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PHOSPHATASE SECRETION MUTANTS IN *ARABIDOPSIS THALIANA*

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by

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ABSTRACT

Phosphatases are important enzymes for understanding plant phosphorus relations. Plant-derived soil phosphatases may help plants during phosphorus deficiency by mobilizing organic phosphorus for plant uptake. In microbes, phosphatases and other responses to phosphorus deficiency are controlled at the transcriptional level by the PHO regulon, and evidence is mounting for the existence of a plant PHO regulon. By isolating *Arabidopsis* mutants for phosphatase secretion, components of a plant PHO regulon may be identified. Identification and characterization of constitutive phosphatase secretion (*cps*) mutants showed that root-bound phosphatase activity can be increased, and that under low-phosphorus conditions this increased activity can preserve higher root phosphate concentrations. However, there may be a physiological cost in growth associated with the *cps* trait. Characterization of the phosphatase under-producing (*pup*) mutants revealed that acid phosphatases can alter whole-plant phosphorus relations, that secreted phosphatases are important for acquiring and/or maintaining phosphate levels when grown in a soil substrate with an organic P component, and that secreted phosphatases are probably under post-transcriptional control. Although the *cps* and *pup* mutants are not defective in P deficiency responses, these mutants give us insight into physiologically important components of plant P regulation.

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CHAPTER 1:
Introduction

Understanding the mechanisms underlying plant responses to phosphate deficiency has important implications for agriculture. Phosphate is mainly taken up by plants in the diprotic form, $H_2PO_4^-$ or orthophosphate (Tate, 1985). Although a macronutrient for plants, the availability of soil inorganic phosphate (P_i) is so limiting that even micronutrients are often more abundant (Bieleski, 1973). In a survey of 135 U.S. soils, P_i levels in solution did not reach above 8 mM (Barber et al., 1963; Bieleski, 1973). P_i is recalcitrant in soils because it is immobilized within soil organic complexes, clay complexes, and precipitated iron, calcium, and aluminum salts (Sample et al., 1980). In agricultural settings, sparingly available soil P_i is ameliorated with rock P fertilizers. Applied fertilizers represent more than 90% of world processed P use (Bieleski and Ferguson, 1983), and modern agriculture relies on crops that provide maximal yields with these fertilizers. However, global resources of extractable P are limited, non-renewable, and increasingly environmentally hazardous to obtain (Cathcart, 1980). As these extractable stores become depleted, agriculture will be forced to adjust to a lack of processed rock P fertilizer while continuing to feed an expanding population. Alternately, the organic P (P_o) component of soil is relatively abundant in agricultural soils, representing 30-70% of total soil P (Marschner, 1995), and P_o soil amendments are sustainable when compared to their mined rock P counterparts. Plants may mobilize P_o during phosphate deficiency by secreting enzymes, including acid phosphatases, into the rhizosphere. Ultimately, increasing the availability of P_o to plants may decrease agriculture's dependence on P_i fertilizers.

Acid phosphatase biochemistry and terminology

Phosphatases (E.C. 3.1.3) are a class of enzymes that have been used as vacuolar markers in cellular compartmentalization studies, protein "fingerprints" in isozyme analysis (Paul and Williamson, 1987 and others), and – ever since Annamaria Torriani-Gorini discovered their secretion from *E. coli* as the first derepressible enzyme expression system in 1953 (reviewed in Torriani-Gorini, 1987)- markers for P deficiency. Phosphatases are non-specific orthophosphoricmonoester phosphohydrolases, cleaving P_i from larger molecules at their ester linkage sites. Phosphatases are divided into two broad groups based on their pH optima; alkaline phosphatases (E.C. 3.1.3.1) and acid phosphatases (E.C. 3.1.3.2). Microbes produce both acid and alkaline phosphatases while higher plants and animals exclusively produce acid phosphatases (Dick and Tabatabai, 1984).

One group of acid phosphatases, termed the purple acid phosphatases (PAPs), contain a metal-binding dinuclear center in their active sites that give them a purple color when concentrated. While some plant acid phosphatase proteins have not been studied sufficiently to determine if they contain the metal-binding residues necessary for this designation (Berger et al., 1995; Gilbert et al., 1999; Trull et al., 1997 and others), other confirmed PAPs from plants have been implicated in P-deficiency responses (Haran et al., 2000; del Pozo et al., 1999; Nakazato et al., 1998). PAPs are glycoproteins, and while sparsely represented in microbes and fungi, these enzymes are widely distributed in plants and animals (Oddie et al., 2000; Schenk et al., 2000). PAPs consist of two groups differentiated by molecular weight. The low-molecular weight PAPs (~35kDa), also known as type 5 or tartrate-resistant PAPs, occur both as monomers and as two-subunit forms derived from disulphide-linked monomer fragments (Ljusberg et al., 1999). Type 5 PAPs have additional peroxidation activity (Hayman and Cox, 1994). Most extensively

studied in human systems, type 5 PAPs are thought to generate reactive oxygen species during both bone resorption in osteoclasts (Hayman et al., 1996) and microbial attack by macrophages (Hayman and Cox, 1994). At least one plant type 5 PAP, *AtACP5*, has been identified in the model plant *Arabidopsis thaliana* (del Pozo et al., 1999). Transcript accumulation of *AtACP5* is induced during senescence and by abscisic acid, peroxide, and low-P conditions. High-molecular weight PAPs (~55kDa) are unique to plants and form homodimers through disulfide bonds. Similarity between the high- and low- molecular weight PAPs is limited to a series of motifs containing the metal-coordinating amino acids of the active sites (Klabunde et al., 1995). The *Arabidopsis* purple acid phosphatase-1 (*PAP1*) gene product is a predicted 55kDa peptide belonging to the high-molecular weight PAP group. *PAP1* has a signal peptide sufficient for secretion of a marker protein, and its promoter is low-P inducible. Expression studies with the *PAP1* promoter linked to GUS show that the gene is transcribed first in leaves, then in the lateral root primordia and emerging lateral meristems after P is removed from the growth media. With prolonged P deprivation, most tissues express *PAP1* except the epidermal layer, root hairs, and apical meristem (Haran et al., 2000). The unique biochemistry of acid phosphatases may make them useful to plants when P_i is limiting.

Phosphatases in the rhizosphere

Phosphatases are active in soil. Their broad substrate specificity makes these enzymes good candidates for action on a variety of P_o substrates found in soils. Although soil P_o is dynamic and difficult to characterize, it is primarily made up of inositol hexaphosphate, glucose-6-phosphate, glycerol phosphate, nucleoside monophosphates, and polynucleotides (Dalal, 1977; Pant et al., 1999). These compounds are all phosphate esters on which phosphatases could act to liberate P_i. While most enzymes are generally short-lived in soil, phosphatases can be immobilized on or within soil clays and humates

that preserve their activity (Burns, 1986), and phosphatases that persist in soils have pH optima in the same range as the surrounding soil pH (Dick and Tabatabai, 1984; Rojo et al., 1990).

Plants alter soil phosphatase activities within the rhizosphere. Tarafdar and Jungk (1987) showed that phosphatase activity increases within 3.1mm of clover and wheat roots, and that this increase significantly correlates ($r=0.97$ and $r=0.99$) with a zone of P_o depletion around these roots. Haussling and Marschner (1989) also showed that soil acid phosphatase activity increases in the rhizosphere of a mature Norway spruce stand when compared to bulk soil, and this increase correlates with a decrease in P_o. The fact that phosphatase activity is higher within the rhizosphere explains observations that P_o declines in the field during the growth season when plants are present and P_i is limiting (Dormaar, 1972; Sharpley, 1985) and that organic P soil amendments (sodium glycerophosphate, lecithin, and phytin) increase plant dry mass (Tarafdar and Claasen, 1988).

Plants can alter their rhizospheric phosphatase activity levels by secreting phosphatases from their roots. Plant-derived acid phosphatases are hardy enzymes that are able to maintain activity in soils. Secreted plant phosphatases maintain >50% activity over a broad pH range (4.0-7.6), maintain >80% activity over a broad temperature range (22-48˚C), and maintain stability at temperatures as high as 60˚C (LeBansky et al., 1991; Li and Tadano, 1996). Plants grown in nutrient solutions can mobilize organic forms of P to fulfill their complete P nutritional requirements (Furlani et al., 1987; Tarafdar and Claasen, 1988; Yan et al., 1996), so (at least under these controlled conditions) the phosphatases that plants secrete are sufficient.

In the soil, plant phosphatases are supplemented with microbial phosphatases. Free-living soil microbes are concentrated around root systems because sloughed off plant cells and root secretions are a source of carbon and nutrients (Marschner, 1995;

Tate et al., 1991). Phosphatases from soil microbes contribute significantly to the phosphatase activity of soils. In two soils fumigated with methyl bromide to decrease their microbial populations, soil phosphatase activity was significantly decreased despite the presence of maize plants (McCallister et al., 1997). However, the plant-microbe-phosphatase interaction is not necessarily advantageous to plants because microbes are both a source and a sink for P_o: while the microbes secrete phosphatases that may liberate P_i for plants, they also compete with plants for that resource.

Mycorrhizal fungi are also a significant source of soil phosphatases. Instead of competing with plants for P, mycorrhizal fungi can be major providers of P_i to plants when available P is limiting (Jayachandran et al., 1992; Rovira et al., 1983). In mycorrhizal associations, the fungus is considered to be an extension of the plant's root system, greatly increasing the soil volume that can be explored by the plant roots alone. In exchange for Pi, the plant supplies the fungus with carbon. Most, but not all, higher plants can form mycorrhizal associations. Uptake of P by plant roots with vesicular-arbuscular mycorrhizae is up to 14 times higher than non-mycorrhizal roots (Rovira et al., 1983), and in field trials where mycorrhizal associations are reduced with fumigation, maize plants have lower P concentrations and appear stunted when compared to controls grown in non-fumigated soil (Jawson et al., 1993). Mung bean, wheat and chick pea produce higher yields when inoculated with high-phosphatase-producing mycorrhizal fungi strains (Tarafdar and Rao, 1996; Tarafdar et al., 1992). However not all plants form mycorrhizal associations, and in non-mycorrhizal roots, acid phosphatase activity is associated with the plant roots rather than microorganisms (Dodd et al., 1987; McLachlan, 1980).

Phosphatases within plants

Within the plant, acid phosphatases are located where they would be effective for either remobilization of stored P or recycling of released P. Vacuolar-localized acid phosphatases can mobilize poly-phosphate stores such as phytate (Duff et al., 1994), and the phosphate-starvation-induced purple acid phosphatase from *Spirodela oligorrhiza* is covalently bound to the outer surface of the plasma membrane (Nakazato et al., 1998). Even P-deficient cells are leaky for P_i, and membrane-bound acid phosphatases may be able to recycle this "lost" P from within the periplasmic space back into the cell as part of a P-transport system complex (Leggewie et al., 1997; Thomas et al., 1999).

Phosphatases and the PHO regulon

Like other microorganisms, *Saccharomyces cerevisiae* responds to low levels of P_i in its surrounding medium by activating transcription of enzymes that can scavenge low amounts of P_i - the PHO regulon. The PHO regulon includes three genes for secreted acid phosphatases (PHO5, PHO10, PHO11), a vacuolar alkaline phosphatase (PHO8), and a high-affinity P_i translocator protein (PHO84). The major acid phosphatase (PHO5) is induced 500-fold, and the P_i -translocator is increased 60-fold, while the alkaline phosphatase is induced only 2- to 3-fold. When coordinately expressed, these genes are thought to serve as an "emergency response system" for Pi stress. The secreted acid phosphatases liberate previously unavailable P_i, and the translocator protein functions to import low levels of P_i from the extracellular surroundings. Meanwhile, the vacuolar alkaline phosphatase serves to mobilize poly-phosphates and other P_i sources from the vacuole (Yoshida et al., 1987).

PHO regulon components are regulated at the transcriptional level by at least five other proteins: Pho81, Pho80, Pho85, Pho2, and Pho4 (Figure 1.1). Pho81, or possibly

another protein upstream in the signal transduction cascade that interacts with Pho81, acts as the P_i -sensitive switch (Hirst et al., 1994). Although the function of Pho81 is unknown, it is thought to participate in protein-protein binding because its structure contains six ankyrin repeats (Ogawa et al., 1993). Pho81 can interact with both Pho80 (independently of P_i status) and Pho4 (only under low- P_i conditions) (Hirst et al., 1994). Pho80 is a cyclin that forms a complex with Pho85, a cyclin-dependent kinase (Kaffman et al., 1994). Pho4 is a basic helix-loop-helix (b-ZIP) transcription factor, and its DNA-binding site is distinct from its transcriptional activation site (Jayaraman et al., 1994). The Pho80-Pho85 complex hyper-phosphorylates Pho4, causing Pho4 to be inactivated (Kaffman et al., 1994). The Pho80-Pho85 complex also binds to Pho4 and masks its activation domain (Jayaraman et al., 1994). When Pho81 binds to Pho4 under low- P_i conditions, Pho4 no longer associates with the Pho80-85 complex. Free Pho4 binds to Pho2, a homeobox protein. The Pho4-Pho2 complex then initiates the transcription of (at least) PHO5 (Hirst et al., 1994; Venter et al., 1994), PHO84 (Tamai et al., 1985), and PHO81 (Ogawa et al., 1993) by binding to the promoters of these genes at their upstream activation sequence(s), 5'-CACGT(G/T)-3' (Hirst et al., 1994; Ogawa et al., 1995). In at least the PHO5 promoter, activation of the gene is precluded by nucleosomal disruption, and the transcriptional activation domain of Pho4 is necessary for the disruption (Fascher et al., 1993; Svaren et al., 1994; Venter et al., 1994). Both Pho4 and Pho2 are constitutively expressed at low levels (although PHO2 can be induced by low levels of P_i and inhibited by its product) (Yoshida et al., 1989). The system is a closed loop because Pho81 indirectly regulates its own transcription (Ogawa et al., 1993; Yoshida et al., 1987).

Evidence for a plant PHO regulon

Although still not elucidated, plants may have a PHO regulon controlling at least some of their -P responses at the transcriptional level because the PHO regulon has been conserved through evolution. There is a prokaryotic PHO regulon that is very similar to the yeast system (Torriani, 1990; Van Dien and Keasling, 1998). Further up the evolutionary chain, the model fungus *Neurospora crassa* controls the expression of phosphatases and high-affinity transporters with a system homologous to the yeast PHO regulon. In *Neurospora*, the PHO system is more complex because it also regulates the transcription of vacuolar and secreted ribonucleases. Mutants in phosphate deficiency responses (*psr*) have recently been identified in the green alga *Chlamydomonas reinhardtii*. Although their mutant genes have not been cloned, these two *Chlamydomonas* mutants have the same genetics and phenotypes as PHO regulon mutants from other model systems: *psr1* is a single recessive mutation that results in a phenotype lacking both inducible secreted phosphatases and high-affinity phosphate transport under P-deficient, while *psr2* is a single dominant mutation that results in increased phosphatase secretion under P-sufficient conditions (Shimogawara et al., 1999). Since the PHO regulon appears to have been conserved up the evolutionary ladder from prokaryotes to unicellular eukaryotes to multicellular fungi and probably even unicellular plants, then one could reasonably expect to find it in higher plants as well.

Evidence for a higher plant PHO regulon is circumstantial but significant. Plant phosphatases (Haran et al., 2000; del Pozo et al., 1999; Nakazato et al., 1998), high-affinity P translocators (Leggewie et al., 1997; Smith et al., 1997; Muchhal et al., 1996), and ribonucleases (Bariola et al., 1994) are all induced by low-P conditions at the transcriptional level. Some of these genes also contain sites within their promoter's 5-bp sequences that are similar to the PHO4 binding site. Recently a family of homoeodomain

leucine zipper proteins were identified by their binding to a P response domain in promoter of a soybean vacuolar-localized acid phosphatase (Tang et al., 2001; Mason et al., 1993). Further study of these proteins may lead to the elucidation of a very complex plant PHO regulon.

The plant PHO regulon may be involved in many adaptations to low P. Just as *Neurospora* is increased in the complexity of its responses to low P, higher plants may control even more responses with their PHO regulon. Examples from at least two other classes of plant genes have increased transcript accumulation under P deficiency – genes also regulated by mycorrhizal associations (Mt4 and At4) (Burleigh and Harrison, 1999; Burleigh and Harrison, 1998) and genes involved in the synthesis of anthocyanin pigments (chalcone synthase) (Trull et al., 1999). Other physiological responses to phosphorus deficiency may be controlled by a plant PHO regulon as well. Some of these responses include the acidification of the rhizosphere, as well as the production of additional root exudates such as organic acids (reviewed in Grinsted et al., 1982). Changes in root morphology during P deficiency may also be controlled by a plant PHO regulon, including increased number and density of root hairs (Bates and Lynch, 1996), shallower root system branching (Williamson et al., 2001; Bonser et al., 1996), and the formation of proteoid roots (Johnson et al., 1996). Shoot growth reduction (relative to root growth) and shoot anthocyanin accumulation during low P conditions (Marschner, 1995) may also be controlled by a plant PHO regulon.

The recent identification of an *Arabidopsis thaliana* mutant defective in multiple aspects of P deficiency responses is additional evidence for a coordinated plant PHO regulon. The *Arabidopsis pho3* mutant has decreased acid phosphatase activity in root and shoot extracts, does not increase its root acid phosphatase activity in response to P deprivation, accumulates less P in roots and shoots when grown in P-sufficient (but not P-deficient) agar media, and accumulates less P in mature (>21 day old) shoots when

grown in P-sufficient media and transferred to soil. The *pho3* mutant has other physiological problems implicated in P deficiency responses, including low fertility, delayed flowering (by 2-3 weeks), less shoot biomass when grown in soil, 50% reduced chlorophyll content when grown in P-sufficient (but not deficient) agar media, starch accumulation, and anthocyanin accumulation. Because the *pho3* mutant lacks the ability to increase its phosphatase activity and anthocyanin accumulation during P deficiency, and because it has so many other defects associated with P deprivation, the *pho3* mutant may be deficient in a regulatory component of the plant PHO regulon (Zakhleniuk et al., 2001).

Arabidopsis mutants may uncover the plant PHO regulon

The starting point for identifying the PHO regulon in each model system has been the isolation of mutants defective in their responses to phosphate deficiency and the subsequent cloning of those mutant genes. *Arabidopsis thaliana* is the model system of choice for studying the genetics of higher plants because of its short life cycle (6-10 weeks), relatively small size (15 cm high X 5 cm wide), large seed output (~1000/plant), and completely sequenced genome. Some *Arabidopsis* mutants in phosphate relations have previously been identified, including the phosphatase under-producing mutants (Trull and Deikman, 1998), a mutant deficient in xylem loading of phosphate (Poirier et al., 1991), and a shoot phosphorus hyper-accumulator mutant (Delhaize and Randall, 1995). Given the complexity involved in root-to-shoot partitioning of phosphate within the plant, a screen focusing on one of the classical PHO regulon responses and targeted to the roots is a much more direct method of screening for mutants in PHO regulon components. The phosphatase under-producing (*pup*) and *pho3* mutants were identified based on *in-vivo* histological staining for seedling root phosphatase activity (Zakhleniuk

et al., 2001; Trull and Deikman, 1998). Two mutants were discovered from the *pup* screening effort, *pup1* and *pup3*. The *pup1* mutant that does not appear to increase its phosphatase secretion on low-P media, lacks one phosphatase isoform, is incompletely dominant and has a decreased root:shoot ratio under -P conditions (Trull and Deikman, 1998). Preliminary characterization of the *pup3* mutant revealed that it is recessive and has decreased activity of another phosphatase isoform (M Trull, pers. comm.). This work set out to further knowledge of the genetics behind plant responses to phosphate deficiency by studying the *pup* mutants and identifying additional *Arabidopsis* mutants for phosphatase secretion.

Objectives of dissertation research

The goal of this research is to further knowledge of plant PHO regulon components by studying the secretion of acid phosphatases using mutants in the model plant *Arabidopsis thaliana*.

Identification and characterization of constitutive phosphatase secretion mutants. Chapter two describes the identification of constitutive phosphatase secretion (*cps*) mutants in *Arabidopsis thaliana*, their genetic characterization, and their phosphatase secretion responses. Phosphatase activity is measured histochemically, in whole root assays, and proteins isolated from root and shoot tissues.

Characterization of the phosphatase under-producing mutants. Chapter three further characterizes the phosphatase under-producing (*pup*) mutants, *pup1* and *pup3*. Root-bound acid phosphatase activity was quantified, and specific acid phosphatase activity measurements were performed on proteins from exudates, roots and shoots. Concentrated exuded proteins were also tested for differences in acid phosphatase

isozyme activity and reactivity with the *Arabidopsis* purple acid phosphatase 1 (PAP1) antibody. Studies of the *pup* mutants grown in soil and nutrient solution were performed to evaluate whole-plant P relations.

