grown under constant light. Plants displayed here are 37 days old.

pho1   *pho1 pap12-1*

Figure 8. Development of *pho1* and *pho1 pap12-1* mutants.

Seeds of *pho1* and *pho1 pap12-1* mutants were germinated directly on soil and grown under constant light. Plants shown here are 3 weeks old.

pho2   *pho2 35S::PAP12*
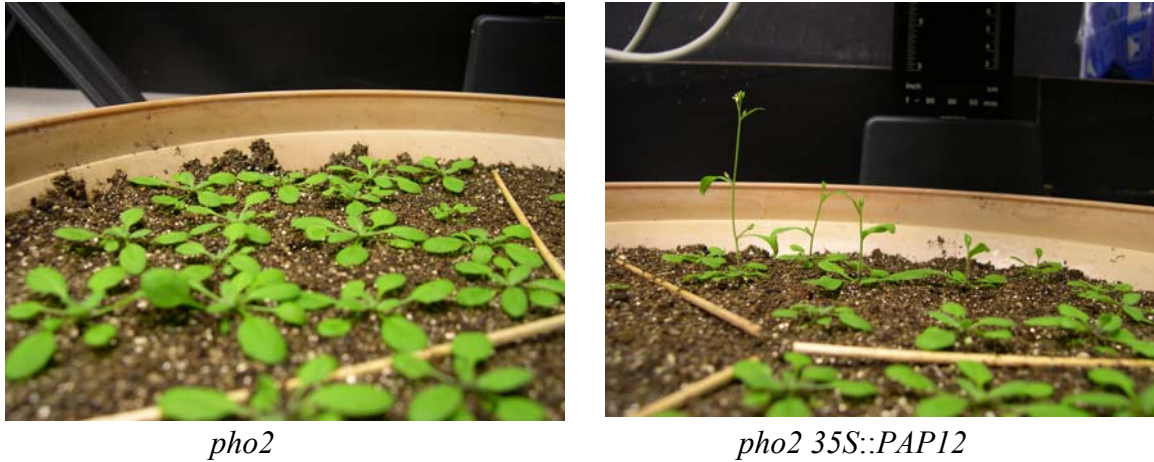
Figure 9. Flowering phenotype of *PAP12* over-expression in the *pho2* background.

Plants were germinated on MS media and transferred to soil. Three weeks old plants are shown here. Plants were grown under 18 hour light/6 hour dark cycle, 22°C and 75 % humidity.

Acid Phosphatase Activity in Arabidopsis

There have been many published reports where acid phosphatase activity is measured using the classical in-gel assay from different species (Pan, 1987; Lefebvre et al., 1990; Asghar and DeMason, 1992; Lebansky et al., 1992; Murray and Collier, 1997; Tso and Chen at al., 1997; Grierson and Comerford, 2000; Turner and Plaxton, 2001; Jiang et al., 2003; Cirkovic et al., 2002; Huttova and Mistrik, 2002; Tejera Garcia et al., 2004; Zimmermann et al., 2004). Several plant acid phosphatase isoforms have been identified in *Arabidopsis* by in-gel staining using napthyl-phosphate as a substrate (Trull and Deikman, 1998; Trull et al., 1997; Yan et al., 2001). To determine which isoform might be PAP12, we performed the same in-gel acid phosphatase assays in the *PAP12* T-DNA insertion line. We also assayed the following tissues for APase activity: *pho1* leaf, *pho2* leaf and wild-type leaf, roots, stem, flower and silique. Proteins were extracted from the indicated tissues and separated on non denaturing poly-acrylamide gels. After the proteins were separated, the gels were stained for acid phosphatase activity with α-Naphthyl Acid Phosphate/Fast Garnet GBC Salt.

Results from the in-gel assay using napthyl-phosphate as a substrate indicate that *PAP12* is responsible for the majority of the phosphate starvation inducible APase activity (Figure 10). In wild-type tissue extracts, a major band of activity was detected. This band of activity increased in response to low phosphate (*pho1* leaf) and decreased in response to high phosphate (*pho2* leaf). In the *pap12-1* extract, this major, phosphate responsive band was not detectable. Acid phosphatase activity from the *pap12-1 pho1* double mutant also did not detect this major band of activity (Figure 11). Gels using two
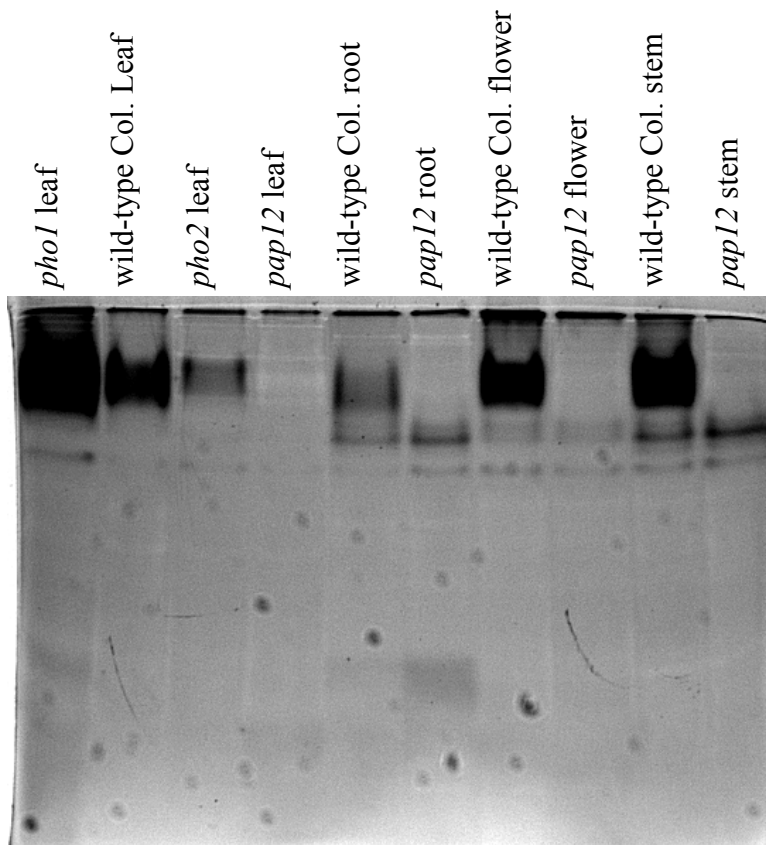
Figure 10. Acid phosphatase activity from various tissues of wild-type and mutants.

A 7.5 % native discontinuous PAGE containing 30 μg of total protein per lane from the indicated tissues was stained to detect Apase activity. Plants were grown on soil. The gel was run under non-reducing conditions at room temperature and stained overnight at room temperature using Fast Garnet GBC Salt and α-Naphthyl Acid Phosphate.
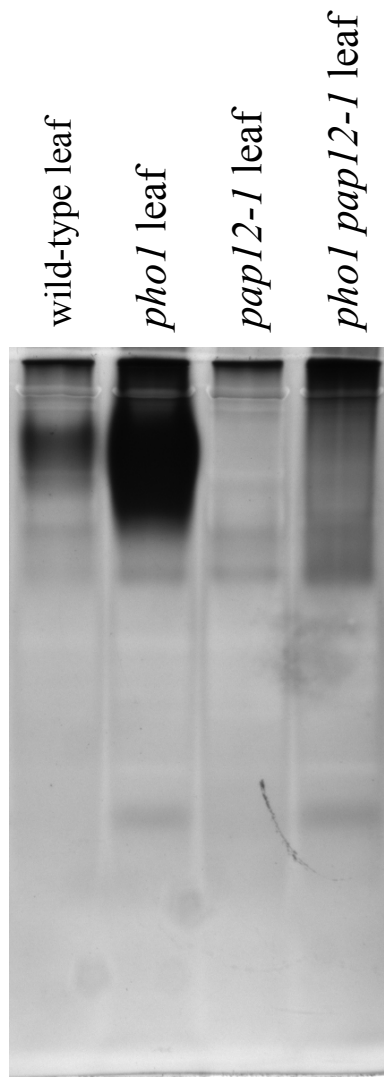
Figure 11. APase activity in wild-type, *pho1*, *pap12-1*, and *pho1 pap12-1* mutants.

Proteins were extracted from leaves of 14 day old plants grown in soil. 50 μg of protein extracts were run on a 7.5% non-denaturing, non-reducing polyacrylamide gel. The gel was run at room temperature and stained overnight at room temperature using Fast Garnet GBC Salt and α-Naphthyl Acid Phosphate.

other substrates, BCIP and Fast Black/β-naphthyl phosphate, showed the same pattern of activity as with α-naphthyl phosphate/Fast Garnet GBC Salt (data not shown).

Detection of PAP12 Protein Levels in Arabidopsis

To determine if *PAP12* is phosphate responsive at the protein level, recombinant PAP12 protein was used to generate polyclonal antibodies in rabbits. Western blots indicated that PAP12 was induced upon P-starvation at the protein level. Two major bands, one at approximately 114 kilodaltons and the second at 47 kilodaltons were detected by αPAP12 antibodies in total protein extracts from leaf, flower, root, stem, and silique of wild-type soil grown plants (Figure 12). The band at 114 kilodaltons correlated with P-status (Figure 13). The intensity of this band was strong in *pho1* leaf extract and low in wild-type leaf extracts and *pho2* leaf extracts. Absence of this band in the *pap12-1* T-DNA insertion line supports the idea that the band detected at 114 kilodaltons is PAP12. A denaturing protein gel stained with coomassie is shown for equal loading (Figure 14).

Discussion

Here, we examined the role of *PAP12* during the PSR of *Arabidopsis*. *PAP12*, which encodes a purple acid phosphatase, belongs to the metallo-phosphoesterase family of proteins (Strater et al., 1995; Li et al., 2002). There are 29 predicted PAPs in *Arabidopsis*, each having a differing expression pattern (Li et al., 2002; Zhu et al., 2005). To understand the role of PAP12 in the PSR and use *PAP12* as a tool to study the

PSR in *Arabidopsis,* functional analysis and expression was performed. Our results

indicate that *PAP12* is induced upon phosphate starvation; is not induced by general

nutrient stress;
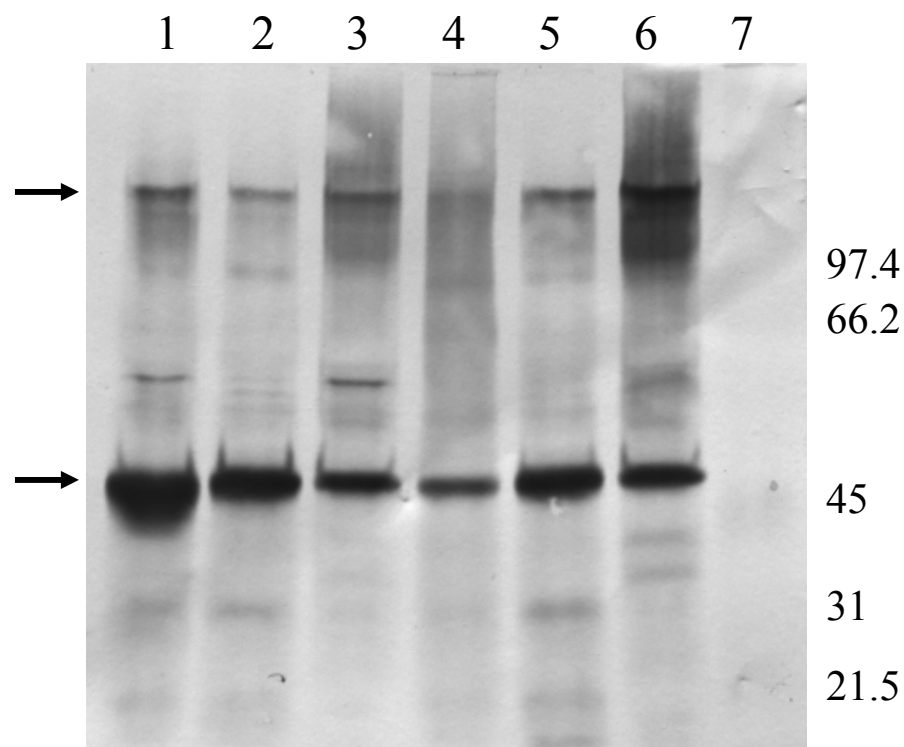
Figure 12. Immunoblot analysis of PAP12 in major organs.