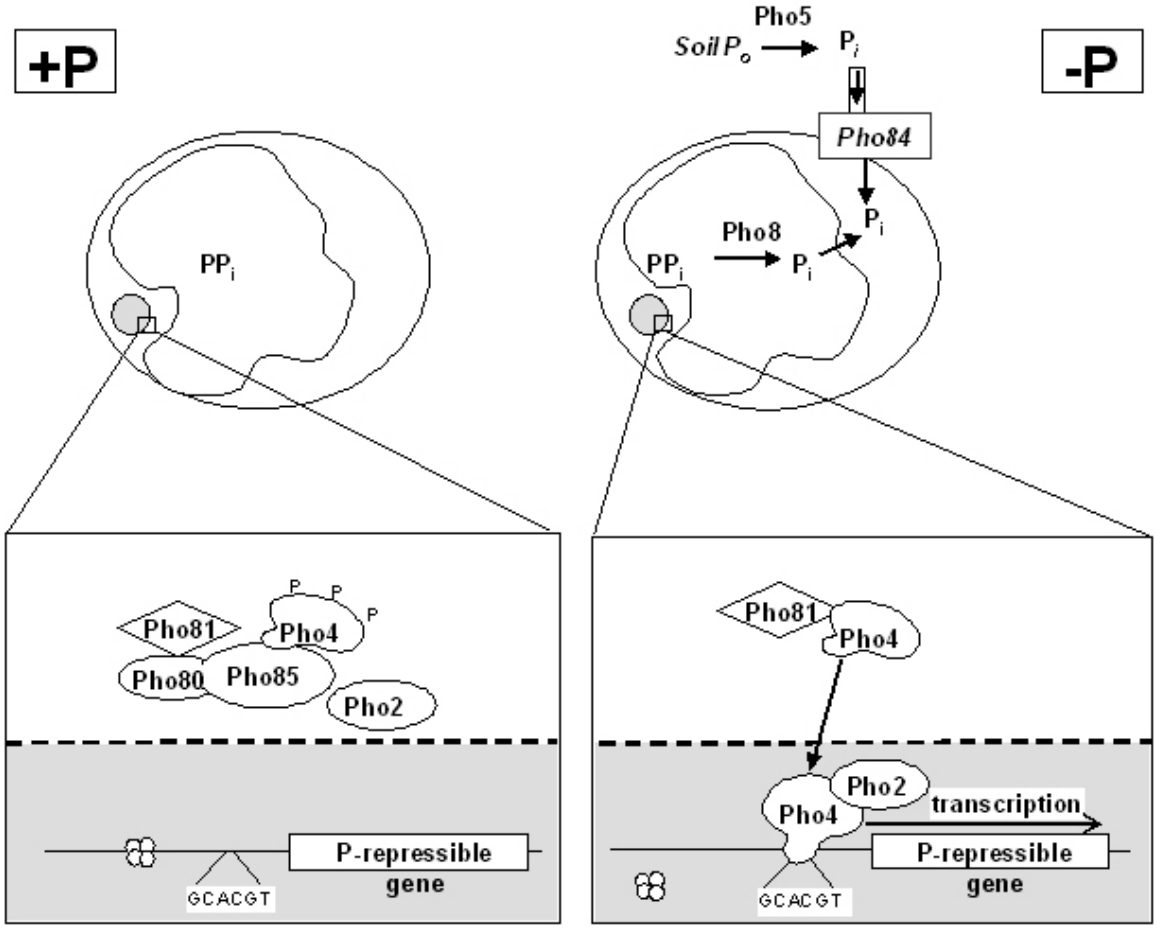

Figure 1.1. The PHO regulon of *S. cerevisiae*. P-repressible genes are controlled by the signal transduction machinery of the PHO regulon. A single yeast cell under +P and -P conditions is represented with a cytoplasm/nucleus border inset. The nuclear envelope is represented by a dashed line and the nucleus is shaded.

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CHAPTER 2:
Identification and characterization of constitutive phosphatase secretion mutants

INTRODUCTION

The PHO regulon controls the transcription of P-repressible genes in microorganisms. In *Saccaromyces cerevisiae* and *Neurospora crassa*, the PHO regulon consists of an ankyrin repeat protein a cyclin/cyclin dependent kinase pair , a basic helix-loop-helix transcription factor, and a homeobox protein. P-sensitive genes controlled by the PHO regulon include high-affinity P_i transporters, ribonucleases, and acid and alkaline phosphatases (Peleg et al., 1996; Peleg and Metzenberg, 1994; Ogawa et al., 1995; Versaw, 1995; Grotelueschen et al., 1994; Hirst et al., 1994; Kaffman et al., 1994; Venter et al., 1994; Kang, 1993; Ogawa et al., 1993; Kang and Metzenberg, 1990; Mann et al., 1989; Yoshida et al., 1987).

Secreted phosphatases were the first de-repressible enzymes discovered, and mutants for phosphatase secretion were important for elucidating PHO regulon components. In *S. cerevisiae*, mutants that constitutively secrete phosphatases are defective in high-affinity P_i transport (PHO84, PHO86, PHO87, PHO88), the cyclin (PHO80), the cyclin dependent kinase (PHO85), a fatty acid synthesis gene (ACC1), and a plasma membrane ATPase (PMA1) (Lau et al., 1998; Bun-ya et al., 1996; Yompakdee et al., 1996; Ueda et al., 1975). Since constitutive phosphatase secretion mutants yielded significant contributions in the elucidation of microbial PHO regulon systems, similar mutants in *Arabidopsis thaliana* may shed light on a plant PHO regulon.

Here I describe the isolation and genetic characterization of a novel class of *Arabidopsis* mutants, the *c*onstitutive *p*hosphatase *s*ecretion (*cps*) mutants. Two *cps1* alleles and *cps2* were subjected to *in-vivo* root-bound acid phosphatase (APase) activity assays to quantify their phosphatase secretion phenotypes, and native gel electrophoresis was carried out on proteins isolated from roots and shoots to determine the APase isoforms affected in the mutants. Further, physiological impacts of the *cps1* mutations were evaluated by growing the plants in a soil mix with an organic P component.

MATERIALS and METHODS

Plant growth conditions

Unless otherwise stated, plants were grown in a growth chamber under the following conditions unless otherwise noted: 22º C, ambient relative humidity, 100 µmol $m^{-2}s^{-1}$, 16 hours light/8 hours dark. Seeds were collected and allowed to dry for 2 weeks before being stored at 4º C. Seeds were sterilized shortly (< 2 weeks) before use. When growing plants on tissue culture media, seeds were plated and stored at 4ºC for 24-36 hours to synchronize germination before transfer to the growth chamber. Two different types of nutrient solutions were used, based on either modified Hoagland's solution (Johnson et al., 1957) or Murashige and Skoog (MS) salts (Murashige and Skoog, 1962). For -P conditions, sulfate salts were used to replace the phosphate salts so that the conjugate ion would remain constant.

cps mutant screen

EMS-mutagenized *Arabidopsis thaliana* seeds were surface sterilized and plated on media containing 0.5X Murashige and Skoog (MS) salts (Murashige and Skoog, 1962), 1% sucrose, 0.05% MES, and 2% phytagel. The media was buffered to pH 5.7. BCIP (5-bromo-4-chloro-3-indoyl phosphate, Sigma, St. Louis, MO) was dissolved in a stock solution of 0.1 M Tris, pH 9.0 to 5mg/mL, filter sterilized, and added to a final concentration of 0.008% to the sterilized media shortly before pouring (Goldstein et al, 1989). After a 2-day cold treatment at 4ºC, plates were positioned 5º from vertical in the growth chamber and seedlings grown until 5 days post germination (dpg). BCIP forms a blue precipitate on growing roots when cleaved by phosphatases. Plants whose roots did not penetrate the media were not scored for BCIP staining. For -P BCIP media, phosphate salts were replaced with sulfate salts so that the conjugate cation concentration remained constant.

Digital images

Digital images of 5dpg BCIP-stained roots were taken from a Stemi SV11 (Zeiss) dissecting scope using a Fostec Ace I EKE dual optic lighting system from above (Figure 2.1.). Plants were removed from the media and their entire length digitally captured. The longer, wild-type (wt) -length roots necessitated curling the roots to fit into the field of view. The images were captured by Adobe® Photoshop® 6.0 (Adobe Systems Incoporated, San Jose, CA) with the following settings: auto gain limit 16, automatic white balance (1.00 red, 1.530 green, 23.087 blue), 1.00 gamma adjust. All images were

taken at the same brightness, contrast, and color settings, and these settings were not altered after image capture.

Genetic Mapping

Mutations were mapped using co-dominant amplified polymorphic sequences (CAPS) located at about 30cM increments throughout the *Arabidopsis* genome. The material used for mapping consisted of F2 mutant segregants from a cross to an ecotype (Landsberg erecta) that is polymorphic when compared to the mutant background ecotype (Columbia). Scoring 28 individuals per locus is the minimum number of plants required to establish linkage to one of the CAPS markers at the 95% confidence limit (Konieczny and Ausubel, 1993). Chi-square analysis was used to determine marker linkage, and once linkage was determined with multiple markers on a chromosome, then the Kosambi method was used to calculate genetic distances from those markers (Koornneef and Stam, 1992).

Root-bound acid phosphatase assay

Seeds were plated on 0.5X modified Hoagland's media and allowed to grow for 8 days. Plants of uniform size were selected and moved to 10mL of liquid media (0.5X modified Hoagland salts with 0.5mM Pi, 3% sucrose, and 2.6mM MES buffered to pH 5.7), 3 plants per 150 mL flask. Flasks were shaken at 150 rpm in the dark to encourage root growth. After 10 days, plants were rinsed in -P media, transferred to fresh media (either +P or -P) and allowed to grow for an additional 48 hours. For the assay, plants were removed from the shaking flasks, rinsed in -P media, and transferred to rocking

Magenta boxes with 10mL reaction buffer (-P media with 19.1mM MES, 1.3%, and 5mM p-nitrophenol phosphate, or pNPP) in a time-dependent manner. Reactions proceeded for 30-50min at ~22°C, then 185µL of the reaction buffer was removed to 832µL 1N NaOH. The accumulation of pNP was read as A_{410} in a standard spectophotometer. Absorbance values were converted to nmol pNP based on a standard curve of pNP (Sigma, St. Louis, MO). Roots and shoots were dissected, transferred to pre-weighed aluminum foil envelopes and dried for 2d at 65°C. Root-bound phosphatase activity was calculated as nmol pNP liberated by the root system/min/root dry weight. Total P determinations were carried out on the dried tissue using the molybdate method (Murphy and Riley, 1962). This experiment was replicated fully twice, and a completely randomized design was used for each replication. In the first replication, N=4 for each treatment, and in the second replication N>=7 for each treatment. After transfer to shaking flasks, the temperature for the second replication (and only the second replication) was increased from 22˚C to 25˚C. Statistical analysis was performed with StatView v.5.0.1 (SAS Institute, Cary, NC).

Protein isolation

Plants were grown under continuous illumination on 300-µm mesh nylon screens placed on 100- X 20-mm petri dishes containing solidified agar supplemented with 1/10 MS salts and 1% sucrose. One week after germination, the nylon filter along with the intact plants were transferred to a sterile floating membrane raft (Life raft, Sigma, St. Louis, MO) and placed in a GA-7 tissue culture box (Sigma, St. Louis, MO) containing 100 mL of 0.5X Hoagland's salts and 1% sucrose. After another week of growth, the

plants were transferred to fresh 0.5X Hoagland's nutrient solution containing either 1 mM P_i or no P_i and allowed to grow for 5 days (Muchhal et al., 1996; Poirier et al., 1991). Root and shoot tissues were harvested, flash frozen in liquid nitrogen and stored at -80°C until proteins were extracted.

Proteins were extracted separately from roots and shoots. Tissues were ground in a liquid-N-chilled mortar and pestle, then transferred to Sarsdedt tubes containing 4 mL extraction buffer per g tissue (100mM potassium acetate, pH 5.5 to buffer the solution; 2 mM EDTA to inhibit metalloproteases; 0.1 mM PMSF to inhibit serine and thiol proteases; 5 mM DTT to protect against protein oxidation, 20mM $CaCl_2$ to release wall-bound proteins), and 60 mg polyvinylpolypyrrolidone (PVPP) per g tissue (to remove polyphenols) (Aarts et al., 1991; Bariola et al., 1999; del Pozo et al., 1999; Gilbert et al., 1999, Trull et al., 1997; Bollag and Edelstein, 1991; Gegenheimer, 1990). The tissue/buffer/PVPP solution was gently agitated at 4ºC for 60 minutes, then centrifuged at 2,7000xG and the pellet discarded.

For all samples, glycerol was added to 20% (v/v), and then samples stored in small aliquots at -80ºC until used. There were 3 separate protein harvests, each harvested from separate plantings staggered by time. All proteins were quantified according to the Bradford technique.

Phosphatase activity gels

Equal amounts of protein (50-100 μg per lane) were loaded onto discontinuous native PAGE (5% (w/v) stacking gel, 10% (w/v) resolving gel). The native gels were run

at low voltage (30-60V) and low temperature (4˚C). Staining was accomplished by the Fast Black/ß-napthyl acid phosphate method (Trull and Deikman, 1998).

P accumulation when grown in a peat/vermiculite soil mix

Seeds were planted in 6 cm diameter pots in a standard 55-65% sphagnum peat moss/perlite mix (Sunshine Aggregate Plus Mix #4, SunGro, Bellevue, WA). The peat mix contained no additional fertilizer. Prepared pots were stored at 4°C for 2 d, then placed in the growth chamber under standard conditions (above). Plants were thinned to a density of 4 plants per pot at 7dpg and thereafter fertilized twice weekly by subirrigation with either +P or -P fertilizer (Somerville et al., 1982). No additional watering was needed. Shoots were harvested at 3 weeks post-germination (or at the first sign of the primary reproductive inflorescence), dried, and total P deteriminations carried out as for the root-bound APase assay.

For the available soil P_i, ~0.3g of soil was suspended in 10mL deionized water and the eluate removed for P determination. To determine the total amount of P in the soil, ~0.1g was converted to ash by baking in a 490°C oven for 12h prior to P determination. Soil tests were repeated with three different samples.

RESULTS

cps *mutant identification*

Constitutive phosphatase secretion *(cps)* mutants were identified based on their blue root phenotype on +P BCIP media. The total number of seeds screened: 11,000 ethylmethane sulfonate (EMS)-mutagenized seeds representing 3800 M1 seeds from seven different parental lines from the Columbia ecotype within the glabrous background (Lehle Seeds, Round Rock, TX) and 65,000 T4 seeds representing the 6500 Feldman T-DNA insertional mutant lines (Feldman, 1991; *Arabidopsis* Biological Resource Center, Ohio State University). Over 230 putative mutants were identified after the initial screen. The candidate mutants were allowed to self and the phenotype confirmed in the M3 generation. Twelve mutant lines were confirmed: eleven from the EMS lines and one from the T-DNA mutagenized lines. The T-DNA mutant was not tagged as confirmed by Southern blot hybridization to the T-DNA left border (data not shown). The mutant isolated from the T-DNA collection has a faint phenotype that is difficult to follow through crosses because its staining pattern resembled that of the controls (the most intense staining is at the top of the root near the hypocotyl junction). Similarly, five of the EMS lines either had severe fertility problems or their BCIP phenotype was too difficult to score reliably through the back-crosses.

Genetic characterization and mapping

Back-crosses to the Columbia ecotype of the remaining 6 *cps* mutants revealed that they are all single-gene recessive mutations. Complementation crosses between

them showed that they represent three distinct genetic loci with four alleles of one locus: *cps1-1*, *cps1-2*, *cps1-3*, *cps1-4*, *cps2*, and *cps3*. The four alleles of *cps1* were isolated from 4 separate family lines and therefore are probably distinct mutations of the same gene. The *cps3* mutant has a stunted root and an associated slow growth cycle compounded by fertility problems. *cps1-1*, *cps1-2*, and *cps2* were back-crossed 3 times to remove or minimize other EMS mutations not associated with their phosphatase secretion phenotype before proceeding with their characterization.

BCIP-staining of the back-crossed *cps* mutants is shown in Figure 2.1. Under +P conditions, *cps1* mutants appear to be dark blue with brown regions, while the -P staining pattern is comparable to wild-type. The staining of *cps2* also has some brown regions but is more clearly blue. In *cps2* there is also a further induction of phosphatase secretion under -P conditions. All of the *cps* mutants have shorter roots than wild-type when grown on BCIP media.

Genetic mapping was carried out with representative alleles of each of the *cps* mutants. The *cps1* mutant is 7.6 +/- 4.3 cM away from LFY3 on chromosome 5, the *cps2* mutant is linked to m305 on chromosome 1 with no recombinations in population of 41 plants, and the *cps3* mutant is 11.5 +/- 6.0 cM away from m305 on chromosome 1 (Table 2.1).

Root-bound APase activity

Root systems from intact plants grown in nutrient culture were assayed for APase activity to quantify the increased APase activity visualized by the histochemical BCIP screen. Two replications of the experiment were performed, and similar trends were seen

in both. Data from the second experiment is presented because the internal repetitions (N) per sample were twice that of the first experiment. The *cps1-1* and *cps1-2* mutants had 2.8- and 2.2-fold root-bound APase activity, respectively when +P treatment is compared to the control (Col) under +P conditions. The *cps2* mutant was not significantly different from the control. The low-P treatment increased APase activity 1.2-fold in the control, and similar induction was seen in the mutants (Figure 2.2, Table 2.2 for ANOVA results).

P concentration when grown in nutrient solution

Root and shoot P concentrations were measured from tissues used in the root-bound APase assay. Results between the two replications were not necessarily consistent, so data from each replication are shown in Figures 2.3 and 2.4, respectively.

Root P concentrations are altered in the *cps* mutants. In replication 1 (Figure 2.3 A), both *cps1* alleles maintained higher P concentrations in the -P treatment, and the *cps2* mutant had lower P concentrations in the +P treatment. The low-P treatment resulted in significantly lower P concentrations in root tissues of both mutant and control plants (ANOVA, Table 2.3). In replication 2 (Figure 2.3B), the *cps1-2* allele had lower P concentrations under +P conditions but higher P concentrations with the -P treatment, and *cps2* had lower P concentrations in the +P treatment. Roots in replication 2 also had lowered P concentrations under the -P treatment, but all genotypes did not respond to the low–P treatment in the same magnitude (significant genotype X P treatment interaction, ANOVA, Table 2.3). In particular, the *cps1-2* mutant had similar P concentrations under both P treatments. Differences between the experimental replications can be seen in the

interaction plot (Figure 2.3 C). Replication 1 had much higher P accumulation under the +P treatment when compared to replication 2. This pattern did not hold for the -P treatment: while replication 1 *cps1-1* mutants had higher P concentrations, *cps1-2* was not different, and both Col and *cps2* had lower P concentrations when compared to replication 2. Despite the sometimes large differences between the two replications, two data points are consistent between them. First, the *cps1-2* mutant maintains higher root P concentrations in the low-P treatment, and second, the *cps2* mutant has lower root P concentrations in the high-P treatment.

Mutant tissues were not consistently altered in shoot P concentrations. In replication 1, *cps1-1* had higher shoot P concentrations in the low-P treatment when compared to the low-P control (Student's T-test, Figure 2.4 A). For replication 2, both *cps1* alleles had lower P concentrations in the +P treatment, and *cps1-2* also had lower P concentrations under the -P treatment (Figure 2.4 B). Trends between replications from the root P concentration data (Figure 2.3 C) did not directly translate into shoot P concentrations (Figure 2.4 C). When comparing the P concentration of each plant between replications, the control and *cps2* did not significantly change from rep1 to rep2. However, the *cps1-1* -P, *cps1-2* +P, and *cps1-2* -P plants all had significantly higher values in rep1 when compared to their values in rep2 (Figure 2.4C).

Biomass accumulation of cps *mutants grown in nutrient solutions*

The *cps1* mutants have dramatic affects on root biomass accumulation when grown in nutrient solution. Root biomass was not significantly affected by the short (2-day) -P treatment for these tissues, so P treatments were pooled (Table 2.3). Although

the relative magnitude of the decreased root biomass varies between replications (Figure 2.5 A and 2.5 B), the *cps1* alleles have a 25-75% reduction in root biomass. The *cps2* mutant has a slight reduction in root dry weight in replication 1 but not replication 2. Control tissues were not different in root biomass accumulation between replications (Figure 2.5 C), however, each *cps* mutant had different patterns of root dry weight accumulation between the two experimental replications. The *cps1-1* and *cps2* mutants both had higher levels of root biomass in replication 2 (relative to replication 1), while the *cps1-2* mutant had 3.5-fold increased biomass accumulation in replication 1 when compared to replication 2. Despite these differences between replications, *cps1* mutants did not accumulate control levels of root biomass in either experimental replication.

Like shoot P concentrations, shoot biomass accumulation was not consistently altered in the *cps* mutants. P treatment did not significantly change shoot biomass in both replications, so P treatments were pooled for analysis (Table 2.3). Both *cps1* alleles had decreased shoot dry weight accumulation in replication 1 (Figure 2.6 A), however, in replication 2 *cps1-1* was normal, and *cps1-2* and *cps2* had increased shoot biomass (Figure 2.6 B). The inconsistency between replications can be seen in the interaction plot (Figure 2.6 C). Replication 1 shoot biomass was higher for control plants and each mutant except *cps1-2*, which was higher in replication 2.

APase isoform analysis

Protein extracts from both root and shoot tissues were run on native discontinuous PAGE to determine which isoform(s) may be affected in the *cps* mutants. Native molecular weight markers were run with the extracts, however, precise molecular weights

cannot be determined on native gels because SDS is not present to linearize and uniformly charge the protein molecules.