Plant tissues were harvested from soil grown plants. Total proteins were separated on a 8% SDS-polyacrylamide gel under non-reducing conditions and analyzed with polyclonal antibodies against PAP12. Lane 1-6 contains 100 μg of total protein from wild-type stem, silique, leaf, root, flower, and *pho1* leaf respectively. Lane 7 contains prestained low range SDS-PAGE standards with sizes indicated at right in kilodaltons. Arrows on the left indicate two major bands detected by αPAP12 antibodies.
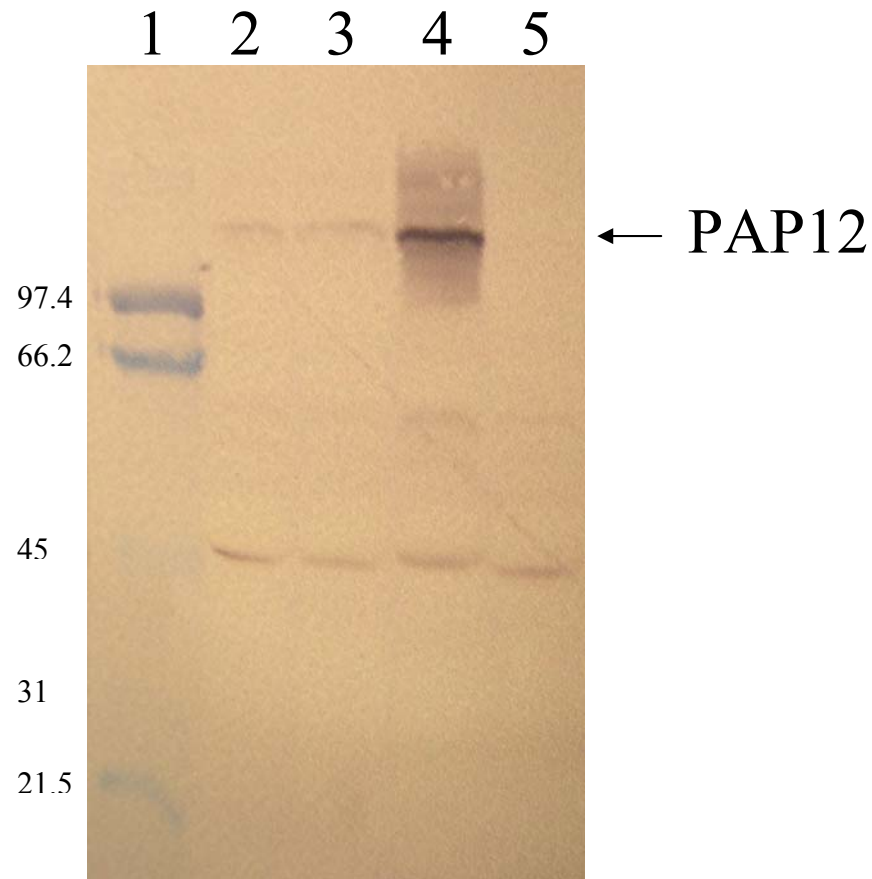
Figure 13. Immunoblot analysis of PAP12 in *pho2*, wild-type, *pho1* and *pap12-1* plants.

Samples were analyzed on an 8% SDS-polyacrylamide gel under non-reducing conditions with polyclonal antibodies against PAP12. Lane 1 contains a prestained low range SDS-PAGE standards with sizes indicated at left in kilodaltons. Lane 2-5 contains 100 μg of leaf proteins from *pho2*, wild-type, *pho1*, and *pap12-1* respectively. PAP12 band is indicated with an arrow.
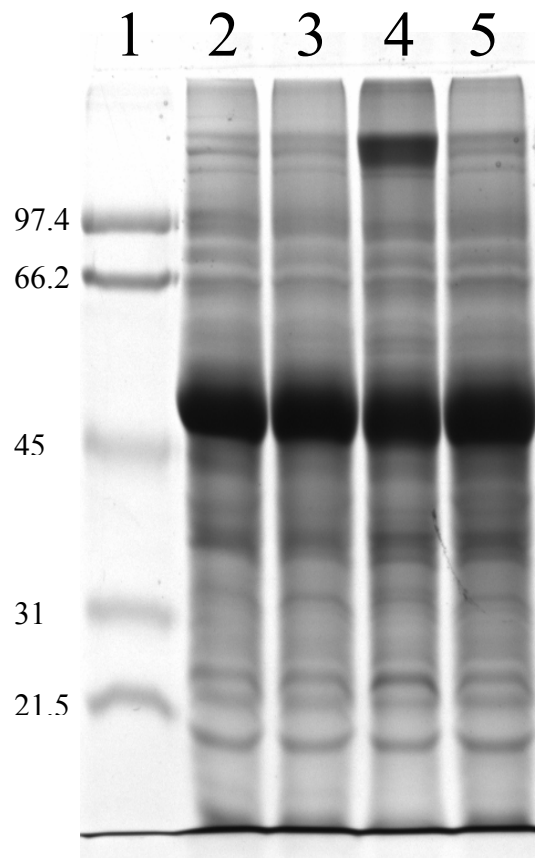
Figure 14. SDS-PAGE analysis of total proteins from *pho2*, wild-type, *pho1* and *pap12-1* leaf tissue.

Total protein extracted from leaf was electrophoresed on an 8% SDS-polyacrylamide gel under non-reducing conditions. Lane 1 contains low range SDS-PAGE standards with sizes indicated at left in kilodaltons. Lane 2-5 contains 100 µg of leaf proteins from *pho2*, wild-type, *pho1*, and *pap12-1* respectively. Proteins were visualized by coomassie blue staining.

is responsible for the majority of the phosphatase activity as determined by the classical in-gel assay; has a broad expression pattern and does not play a major role in plant adaptation to phosphorus deficiency under the conditions tested. *PAP12* does, however, have a strong effect on flowering time in *pho1*, a mutant with low endogenous P_i concentrations.

To use *PAP12* as a tool to study the PSR in *Arabidopsis*, we need to first establish that it is responsive to phosphate and not other macronutrients. RNA gel blots indicate that *PAP12* is induced in a phosphate dependent manner (Figure 1). The response is specific to phosphate and not a general nutrient starvation response. *PAP12* is not significantly induced upon starvation to potassium or iron (Figure 1). In two mutants analyzed, *PAP12* is also expressed in a phosphate dependent manner. In the *pho1* background, whose leaves are deprived of phosphate compare to wild-type plants, *PAP12* is highly expressed. The *pho2* mutant, which has about 2.5 times more P_i than wild-type plants, had less *PAP12* transcript present in the leaves than wild-type. Taken together, the expression of *PAP12* was responsive to phosphate, and its pattern of expression was consistent in *pho1* and *pho2* mutants with that of plants grown in media with or without P_i. Its expression in response to phosphate supports a presumed role in phosphate metabolism, despite the minimal effect of a *PAP12* null mutation in the Columbia background.

PAP12 was expressed in all the major tissues as indicated by RNA gel blot analysis and western blots using αPAP12 antibodies (Figure 2, 12). Thus, it may play or have a broader role in P metabolism than previously thought. It may accumulate in

vacuoles to scavenge phosphorus (Duff et al., 1994). The sub-cellular localization of PAP12 will be important to elucidate its role in plant processes.

Numerous papers have used the classical in-gel assay to detect APase activity. We showed that PAP12 is responsible for the most abundant inducible isoform detected by the in-gel assay. Using another substrate, p-nitrophenylphosphate (pNPP), showed that total APase activity is unchanged in wild-type leaf and *pap12-1* leaf (data not shown). It seems that the activity that other groups have reported is mostly that of *PAP12* and not the other 28 PAPs in *Arabidopsis*. This is a surprise because it was thought these enzymes have broad substrate specificity, but it seems that PAP12 has specificity for naphthyl phosphate, while most of the other PAPs cannot use naphthyl phosphate as a substrate or they are not expressed at a significant level. Thus these in-gel assays cannot be used to observe or determine the total APase activity. A new assay or other substrates will be needed to observe the activity of the other PAPs.

PAPs are abundant in plant tissues, capable of hydrolyzing a broad spectrum of substrates. Although their function is unknown, it has been proposed that their expression, regulated by phosphate levels in the medium and soil, suggests a role in phosphate acquisition. They can be secreted outside root cells to hydrolyze phosphate esters present in the soil. PAPs localized to vacuoles could be involved in the utilization of some vacuolar metabolites. They could also be involved in remobilizing phosphate from senescent tissue to growing tissue (Smith et al., 1990; Snapp and Lynch, 1996). Thus, remobilization of phosphorus from old to new tissue is an important determinant of phosphorous efficiency. Since PAP12 is expressed in all major plant tissues and is

only one of the two PAPs induced upon phosphate starvation (out of 29 PAPs present in *Arabidopsis*), we wanted to see if its activity confers adaptation to low phosphorus availability in *Arabidopsis*. We obtained a T-DNA line which has an insertion in the third intron, resulting in a truncated *PAP12* mRNA (Figure 5). There was no difference in germination, plant growth, total and free phosphate concentration in the *PAP12* T-DNA insertion line compared to wild-type plants when grown in soil (Table 1, Figure 6).

This is not surprising, as there are 28 other predicted PAPs in *Arabidopsis* with overlapping expression pattern to that of *PAP12*. Any one of these other PAPs could compensate for the loss of *PAP12*. Additionally, there could be compensation by other responses: morphological, biochemical, molecular, and physiological. To minimize the contribution from these other responses, we made a *pap12 pho1* double mutant. The rationale is that in the *pho1* background there is no further contribution from these responses, as they are already compensating due to the defect in xylem phosphate loading. Loss of *PAP12* activity in the *pho1* background was predicted to be magnified, possibly revealing a function or phenotype.

Analysis of the *pho1 pap12-1* double mutant showed a two-week delay in flowering time (Figure 7). There was no significant difference in total phosphate content of leaves from *pho1* and *pho1 pap12-1* nor were there any changes in leaf number, germination or seed set (Table 1, Figure 8). The double mutant took 11-14 days longer to flower than *pho1*. It may be that purple acid phosphatases play a role in the transition from vegetative to reproductive stage. Environmental and endogenous factors control timing of flowering in plants. However, some environmental stress factors disrupt this

tight control and cause acceleration of flowering. It has been proposed that any type of stress may cause plants to set seeds. This has been observed in two soybean cultivars. Here the authors observe that drought stress seemed to trigger an early switch from vegetative to reproductive development (Desclaux et al., 1996). Another relevant observation is that although 28 members of the PAP gene family differ in there expression patterns in vegetative tissues; they are all expressed in flowers (Zhu, 2005).