Five major APase isoforms were detected in *Arabidopsis* root extracts (Figure 2.7). The largest band with the most activity, the R1 isoform, is increased in control tissues during low-P conditions. Activities of the other four major APase isoforms are not affected by P status. The *cps1-2* mutant has a large reduction in activity of the R1 isoform in both +P and -P extracts. Smearing from this band to the top of the gel implicates that the mobility of this isoform may have been decreased. The *cps1-1* and *cps2* root APase isoforms do not appear to have significantly different levels of activity or mobility.

Three major APase isoforms are present in *Arabidopisis* shoot protein extracts (Figure 2.8). The largest isoform, S1, slightly increases APase activity under the -P treatment in control tissues. The activities of two other major isoforms are not affected by P treatment in control tissues. A novel isoform is present in *cps1-2* mutant protein extracts that runs higher than the S1 protein in both +P and -P conditions. This is accompanied by a marked reduction in *cps1-2* activity of the S1 isoform under +P but not -P conditions. As with the root proteins, the *cps1-1* and *cps2* shoot APase isoforms appear normal.

cps *mutants grown on a peat/vermiculite soil mix*

The *cps1* mutants were grown in a peat/vermiculite soil mix to ascertain the effects of their mutations on P accumulation and growth when an organic form of P is available. The soil mix had 2.67 +/- 0.14 μg P/g soil, however; only roughly a third of

that amount, 0.88 +/- 0.02 μg P/g soil, was available as P_i. Plants were irrigated with either +/-P fertilizer.

As with the nutrient solution-grown plants, shoot P concentrations were not consistently different in *cps1* mutants (Figure 2.9). In rep1, both *cps1* mutants had lower shoot P concentrations when compared to their respective controls in both P treatments (Figure 2.9 A), however, in rep2 *cps1-1* had higher P concentrations in the +P treatment and *cps1-2* was not different from control tissues in either P treatment (Figure 2.9 B). Shoot P concentrations in all plants in both replicates were decreased by the low-P fertilizer treatment (Table 2.4). When comparing the two replications to each other, the control tissues were not different between replicates but *cps1* tissues had higher P concentrations in rep 2 (Figure 2.9 C).

Shoot biomass is reduced in *cps1* mutants when grown on a peat/vermiculite soil mix. Although the magnitude of the decrease differed between replications, the trend held that *cps1* shoot biomass was approximately 60% of control levels in both +P and -P fertilized plants. One representative replication is shown in Figure 2.10.

DISCUSSION

A histochemical screen for increased APase activity under +P conditions was attempted to identify components of the plant PHO regulon. Four alleles of the same mutantion, *cps1*, were identified. Mutants in two other genes were also identified, *cps2* and *cps3*. These mutants had dramatic phenotypes that made them amenable to further genetic characterization while other mutant lines had fainter phenotypes that were more difficult to follow through genetic crosses. Unfortunately this dramatic phenotype is also

associated with stunted root growth in *cps1* and *cps3* that makes physiological evaluation of the mutant's P relations difficult. In *cps3* the stunted root phenotype was so severe that it slowed the growth and reproductive cycles, limiting its use for further characterization. Despite the *cps2* mutant's dramatic APase histochemical staining, and aside from its decreased root P concentrations when grown in +P nutrient media (Figure 2.3), *cps2* had a phenotype indistinguishable from wild-type in other studies related to its APase activity. Altered morphology of the root, especially in the root cap and elongation zone (Figure 2.1), may indicate that the increased histochemical APase staining is due to an increase in permeability to the stain. The *cps2* staining may be time-dependent since the seedlings were grown for five days in this substrate: this is a very long time when compared to the root-bound assay, where plants were exposed to the pNPP substrate for less than an hour and the *cps2* mutant did not differ from controls.

Early work with crude protein extracts from these and another phosphatase secretion mutant (*pup1*) showed that APase activity in extracted proteins may not accurately measure the proteins I was interested in quantifying - the relatively small subset of proteins at the root surface. Instead, the traditional APase activity assay, consisting of pNPP in a sodium acetate buffer (Bessey et al., 1946), was modified extensively for use with intact *Arabidopsis* roots. Although other root systems had previously been assayed successfully for APase activity using the sodium acetate buffer, the extremely fine *Arabidopsis* roots did not show any -P inducible response when this buffer was used, APase activity levels were very high, and the root tissues appeared to be wilty and damaged (data not shown). The damage was not the result of a change in osmoticum, but may have been due to sodium toxicity. Increasing the MES buffering

capacity of the -P culture media and adjusting sucrose levels to maintain the osmotic balance between culture media and assay buffer solved this problem. The two *cps1* alleles characterized here were shown to have more than a 200% increases in APase activity at the root surface with this assay.

Tissues from the root-bound APase assay were analyzed for dry weight accumulation and P concentration to further characterize the *cps* phenotypes. Differences between replications in root P uptake led to changes in tissue P concentration and shoot biomass accumulation in controls. Replication 1 of this experiment had significantly higher +P treatment root P concentrations when compared to replication 2 in both mutant and control plants (Figure 2.3C). This may have been the result of above-optimal temperatures (25 degrees C) during replication 2. In control tissues, the higher replication 1 root P concentration can explain other physiological differences between the replications. With higher root P concentrations, more P is transported to the shoots, shoot P concentrations are maintained (Figure 2.4C), and shoot biomass is greater (Figure 2.6C). Root biomass in control tissue is not responsive to P concentrations in this experiment because, even within the 2 d low-P induction period, P pools are not limiting (Figure 2.5C).

This rationalization of replication P uptake differences in the controls does not explain the erratic behaviour of the *cps1* alleles in this experiment. Just as in control tissues, *cps1* alleles have increased replication 1 +P treatment root P concentrations. However, *cps1* alleles have much smaller root systems than control plants, and their root biomass was dramatically different between replications (Figure 2.5). *cps1* mutants may have increased sensitivity to temperature changes, bringing about differences in root

growth between the replications, but this does not explain why the two alleles would have opposite reactions between replications (Figure 2.5C). Since limiting root tissue can control the amount of P that can be transported to the shoot, shoot P concentrations (Figure 2.4C) and biomass accumulation (Figure 2.6C) are also erratic in the *cps1* alleles. This same inconsistent behavior was seen in shoot P accumulation of *cps1* alleles when grown in a peat/vermiculite soil mix, and temperatures were constant between replications in that experiment (Figure 2.9). Other environmental conditions not accounted for in these studies may result in changes in *cps1* root biomass between replications, and these changes may result in abberrant shoot biomass and shoot P concentrations. It is impressive that relative root-bound phosphatase activity measurements are consistent between replications even when tissue biomass and P concentrations are not.

The *cps1* mutants have severe root growth defects that make P relation studies difficult to evaluate, but some consistent physiological phenotypes were observed. The *cps1-2* mutant had higher root P concentrations when exposed to a two-day P deprivation treatment in nutrient culture (Figure 2.3), and root biomass was reduced in *cps1-1* and *cps1-2* by 20-75% regardless of P treatment when plants were grown in nutrient culture (Figure 2.5). Finally, when grown in a peat/vermiculite soil mix *cps1* shoots accumulated 40% less biomass than controls (Figure 2.10).

The *cps* mutants do not appear to be defective in their overall low-P deficiency responses. P deficiency heightens their APase activity staining (Figure 2.1), increases their root-bound APase activity (Figure 2.2), and decreases tissue P concentrations

(Figures 2.3, 2.4, and 2.9). Transcript accumulation of low-P induced APase genes is also normal in the mutants (data not shown).

One of the *cps1* alleles, *cps1-2*, exhibited alterations in APase isoforms when run on native activity gels. In roots, a major low-P inducible APase is reduced in *cps1-2* protein extracts isolated from both +P and -P treated plants (Figure 2.7). Residual activity remains in the sides of the wells and is smeared along the wells above the band to the origin of the resolving gel. A similar phenotype is present in *cps1-2* shoot extracts. A major low-P inducible isoform is reduced in *cps1-2* +P extracts but not -P extracts, and a novel band that does not migrate well in the gel appears in extracts from both P treatments (Figure 2.8). It is possible that the same protein is affected in both roots and shoots, and that either it is more abundant in shoots and more tightly regulated there by low-P, or that a second isoform very tightly regulated by -P conditions co-migrates with this protein in shoots.

The *cps1-1* allele did not have the shifted isoforms present in *cps1-2*. The two alleles are likely the result of different mutations since they were isolated from different mutagenized parental pools. The *cps1-2* mutation could result in an altered amino acid that resulted in a change in charge, glycosylation, or conformation in this structural gene. Since proteins migrate according to native charge and conformation on non-denaturing gels, this could account for the shifted mobility of S1 and R1 in *cps1-2* APase activity gels. The fact that *cps1-1* does not share this activity gel phenotype indicates that the *cps1-1* mutation does not lead to this same change in amino acid charge, protein glycosylation, or conformation change. This in no way rules out that the *cps1-1* is

defective in the same gene product; instead it shows that *cps1* has at least two sites of regulation that generate similar increased root-bound phosphatase activity phenotypes.

Since this work was begun, a second group looking for constitutive phosphatase secretion mutants in the green alga *Chlamydomonas reinhardtii* identified a dominant mutant with elevated phosphatase secretion, *psr2*. The two periplasmic phosphatases induced in *C. reinhardtii* are normally regulated in *psr2*, and other results fit a model in which *psr2* either has high levels of a normally low-abundant extracellular phosphatase or exports a normally intracellular phosphatase (Shimogawara et al., 1999). Similarly, *cps1* may be the result of altered targeting of a normally intercellular protein to the cell wall.

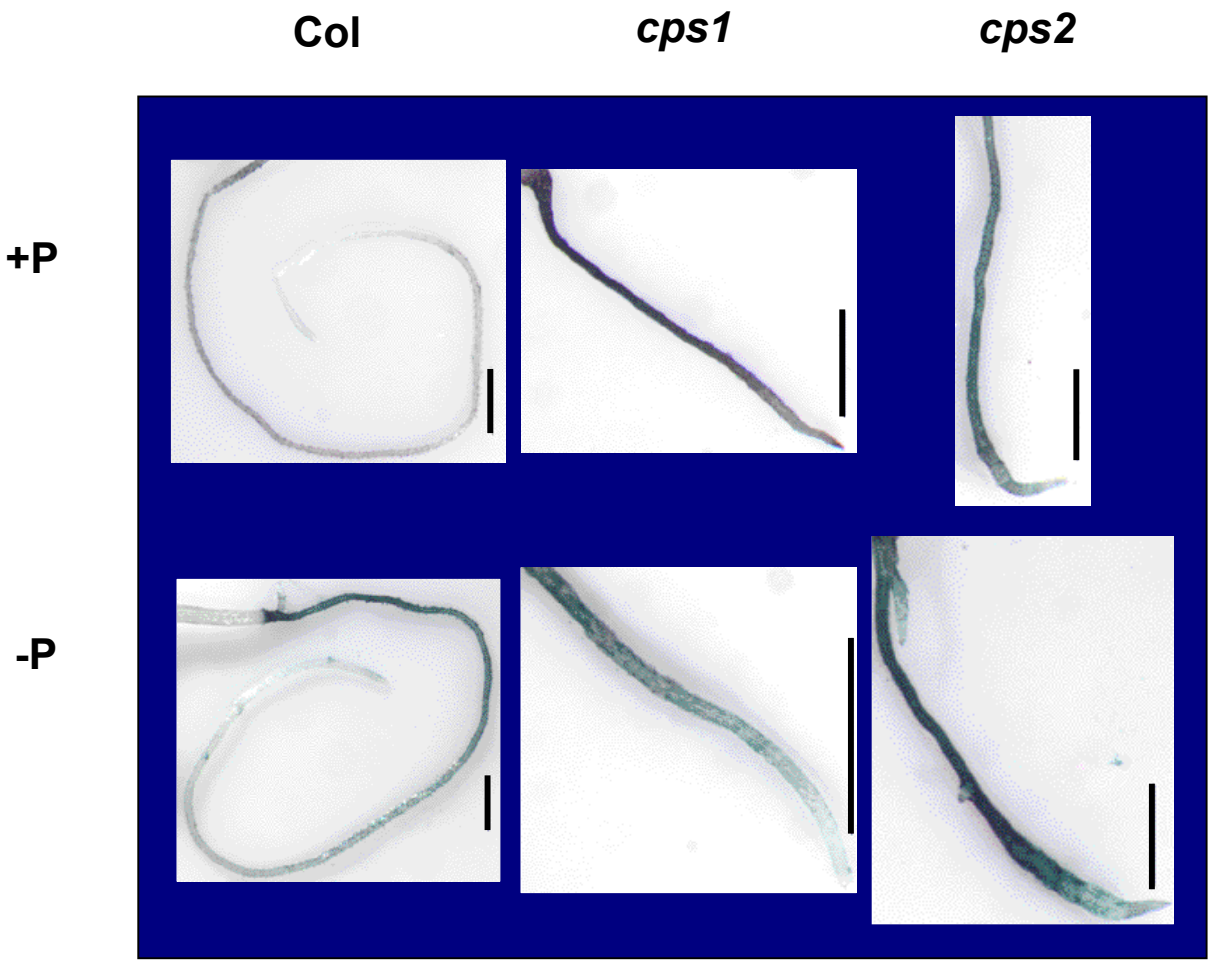

Figure 2.1. *In-vivo* phosphatase activity staining of the *cps* mutants. When cleaved by phosphatases, the substrate BCIP forms a blue precipitate on the root surface. *Arabidopsis* seedlings were grown on media with (+P) or without (-P) phosphate containing BCIP to 5dpg before visualization. Standard bars are 1mm.

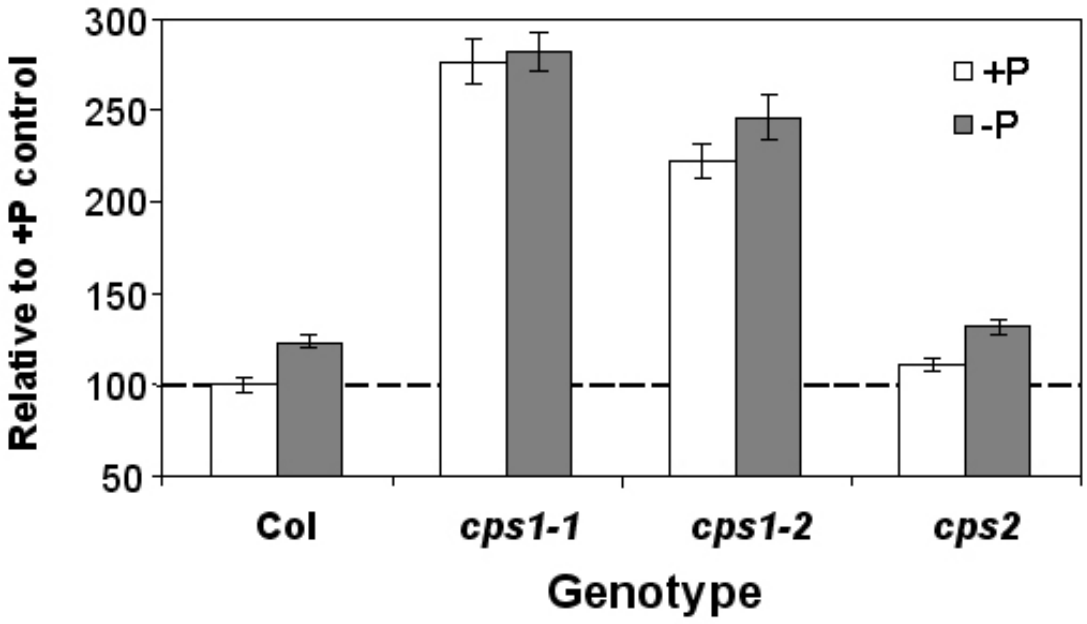

Figure 2.2. Root-bound acid phosphatase activity in the *cps* mutants. Intact root systems were assayed for acid phosphatase activity by hydrolysis of pNPP, corrected for root dry weight and converted to relative units compared to the +P control. Error bars are standard error, and N>=7 for each measurement.

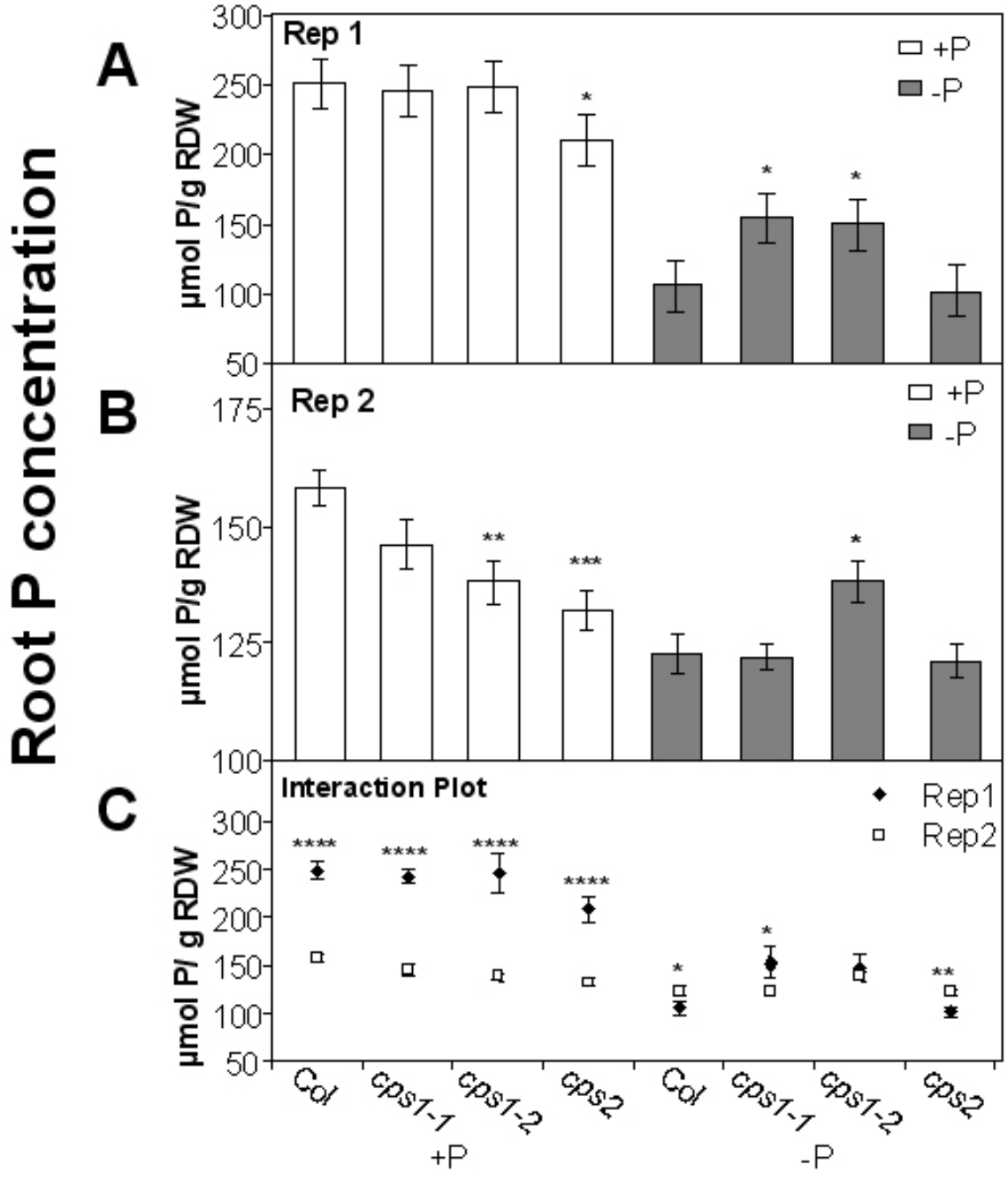

Figure 2.3. Root P concentration of tissues grown in nutrient solution. Tissues from the root-bound APase assay were dissected for P determinations using the molybdate method. Replication effects were significant, so experimental replications are presented separately. Replication 1 had N=4 per data point (panel A), and replication 2 had N>=7 per data point (panel B). Both replications are plotted in panel C for comparison. In panel A, error bars represent Fisher's Least Significant Difference values at P<0.05 and asterisks denote significant difference from the appropriate +P or -P control. In panels B and C, error bars represent standard error and asterisks denote significant difference between the same measurements between the two experimental replications as determined by Student's t-test. Refer to Table 2.3 for ANOVA summaries using this data. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

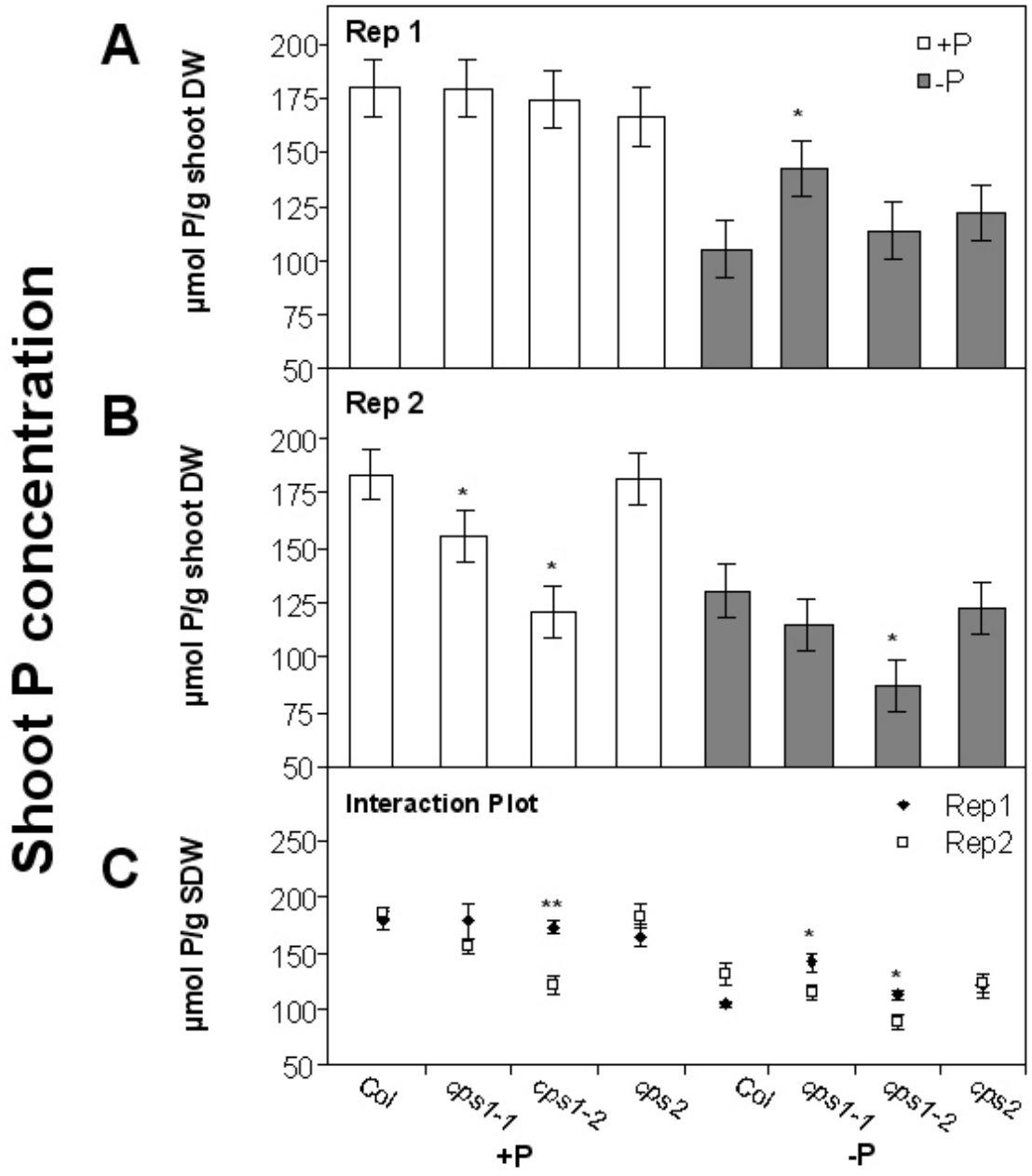

Figure 2.4. Shoot P concentration of tissues grown in nutrient solution. Tissues from the root-bound APase assay were dissected for P determinations using the molybdate method. Replication effects were large and significant, so experimental replications are presented separately. Replication 1 had N=4 per data point (panel A), and replication 2 had N>=7 per data point (panel B). Both replications are plotted in panel C for comparison. In panels A and B, error bars represent Fisher's Least Significant Difference values at P<0.05 and asterisks denote significant difference from the appropriate +P or -P control. In panel C, error bars represent standard error and asterisks denote significant difference between the same measurements between the two experimental replications as determined by Student's t-test. Refer to Table 2.3 for ANOVA summaries using this data. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

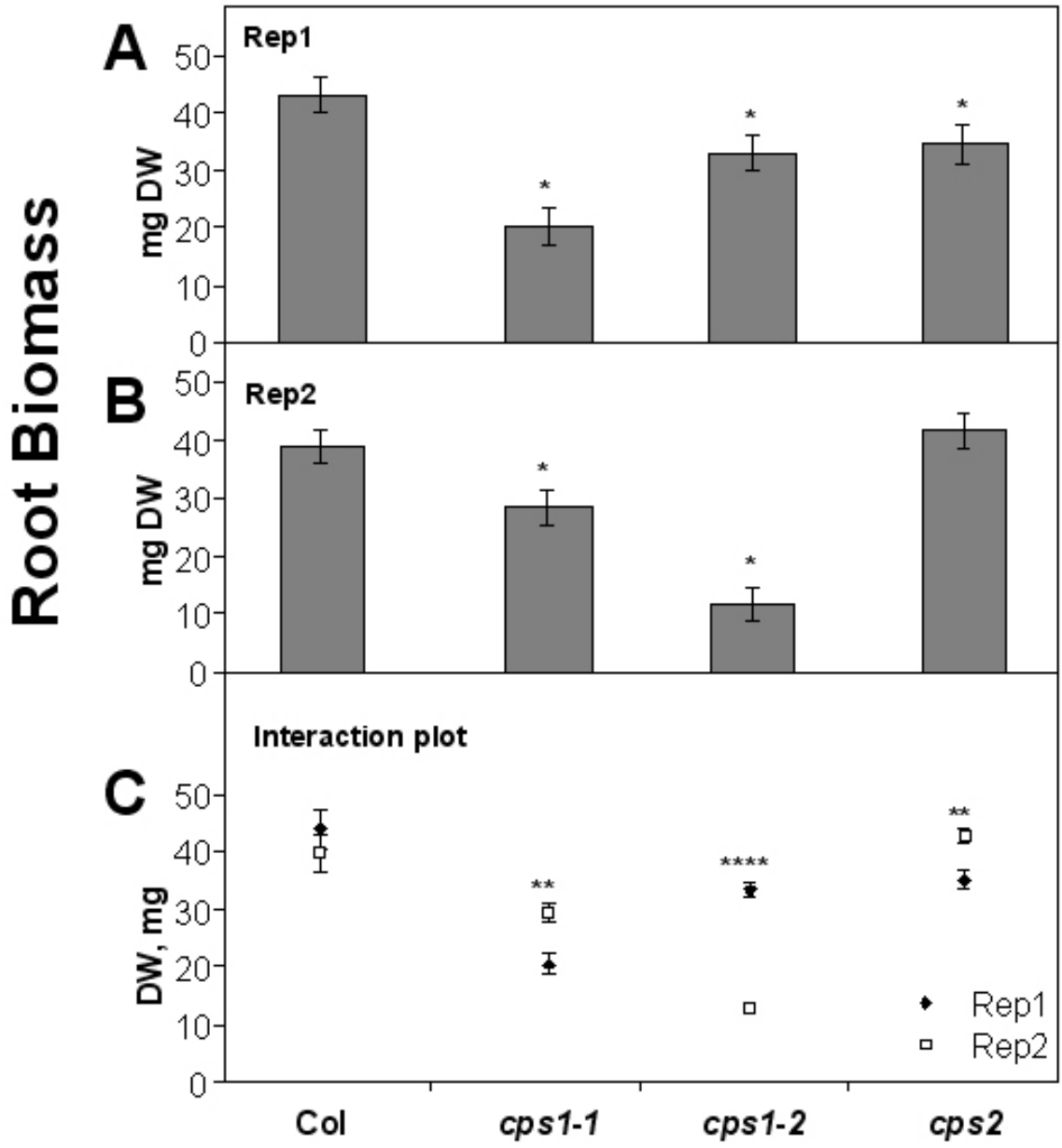

Figure 2.5. Root biomass accumulation of tissues grown in nutrient solution. Tissues from the root-bound APase assay were dissected and dried in pre-weighed aluminum foil envelopes to obtain plant dry weight. Replication effects were large and significant. Replication 1 had N=8 per data point (panel A), and replication 2 had N>=14 per data point (panel B). Both replications are plotted in panel C for comparison. In panels A and B, error bars represent Fisher's Least Significant Difference values at the P>0.05 level and asterisks denote significant difference from the control (Col). In panel C, error bars represent standard error and asterisks denote significant difference between the same measurements of the two experimental replications as determined by Student's t-test. P treatments were pooled because there was no effect of P on root biomass (Table 2.3). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

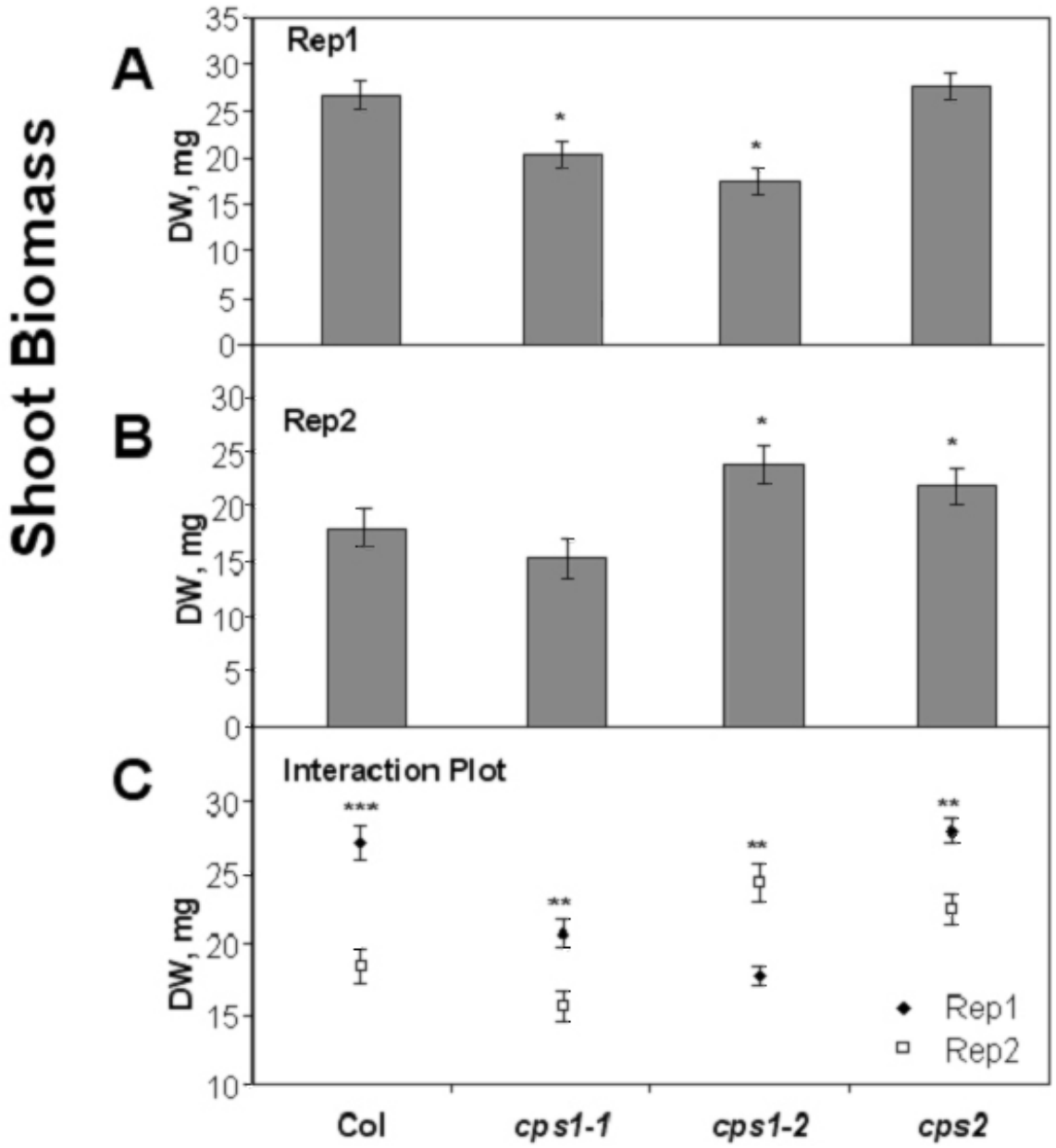

Figure 2.6. Shoot biomass accumulation of tissues grown in nutrient solution. Tissues from the root-bound APase assay were dissected and dried in pre-weighed aluminum foil envelopes to obtain plant dry weight. Replication effects were large and significant. Replication 1 had N=8 per data point (panel A), and replication 2 had N>=14 per data point (panel B). Both replications are plotted in panel C for comparison. In panels A and B, error bars represent Fisher's Least Significant Difference values at P<0.05, and asterisks denote significant difference from the control (Col). In panel C, error bars represent standard error and asterisks denote significant difference between the same measurements of the two experimental replications as determined by Student's t-test. P treatments were pooled because there was no effect of P on root biomass (Table 2.3). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

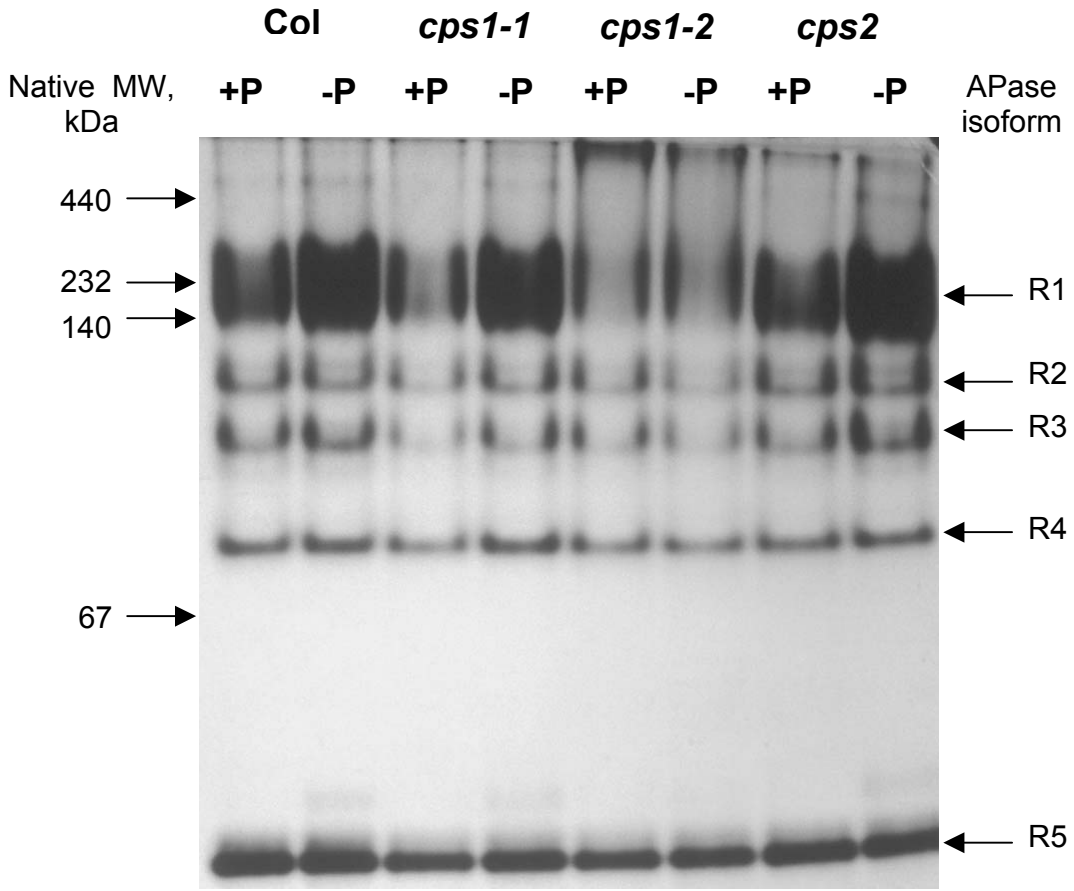

Figure 2.7. APase activity isoforms in *cps* roots. Proteins were extracted from roots of plants either grown in +P nutrient media for 21 days (+P) or in +P media for 14 days followed by -P media for 7 days (-P). Equal amounts of protein (30μg) were loaded per lane, and gels were run under native conditions at 4˚C. APase activity was detected by incubation in Fast Black K with β-naphthyl acid phosphate. N=9 root systems per extract. There were two replications, and plants were grown separately for each replication.

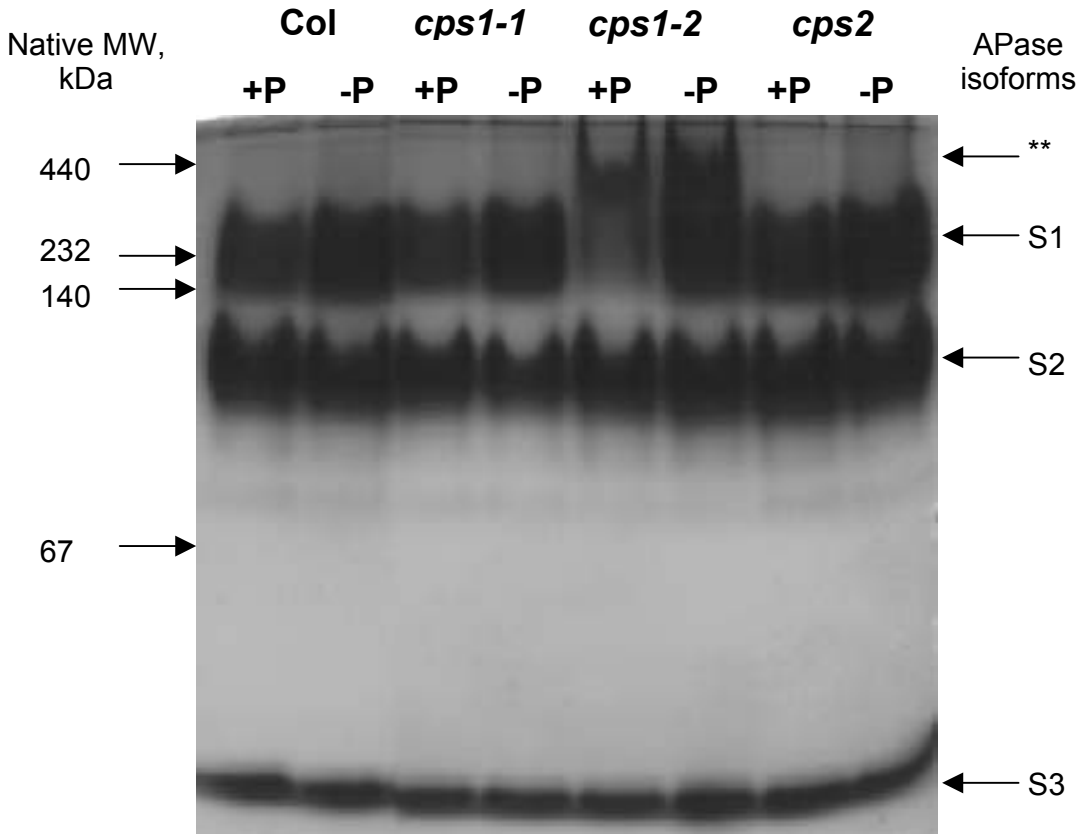

Figure 2.8. APase activity isoforms in *cps* shoots. Proteins were extracted from shoots of plants either grown in +P nutrient media for 21 days (+P) or in +P media for 14 days followed by -P media for 7 days (-P). Asterisks indicate the novel *cps1-2* isoform. Equal amounts of protein (25µg) were loaded per lane, and gels were run under native conditions at 4˚C. APase activity was detected by incubation in Fast Black K with β-naphthyl acid phosphate. N=9 shoots per extract. There were two replications, and plants were grown separately for each replication.