In the *pho2* background, expression of *PAP12* is repressed by high levels of phosphate, but expression of *PAP12* by the *35S* promoter, derived from the common plant virus, cauliflower mosaic virus (CaMV), overrides this regulation. We predict that the earlier flowering phenotype may not be observed in a wild-type background over expressing *PAP12*. This is because there will not be enough phosphate to support the shift into reproductive phase. Phosphorus is a limiting factor, after nitrogen, for plant growth. High phosphate levels will allow plants to flower earlier as their growth and development is no longer limited by phosphate, provided they have adequate amounts of the other macro and micro nutrients and optimum growth conditions (water, light, temperature, etc.). Only in the *pho2* background was a clear early flowering phenotype observed, as this mutant has 2.5 times higher levels of phosphate and flowers earlier than wild-type plants. We consistently observe the *pho2* mutant flowers much earlier than wild-type Col-O plants, while the *pho1* mutant takes much longer to flower under our growth conditions (24 hour light, 22° C, RediEarth soil). This may be due to pleiotropic effects of the *pho1* and *pho2* mutations, which needs to be investigated or a high correlation of flowering time and phosphate levels in these backgrounds.

Except for the striking effect on flowering times in *pho1* and *pho2* mutants our results did not indicate a major role for *PAP12* in helping plants adapt to low phosphorus availability, despite its expression in all major plant tissues and being up-regulated upon P starvation. This is consistent with results reported by a group working with common bean (Yan et al., 2001). Here the authors find that induction of a major leaf acid phosphatase, detected by the classical in-gel assay, does not confer adaptation to low phosphorus availability in common bean.

Analysis of another PAP, *PAP23*, also failed to identify its function in plant development (Zhu et al., 2005). In order to elucidate the biological functions of PAPs in plant development and their role in the phosphate starvation response, knockouts will have to be obtained for the remaining PAPs, and their sub-cellular location determined.

CHAPTER III

IDENTIFICATION OF A PHOSPHATE RESPONSIVE ELEMENT (PRE) IN

THE *PAP12* PROMOTER

Introduction

We know much about the molecular responses to P_i starvation in plants, but have very little knowledge of the components that regulate these responses (Hammond et al., 2003). Two groups have performed genome-wide transcription expression analysis to phosphate deprivation in plants (Wasaki et al., 2006; Misson et al., 2005; Hammond et al., 2003). Several motifs (PHO-like, TATA box-like, PHO element, NIT2, and helix-loop-helix) have been identified as potential phosphate starvation regulatory sites by *in-silico* methods, but few have actually been tested *in-planta* (Rubio et al., 2001, Hammond et al., 2003; Mukatira et al., 2001; Schunmann et al., 2004). The P1BS motif (GNATATNC) is present in many P_i-starvation responsive genes from *Arabidopsis* (Rubio et al., 2001).

The *PAP12* promoter sequence was analyzed to identify promoter elements that regulate *PAP12* expression during phosphate starvation. We established by RNA gel blots and western blots that *PAP12* is phosphate responsive and not induced by general nutrient stress other than phosphate. This now allows us to use the *PAP12* promoter to isolate regulatory *cis*-elements that are important for its induction upon phosphate starvation. The approach we have taken is to make various promoter fragments fused to

a reporter gene, *GUS*. The activities of the reporter gene were then assayed in the wild-type and *pho1* genetic backgrounds.

The analysis here will help identify additional *cis*-elements, which can be used to identify factors that regulate the expression of P_i-responsive genes. Clustering of P_i-responsive genes based on whether they share the same *cis*-elements will help understand and identify transcription regulatory networks that control the phosphate starvation response (PSR) in *Arabidopsis*.

Materials and Methods

Arabidopsis Seed Sterilization and Growth

Arabidopsis thaliana seeds were first rinsed in 70% ethanol then sterilized in 50% bleach, 0.1% Triton X-100 solution for seven minutes on a rotating platform. The seeds were then rinsed three times with sterile MQ water and plated on Murashige and Skoog (MS) media with 1% Sucrose. Plants were grown on MS media with 1% sucrose purchased from Caisson Laboratories, Inc. (catalog #: MSP009) in growth chambers set at 24 hour light, 22°C and 22 μmol m^{-2} s^{-1}. Soil-grown plants were grown in growth chambers using RediEarth soil under conditions of 24 hour light/0 hour dark (152 μmol m^{-2} s^{-1}), at 22°C and 74% humidity.

Histochemical GUS Staining

Plant tissue was stained in 1mM X-Gluc, 1mM EDTA, 0.1%Triton X-100, 1x FeCN

Stock, 50 mM NaHPO$_4$ pH 7.0. X-Gluc was prepared by dissolving in

dimethylformamide to a final concentration of 1mg/ml. A 50x FeCN stock contained 25

mM potassium ferricyanide, 25 mM potassium ferrocyanide and 0.5M EDTA.

Plant tissue was covered with the X-Gluc solution, briefly infiltrated for a few minutes

by applying a vacuum and stained overnight at 37°C.

Promoter Deletion Constructs

Promoter deletion constructs (containing approximately 500, 400, 300, 200, and100 bp

upstream from the start codon) were created by using PCR primers that amplified

corresponding regions of the *PAP12* promoter and introduced a 5' HindIII site and a 3'

NcoI site for cloning into an Nco-Gus vector (Table 2). After *PAP12* promoter

fragments were cloned into the Nco-Gus vector, the *PAP12* promoter::*GUS*::*NosT*

construct was digested with HindIII and EcoRI and cloned into the binary vector

pBIN19. A minimum of 10 independent transformants were analyzed for each

construct. Images representing the consensus staining of the transformants are shown.

PAP12 Minimal Promoter Constructs

PAP12 minimal promoter was amplified using primers containing a 5' EcoRI site and a

3' XbaI site (Table 2). The PCR product was cloned into pCR2.1-TOPO (Invitrogen

Life Technologies) and sequenced. The *PAP12* minimal promoter in pCR2.1-TOPO

was digested with EcoRI and XbaI and cloned into p35S14 (-64*35S*::*GUS*::*NosT*) vector

to create a *PAP12* minimal promoter::-64*35S*::*GUS*::*NosT* construct. The p35S14

(-64::*GUS*::*NosT*) vector containing the minimal *PAP12* promoter was sub-cloned into

pBin19 using HindIII and EcoRI. A minimum of 10 independent transformants were

analyzed for each construct. Images representing the consensus staining of the

transformants are shown and were taken with a Nikon Cool Pix 4300 digital camera.

Table 2. Primers used for the design of the *PAP12* promoter reporter constructs

Primer	Sequence
PAP12_0	gcgccatggCTTCAAGATTAG
PAP12_-106	gcgaagcttCATATGATTGTGTCTGGCC
PAP12_-207	gcgaagcttGAGATGGTCTTAGAAGCC
PAP12_-318	gcgaagcttGGACAAATGTCATCATAC
PAP12_-403	gcgaagcttTATGAGAGGAGGGAG
PAP12_-488	gcgaagcttCGATATTAAAAAAAAAAAAAAAGATAGTTAGAGC
PAP12_minimal_promoter_5'	ggaattcGAAAGCAACACTATCAATTG
PAP12_minimal_promoter_3'	gtctagaTTTCTCCTTCCTCTC

Sequences are written in the 5' to 3' direction. Restrictions sites used for cloning the
promoter fragments are in lowercase.

Arabidopsis Transformation

Arabidopsis (ecotype Columbia) plants were transformed by the floral dip method of

Bent et al., 1994. *Agrobacterium*, strain GV3101, was transformed by electroporation.

Transformants, carrying *PAP12* promoter deletion constructs were selected on Phytagar

(Gibco-BRL)-Murashige and Skoog (MS) medium (Caisson Laboratories, Inc)

containing 1% sucrose, kanamycin (50 mg/L) and carbenicillin (500 mg/L).

Results

PAP12 Promoter Sequence

Several sequenced cDNA clones indicated the 5'UTR was at least a minimum of 101

base pairs upstream of the start codon. Sequences used for promoter analysis and

construction of promoter deletions are shown in Figure 15.

Analysis of Promoters of Additional Genes in Arabidopsis Containing Sequences Similar

to Sequences Present in the PAP12 Promoter

A BLAST search (with low-complexity filter turned off) of the *PAP12* promoter against

a *Arabidopsis* database containing 1000 bp of sequence immediately upstream of the

5'UTR for those genes with annotated UTRs and upstream of the translational start for

the remainder, was performed using the following dataset: Loci upstream Sequences

-1000bp (DNA). We found that a region of the *PAP12* promoter sequence (481 bp

upstream of the start codon) is present within 1000bp upstream of several genes (Table

3). We analyzed a few of these genes (At1g63430, At3g09770, and At2g31450) by

-482
cactttttaatcgatattaaaaaaaaaaaaaaagatagttagagcattttcattggaagtatctcaatgagatacccaa

-403
caaaaaaaaaaatatgagaggaaggagaaaaaataggagagaaggagagttgagtttctcttccgagaaacttt

-320
gagaaactattctcatccaatttggacaaatgtcatcatactattagttcaattattaaataataattaatatatttgttta

-211
gttaaaatattaatatatatttttttttagaaactttcttgtttttagatagagatggtcttagaagccttcattttcaaaggttg

-106
attaaaaataaaataaaaatttggaatatccacttgcgatcctttcttggaaaaaaaaaaaaaaaatcatatgattgtg

tctggccagacaagctagctatctcatttaatttcccaaaaagaaaatataaagcacgttcttcgtctcggattcaga

0
gaaactaatcttgaagATG

Figure 15. *PAP12* promoter sequence.

Arrows indicate the position of the deletions used in *GUS* reporter fusions. The
beginning of the start codon is designated as 0 bp. The 5'UTR is underlined. The PHR1
binding site, P1BS element, is boxed.

RNA gel blot analysis to see if they were also responsive to phosphate starvation. None of the genes were up-regulated in wild-type plants starved for phosphate or in the *pho1* mutant (data not shown).

Further sequence analysis of the *PAP12* promoter indicated that the region between -459 and -196 is a non-autonomous DNA transposon element called *RP1_AT.I* predicted by Repbase (Jurka et al., 2005), and apparently is not involved in the phosphate starvation response.

Identification of Known P_i Starvation Motifs in the PAP12 Promoter

To identify previously identified P_i starvation elements in the *PAP12* promoter, we analyzed a sequence 1000 bp upstream of the start codon using the PLACE program (Higo et al., 1999). Only one motif, P1BS, was identified at position -149 relative to the start codon (Figure 15). This motif is a PHR1-binding sequence found in the upstream regions of phosphate starvation responsive genes (Rubio et al., 2001).

To test whether *PHR1* is important for *PAP12* expression under Pi-deficient conditions, *phr1* mutants were grown on MS media with phosphate and without phosphate. RNA gel blot analysis was carried out using RNA from wild-type and *phr1* mutant plants that were starved for phosphate for 48 hours.