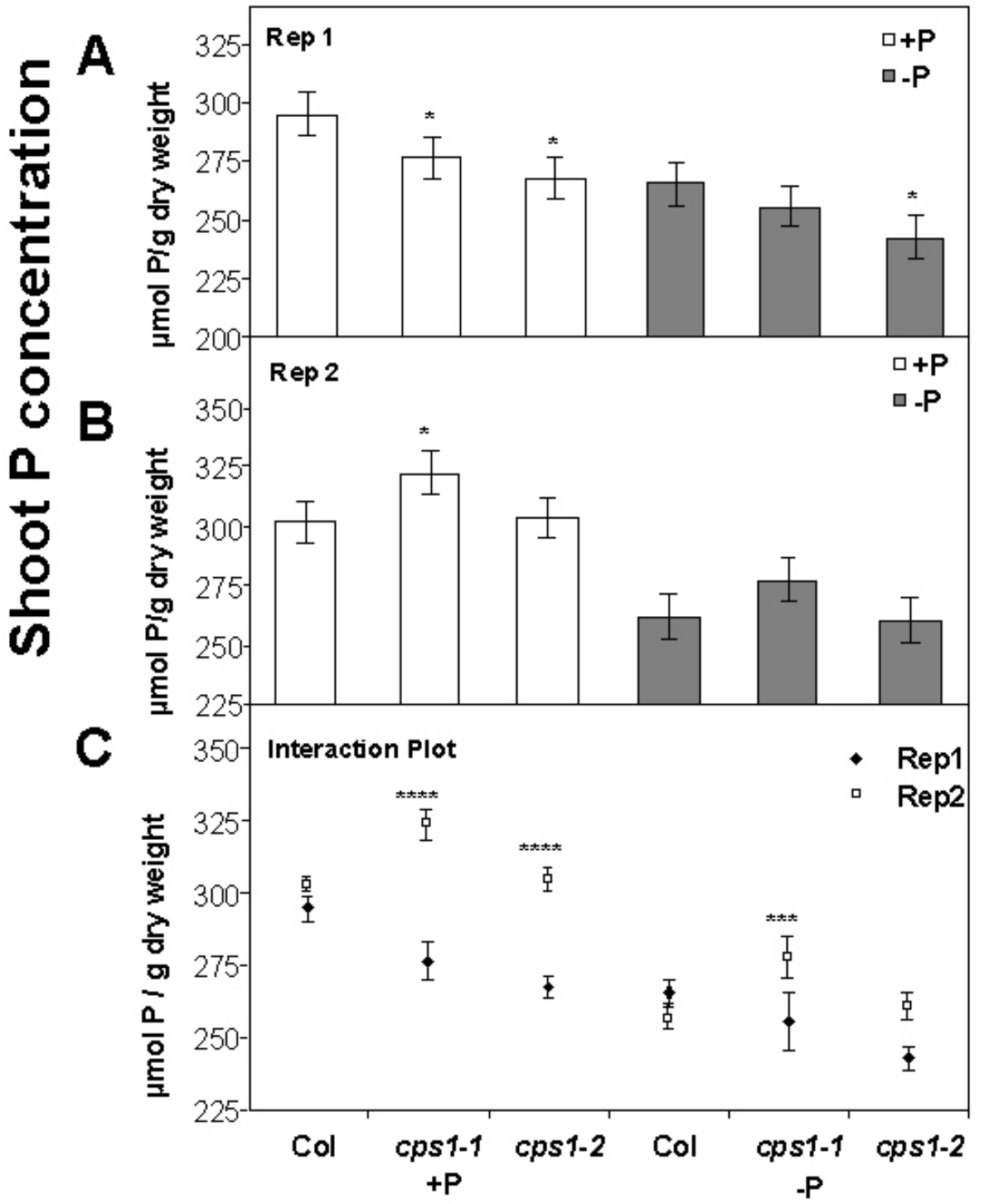

Figure 2.9. Shoot P concentrations in *cps1* mutants grown in a peat/vermiculite soil mix. Plants were grown in a peat/vermiculite mix with (+P) or without (-P) fertilizer irrigation until the first sign of the primary inflorescence. Replication effects were large and significant. Each replication had N>=9 per data point. In panels A and B, error bars represent Fisher's Least Significant Difference values at P<0.05 and asterisks denote significant genotype difference from the control (Col). In panel C, error bars represent standard error and asterisks denote significant difference between the same measurements of the two experimental replications as determined by Student's t-test. Refer to Table 2.4 for ANOVA results from this data. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

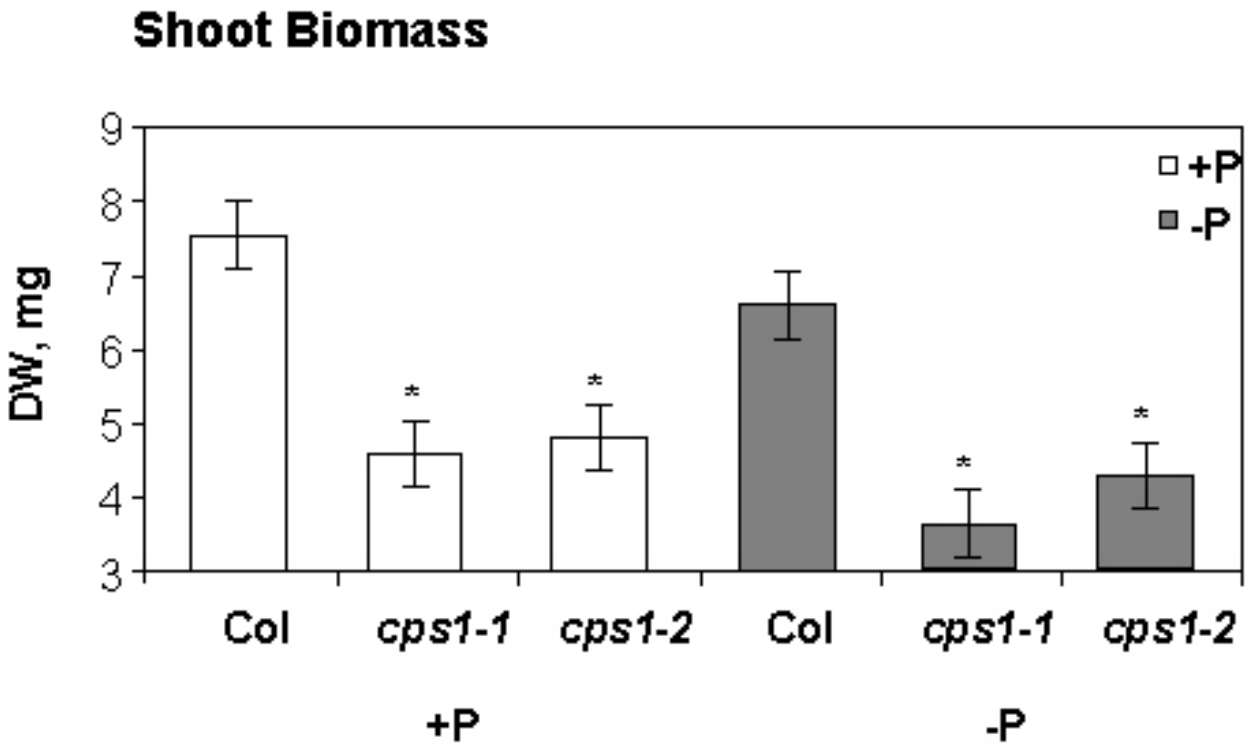

Figure 2.10. Shoot biomass accumulation in *cps1* mutants grown in soil. Plants were grown in a peat/vermiculite mix with (+P) or without (-P) fertilizer irrigation until the first sign of the primary inflorescence. Data from one representative replication is shown. N>=9 per data point. Error bars represent standard error. Asterisks denote significant genotype difference (mean of both P treatments) from the control (Col) as determined by Fisher's Protected Least Significant Differenct test. Refer to Table 2.4 for ANOVA results from this data. ****P<0.0001

Table 2.1. Genetic mapping of the *cps* mutants. CAPS mapping results are shown for markers throughout the *Arabidopsis* genome. The segregation ratio is the number of F2 mutant segregants from an outcross to the Landsberg erecta (Ler) ecotype showing either the Colombia polymorphism (Col), Landsberg polymorphism (Ler) or both (Het) for each locus. Significant deviation from the expected segregation ratio of 1Col:2Het:1WS (P-value<0.1, Chi square)) indicates that the marker is linked to the mutation. Once linkage was established, distance from the mutation was calculated based on the Kosambi function (Koornneef and Stam, 1992).

Mutant	Marker	Marker location[a]	Segregation Ratio, Col:Het:Ler	Distance from mutation, cM
cps1	m235	34.01 cM, chr1	5:10:6	
	GAPB	61.21 cM, chr1	5:12:10	
	m305	91.89 cM, chr1	5:9:5	
	PhyB	34.45, chr2	3:9:9	
	BglI	75.23 cM, chr3	3:9:5	
	Det1	31.44, chr4	7:17:8	
	g4539	57.64 cM, chr4	11:20:12	
	CAT2	85.75 cM, chr4	7:10:9	
	ASAI	18.35 cM, chr5	9:9:7	
	NIT4	45 cM, chr5	12:12:6	
	PHYC	71.13 cM, chr5	14:16:5*	47.8 +/- 18.2 cM above
	ASB2	115 cM, chr5	25:5:0***	8.4 +/- 5.2 cM
	LFY3	116.88 cM, chr 5	35:4:1***	7.6 +/- 4.3 cM
cps2	m235	34.01 cM, chr1	11:20:11	
	GAPB	61.21 cM, chr1	20:19:2***	31.7 +/- 10.2 cM above
	m305	91.89 cM, chr1	41:0:0***	0.0 +/- 0.0 cM
	PhyB	34.45, chr2	6:17:6	
	cop1	63.34 cM, chr2	6:15:6	
	Det1	31.44, chr4	15:18:8	
	g4539	57.64 cM, chr4	8:16:4	
	NIT4	45 cM, chr5	8:17:3	
cps3	m235	34.01 cM, chr1	6:16:1*	52.6 +/- 26.3 cM above
	m305	91.89 cM, chr1	25:5:1***	11.5 +/- 6.0 cM
	PhyB	34.45, chr2	1:8:1	
	g4539	57.64 cM, chr4	2:10:10	
	ASAI	18.35 cM, chr5	7:11:8	

*P<0.1, **P<0.05, ***P<0.001 (Chi-square)

[a] As of June, 2001, www.arabidopsis.org

Table 2.2. ANOVA summary of the *cps* root-bound APase study. Root systems from intact plants were assayed for APase activity. The uneven number of internal repetitions (N) per data point prevented bulking the two experimental replications together for analysis. Analysis of variance (ANOVA) was carried out separately for each experimental replication, and similar trends were seen in each. Data presented in this table is from the second replication as presented in Figure 2.2.

Components of variance	Error df	F-Value
Genotype	55	189.5****
P treatment		9.6**
Genotype X P treatment		0.5

**P<0.01, ****P<0.0001

Table 2.3. ANOVA summary of *cps* P concentrations when grown in nutrient solution. Root and shoot tissues were collected from tissues assayed for root-bound APase (see Figure 2.2, Table 2.2) and P concentrations measures. Significant interactions prevented pooling the experimental replications. Analysis of variance (ANOVA) was carried out separately for each experimental replication. Data presented in this table is graphically presented in Figures 2.3- 2.6.

Experiment	Rep	Components of variance	Error df	F-Value
Root P concentration	1	Genotype P treatment Genotype X P treatment	24	5.6** 160.1**** 1.9
	2	Genotype P treatment Genotype X P treatment	54	4.2** 35.0**** 6.8***
Shoot P concentration	1	Genotype P treatment Genotype X P treatment	24	1.9 71.4**** 1.7
	2	Genotype P treatment Genotype X P treatment	53	19.0**** 71.3**** 1.1
Root Biomass	1	Genotype P treatment Genotype X P treatment	24	17.3**** 0.4 0.1
	2	Genotype P treatment Genotype X P treatment	54	47.9**** 0.8 0.6
Shoot Biomass	1	Genotype P treatment Genotype X P treatment	24	24.7**** 0.6 0.7
	2	Genotype P treatment Genotype X P treatment	54	11.6**** 3.2 1.5

**P<0.01, ***P<0.001, ****P<0.0001

Table 2.4 ANOVA summary of *cps1* P concentrations and shoot biomass when grown in a peat/vermiculite soil mix. Refer to data presented in Figures 2.9 and 2.10.

Experiment	Rep	Components of variance	Error df	F-Value
Shoot P concentration	1	Genotype P treatment Genotype X P treatment	51	6.5** 80.0**** 0.1
	2	Genotype P treatment Genotype X P treatment	54	9.0*** 26.5**** 0.3
Shoot Biomass	2	Genotype P treatment Genotype X P treatment	51	55.1**** 10.6*** 0.4

**P<0.01, ***P<0.001, ****P<0.0001

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CHAPTER 3:
Characterization of the phosphatase under-producing mutants

INTRODUCTION

Phosphorus (P) deficiency is a major limitation to plant growth. P is mainly taken up by plants in anion form, P_i or orthophosphate ($H_2PO_4^-$). Although a macronutrient for plants, the availability of soil P_i is often below that of micronutrients. Soil P_i levels in solution rarely reach above 1.5 μM (Bieleski, 1973) because P_i is immobilized within soil organic complexes, clay complexes, and precipitated iron and calcium salts (Marschner, 1995). Sparingly available soil P_i can be ameliorated with P_i fertilizers. Applied fertilizers represent more than 90% of world P_i use (Bieleski and Ferguson, 1983), and modern agriculture relies on crops that provide maximal yields with these fertilizers. However, world resources of extractable P_i are limited, non-renewable (Cathcart, 1980), and increasingly ecologically hazardous to mine. As extractible world P_i stores become depleted, modern agriculture will be forced to adjust to a lack of P_i fertilizer while continuing to feed an expanding population. Alternatively, the organic P (P_o) component of agricultural soil is abundant, representing up to 80% of total soil P (Schachtman et al., 1998). Plants may mobilize P_o during P deficiency by secreting enzymes into the rhizosphere, including acid phosphatases (Gilbert et al., 1999; Li and Tadano, 1996; Goldstein et al., 1988; Tarafdar and Claasen, 1988; Caradus and Snaydon, 1987; McLachlan, 1980), ribonucleases (Bariola et al., 1999; Nurnberger et al., 1990) and deoxyribonucleases (Chen et al., 2000).

Acid phosphatases (APases) may be active against a wide array of organic molecules present in soil P_o. These enzymes are non-specific orthophosphoricmonoester phosphohydrolases (E.C. 3.1.3.2), cleaving P_i from larger molecules at their ester linkage sites. Secreted plant phosphatases preserve >50% activity over a broad pH range (4.0-7.6), maintain >80% activity over a broad temperature range (22-48°C), and are stable at temperatures as high as 60°C (Li and Tadano, 1996; LeBansky et al., 1991), making them ideal candidates for active soil enzymes. While most soil enzymes are generally short-lived, APases can be immobilized on or within soil clays and humates that preserve their activity (Burns, 1986). Soil APases are important for P_i acquisition by plant roots (Haussling and Marschner, 1989), and plants can use P_o as a sole source of P_i nutrition in sterile cultures (Chen et al., 2000; Richardson et al., 2000; Yan et al., 1996; Tarafdar and Claasen, 1988; Furlani et al., 1987). Therefore, plant-derived secreted APases have the potential to facilitate breakdown of soil P_o to P_i for plant uptake.

Purple acid phosphatases (PAPs) are the best characterized class of APases to date. They are glycoproteins and contain metal-binding dinuclear centers in their active sites that give them a purple color when concentrated. While sparsely represented in microbes and fungi, these enzymes are widely distributed in plants and animals (Oddie et al., 2000; Schenk et al., 2000). PAPs consist of two groups differentiated by molecular weight. The low-molecular weight PAPs (~35kDa) occur both as monomers and as two-subunit forms derived from disulphide-linked monomer fragments (Ljusberg et al., 1999). High-molecular weight PAPs (~55kDa) are unique to plants and are homodimers with disulfide bond interactions between the monomers. Similarity between the high- and low- molecular weight PAPs is limited to a series of motifs containing the metal-

coordinating amino acids of the active sites (Klabunde et al., 1995). Plant PAPs have been implicated in low-P responses because some are transcriptionally up-regulated during P deficiency (Haran et al., 2000; del Pozo et al., 1999; Nakazato et al., 1998). The biochemistry of kidney bean PAP (KBPAP) has been intensely studied. KBPAP is a dimeric glycoprotein of molecular mass 110kDa with a single disulfide bond linkage (Cashikar and Rao, 1995). Its crystalline structure has been solved, and the metals within its active sites identified (Strater et al., 1995). The KBAP protein localizes to the cytoplasm of dried beans (Grote et al., 1998), and has a high specific activity for ATP and poly-P (Cashikar and Rao, 1996).

Two PAPs have been studied in *Arabidopsis thaliana*: *AtACP5* (del Pozo et al., 1999) and *PAP1* (Haran et al., 2000). The transcription of *AtACP5* is induced during senescence and by low-P conditions, abscisic acid, and peroxide. At 35kDa, it is similar in size to the animal PAPs. Most plant PAPs have an additional N-terminal domain that increases their predicted monomeric size to ~55kDa, and the *PAP1* gene product belongs to this group. The *PAP1* protein encodes a signal peptide sufficient for secretion of a marker protein, and its promoter is low-P inducible. Expression studies with the *PAP1* promoter linked to GUS show that the gene is transcribed first in leaves, then in the lateral root primordia and emerging lateral meristems after P is removed from the growth media. With prolonged P deprivation, most tissues express *PAP1* except the epidermal layer, root hairs, and apical meristem (Haran et al., 2000). Despite their differences, the *PAP1* and *AtACP5* proteins have 23% identity and 36% similarity when compared to each other (BLASTP 2.2.1, NCBI).

Trull and Deikman (1998) previously reported the identification and characterization of *phosphatase-underproducer 1 (pup1)*, an *Arabidopsis* mutant that does not appear to increase its phosphatase secretion on low-P BCIP media and lacks one phosphatase isoform. The *pup1* mutant is incompletely dominant and has a decreased root:shoot ratio under -P conditions. Further characterization of the *pup1* mutant will be presented here, along with a second mutant whose identification was reported from that screening effort, *pup3*.

MATERIALS and METHODS

Plant material and growth conditions

The *pup1* and *pup3* mutant identification has been previously described (Trull and Deikman, 1998). The *pup1* mutant originated from *Arabidopsis* T-DNA insertion lines in the Wassilewskija (WS) ecotype background (unlinked to an insertion), and the *pup3* mutant was identified from EMS mutagenized *Arabidopsis* seeds from the Colombia (Col) ecotype background. The *pup3* mutant was back-crossed at least 3 generations prior to this study to remove unrelated EMS mutations. Plants were grown in a growth chamber under the following conditions unless otherwise noted: 22°C, ambient relative humidity, 100 μmol $m^{-2}s^{-1}$, 16 h light/8 h dark. Seeds were collected and allowed to dry for >2 weeks before being stored at 4°C. Seed surface sterilization was accomplished by soaking <100μL volume of seeds successively in approximately 1mL volumes of water for 30min, 95% (v/v) ethanol for 5min, and 10% (v/v) sodium hypochlorite for 5min, followed by 5 rinses in sterile water. After sterilization seeds were placed on sterilized

filter paper to dry and stored at 4°C with dessication. Seeds were sterilized shortly (< 2 weeks) before use.

In-vivo phosphatase staining

Seeds were surface sterilized and plated on either +P or -P 0.5X modified Hoagland's media containing BCIP (Trull and Deikman, 1998). After a 2 d cold treatment at 4°C, plates were positioned 5° from vertical in the growth chamber and seedlings grown until 5 dpg. Plants whose roots did not penetrate the media were not scored for BCIP staining. Digital images were taken with a Stemi SV11 (Zeiss, Thornwood, NY) dissecting scope using a Fostec Ace I EKE dual optic lighting system from above with white background correction, and all subsequent manipulations of images were identical.

Root-bound phosphatase activity

Seeds were plated on solid 0.5X modified Hoagland's media and allowed to grow for 8 d. Plants were moved to 10mL of liquid media (0.5X modified Hoagland's media with 0.5mM P_i, 3% sucrose), 3 plants per flask and shaken at 150 rpm in the dark. As with -P treatments, P salts were replaced with appropriate sulfate salts. After 10d, plants were rinsed in -P media, transferred to 15mL fresh media (either +P or -P) and grown for 48h. For the assay, plants were rinsed in -P media and transferred to 15mL reaction buffer (-P media + 19.1mM MES, 1.3% sucrose, 5mM *p*-nitrophenyl phosphate, pNPP) in a time-dependent manner. Reactions proceeded for 30-90min at 22°C, then 185µL of the reaction buffer was removed to 832µL 1N NaOH. The accumulation of *p*-nitrophenol

(pNP) was read as A_{410}. Absorbance values were converted to nmol pNP based on a standard curve of pNP (Sigma, St. Louis, MO). Roots and shoots were dissected, transferred to pre-weighed aluminum foil envelopes and dried for 2d at 65°C. Root-bound phosphatase activity was calculated as nmol pNP/min/root dry weight. Total P determinations were carried out on the dried tissue with the molybdate method (Murphy and Riley, 1962). A completely randomized design was used with N>=8 for each treatment. Separate experiments were performed with each mutant and its respective control, and each of these experiments were replicated twice. Statistical analysis was performed with StatView v.5.0.1 (SAS Institute, Cary, NC).

Tissue protein extraction

Plants were grown as described (Muchhal et al., 1996) with notable exceptions. Plants were plated on 3x3cm 300-µm mesh nylon filters (Spectra/Mesh®, Spectrum Laboratories, Los Angeles, CA) at a density of 9 seeds per filter on standard 0.1X MS media + 1% sucrose solidified with 1% agar. After a 2 d cold treatment, plants were grown under continuous light until 7 d post germination, then the nylon mesh filters transferred to floating membrane rafts (LifeRafts™, Sigma Aldrich, St. Louis, MO) over 100mL liquid 0.5X modified Hoagland's media + 1% sucrose. Plants were grown for 7d under continuous illumination, transferred to fresh +P (1mM) or -P (0mM) 0.5X modified Hoagland's media + 1% sucrose, and grown for an additional 5d.

For the protein extraction, roots and shoots were ground in liquid N, then ice cold extraction buffer (0.1M K-acetate pH 5.5, 20mM $CaCl_2$, 2mM EDTA, 0.1mM PMSF) added at 4mL/g tissue. Polyvinylpolypyrrolidone was added at 60mg/g tissue and the

samples were gently agitated at 4 degrees C for 1h. Samples were centrifuged at 27,000xg and 4 degrees C for 30min, the supernatant removed to a fresh tube, and glycerol added to 20% (v/v). Proteins were quantified according to the Bradford method (Bradford, 1976) using Coomassie Plus protein assay reagent (Pierce, Rockford, IL). Proteins were stored at -80 degrees C. Proteins were isolated from three separate plantings.

Exudate protein concentration

Growth media from the 2-d induction period of the root-bound phosphatase study was concentrated 200-fold with Centriplus-10 centrifugal concentrators (Millipore, Bedford, MA). Growth media from three separate flasks were bulked together per exudate sample (N=9 plants per sample). Buffer exchange was carried out with protein extraction buffer during the concentration, and glycerol added to 20% (v/v) after concentration was complete. Exuded proteins were stored at -80 degrees C. Exudates were collected from three separate experiments.

Specific APase activity

Extracted proteins were dialyzed overnight in dialysis tubing (6,000-8,000 MW cut-off, Spectra/Por, Spectrum Laboratories, Los Angeles, CA) against protein extraction buffer + 20% glycerol to remove P_i and other possible APase inhibitors. To measure the specific APase activity, 0.5µg protein (< 40µL) was added to 300µL pre-warmed 10mM pNPP in 50mM sodium acetate, pH 5.5. Reactions proceeded for 10min at 25˚C, were

stopped with 600µL 1N NaOH, and were quantified as for the root-bound APase assay. Samples from each of the 3 protein harvests were assayed simultaneously.

APase activity isoform analysis

Protein electrophoresis was carried out on 5% stacking/10% resolving (w/v) native acrylamide gels at low voltage (30-60V) and low temperature (4 degrees C). Equal amounts of concentrated exuded proteins (7.5 µg) were loaded for comparison between samples. APase staining was carried out with Fast Black K salt and β-naphthyl acid phosphate as previously described (Trull et al., 1997). High molecular weight native electrophoresis markers (Amersham Pharmacia Biotech, Piscataway, NJ) were used to approximate molecular weight values.

∝PAP1 immunodetection

∝PAP1 polyclonal antibody, generated from recombinant *Arabidopsis* purple acid phosphatase (*PAP1*) gene product (GenBank #U48448), was obtained from Dr. Thomas McKnight (Dept. of Biology, Texas A&M University). Equal amounts of exuded protein (7µg) were separated by native PAGE on a BioRad MiniGel apparatus, then blotted to a PVDF membrane using a semi-dry electroblotter according to manufacturer's instructions (Panther™, Owl Scientific, Woburn, MA). Detection of the antibody/antigen interaction was carried out with the ECL™ Western blotting analysis system (Amersham Pharmacia Biotech, Piscataway, NJ) using a 1:2500 dilution of ∝PAP1 primary antibody and a 1:1000 dilution of peroxidase-linked anti-rabbit secondary antibody. Washes were

conducted with 1X TBS-T (20mM Tris base, 137mM NaCl, 0.1% Tween 20 pH7.6) according to manufacturer's recommendations.

Isoform silver staining

Equal amounts of exudate protein (3.5µg) were separated by native PAGE on a BioRad MiniGel apparatus. Proteins were fixed by 30min sequential 100mL incubations in pre-fix A (50% methanol, 10% acetic acid), pre-fix B (5.4% methanol, 7.6% acetic acid), and 1% glutaraldehyde. After shaking overnight in 250mL water, the fixed protein gel was incubated for 30min in 100mL 33µM DTT, then 100mL 1% silver nitrate. The protein gel was developed with 150mL developer (3% w/v sodium carbonate, 0.02% v/v formaldehyde) until proteins were clearly visible, when development was stopped by the addition of 5mL 72% (w/v) citric acid.

RNA accumulation

RNA was isolated from 16d post germination plants grown vertically on either +P (1.2mM) or -P (9µM) 0.5X modified Hoagland's media with P-purified phytagel (Trull et al., 1997). Equal amounts of RNA (9.25µg) were run on a 1.5% agarose gel for 4h at 95V. The running buffer consisted of 20mM MOPS, 5mM sodium acetate, 100µM EDTA brought to pH 7.0 with glacial acetic acid. The gel was made up of the same buffer with an additional 6.7%(v/v) formaldehyde, and the RNA was suspended in loading buffer at final concentrations of 64%(v/v) formamide, 8.5%(v/v) formaldehyde, 5%(v/v) gycerol, 0.1mM EDTA, and 0.04%(w/v) xylene cyanol. The RNA gel was blotted to a nylon membrane (GeneScreen, PerkinElmer Life Sciences, Boston, MA)

overnight with 10X SSPE buffer (1X: 150mM NaCl, 10mM NaH_2PO_4, 1mM EDTA, final pH 7.4). The membrane was rinsed in 2X SSPE, then dried in an 80°C oven for 1h. All hybridizations and washes were carried out in 3.5mm diameter hybridization tubes in a rotating hybridization oven (Robbins Scientific). The RNA blot was pre-hybridized for 4h at 35°C in 5mL of hybridization solution (5X Denhardt's solution, 5X SSPE, 50% formamide, 1% SDS, 100μg/mL denatured salmon sperm DNA), then 100ng of a ^{32}P-labeled expressed sequence tag corresponding to *PAP1* (EST#155C5T7) added and hybridized for 15h at 35°C. Probes were radioactively labeled with the Random Primers DNA Labeling System using [^{32}P]-dATP according to manufacturer's instructions (GibcoBRL, Carlsbad, CA), then run over a Sephadex G-50 spin column to remove unincorporated radioactivity (Feinberg and Vogelstein, 1983). All washes were carried out in 50mL volumes. The RNA blot was washed once for 30 min at 35°C in low stringency buffer (2X SSPE, 0.05%w/v sarcosyl, 0.01% w/v sodium pyrophosphate), again for 30 min at 45°C in low stringency buffer, and then exposed to Kodak XAR autoradiography film. The blot was stripped of the *PAP1* signal by pouring 500mL of boiling stripping solution (1mM Tris, 0.5mM EDTA, 1% SDS pH 8) directly onto the blot and allowing the solution to cool to room temperature. After briefly rinsing the blot in 2X SSPE followed by 4h of pre-hybridization, 100ng of ^{32}P-labeled β-tubulin cDNA was hybridized to the blot as a loading control. Hybridization conditions and washes were the same except that an additional three washes for 30 min each at 45°C in high stringency wash buffer (0.1X SSPE, 0.05%w/v sarcosyl, 0.01% w/v sodium pyrophosphate) was necessary. This experiment was carried out a total of three times

with identical results but under different plant growth conditions and experimental protocols.

P accumulation when grown in a peat/vermiculite soil mix

Seeds were planted in 6cm diameter pots in a standard 55-65% sphagnum peat moss/perlite mix (Sunshine Aggregate Plus Mix #4, SunGro, Bellevue, WA). The peat mix contained no additional fertilizer. Prepared pots were stored at 4°C for 2d, then placed in the growth chamber under standard conditions (above). Plants were thinned to a density of 4 plants per pot at 7dpg and thereafter fertilized twice weekly by subirrigation with either +P or -P fertilizer (Somerville et al., 1982). No additional watering was needed. Shoots were harvested at 3 weeks post-germination (or at the first sign of the primary reproductive inflorescence), dried, and total P determinations carried out as for the root-bound APase assay. At least 9 shoots of each genotype per P fertilizer treatment were harvested in each experiment, and the experiment was replicated fully twice.

For the available soil P_i, ~0.3g of soil was suspended in 10mL deionized water and the eluate removed for P determination. To determine the total amount of P in the soil, ~0.1g was converted to ash by baking in a 490°C oven for 12h prior to P determination. Soil tests were repeated three times each.

Genetic mapping

A *pup3* mapping population was generated by outcross to the WS ecotype. F2 mutant segregants were identified first by their decreased APase activity in crude shoot protein extracts. Crude proteins were isolated by grinding individual small leaves from 10 dpg seedlings (grown in peat-vermiculite soil mix) in 400 µL protein extraction buffer (above), pelleting debris by centrifugation at 14,000 X G at 4 degrees C for 10 min, and collecting the supernatant. The crude proteins were quantified using the Bradford method (Bradford, 1976), and specific activity measurements carried out exactly as with the dialyzed protein samples (above). Candidate *pup3* protein samples with <75% specific APase activity compared to Col and WS control samples were run on native acrylamide gels to confirm the pup3 phenotype by a slight reduction in a major APase isoform when compared to control extracts (data not shown). The *pup3* mutation was mapped using co-dominant amplified polymorphic sequences (CAPS) as markers (Konieczny and Ausubel, 1993). Chi-square analysis was used to determine marker linkage, and once linkage was determined with multiple markers on a chromosome, then the Kosambi method was used to calculate genetic distances from those markers (Koornneef and Stam, 1992).

RESULTS

In-vivo root phosphatase activity

The screening and identification of both the *pup1* and *pup3* mutants have been previously described (Trull and Deikman, 1998). The *pup* mutants were identified based

on their reduced histochemical staining for root-bound and secreted APase when grown in media lacking P. The *pup1* phenotype very clearly shows no increase in blue color under -P conditions, while the *pup3* phenotype is only slightly lighter than the -P controls (Figure 3.1).

Root-bound phosphatase activity

Root-bound APase activity was measured to confirm and quantify the *pup1* and *pup3* reduced APase histochemistry phenotypes (see Figure 3.2 for a graphical representation of the data and Table 3.1 for the statistical analysis). Seedlings were germinated on solid +P media with normal light/dark conditions until 8 dpg, then transferred to liquid +P media shaking flasks in the dark for 10 d, and finally moved into either +P or -P liquid media for a 2 d induction period before the assay. The assay was performed by moving the plants to buffered -P media containing the colorimetric substrate for APase activity, pNPP, and measuring the amount of pNPP cleaved by the root systems from the intact plants.

Both *pup* mutants have significantly lower root-bound APase activity when compared to their controls (16% less for *pup3* and 25% less for *pup1*). The relatively short (2 d) -P treatment increased root-bound APase activity by 22% in the Col/*pup3* experiments and 25% in the WS/*pup1* experiments, however, this level of induction was not altered in the *pup* mutants.

To rule out the possibility that the mutants' reduction of APase activity is due to increased levels of P sequestration (therefore a decreased P starvation response), P concentration was measured from tissues used in these assays (see Figure 3.3 for a

graphical representation of the data and Table 3.1 for the statistical analysis). The 2 d -P treatment dramatically lowered P tissue concentrations in both roots (27% decrease in Col/*pup3*, 25% in WS/*pup1*) and shoots (32% decrease in Col/*pup3*, 37% in WS/*pup1*), but mutant plants did not differ from their controls in this respect.

Specific APase activity

Relative to Col control plants, the *pup3* mutant had consistently lower specific APase activity in all extracts tested (Figure 3.4). The *pup3* mutant had 49% less specific APase activity in exudates, 38% less activity in roots, and 37% less activity in shoots. Specific APase activity was not increased by -P conditions in either the exudates, roots, or shoots, so +P and -P samples were pooled.

Exudates from the *pup1* mutant showed no change in specific APase activity when compared to controls (data not shown), and previous work with *pup1* extracted proteins from roots and shoots similarly showed no differences in APase activity (Trull and Deikman, 1998).

There was no difference in the protein concentrations in mutant tissues or exudates (data not shown).

Isoform analysis

Concentrated exudates were run on native PAGE for APase activity staining, immunodetection against the PAP1 antibody, and silver staining. Four major APase activity isoforms are present in *Arabidopsis* exudates (Figure 3.5). Molecular weights can only be roughly estimated on a native gel because SDS is not present to linearize and

uniformly charge the proteins, so major APase isoforms are named here as “E1” “E2”, etc. for the sake of discussion. The highest-running major secreted APase isoform, E1, is -P inducible. The next two bands of APase activity, E2 and E3, are reduced in *pup3* exudates. Two lower APase activity isoforms, E4 and E5, are constitutive and not affected in the *pup3* mutant. Protein extracts from *pup3* roots and shoots showed slight APase activity reductions in isoforms running at about the same locations as E2, and APase isoforms from *pup1* exudates were not different from controls (data not shown).

Only one APase isoform, E3, is recognized by the PAP1 antibody. The decreased APase activity of E3 in *pup3* extracts correlates with reduced reactivity of E3 with the PAP1 antibody (Figure 3.6). There is no difference in immunoreactivity of *pup1* proteins with the PAP1 antibody (data not shown).

Silver staining is a very sensitive method, detecting as little as 0.1ng of protein per lane on a gel. While many other proteins appear when native exudate gels are silver stained, no differences in protein accumulation can be seen in the *pup3* mutant (Figure 3.7).

RNA accumulation

PAP1 transcript accumulation is induced under -P conditions, and the *pup3* mutant does not differ from control tissues in its accumulation (Figure 3.8). The *pup1* mutant also shows no differences in *PAP1* transcript accumulation (data not shown).

P accumulation when grown in a peat/vermiculite soil mix

The *pup* mutants exhibit altered P relations when grown in a peat/vermiculite soil mix. The soil mix had 2.67 +/- 0.14 μg P/g soil, however, only 0.88 +/- 0.02 μg P/g soil was available as P_i. Graphical representation of the data is present in Figure 3.9, and the ANOVA results are in Table 3.2.

The *pup3* mutant had an overall 17% decrease in shoot P concentration. Although applying P fertilizer resulted in significantly higher shoot P concentrations, the *pup3* mutant was not different in the relative magnitude of this increase (no genotype X P interaction).

The *pup1* mutant had an overall decrease of 10% in shoot P concentration when compared to the WS control, and in the WS/*pup1* experiment P fertilizer application had no effect on shoot P concentration.

Genetic mapping

The *pup1* mutant was previously mapped to chromosome 2, and the semi-dominant nature of the mutation reported (Trull and Deikman, 1998). The *pup3* mutant was also previously shown to be recessive (MC Trull and J Deikman, personal communication).

CAPS mapping was performed to localize the *pup3* mutation to a chromosomal region within the *Arabidopsis* genome (Table 3.3). If normal segregation ratios did not hold for any given marker ($P<0.1$, Chi-square), then distance from the mutation was calculated based on the recombination frequency using the Kosambi function (Koornneef

and Stam, 1992). Three markers on chromosome 5 (NIT4, RBCS-B, and ASB2) are linked to the *pup3* mutation. The RBCS-B marker is the closest to *pup3*, so based on the directionality from the other two markers the mutation is located at 68.4+/-6.0cM on chr5 (Table 3.3). *PAP1* is located on BAC T22013 from chromosome 2.

DISCUSSION

The *pup* mutants have reduced APase activity as demonstrated by histochemistry, root-bound assay, and specific activity. As previously reported (Trull and Deikman, 1998), *pup1* has obviously lower histochemical staining on the substrate BCIP under -P conditions. The *pup3* phenotype on the BCIP substrate is slight but significant. Root-bound APase activity is decreased by 25% in *pup1* and 16% in *pup3*. Specific activity measurements of *pup1* exudates showed no differences when compared to controls, as did similar measurements of root and shoot extracts presented in an earlier work (Trull and Deikman, 1998), however, the *pup3* mutant is defective in a major component of the overall APase activity pool. *pup3* root exudates, roots, and shoots have a 49%, 37%, and 38% respective reduction in specific APase activity. Therefore, the *pup3* mutation affects a major pool of phosphatase activity present in roots, shoots, and exudates, while the *pup1* mutation significantly affects the root-bound phosphatase activity pool.

APase isoform analysis was carried out as a first step in determining the nature of the decreased APase activity in the mutants. The *pup1* mutant was previously shown to be defective in a 160kDa isoform that is only discernable when extracts are run on SDS PAGE under non-reducing and otherwise native conditions (Trull and Deikman, 1998).

In *pup3* exudates, the decreased specific APase activity appears to correspond to the lessened activity of two APase isoforms that run at approximately 93-107kDa and 135kDa on native PAGE. The reduced isoforms are not induced by P deficiency in either control or mutant plants.

When grown in soils, the *pup* mutants show altered P relations (Figure 3.9). The *pup* mutants have lower shoot P concentrations when grown in soil independent of P fertilization. This decreased P uptake is not seen when plants are grown in nutrient solution, where P_i is the sole source of P (Figure 3.2). Therefore, the *pup1* and *pup3* phenotypes are functionally important when P_o can be utilized as a P source.

The *pup* mutants are probably not components of a low-P signal transduction pathway. The mutants did not differ in relative -P responsiveness in each experiment where P treatment was significant, proving that their defects are neither in their overall responses to P deficiency nor are they -P inducible. It seems more likely that at least the *pup3* mutant is defective in post-translational protein modification since *pup3* is defective in at least two distinct secreted phosphatases and the PAP1 antibody only recognizes one of those proteins. The *pup3* mutation cannot be in *PAP1* because not only does *pup3* have normal *PAP1* transcript accumulation but it also maps to a different chromosome. The fact that protein APase activity and PAP1 antibody reactivity are both decreased in these two isoforms but that their protein accumulation is not different points to altered conformation affecting the active sites of these proteins.

The proteins affected by the *pup* mutants are important for obtaining and maintaining shoot P concentrations when grown in soil. Further physiological work with the mutants may entail studying more precisely their defects in P regulation. Plants

tightly regulate P_i uptake, vacuolar P_i storage pools, and transport between organelles, cells, and organs (reviewed in Marschner, 1995). Since both mutants have shoot and root phenotypes, they may be defective in P partitioning at any of these regulatory levels. Because the *pup* gene products are unknown and cloning point mutations is difficult, further molecular work with these mutants should start with the purification, biochemical characterization, and sequencing of the affected proteins. These secreted phosphatases are functionally important and their manipulation may lead to developing plants with improved plant P relations.

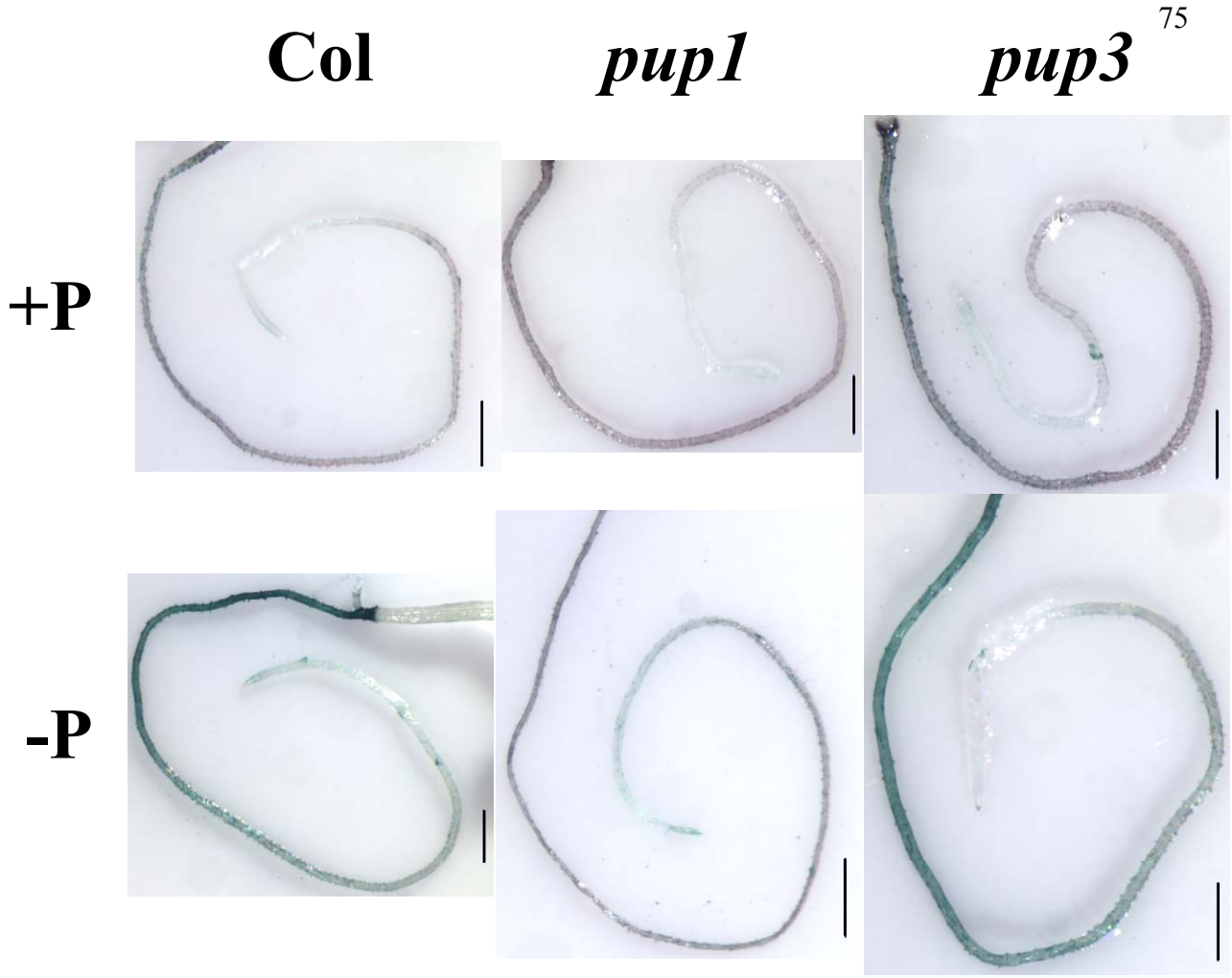

Figure 3.1. *In-vivo* root phosphatase staining of the *pup* mutants. When cleaved by phosphatases, the substrate BCIP forms a blue precipitate on the root surface. Seedlings were grown on media with (+P) or without (-P) phosphate containing BCIP to 5dpg before visualization. Standard bars are 1mm.