Table 3. Gene IDs with promoter sequences similar to the *PAP12* promoter

AGI	Location and Orientation	E-Value
AT2G27190	chr2:11631534-11630535 REVERSE	0.0
AT2G05130	chr2:1851493-1850494 REVERSE	2e-69
AT3G09770	chr3:2999195-2998196 REVERSE	1e-67
AT2G31460	chr2:13410853-13411852 FORWARD	3e-62
AT2G31450	chr2:13412254-13411255 REVERSE	3e-62
AT1G42190	chr1:15762523-15763522 FORWARD	3e-62
AT1G63430	chr1:23525273-23526272 FORWARD	8e-41
AT3G13710	chr3:4494838-4493839 REVERSE	7e-35
AT3G13700	chr3:4494744-4493745 REVERSE	7e-35
AT1G48060	chr1:17730964-17731963 FORWARD	6e-17
AT1G69080	chr1:25977934-25976935 REVERSE	9e-16
AT2G20650	chr2:8915420-8914421 REVERSE	9e-13
AT1G58330	chr1:21643795-21644794 FORWARD	2e-07
AT3G50500	chr3:18756054-18755055 REVERSE	1e-05
AT3G44170	chr3:15904777-15905776 FORWARD	5e-05
AT1G64770	chr1:24060102-24061101 FORWARD	2e-04
AT1G64760	chr1:24061077-24060078 REVERSE	2e-04
AT1G09980	chr1:3262575-3261576 REVERSE	8e-04

The blot was then hybridized with a *PAP12* probe. RNA gel blot analysis showed that *PAP12* is still up-regulated in the *phr1* mutant when starved for phosphate. Both wild-type plants and the *phr1* mutant show the same level of *PAP12* induction (Figure 16). Expression of *PAP12* is still phosphate dependent in the *phr1* mutant.

Deletion Analysis of PAP12 Promoter

In order to identify *cis*-acting elements required for *PAP12* up-regulation upon P_i starvation, promoter deletion analysis was carried out. A series of *PAP12* promoter-*GUS* constructs with various degrees of deletion from the 5' end of a -482 bp *PAP12* promoter were generated. For each deletion construct, 10 independent T_2 lines were analyzed by staining for GUS activity. GUS expression from the *PAP12*::*GUS* reporter series in wild-type and *pho1* plants was compared (Figure 17). Plants were grown on MS plates with phosphate until they developed two to three sets of true leaves and stained for GUS activity. We used the *pho1* background because the leaves have lower phosphate than wild-type, thus making it easy to assay the promoter constructs in normal (wild-type) and low phosphate (*pho1*).

Only the -482 *PAP12*::*GUS* construct was expressed in a phosphate dependent manner based on GUS histochemical staining. There was strong uniform staining in the
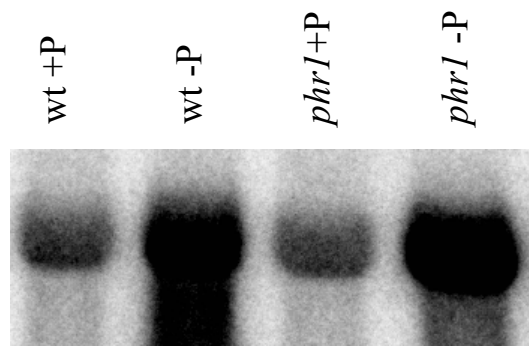
Figure 16. *PAP12* expression is unaltered in wild-type and *phr1*.

Wild-type (wt) and *phr1* plants were grown in MS media with phosphate and grown in MS media without phosphate for 48 hours. RNA gel blot containing 10 μg of total RNA was hybridized with a *PAP12* probe.
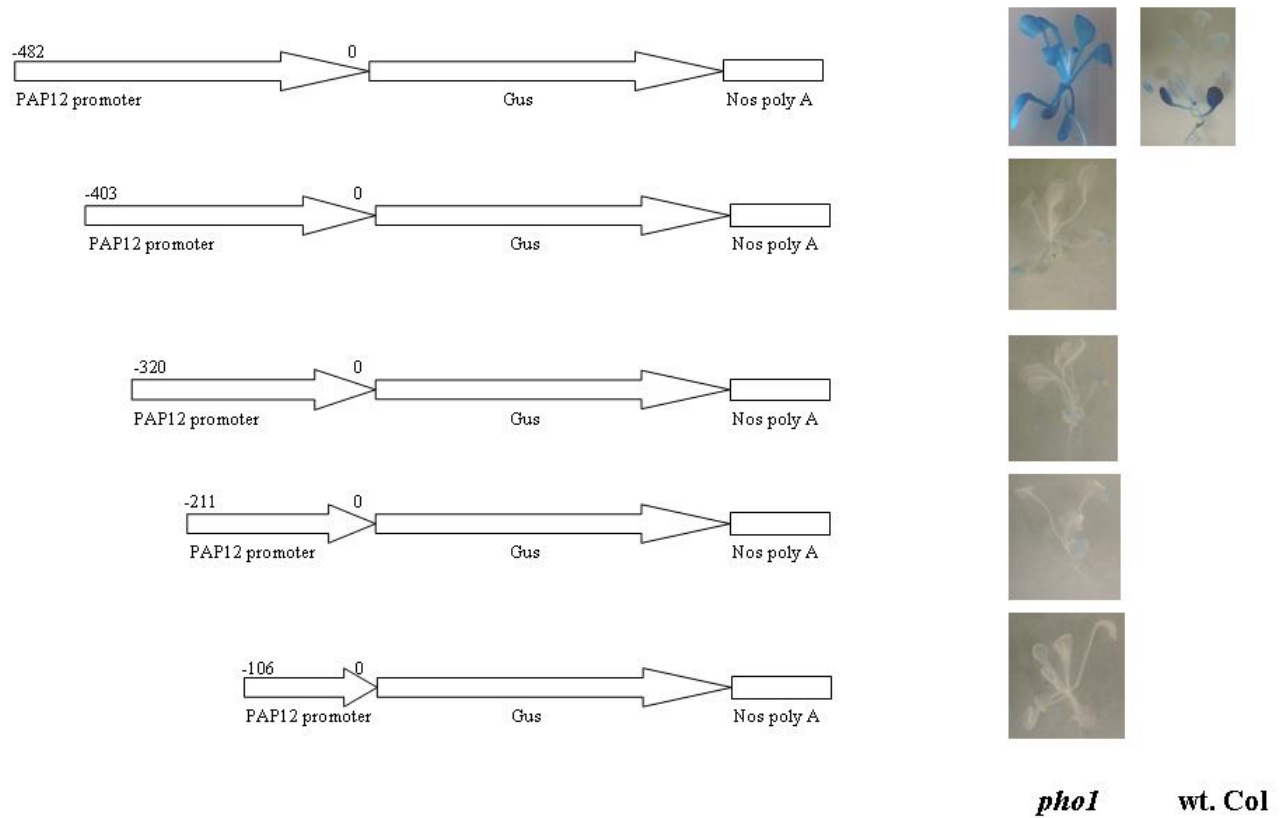
Figure 17. *PAP12* promoter driven expression of *GUS* in *pho1* and wild-type plants.

Five different constructs were analyzed. Plants were grown on MS plates till 2-3 sets of true leaves developed (10 days) and stained for GUS activity.

pho1 leaves, but no staining in the wild-type leaves. The -482 *PAP12*::*GUS* construct in the wild-type background showed staining in the cotyledons. The -403 *PAP12*::*GUS*, -320 *PAP12*::*GUS*, -211 *PAP12*::*GUS*, -106 *PAP12*::*GUS* constructs showed no staining in *pho1* leaves or wild-type *Arabidopsis* (ecotype Columbia) leaves (Figure 17). Deletion of sequences from -482 to -403 greatly reduced GUS expression in the *pho1* background, indicating this region is important for P-starvation inducibility of *PAP12*. Thus this region may contain a Phosphate Responsive Element (PRE), responsible for P_i inducibility of *PAP12*.

A 96-bp Region of the PAP12, When Fused to the 35S Minimal Promoter Confers

Phosphate Dependent Regulation of a Reporter Gene

We showed that a 79-bp region from the *PAP12* promoter (-482 to -403) when deleted failed to express GUS in a phosphate dependent manner (Figure 17). Thus a putative *cis*-regulatory element, Phosphate Responsive Element (PRE), is present between -482bp and -403bp of the *PAP12* promoter. To obtain additional evidence of a PRE in this region, we fused a -482 to -386 region of the *PAP12* promoter to a minimal 35S::*GUS* (-64 *35S*::*GUS*) construct and analyzed its expression in *pho1* and wild-type plants. The minimal promoter alone showed no detectable GUS activity based on histochemical staining in the *pho1* or wild-type leaves. However, the minimal promoter when fused with the -482 to -386 region of the *PAP12* promoter was able to drive the expression of GUS in a phosphate dependent manner (Figure 18). Wild-type plants transformed with this construct showed little GUS staining compared to *pho1* plants
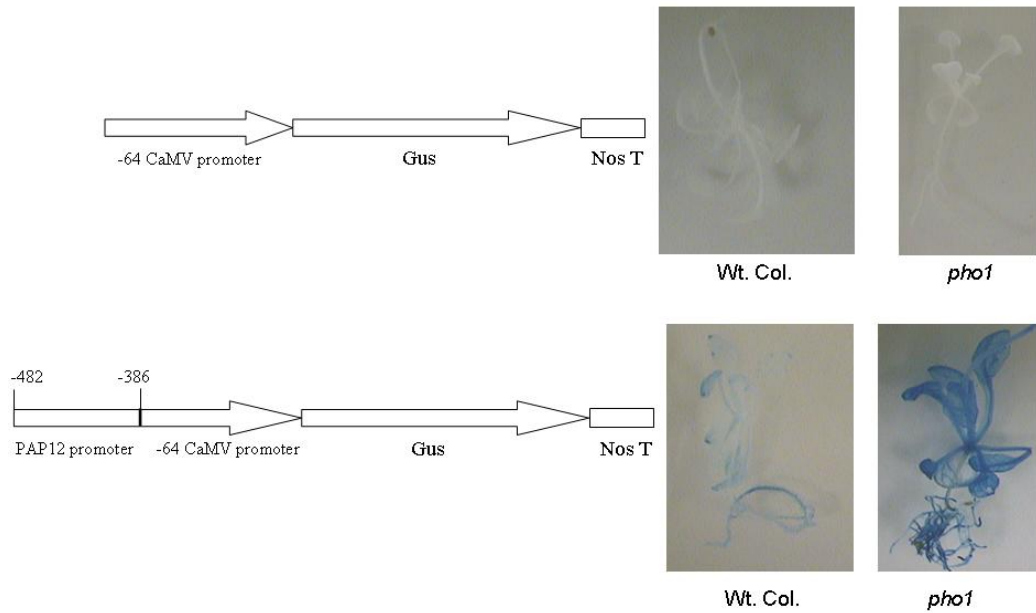
Figure 18. A 96-bp region of the *PAP12* promoter fused to a -64 minimal *35SCaMV* promoter.

A -482 to -386 bp region of the *PAP12* promoter was fused to a minimal 35S *CaMV* promoter. Two constructs, one containing a minimal 35S *CaMV*::*GUS* construct and another containing a 96 bp region of the *PAP12* promoter fused to the minimal *35S CaMV*::*GUS* were transformed into *pho1* and wild-type plants. Plants were grown on MS plates until 2-3 sets of true leaves developed (10 days) and stained for GUS activity.

containing this same construct. The *pho1* plants showed strong, uniform GUS staining of the shoots and roots. This is consistent with the expression of endogenous *PAP12* in these two backgrounds.

PAP12 Expression Profile

The expression profile of *PAP12* was first examined by RNA gel blot. Transcripts were detected in root, flower, leaf, stem and silique. Also, transcripts levels were higher in the *pho1* background and lower in the *pho2* when compared to wild-type. The expression profile of *PAP12* was further analyzed by assaying a promoter reporter gene construct in wild-type, *pho1* and *pho2* background (Figure 19). This allowed us to better determine the spatial expression pattern of *PAP12* and possible function and role in the PSR. *Arabidopsis* plants containing the -482 *PAP12*::*GUS* construct were grown on MS media with phosphate and in soil. GUS expression was detected in all of the tissues in which PAP12 expression was detected by northern blot, except stem and silique (Figure 2, 19).

There were several patterns of expression of the -482 *PAP12*::*GUS* construct in wild-type, *pho1* and *pho2* plants (Figure 19). In wild-type roots, GUS was expressed in the root tip and at sites of lateral root initiation. Wild-type shoots showed staining in the cotyledons and diffuse staining throughout the leaf and vascular tissue. In wild-type flowers, GUS activity was found in pollen grain, sepals, anthers and stigma apex. The *pho2* mutants had more staining in the roots than in the shoots. GUS expression in *pho2*
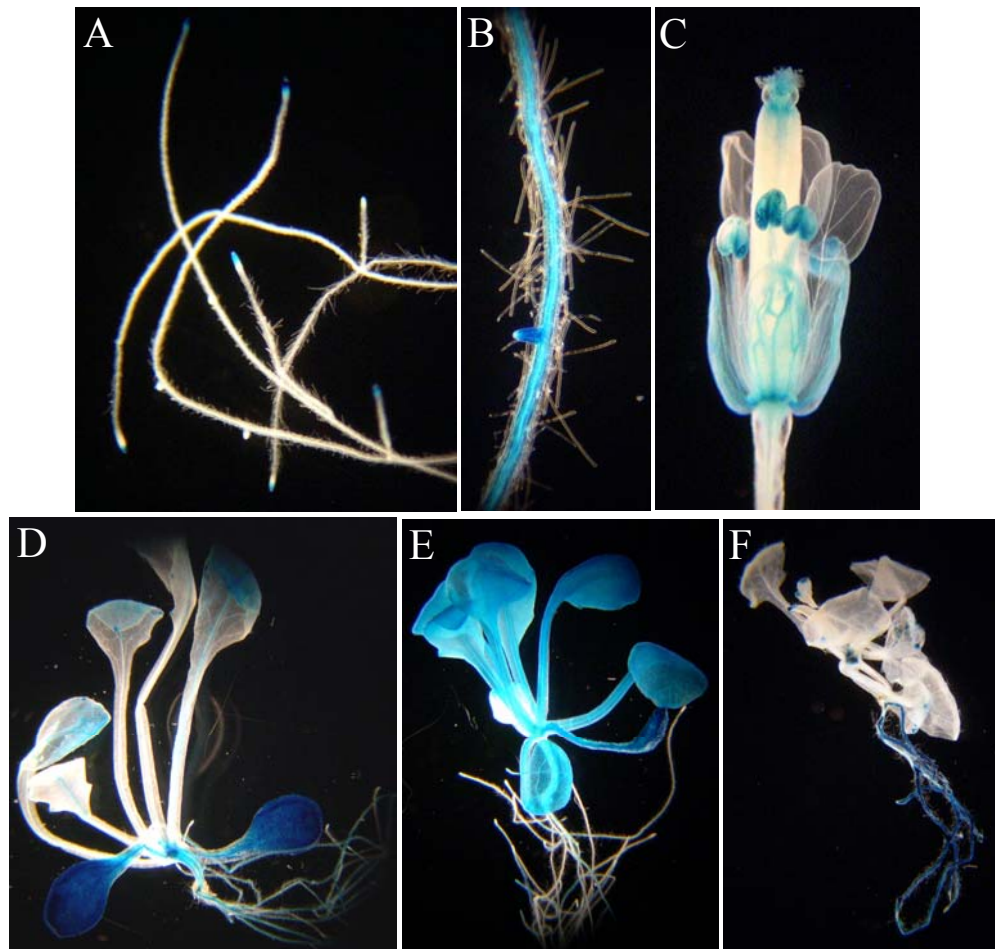
Figure 19. Histochemical localization of *GUS* expression under control of the *PAP12* promoter in wild-type, *pho1* and *pho2* mutants.

A -482bp *PAP12*::*GUS* construct was transformed into wild-type, *pho1* and *pho2* plants. Plants were grown on MS media with phosphate. Promoter activity in root tips of wild type plants (A), root vascular tissue and lateral root initiation (B), flower (C), wild-type plant (D), *pho1* plant (E), and *pho2* plant (F).

leaf was also observed in the hydathodes. The *pho1* mutant showed strong uniform expression in leaves and but very weak staining in the roots.

Discussion

To study tissue specificity and the expression pattern of *PAP12* in *Arabidopsis*, we analyzed a -482 *PAP12*::*GUS* construct in wild-type, *pho1* and *pho2* plants. Our results agree with those of Haran et al., 2000. Strong GUS staining was observed in the lateral root initiation and root apical meristem, possible suggesting a role for PAP12 in cell wall modification at these rapidly growing sites. This role also has been suggested in tobacco cells by two PAP gene products, *NtPAP12* and *NtPAP21*. These two gene products are wall-bound PAPs at the early stage of regenerating walls in tobacco protoplasts (Kaida et al., 2003). GUS staining was also observed in cotyledons of wild-type and *pho2* plants, suggesting that PAP12 may play a role in remobilizing phosphate from cotyledons to the rest of the plant. We also observed expression in the vascular tissues of roots, leaves and flowers as well as non vascular tissues, hydathodes, and root tip. PAP12 may be involved in moving P_i to or from the vascular tissues. The expression pattern of *PAP12* reported here has considerable overlap with other know P_i-responsive genes, such as *PHO1*. Expression pattern of all *PHO1* homologs reveals that a broad range of tissues can express members of the *PHO1* family, with strong expression in vascular tissue (Wang et al., 2004).

Analysis of the *PAP12* promoter sequence revealed a previously identified phosphate responsive element, P1BS. This element is 149 bp upstream of the *PAP12* start codon. A conserved MYB transcription factor involved in phosphate starvation signaling in vascular plants and *Chlamydomonas reinhardtii* binds to this element. This sequence is present in promoters of P_i-starvation responsive genes, indicating this transcription factor may act downstream in the P_i starvation signaling pathway (Rubio et al., 2001). We show by promoter analysis the region containing the P1BS element was not essential or necessary for phosphate dependent expression of *PAP12*. To further validate that the P1BS site is not important for the P-dependent regulation of *PAP12* expression under P-sufficient and P-deficient conditions in the *phr1* mutant, we analyze the expression of *PAP12* in the *phr1* mutant. PHR1 is a MYB transcription factor involved in phosphate starvation signaling that binds to the P1BS motif (Rubio et al., 2001). P_i-starvation induced genes, such as *AtIPS1*, *At4* and *RNS1*, contain P1BS binding sites in their promoters and show a reduced expression in the *phr1* mutant. Our results show that *PAP12* is still responsive in the *phr1* mutant, supporting the statement that the P1BS site and PHR1 are not necessary for *PAP12* expression under P_i-deficient conditions. Thus, a separate factor is responsible for the expression of *PAP12* under phosphate limiting conditions.

Deletion analysis identified a region of the *PAP12* promoter between -482 and -403 associated with P_i responsiveness. This 79 base pair region, when deleted from a -482 *PAP12*::*GUS* construct, no longer expressed *GUS* in a phosphate dependent manner (Figure 17). In addition, this same region when fused upstream to a minimal *35S*

promoter (-64 *35S*::*GUS*) was able to drive the expression of *GUS* in a phosphate

dependent manner (Figure 18). Thus, this sequence plays an important role in the

transcription of *PAP12* during P_i starvation. One possibility is that *PAP12* expression

under phosphate limiting conditions is not regulated by PHR1, but by a yet unidentified

transcription factor, which binds in a region between -482 and -403 with respect to the

start codon of the *PAP12* promoter. Additional analysis of this region will be required to

narrow the *cis*-element phosphate responsive element. A consequence of this is that

more than one transcription factor appears to regulate P_i-responsive genes in

Arabidopsis. In yeast the PHO regulon is regulated by one transcription factor, *PHO4*, a

basic helix-loop-helix transcription factor and its partner *PHO2*, a homeobox protein

(Lenburg and O'Shea, 1996). Their maybe greater a complexity and a greater

coordination in plants required to regulate P_i-responsive genes.

Further analysis of the *PAP12* promoter revealed a non-autonomous DNA

transposon element between -459 to -196. Thus, the region between -482 and -403,

which is important to drive the expression of *PAP12* in a phosphate dependent manner,

overlaps with this transposon, leaving 23 bp that does not belong to this non-autonomous

DNA element. We also performed a blast of the *PAP12* promoter against the dataset:

Loci upstream Sequences -1000bp (DNA). We found several genes containing similar

sequences (Table 3). This may be due to the transposon being active at some time

during the evolutionary history of *Arabidopsis*. If these genes are phosphate repressed,

then we predict that they might share the same *cis*-element as *PAP12* for induction upon

phosphate deprivation. We analyzed three genes: At1g63430, At3g09770, and

At2g31450, and showed that they were not phosphate responsive as indicated by RNA

gel blots (Figure 16). Despite having good identity, they were all missing the sequence

from -487 to -452. This 35-bp region might contain the PRE for PAP12, which is

different from previously reported PREs and will be the focus of further analysis.

CHAPTER IV

PURIFICATION OF A NUCLEAR FACTOR THAT BINDS THE PAP12 PRE

Introduction

Although a number of Phosphate Responsive Elements (PREs) have been identified, only one factor has been characterized that bind to these elements (Rubio et al., 2001). This factor (PHR1), a MYB-domain containing transcription factor, was identified in a screen for mutants altered in P_i-starvation regulation. The PHR1 protein binds to a imperfect-palindromic sequence designated as P1BS. The *phr1* mutant has reduced expression of P_i starvation-induced genes, such as *AtIPS1*, *At4* and *RNS1* (Rubio et al., 2001).

Analysis of the *PAP12* promoter revealed a putative binding site for PHR1 at position -149 with respect to the start codon. However, our analysis indicated that this P1BS *cis*-element does not play a role in the induction or expression of *PAP12* during P-starvation. Instead, our results indicate a 79-bp region (482 to -403) of the *PAP12* promoter is important for its induction upon P starvation. We refined the 79-bp regulatory region of the *PAP12* promoter to 35-bp, which we call PAP12PRE, by gel shift assays and analyzed the interaction of this 35-bp region with nuclear protein factors from phosphate-starved plants. We also purified a factor that binds to this 35-bp PRE by using an avidin-biotin method. These experiments identified an additional phosphate responsive *cis*-element and proteins that are important for *PAP12* expression under low phosphate conditions.

Materials and Methods

Arabidopsis Nuclei Isolation

Tissue was ground to a fine powder using liquid nitrogen and added to nuclei isolation

buffer (10 mM HEPES (pH 8.0), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM

PMSF) at a ratio of 3mls/g of tissue. The suspension was filtered through a 76 micron

mesh filter. Triton X-100 was added to a final concentration of 0.3% to the filtered

sample. The filtrate was then layered on top of a 1.5 M sucrose cushion (sucrose

dissolved in nuclei isolation buffer) and centrifuged at 12000 x *g* for 10 minutes. The

upper green phase and sucrose cushion were removed, and the nuclei pellet was washed

twice by gently re-suspending in nuclei extraction buffer. Nuclei were either stored in

nuclei storage buffer [50% glycerol, 20 mM Tris (pH 7.9), 75 mM NaCl, 0.5 mM EDTA

(pH 8.0)] or extracted for nuclear proteins.