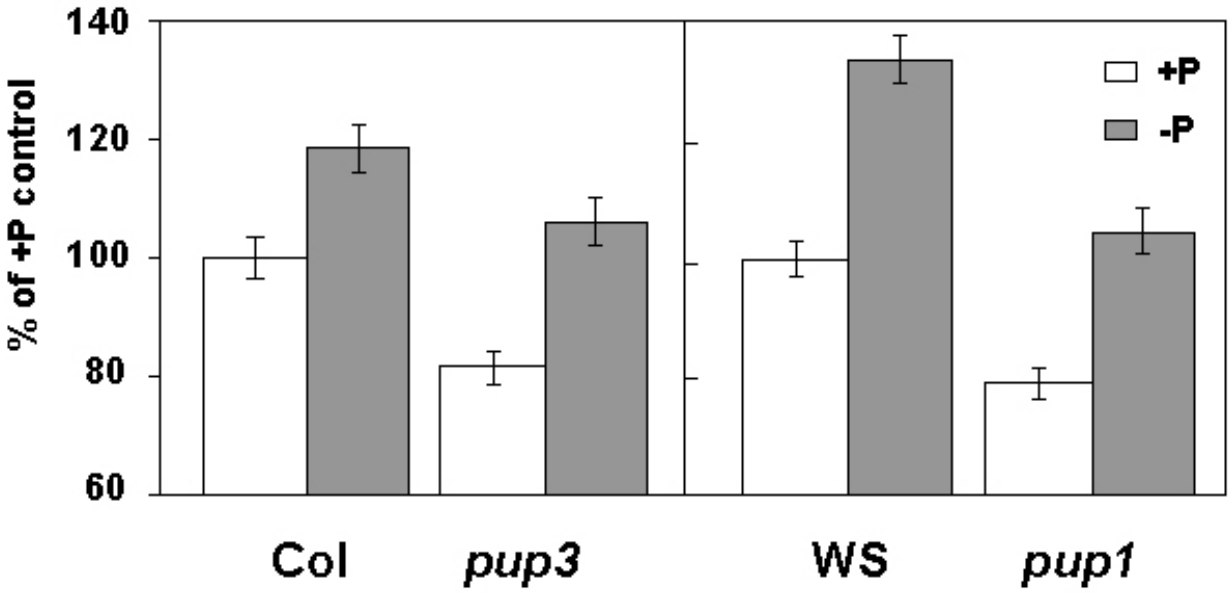

Figure 3.2. Root-bound APase activity of the *pup* mutants. After 2 days of -P induction, root systems from intact plants were placed in buffered -P growth media containing pNPP and its hydrolysis measured per unit root dry weight. Error bars are SE. Each bar represents a total of N>=16 measurements (pooled data from 2 experiments with N>=8 for each treatment in each experiment). Refer to Table 3.1 for ANOVA results from this data.

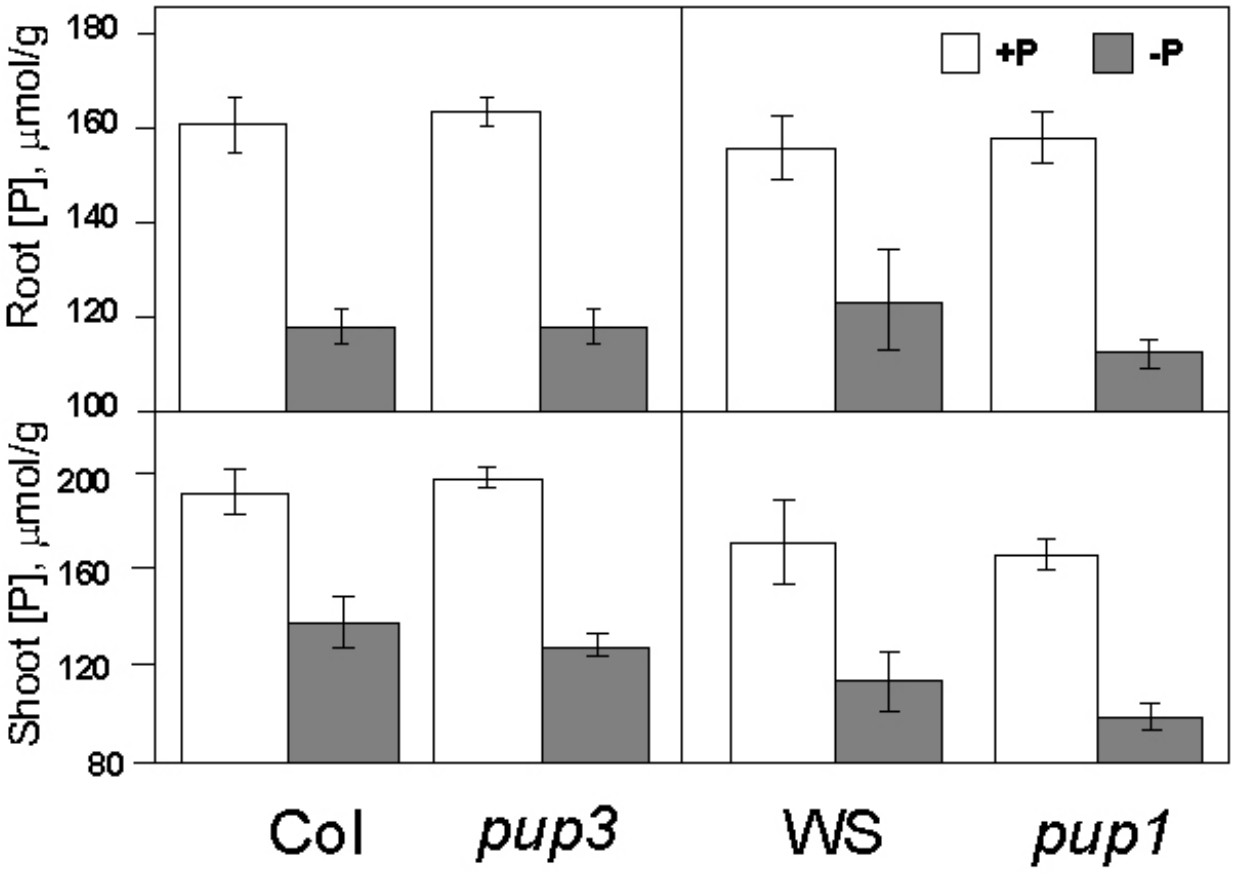

Figure 3.3. P concentrations of tissues used in the root-bound APase activity experiments. P concentrations are normal in mutant tissues when grown in nutrient solution, so the decreased mutant APase activity is not due to changes in internal P. Error bars are SE. Each bar represents a total of N>=16 measurements (pooled data from 2 experiments with N>=8 for each treatment in each experiment). Refer to Table 3.1 for ANOVA results using this data.

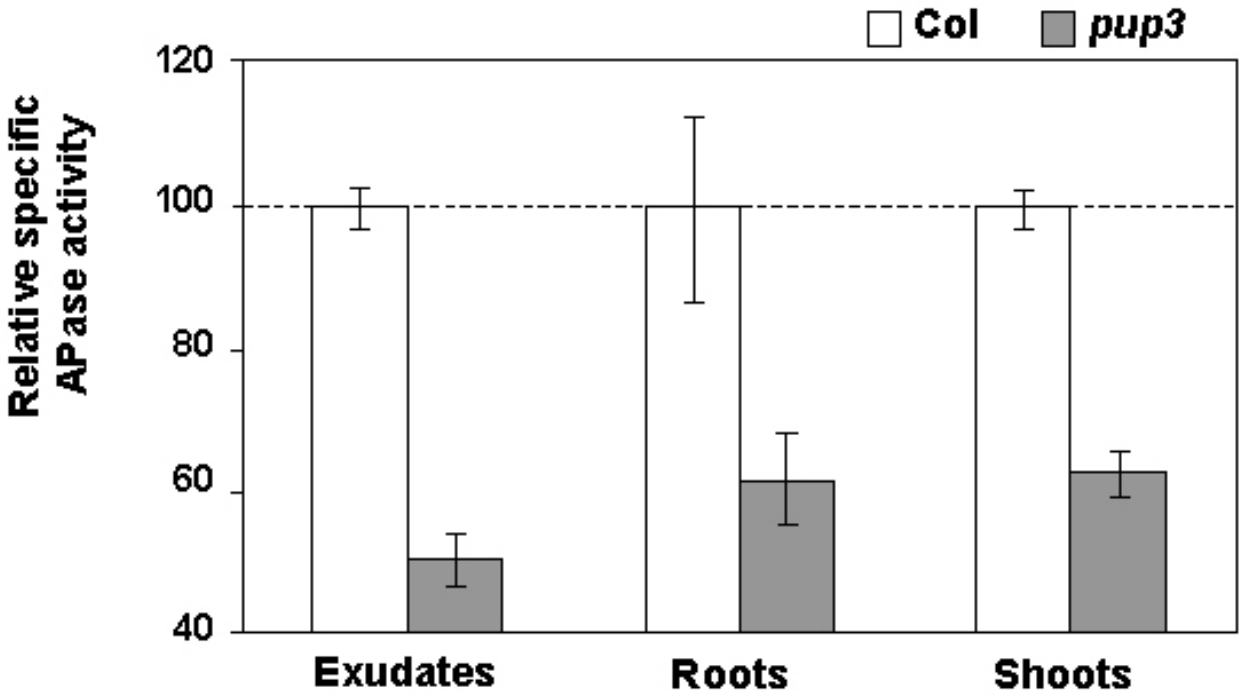

Figure 3.4. Specific APase activity. Proteins from concentrated exudates, roots, and shoots were assayed for specific phosphatase activity. Buffer exchange was carried out on protein samples to remove P and other inhibitors of the reaction prior to the assay. The hydrolysis of pNPP at 25°C over 10 minutes was measured for 0.5µg of protein, and activity is expressed as a % of the Col control reactions. N=12 for exudates, N=6 for roots and N=6 for shoots. Error bars are SE.

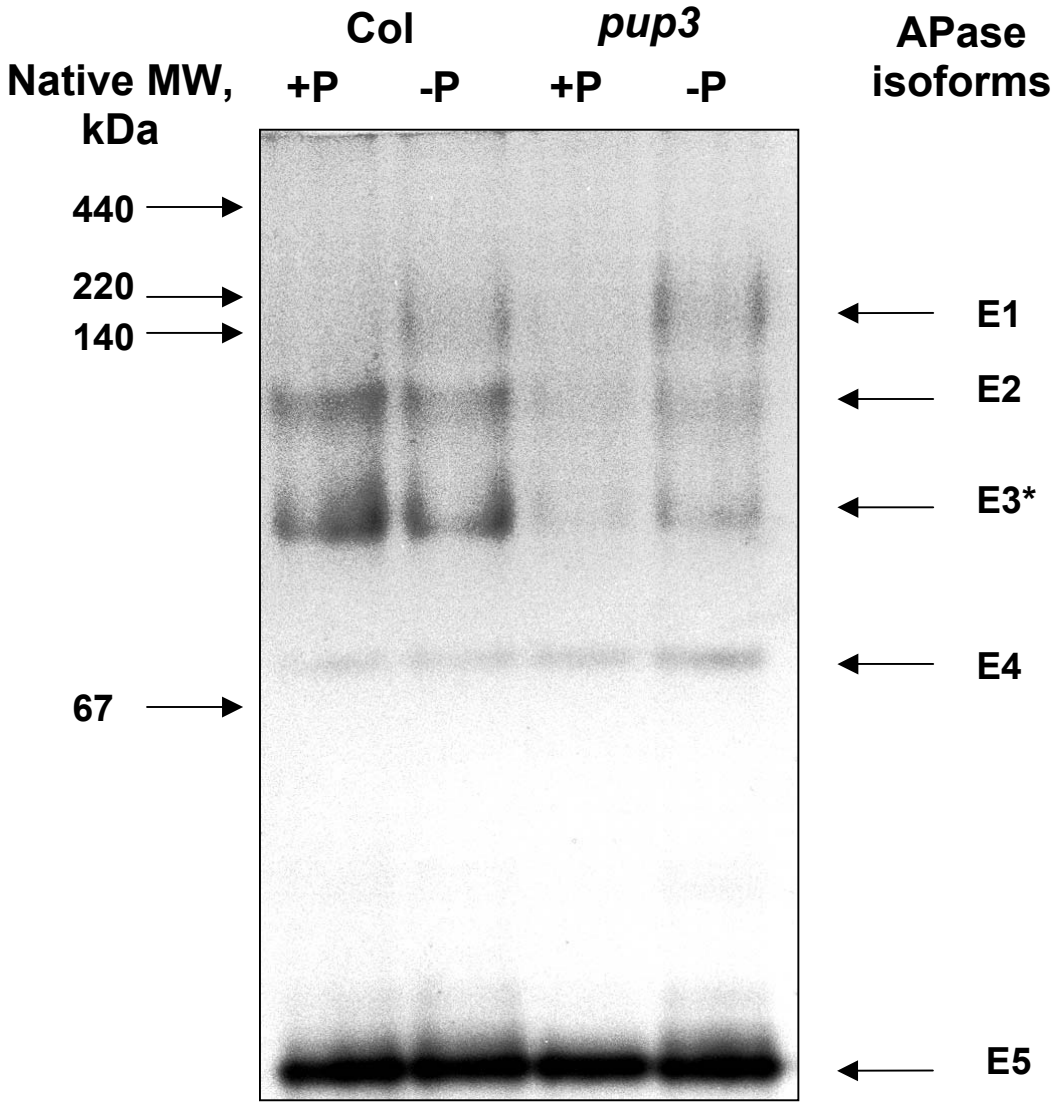

Figure 3.5. Acid phosphatase activity isoforms in *pup3* exudates. Concentrated exudates (7.5µg) were run under native discontinuous PAGE conditions and stained for APase activity using Fast Black K and β-naphthyl acid phosphate. Markers are native protein electophoresis markers (far left), and APase isoforms are noted on the right. Isoforms designated with an asterisk (*) have decreased activity in *pup3* exudates.

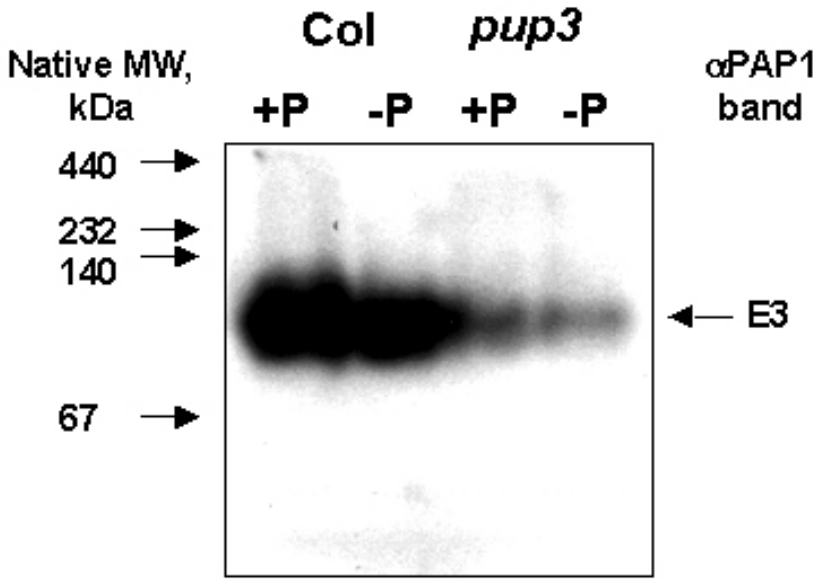

Figure 3.6. Immunoreactivity of *pup3* exudates against the αPAP1 antibody. Concentrated exudates (7μg) were run under native discontinuous PAGE conditions and blotted to a PVDF membrane. The membrane was hybridized with a polyclonal antibody raised against recombinant *Arabidopsis* purple acid phosphatase-1 protein (PAP1). Markers are native protein electophoresis markers (far left).

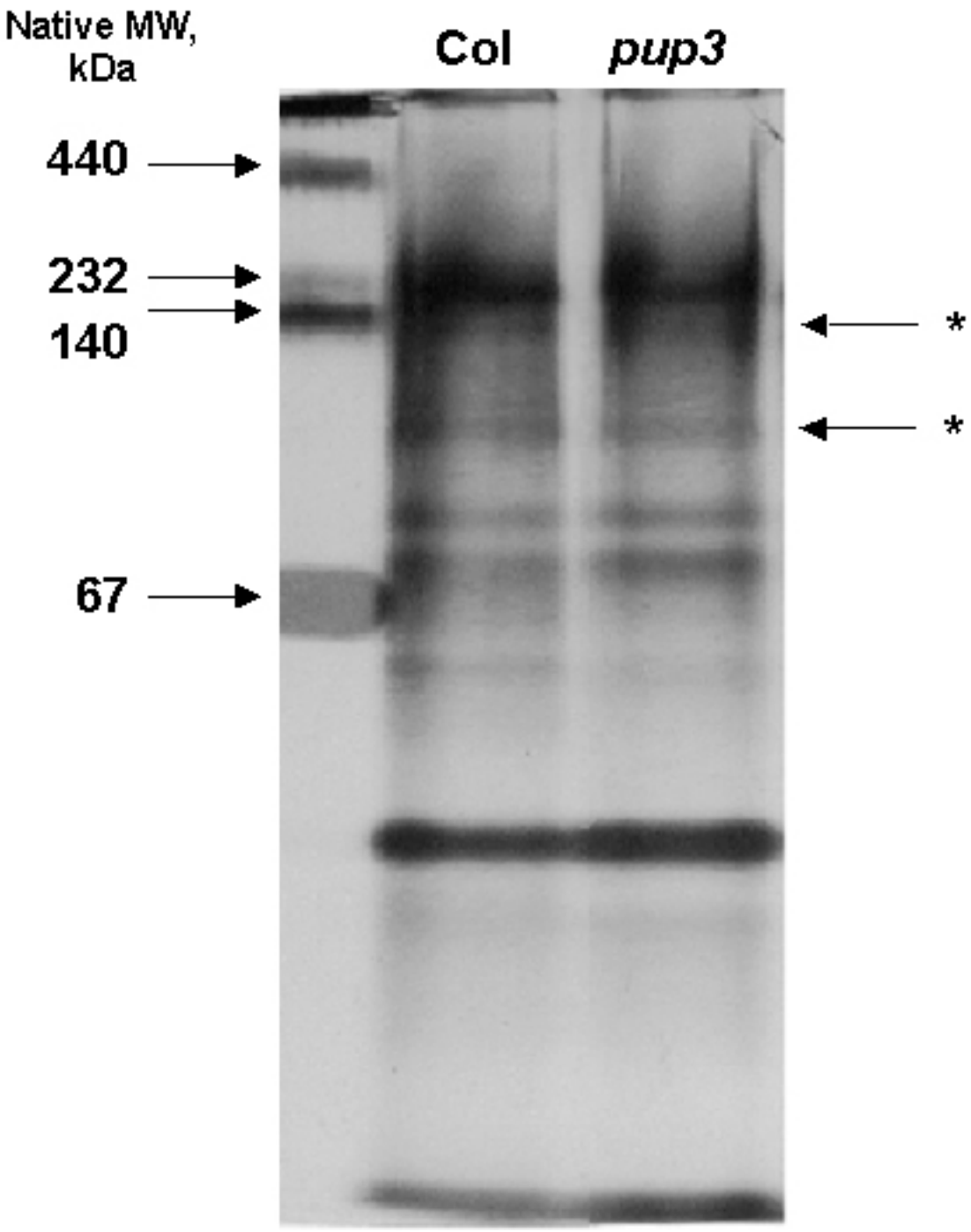

Figure 3.7. Total exudate proteins. Concentrated exudates (3.5µg) were run under native discontinuous PAGE conditions, fixed, and stained with silver nitrate. Markers are native protein electophoresis markers (far left), and candidate proteins with defective APase activity in the *pup3* mutant (see Figure 3.5) denoted with an asterisk (*).

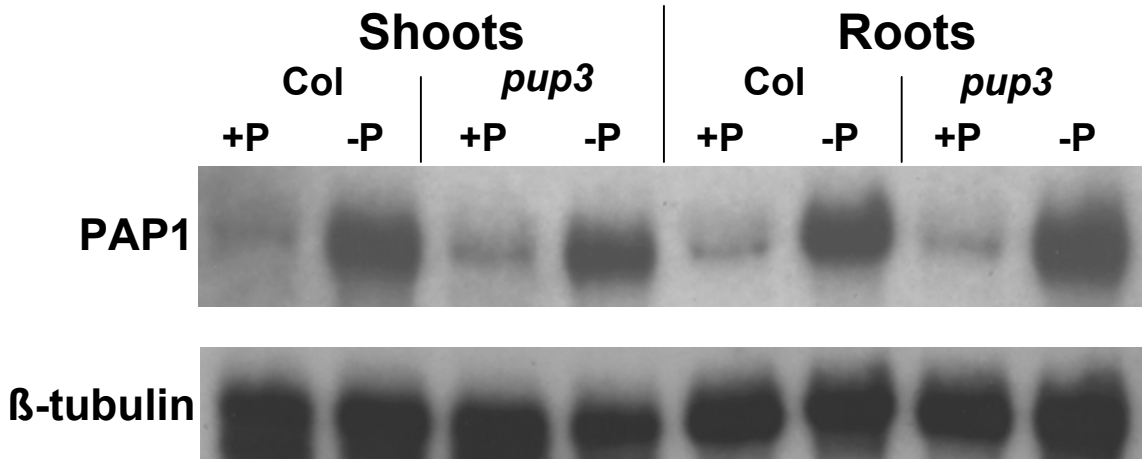

Figure 3.8. PAP1 transcript accumulation is not altered in the *pup3* mutant. Total RNA (9.25μg/lane) isolated from 16dpg plants grown on a continuous treatment of either high- or low-P was probed sequentially with an EST corresponding to PAP1 or β-tubulin (loading control).