Nuclear Protein Extraction

An equal volume of 0.8M ammonium sulfate was added to nuclei resuspended in nuclei

storage buffer, briefly vortexed and incubated on ice for 15-20 minutes. The nuclei were

then centrifuged at 16000 x *g* for 10 minutes and spin-dialyzed thorough a Bio-Spin 6

chromatography column (Bio-Rad, catalog 732-6002) equilibrated in P6 buffer (20%

glycerol, 20 mM HEPES (pH 8.0), 60 mM KCl, .5 mM EDTA pH 8.0, 1 mM DTT).

Nuclear extracts were then aliquoted, frozen in liquid nitrogen and stored at -80°C.

DNA Binding Reactions

Binding reactions (20µl) were composed of 20 % glycerol, 20mM HEPES, 60 mM KCl, 0.5mM EDTA, 1.6 mM MgCl2, 125 ng/µl poly dI/dC (Amersham Biosciences, 27-7880-02) and nuclear extract. Nuclear proteins were first incubated with poly dI/dC for 10 minutes at room temperature. Next, a ^{32}P labeled oligonucleotide was added to the binding reaction and allowed to incubate at 25°C for 15 minutes before loading onto the gel. For the competition assays, indicated molar excess of unlabeled oligo or DNA fragments were added to the nuclear extracts, allowed to incubate for 10 minutes at 25°C before the addition of the probe.

Electrophoretic Mobility Shift Assay (EMSA)

The samples were run at room temperature on a 5% polyacrylamide, 0.5 x TBE gels at a constant voltage of 7 V/cm. Gels were dried and autoradiographed on a phosphoimager plate. Gels were pre-run for 1 hour at 70 V.

Purification of PAP12PRE Binding Nuclear Factors

Two milligrams of *pho1* leaf nuclear extract in 20 % glycerol, 20mM HEPES, 60 mM KCl, 0.5mM EDTA, 1.6 mM MgCl2, was incubated with 125ng/µl poly dI/dC on ice for 20 minutes. 100 pmole of the biotinylated PAP12PRE was added and allowed to incubate for 30 minutes at room temperature. This mixture was then added to a column containing streptavidin-agarose beads (SAA). The flow through was reapplied to the

column 3 times to ensure all the biotinylated PAP12PRE was bound to the SAA beads.

The column was washed with binding buffer, 0.1 M KCl binding buffer and 0.15 M KCl

binding buffer (4 bed volumes of each). PAP12PRE nuclear binding factors were eluted

from the column with 1 bed volume of 0.5 M KCl. The eluted extracts and salt washes

were desalted using P-6 spin columns equilibrated with P6 buffer. Samples were

fractionated on a SDS-PAGE gel and tested for PAP12PRE binding activity by gel shift

assay.

Probes for Electrophoretic Mobility Shift Assay

Oligos (purchased from Invitrogen) were annealed in 1x annealing buffer (10 mM Tris

pH 8.0, 50 mM NaCl, 1 mM EDTA) by heating to 95°C for 5 minutes and allowed to

cool slowly to 25°C. They were end labeled with [γ-^{32}P] ATP using T4 polynucleotide

kinase. Sequences of oligonucleotides used as probes for gel shift assays and cold

competitors are given in Table 4.

Table 4. Primer sequence used for mobility shift assay

Primer	Sequence
PAP12-407/-385	AAAATATGAGAGGAAGGAGAAAA
PAP12-465/-410	TTTTGTTGGGTATCTCATTGAAATACTTCCAATGAAGATGCTCTAACTATCTTTT
PAP12-466/-445	AAAAAAGATAGTTAGAGCATC
PAP12-487/-452 (PAP12PRE)	TTTAATCGATATTAAAAAAAAAAAAAAGATAGTTAG
TERT_Promoter 5'	CATACACTGTAACTAGAGTGG
TETR_Promoter 3'	TGCATCATTTCAATAATCCTTTCAAC
rns2 (at2g39780) promoter; -1 to -277	GTAACATTCAAAATTCAATAGTAGTC
rns2 (at2g39780) promoter; -1 to -277	TGACTTTAATTCGATTGAAACCG
rns2 (at2g39780) promoter; -256 to -500	GCCAGAGATAGTGATGTGAAAATG
rns2 (at2g39780) promoter; -256 to -500	CAATGACTACTATTGAATTTTGAATG
BiotinPAP12PRE	B-TTTTAATCGATATTAAAAAAAAAAAAAAGATAGTTAGAGCA

Sequences are written in the 5' to 3' direction. B indicates a biotin modification to the oligo.

Results

Nuclear Proteins Interaction with a 35-bp Region of the PAP12 Promoter

To further refine the 79-bp *PAP12* promoter region (-482 to -386), containing a putative

PAP12 phosphate responsive element (PAP12PRE), we performed gel shift assays using

nuclear extracts from phosphate-starved plants or *pho1* leaves. Since *PAP12* is induced

upon phosphate starvation in leaf, we chose to use this tissue as the source of nuclear

protein for the gel shift assays. EMSAs were performed using a 35-bp region (-487/-

452) of the *PAP12* promoter as the probe. This region was predicted to contain the PRE

because it was absent in the promoters of other genes, which were not induced upon

phosphate starvation even though their promoter had high sequence identity with the

PAP12 promoter. Nuclear extracts were incubated for 10 minutes at room temperature

with poly dI/dC in binding buffer. Poly dI/dC was added to all binding reactions to

prevent non-specific binding. ^{32}P-labeled probes were then added and allowed to

incubate for 15 minutes at room temperature before loading onto a 0.5X TBE 5%

polyacrylamide gel. The EMSA in Figure 20 shows a single binding activity present in

pho1 leaf extracts with specific binding to the PAP12PRE. This activity can be

competed away by PAP12PRE (-487/-452) but not by other fragments (-484/-429, -486/-

468, -407/-386) within this 79-bp *PAP12* promoter region. Division of this 35-bp region

into two fragments (-466/-446, -484/-471) failed to compete away the shift when the 35-

bp fragment was used as the probe (data not shown). We conclude that the PAP12PRE

is contained within this (-487/-452) region by gel shift analysis.
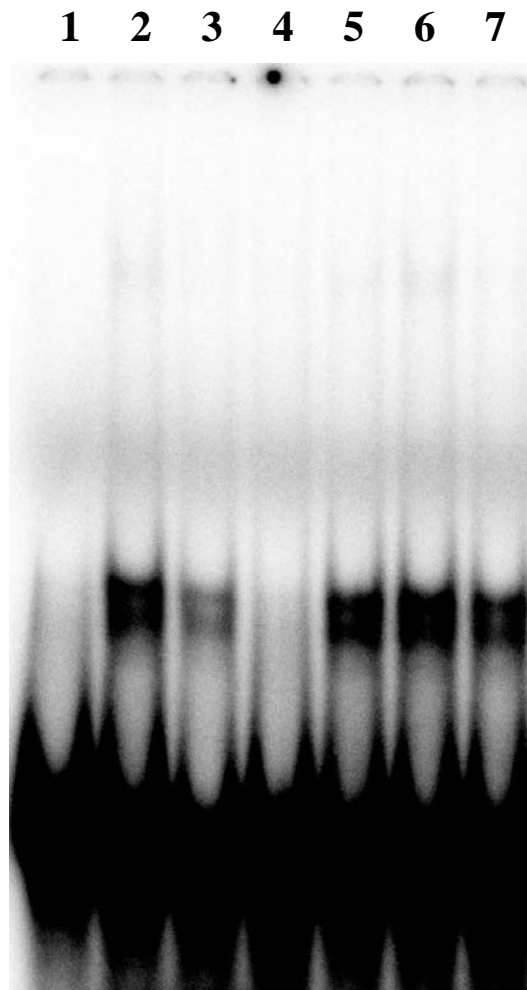
Figure 20. A nuclear factor from *pho1* leaf nuclei extract binds to a 35-bp region of the *PAP12* promoter.

Extract from *pho1* leaf (2.5 μg) was used for a mobility shift assay with a ^{32}P-PAP12PRE probe (-487/-452; 33 fmole). All binding reaction contained poly dI/dC (125 ng/μl) as a non-specific competitor except lane 3 contained poly dA/dT (125ng/μl). Lane 1: no extract; lane 2: *pho1* extract; lane 3: *pho1*extract; lane4: *pho1* extract + cold PAP12PRE (121x); lane 5: *pho1* extract + -465/-410 cold oligo (90x); Lane 6: *pho1* extract + -407/-385 cold oligo (151x); Lane 7: *pho1* extract + -466/-447 cold oligo (90x).

PAP12PRE-Binding Activity is Phosphate Dependent

Since the expression of *PAP12* is phosphate dependent as indicated by RNA gel blots,

the PAP12PRE binding by nuclear factors might also be phosphate dependent. We

predicted that nuclear extracts from wild-type plants grown in MS media with phosphate

should have little to no binding activity, while extracts from wild-type plants grown in

MS media without phosphate should show more or stronger binding than from extracts

of plants grown in MS media with phosphate. We performed gel shift assay with

extracts from plants grown in sufficient phosphate and phosphate deficient media.

Figure 21 shows that binding to PAP12PRE is phosphate dependent. An equivalent

amount of extract from plants grown in MS media without phosphate displays a stronger

shift than from an extract of plants grown in MS media with phosphate. Thus, the

PAP12PRE shifts in a phosphate dependent manner, which is consistent with *PAP12*

expression in plants grown in MS media with and without phosphate. An SDS-PAGE

gel was run to show that equivalent amounts of nuclear protein extracts were used for

binding assay (Figure 22).
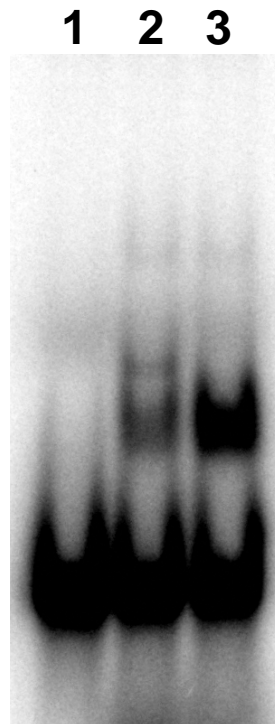
Figure 21. A 35-bp region of the *PAP12* promoter shifts in a phosphate dependent manner.

Nuclear extracts were prepared from wild-type plants grown in MS media with phosphate and from plants grown in MS media without phosphate for 48 hours. 10 μg of each nuclear extract was used for the gel shift assay. Lane 1 contains no extract, lane 2 contains 10 μg of extract from plants grown in MS +P, and lane 3 contains 10 μg of extract from plants grown in MS –P.
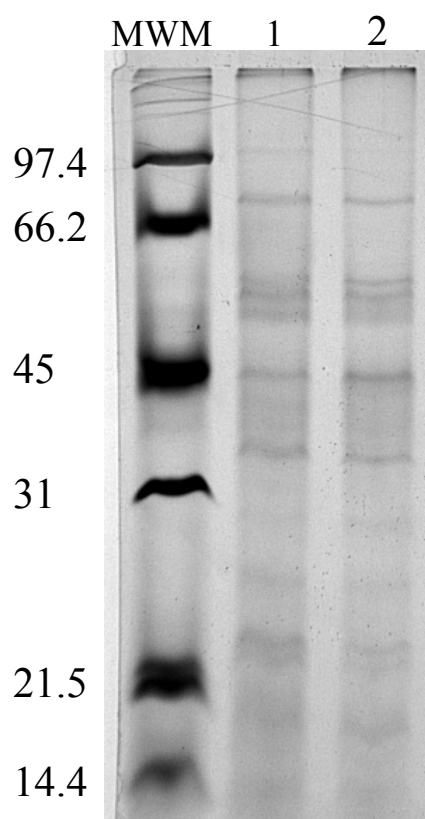
Figure 22. 10% discontinuous SDS-PAGE of nuclear extracts used for gel shift assay.

A 10 % SDS-PAGE containing 1 μg of nuclear extract from wild-type plants grown in MS media with phosphate (lane 2) and 1μg from plants grown in MS media without phosphate for 48 hours (lane 2). The gel was run at 100V and silver stained. Protein concentrations were determined by the Bradford method. The molecular weight standards (MWM) are in kilodaltons.

Purification of a Nuclear Protein Factor that Binds to the PAP12PRE

We have identified a phosphate responsive element by gel shift assay in the *PAP12*

promoter. To isolate the PRE-binding proteins (PREBP) from nuclear extracts, we used

an affinity purification method, in which a biotinylated probe containing the PAP12PRE

is immobilized on streptavidin-agarose beads. Nuclear protein extracts preincubated

with polydI/dC, from *pho1* or wild-type plants starved for phosphate are then added to a

column containing a complex formed between the biotinylated PAP12PRE and

streptavidin-agarose beads. The column was then washed with 4 column volumes of

binding buffer containing 60mM KCL, followed by binding buffer containing 100mM

KCl, and 150 mM KCl, and the PAP12PREBP was eluted from the column by washing

with binding buffer containing 500 mM KCl. Figure 23a shows the SDS-PAGE elution

profile from the avidin-biotin column purification. Most of the nuclear proteins were

removed by the salt washes. The 500mM KCl elution shows an enrichment of a 23.5

kilodalton band (Figure 24). Some high molecular weight bands, which were not

enriched, compared to the starting nuclear extract are also present. Next we tested the

eluted fraction for DNA binding activity by gel shit assays to the PAP12PRE (Figure

23b). We were able to detect binding activity for the PAP12PRE in the eluted fraction

and show it is sequence specific, as it is competed away with cold specific competitor

(Figure 25). This is the same band of activity seen in the starting nuclear extract.

An RNS2 Promoter Fragment Compete away the Shift Observed Between Nuclear

Factors and PAP12PRE

Analysis of additional P$_i$-starvation induced genes by computational methods failed to

find PAP12PRE like *cis*-elements in their promoters. This could be due to the limitation

of the computational methods used, small dataset, degeneracy and flexibility in spacing

of the PAP12PRE, or the possibility that the PAP12PRE is unique to *PAP12*. Many

binding sites for transcription factors are degenerate. Promoters with the same motif

may contain different instances of that motif, making it difficult to identify similar *cis*-

elements. To test whether PAP12PRE is present in the promoter or other P$_i$-starvation

induced genes, we performed gel shift assays using promoter fragments as cold

competitors. If a binding site similar to PAP12PRE is present, it should compete away

the sequence specific shift observed between nuclear factors and PAP12PRE. We tested

a promoter fragment of the *RNS2* gene, a phosphate repressed gene (Taylor et al., 1993).

Figure 26 shows the *RNS2* promoter can compete away the shift between nuclear factors

and PAP12PRE. A *cis*-element similar to PAP12PRE exists between -256/-500 bp of

the *RNS2* promoter, as this fragment can compete away the shift; a fragment from -1/

-277 could not compete away the shift. A 900 bp *TERT* promoter fragment containing

the first exon and intron, a gene known not to be induced by P$_i$-starvation, could not

compete away the shift between nuclear factors and PAP12PRE.
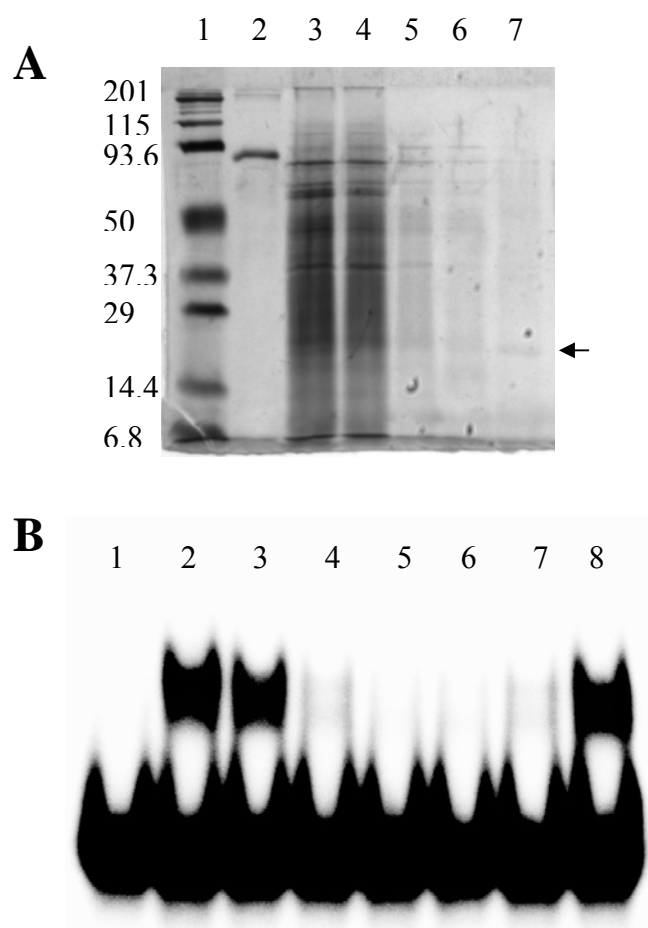
Figure 23. The purification of PAP12PREBP by avidin-biotin method.

The purification of a PAP12PRE binding factor on SAA column was monitored by gel SDS-PAGE (A) and gel shift assay (B). Proteins were analyzed on a 7.5% SDS-polyacrylamide gel and visualized by silver stain. Lane 1A shows the MWM in kDa, 2A 200ng BSA, 3A starting material, 4A flow through, 5A binding buffer wash, 6A pooled 100 mM, 150 mM, and 200 mM KCl washes, 7A elution. Binding reactions containing starting material (Lane 2B), flow-through material (Lane 3B), binding buffer wash (Lane 4B), 100 mM KCl wash (Lane 5B), 150 mM wash (Lane 6B), 200 mM wash (Lane7B), elution (Lane8B) were carried out using PAP12PRE as probe.
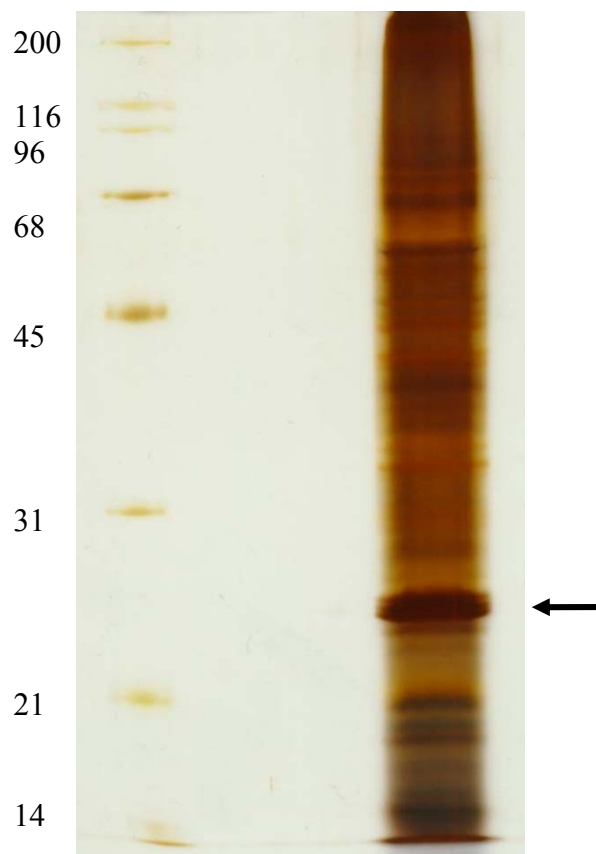
Figure 24. SDS-PAGE analysis of eluted fraction.

The eluted fraction from the avidin-biotin method was analyzed on a 12% SDS-polyacrylamide gel and silver stained. An enriched band at 23.5 kDa is indicated by an arrow. Protein standards in kDa are indicated on the left.
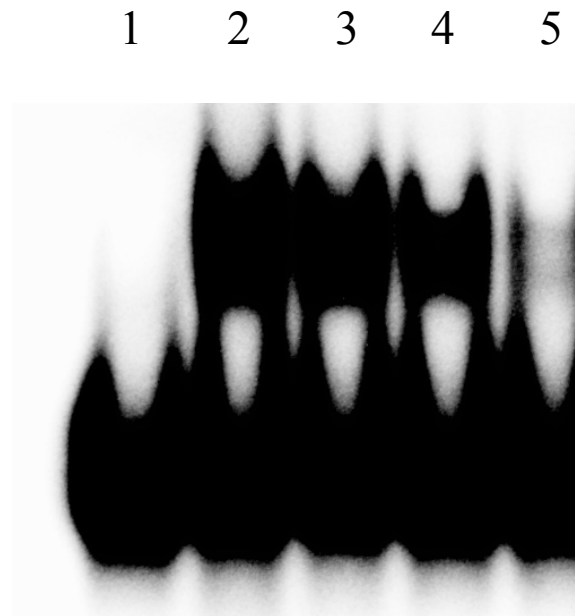
Figure 25. Sequence specific binding activity of eluted fraction.

Gel shift assays were performed using ^{32}P-PAP12PRE probe (-487/-452; 33 fmole). All binding reaction contained poly dI/dC (125 ng/μl) as a non-specific competitor. Lane 1 is free probe (no extract); Lane 2 contains 5 μg of starting amount used (*pho1* extract); Lane 3 contains 10 μg of sample from the flow through fraction; Lane 4 contains a sample of the eluted fraction and Lane 5 contains the same amount of sample as in lane 4 plus 50x cold PREPAP12 as competitor.
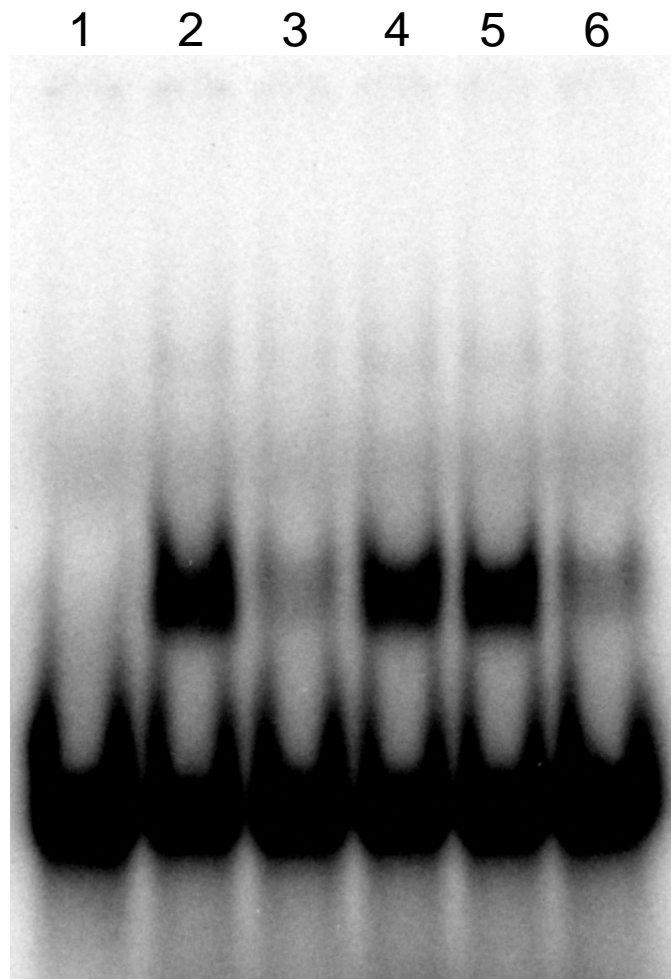
Figure 26. *RNS2* promoter can compete away the shift between *pho1* nuclear extracts and PAP12PRE.

7 µg of *pho1* nuclear extract were used in the binding reactions and incubated with 6.66 fmole of probe (60,000 CPM). The PAP12PRE was used as probe. Lane 1 contains only the probe, lane 2 contains nuclear extract plus probe. Lane 3 contains 50x molar ratio of a cold specific competitor, PAP12PRE. Lane 4 contains cold 50x molar ratio of a -900/-1 *TERT* promoter fragment. Lane 5 contains cold 50x molar ratio of a -277/-1 fragment of the *RNS2* promoter, while lane 6 contains cold 50x molar ratio of a -500/-256 bp *RNS2* promoter fragment.

Discussion

PAP12 transcript levels are low in plants grown in MS media with phosphate and are induced in plants grown in MS media without phosphate. This may be due to the fact that under low phosphate, a protein factor or factors are induced which can bind to the PAP12PRE or the protein factor or factors are modified such that they bind to the PAP12PRE. Binding of nuclear protein extracts to the PAP12PRE should be phosphate dependent. Nuclear extracts from plants grown in MS media with phosphate should have little to no binding activity, while extracts from plants grown in MS media minus phosphate should show more binding than from extracts of plants grown in MS media with phosphate. Our results support this prediction; nuclear extracts from plants grown in low phosphate gave a stronger shift than extracts from plants grown in sufficient phosphate. Our result suggests that *PAP12* is under positive regulation because DNA binding activity increases upon P starvation. This is different from results obtained in studies looking at DNA-protein interaction of promoter regions of other P_i-starvation induced genes (Mukatira et al., 2001). In that case, the DNA binding activity disappeared during P_i-starvation, indicating that some P_i-starvation induced genes may be under negative regulation. It is possible that some P_i-starvation induced genes are under positive regulation while others are under negative regulation.

Using mobility shift assays, we were able to further refine a 79-bp region (-482/-403) of the *PAP12* promoter. Nuclear factors from *pho1* leaf or wild-type plants starved for phosphate were able to shift a 35-bp region (-487/-452) contained within this 79-bp region (-482/-403) in a phosphate dependent manner. This shift with this 35-bp region

was sequence specific. Sequences from -484/-429, -486/-468, and -407/-386 could not compete away the shift when used as cold competitors in a competition assay where the 35-bp region (-487/-452) was used as a probe. Further attempts to narrow the 35-bp region failed (data not shown), and we thus conclude that at least a minimum of 35-bp are required to detect binding activity. Using gel shift assays to precisely identify the PRE may have limited use, as issues of degeneracy and spacing cannot be avoided. Two possible solutions are to use *in vitro* footprinting, which may narrow this element by identifying the nucleotides that are recognized by the PAP12PRE binding protein or to perform a mutagenesis scan on the PAP12PRE.

Genes induced by common stimuli may share common *cis*-elements. This is supported by sequences analysis of promoters of genes that respond to phosphate deprivation (Misson et al., 2005; Rubio et al., 2001). Promoters of P_i-responsive genes were enriched with the PHR1 binding sequence. The *PAP12* promoter contains a PHR1 binding site, but our results indicate it is not important or does not play a role in responding to phosphate deprivation for this gene. Instead we identified a 35-bp region, designated PAP12PRE, required by *PAP12* to respond to phosphate deprivation. To see whether this element was present in additional genes confirmed to be P_i-responsive, we analyzed sequences located upstream of the start codon. We could not find *cis*-elements similar to PAP12PRE with a high degree of identity in other P_i-responsive genes. This could be because we do not know exactly which individual nucleotides within the PAP12PRE interact with the PAP12PREBP (PAP12 phosphate responsive element binding protein). The PAP12PREBP may have some flexibility for its target sequence

and certain base substitution maybe allowed. One way to overcome these limitations is to use various promoter fragments as cold competitors and see if they can compete away the shift between PAP12PRE and nuclear factors. In addition, this approach avoids any bias one may have towards a specific sequence. We used this approach with the *RNS2* promoter. We chose this gene because it is P_i-responsive in wild-type plants starved for phosphate, induced in *pho1* leaves, repressed in *pho2* leaves and contains a relatively short promoter (Taylor et al., 1993; Bariola et al., 1994). An *RNS2* promoter fragment (-256/-500) was able to compete away the shift between nuclear factors (PAP12PREBP) and PAP12PRE. As a control we used a non P_i-responsive gene, *TERT* (At5g16850). A 900 bp promoter fragment from this gene could not compete away the shift between PAP12PRE and PAP12PREBP. These results support our ideas that despite having low sequence identity, PREs can still be detected by gel shift assays.

We also enriched a nuclear factor that binds to the PAP12PRE using an avidin-biotin method, in which a complex formed between a biotinylated PAP12PRE probe and PAP12PREBPs are trapped by streptavidin-agarose beads. In the eluted fraction, a 23.5 kDa nuclear factor was enriched as indicated by SDS-PAGE analysis. This factor, when present, was able to shift the PAP12PRE in a sequence specific manner. In fractions where this band was missing, no DNA binding was detected. Thus a fraction enriched for this 23.5 kDa factor binds to the PAP12PRE. We attempted to identify this factor by MALDI-TOF mass spectrometry. The results obtained from the MALDI-TOF mass spectrometry could not identify a unique factor from the *Arabidopsis* genome. This method is useful for the isolation of PAP12PRE binding proteins and can be applied to

other PREs. In the future, other methods for protein identification will be performed to identify the 23.5 kDa band. The identification of this band will help understand the molecular response of *Arabidopsis* during phosphate deficiency.

CHAPTER V

CONCLUSIONS AND PERSPECTIVES

Phosphate is a limiting macronutrient for plant growth and development. Thus, plants have developed strategies to adapt to P_i stress such as root growth, increase root uptake of P_i, secretion of organic acids, remobilization of phosphate from old tissue to growing tissue, and secretion of phosphatases and nucleases to release P_i from organic sources. Transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips and small microarrays have been used to identify phosphate-responsive genes (Misson et al., 2005; Hammond et al., 2003).

Several proteins have been identified that are involved in P_i-starvation signaling, yet we do not know the molecular details of how this process is regulated. Mutants defective in the induction of P_i-responsive genes have been isolated (*phr1* and *pdr2*; Rubio et al., 2001; Ticconi et al., 2004). PHR1 encodes a MYB domain containing transcription factor, while the gene responsible for the *pdr2* mutant remains to be identified.

Additional mutants with altered levels of P_i have also been identified. These include *pho1* and *pho2* (Poirier at al., 1991; Delhaize and Randall, 1995). The *PHO1* gene encodes a novel protein involved in xylem loading of phosphate (Hamburger et al., 2002). It contains SPX and EXS domains which may be involved in signaling (Wang et al., 2004). The *pho2* mutant has recently been cloned, and it encodes an E2 conjugase. This gene is regulated by a P_i-dependent micro RNA, miR399 (Bari et al., 2006; Aung et al., 2006).

Here we used a reverse genetics approach to elucidate the role of *PAP12* (a purple acid phosphatase) during the phosphate starvation response (PSR), identify a *cis-*element (PAP12PRE) important for its induction during P_i-limiting conditions and purified a transcriptional regulator that binds to the PAP12PRE.

PAP12 is a member of the purple acid phosphatase family. These acid phosphatases are enzymes secreted by plant cells in response to phosphorus-deficient conditions. They are thought to liberate phosphate from organic sources (Tarafdar and Claassen, 1988). We show that *PAP12* expression is controlled in response to phosphate limitation. It is expressed in leaf, root, stem, silique, and flower, indicating that PAP12 may play a broader role in the PSR than just scavenging phosphate from organic sources in the soil.

To determine the role of *PAP12* in the PSR, we obtained a T-DNA insertion line in the *PAP12* gene. We measured total and free phoaphate in wild-type and the *pap12* mutant, assayed its ability to grow on different phosphate sources, and looked at the effect of loss of PAP12 activity in the *pho1* background. Our results do not support a major role for PAP12 during P_i-limiting conditions. We did not observe any visible phenotype other than the *pho1 pap12* double mutant took longer to flower than the *pho1* mutant alone. We still do not know the role of *PAP12* or the other 28 secreted acid phosphatases, present in *Arabidopsis*, in plant adaptation to low phosphorus conditions. Knockouts for the other PAPs will need to be identified and several different PAP mutant combinations will have to be analyzed.

There may be other functions for PAPs. The first is that an *Arabidopsis* phospholipase D gene family (*PLDZ2*) is gradually induced upon Pi starvation in both shoots and roots (Cruz-Ramirez et al., 2006). The second is that bacterial PAPs may act as Fenton-type catalysts in order to prevent damage caused by reactive oxygen species (Schenk et al., 2000). The third is that genes involved in oxidative stress are strongly induced in P_i-starved tissue (Misson et al., 2005). Based on these observations, we propose PAPs may play a role in regulating membrane stability or permeability or in controlling the level of reactive oxygen species generated during P_i-deficiency stress. PAP12 could also be involved in the activation of a stress-induced flowering pathway. Intrinsic (genetic) and extrinsic (environmental) factors control the timing of flowering in plants. However, stress can disrupt this control and cause plants to set seeds. This may be a strategy that all organisms use, as organisms faced with severe stress should evolve to reproduce more quickly to ensure future generations. This hypothesis will be the focus of further analysis. We provide some evidence that *PAP12* is involved in regulating flowering in *Arabidopsis* under phosphate stress. *PAP12* over-expression lines flower earlier while knockout for *PAP12* flower later. Now that the gene responsible for the *pho2* phenotype is cloned, we can make a *pap12 pho2* double mutant (Aung et al., 2006). We predict that *pho2* over-expressing PAP12 will lead to flowering earlier than *pho2* plants, and a *pho2 pap12* double mutant will take longer to flower than *pho2* plants when stressed for phosphate.

To understand how *PAP12* is transcriptionally regulated, we used promoter deletions and gel shift assay to identify a P-responsive *cis*-element, PAP12PRE, in the

PAP12 promoter. Using an avidin-biotin method we also isolated and attempted to identify the factor that binds to the PAP12PRE. A 35-bp region of the *PAP12* promoter shifted in a phosphate dependent manner. Although we could not find this exact *cis-*element in other P_i-responsive genes, we were able to show that promoter fragments from *RNS2*, a P_i-responsive gene, can compete away the shift observed between PAP12PRE and an unidentified nuclear factor. We enriched a 23.5 kDa nuclear factor that binds specifically to the PAP12PRE and believe this is the factor responsible for the induction of *PAP12* upon phosphate starvation. An attempt was made to identify this protein by MALDI-TOF Mass Spectrometry. We could not positively identify the factor with high confidence. Thus, further attempts will be made to identify this factor. The work presented here will add to our knowledge about the molecular processes which regulate phosphate nutrition and provide a foundation on which to build upon. This will be important in improving P_i use by crop species.

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