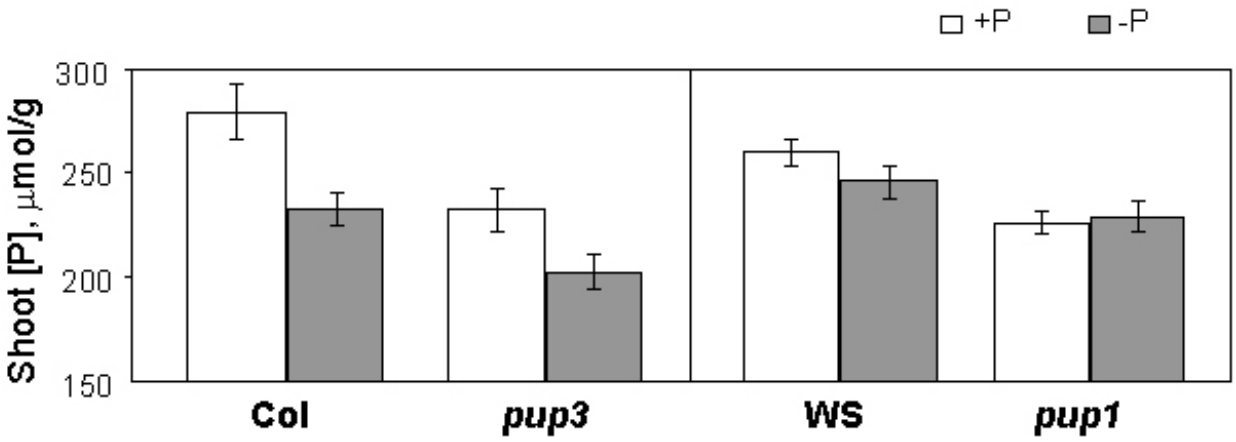

Figure 3.9. P accumulation when grown in soil. *pup* mutants and their respective control plants were grown in a peat/vermiculite soil mix for 3 weeks or until the first sign of the primary reproductive inflorescence, then shoots harvested for P determinations. Shoot P concentration is expressed on a per g shoot dry weight basis. This experiment was repeated twice, with N>=9 per treatment per replication. Data presented here is pooled from both replications.

TABLE 3.1. Statistical analysis of the root-bound APase experiments.

Variable	Components of variance	Error df	F-Value
Root-bound APase (WS/*pup1*)	Genotype P treatment Genotype X P treatment	64	28.73**** 41.15**** 0.58
Root-bound APase (Col/*pup3*)	Genotype P treatment Genotype X P treatment	68	20.52**** 40.05**** 0.78
Root P concentration (WS/*pup1*)	Genotype P treatment Genotype X P treatment	62	0.17 18.87**** 0.89
Root P concentration (Col/*pup3*)	Genotype P treatment Genotype X P treatment	61	0.10 116.47**** 0.11
Shoot P concentration (WS/*pup1*)	Genotype P treatment Genotype X P treatment	64	0.82 30.28**** 0.17
Shoot P concentration (Col/*pup3*)	Genotype P treatment Genotype X P treatment	63	0.28 158.90**** 0.02

****P<0.0001

TABLE 3.2. ANOVA summaries of plants grown in soil under different fertilizer treatments.

Variable	Components of variance	Error df	F-Value
P concentration (WS/*pup1*)	Genotype	75	14.0***
	P treatment		0.7
	Genotype X P treatment		0.2
P concentration (Col/*pup3*)	Genotype	69	14.3***
	P treatment		14.1***
	Genotype X P treatment		0.7
Total P accumulation (WS/*pup1*)	Genotype	79	5.8*
	P treatment		1.2
	Genotype X P treatment		8.2**
Total P accumulation (Col/*pup3*)	Genotype	73	10.6**
	P treatment		0.1
	Genotype X P treatment		<0.1

* P<0.05, **P<0.01, ***P<0.001

TABLE 3.3. Genetic location of the *pup3* mutation. CAPS mapping results are shown for markers throughout the *Arabidopsis* genome. N is the number of F2 plants scored from an outcross to another *Arabidopsis* ecotype (WS). Significant deviation from the expected segregation ratio of 1Col:2Het:1WS (P-value<0.1) indicates that the marker is linked to the mutation.

Marker	**Marker Location**	**N**	**Segregation Ratio, Col:Het:WS**	**Distance, cM from mutation**
m246	11cM, chr2	22	7:9:6	
GPA1	49cM, chr2	31	8:18:5	
Bgl1	75cM, chr3	25	6:15:4	
g4539	55 cM, chr4	24	5:16:3	
ASA1	15, chr5	25	8:14:3	
NIT4	45, chr5	32	16:15:1***	29.6+/-10.9
RBCS-B	80.8, chr5	33	25:8:0***	**12.4+/-6.0**
ASB2	115, chr5	26	10:14:2*	42.6+/-17.9

*P<0.1, **P<0.05, ***P<0.01

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CHAPTER 4:
Summary and context

Considerable progress has been made in understanding plant P deficiency since the work presented in this thesis was begun. It has been an exciting field to work in, and this thesis adds to the work of other active groups in the field. This chapter summarizes significant contributions made by this thesis, places them in context with other recent developments in the field, and sets them in context with the completed *Arabidopsis* genome sequencing project. A model for the plant PHO regulon and suggestions for future work conclude the chapter.

Contributions in this thesis

First, this thesis introduces a new class of *Arabidopsis thaliana* mutants for phosphatase secretion , the *constitutive phosphatase secretion* (*cps*) mutants. *cps1* mutants have increased root-bound phosphatase activity under both +P and -P conditions. The *cps1* mutation is associated with a severe root growth reduction that, when plants are grown in soil, results in decreased shoot biomass accumulation. The *cps1* mutation affects a major acid phosphatase isoform present in shoots and roots, and its defect may be the result of defective targeting of a normally intercellular acid phosphatase to the cell wall.

Here I also present further characterization of the *phosphatase-underproducer-1* (*pup1)* mutant. When *pup1* was initially reported by Trull and Deikman (1998), its decreased histochemical acid phosphatase staining was shown to be the result of a co-

dominant mutation. *pup1* was also shown to have a reduced root:shoot ratio when grown under low-P conditions, and *pup1* lacks a 160 kDa acid phosphatase isoform when root and shoot extracts are run in a gel system with SDS but without reducing agents. Solution assays for phosphatase activity had also been shown to be normal, as were acid phosphatase isoforms when run under completely non-denaturing conditions. In this work, data is presented that demonstrate that the *pup1* mutant indeed has lower root-bound phosphatase activity, and that this defect has a physiological cost in shoot P concentration levels when grown in a soil substrate with an organic P component. This new data fits the original hypothesis by Trull and Deikman concerning PUP1 protein function: that *pup1* is defective in a structural acid phosphatase gene such that the gene product is unable to dimerize for subsequent secretion (Trull and Deikman, 1998).

Previous to this work, the *pup3* mutant's isolation had been published (Trull and Deikman, 1998) but its characterization not reported. Work with it in the Deikman Lab by Melanie Trull had shown that the phenotype included a slight reduction in acid phosphatase histochemical staining under low-P conditions, reduced acid phosphatase activity in crude protein extracts, recessive genetic inheritance, and a faint reduction in activity of a major shoot acid phosphatase isoform (personal communication). In this work, I determined the genetic map location of the *pup3* mutation and show that it results in reduction of a major pool of specific acid phosphatase activity in roots, shoots, and root exudates. Further, I demonstrate that it has decreased activity of two secreted acid phosphatase isoforms. One of these isoforms is recognized by the purple acid phosphatase-1 (PAP1) antibody, and this isoform has reduced reactivity with the PAP1 antibody in *pup3* exudates. This finding implies that PUP3 functions to either preserve or

augment the activity of two distinct secreted acid phosphatases. I also show that the *pup3* mutation results in decreased P accumulation when grown in a soil mix with an organic P component but not in nutrient solution without an organic P component, proving that the activity of these secreted phosphatases is important for maintaining normal P concentrations when organic P is available.

This work also includes other significant contributions to the plant science community in the form of methods development. An assay for root-bound acid phosphatase activity was modified for use with *Arabidopsis thaliana*, and root protein exudates concentrated for APase isoform analysis for the first time in this model organism.

This new subset of APase isoforms leads to comparison between roots, shoots, and exudates (Figures 2.7, 2.8, and 3.9, respectively). The highest migrating phosphatase isoform in each (R1, S1, and E1) migrates at about the same location and with the same characteristic shape and low-P responsiveness. These isoforms could be products from the same gene. The relative intensity of E1 to other exudate isoforms is not as dramatic as R1 is to other root phosphatase isoforms In roots, R1 is the most intense isoform, and in shoots, S1 is one of two most reactive isoforms, but in exudates E1 activity is much less than E2, E3, and E5. If S1, R1, and E1 are the same protein, then the primary location of this protein is probably intercellular. Along these same lines, root isoforms R2- R5 could be secreted as E2-E5. The shoot S2 isoform is broad and seems to be made up of multiple proteins, possibly modified (heavily glycosylated) versions of proteins that run separately as R2 and R3 in roots. The root isoform R4 may also have activity in shoots as a minor isoform between S2 and S3.

Concurrent relevant work from other groups

Two other *Arabidopsis* mutants have recently been reported that shed light on the plant PHO regulon. The *pho3* mutant was identified in the same histochemical screen used to identify the *pup* mutants (Zakhleniuk et al., 2001). *pho3* has decreased acid phosphatase activity in root and shoot extracts, does not increase its root acid phosphatase activity in response to P deprivation, accumulates less P in roots and shoots when grown in +P (but not -P) solid nutrient media, and accumulates shoot P when grown in soil. The *pho3* mutant has other physiological problems implicated in P deficiency responses, including low fertility, delayed flowering (by 2-3 weeks), less shoot biomass when grown in soil, 50% reduced chlorophyll content when grown in +P (but not -P) solid nutrient media, decreased starch accumulation, and decreased anthocyanin accumulation. Because the *pho3* mutant lacks the ability to increase its phosphatase activity and anthocyanin accumulation during P deficiency, and because it has so many other defects associated with P deprivation, the *pho3* mutant may be deficient in a regulatory component of the plant PHO regulon. Although genetic mapping information was not reported for *pho3*, it is distinct from *pup3* because *pup3* does not have altered P concentrations when grown in media lacking an organic P component, and *pup3* is not defective in other P deficiency responses.

The *phosphate starvation response 1* (*phr1*) mutant was isolated by EMS mutagenesis of a transgenic line with the P-responsive reporter gene fusion AtIPS1::GUS (Rubio et al., 2001).. Unlike the *pup* mutants (but similar to *pho3*), *phr1* has decreased P concentrations under +P conditions in a solid medium without an organic P component.

phr1 also has reduced low-P responsiveness in anthocyanin accumulation and gene induction (including reduced -P induction of AtACP5). Although the reporter gene is uniformly repressed in all *phr1* plant parts, -P regulated root hair elongation is normal in the mutant. The *phr1* mutation was mapped by positional cloning and the gene identified by homology to the *Chlamydomonas reinhardtii* phosphate starvation responsive 1 gene (*PSR1*), a MYB-coil-coil transcription factor (Shimogawara et al., 1999). PHR1 was shown to bind as a dimer to an imperfectly palindromic 10-bp sequence found in P-sensitive promoters (including AtACP5 and PAP1), GNATATNC (Rubio et al., 2001). Unlike the *S. cerevisiae* and *N. crassa* PHO regulon b-zip transcription factors, PHR1 is localized to the nucleus during both +P and -P conditions. PHR1 is part of a 15-member family in *Arabidopsis*, and the authors suggest that other members may also contribute to the low-P response (Rubio et al., 2001).

The sequenced Arabidopsis genome

Complete genome analysis of other possible PHO regulon genes can now be accomplished with the fully sequenced *Arabidopsis* genome (www.arabidopsis.org). Eight predicted *Arabidopsis* genes have significant homology with PHO80 (Madden et al., 1988), PHO85 (Uesono et al., 1987), and PREG (Kang and Metzenberg, 1993), transducers of the -P signal in *S. cerevisiae* and *N. crassa* (Figure 4.1). Searching with the "phosphate switch" ankyrin repeat proteins PHO81 (Coche et al., 1990) and NUC-2 (Poleg et al., 1996) identified proteins with homology only in the ankyrin repeat portion of their genes. None were significantly related to PHO81 and NUC-2 in a CLUSTALW

analysis (bootstrap values >45 out of 100 samples). Searching for acid phosphatases with AtVSP (Berger et al., 1995), AtACP5 (del Pozo et al., 1999), and PAP1 (Patel et al., 1996) revealed a 30-member superfamily of predicted genes (Figure 4.2). There are nine AtVSP-like genes, four AtACP5-like genes, and thirteen PAP1-like predicted genes. The AtVSP and AtACP5 groups are more related to each other than to the PAP1 group of phosphatases.

The *pup* and *cps* mutants can be placed on the newly sequenced *Arabidopsis* genome. Each *Arabidopsis* open reading frame (ORF) is named by position along a chromosome, facilitating comparisons between mapping regions and candidate genes. The *cps1* mutant is either between At5g49010 and At5g57780, or between At5g63560 and At5g67460. This region spans 5.1Mb of sequence. One of the predicted AtVSP homologs, At5g51260, is within this region and should be considered a possible candidate for CPS1. Assuming that *cps2* is within 1 cM of its tightly linked marker, this mutation lies between At1g63440 and At1g66210, a region encompassing 1.1Mb of sequence data. This area does not include any of the predicted phosphatase genes. The *pup3* mutation maps to between At5g29584 and At5g36210, an area encompassing 2.7Mb of sequence. This area includes the PAP1-like predicted protein At5g34850, however, according to the prediction for PUP3 function this gene should not be a structural phosphatase gene. The *pup1* gene was previously mapped to between 34.0 cM and 54.8 cM on chromosome 2 (Trull and Deikman, 1998). This is an area of 4.6Mb, from At2g18200-At2g28940, which is very close to the PAP1-predicted protein At2g18130 and also includes PAP1 itself. None of the cps or pup mutants map to a location consistent with *Arabidopsis* PREG, PHO80 or PHO85 homologs. In summary,

analysis of the *Arabidopsis* genome revealed candidate phosphatase genes for *cps1* and *pup1*.

Plant PHO regulon model

One possible model for a plant PHO regulon based on this and other recent work is shown in Figure 4.3. Acid phosphatase and high-affinity transporter regulation is pictured in this model, but other genes are similarly regulated. Under +P conditions, constitutive phosphatases such as AtVSP and possibly CPS1 are active in the vacuole regulating intercellular P levels by breaking down phytic acid and other P stores. PUP3 modifies PAP1 and at least one other acid phosphatase before their secretion. Once secreted, PAP1 and PUP1 liberate organic P for uptake. The transcription factor PSR1 is localized to the nucleus but not active. Under low-P conditions, PSR1 is activated by a signal transduction cascade that may include PHO3 and Arabidopsis PREG homologs. Once active, PSR1 binds to the promoters of P-sensitive genes such as PAP1 and the high-affinity transporters AtPT1 and AtPT2 to upregulate their transcription.

Future directions for this work

Many unanswered questions remain in this field of study. The central question that I began with still remains: How do plants sense and respond to P deficiency? PSR1 is nuclear localized, therefore there must be some other P sensor. Studies on the putative PREG-like proteins and cloning the *pho3* mutant may help to identify the P-responsive signal transduction cascade.

Insights into functionally important secreted phosphatases were discovered by the identification and characterization of the *pup* and *cps* mutants. A candidate for the *cps1* gene was identified. Sequencing this locus in the *cps1* mutants and complementation of the mutant phenotype by transformation with wild-type copies of the gene would confirm that this is in fact the defective gene. If this protein is normally intercellular, then identification of CPS1 and further study of the defects of *cps1* mutants may lead to further progress in protein sorting. Similarly, the candidate *pup1* gene could be confirmed by sequence analysis and transformation. Identification of the PUP1 protein would add to our understanding of how this phosphatase contributes to whole-plant P relations. Of the *cps* and *pup* mutants, *pup3* seems to have the most dramatic affect on whole-plant P relations, probably because it is defective in at least two different phosphatases. While cloning *pup3* would give important information about how these phosphatases are regulated, identifying the affected phosphatases themselves would lead to possible candidates for genetic engineering that may help plants to mobilize P from organic soil sources more efficiently. This could be accomplished by cutting the phosphatase isoforms from activity gels and sequencing the proteins. Further study of the *pup* and *cps* mutants and their affected proteins may continue to give valuable insight into plant P relations.

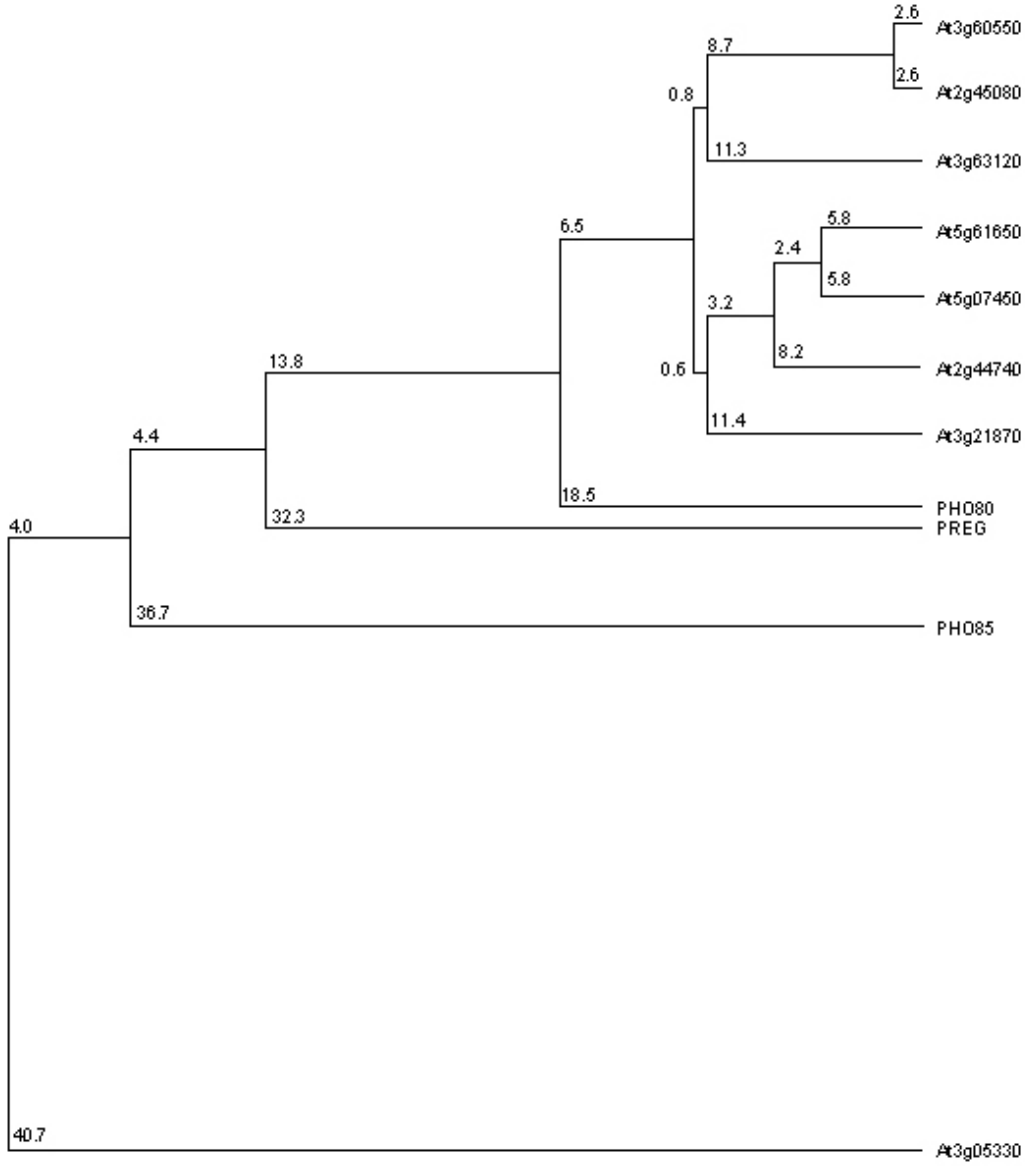

Figure 4.1. Putative PHO regulon signal transduction components in *Arabidopsis thaliana*. Predicted proteins from the fully sequenced *Arabidopsis thaliana* genome were searched by homology to PREG, PHO85, and PHO80 proteins using BLASTP (www.tair.org). Proteins with significant homology (expected values $P<0.01$) were aligned using Clustal W from the European Bioinformatics Institute (www.ebi.ac.uk), which returned this average distance phylogenetic tree calculated by PID (a measure of percent identity with a consensus sequence).

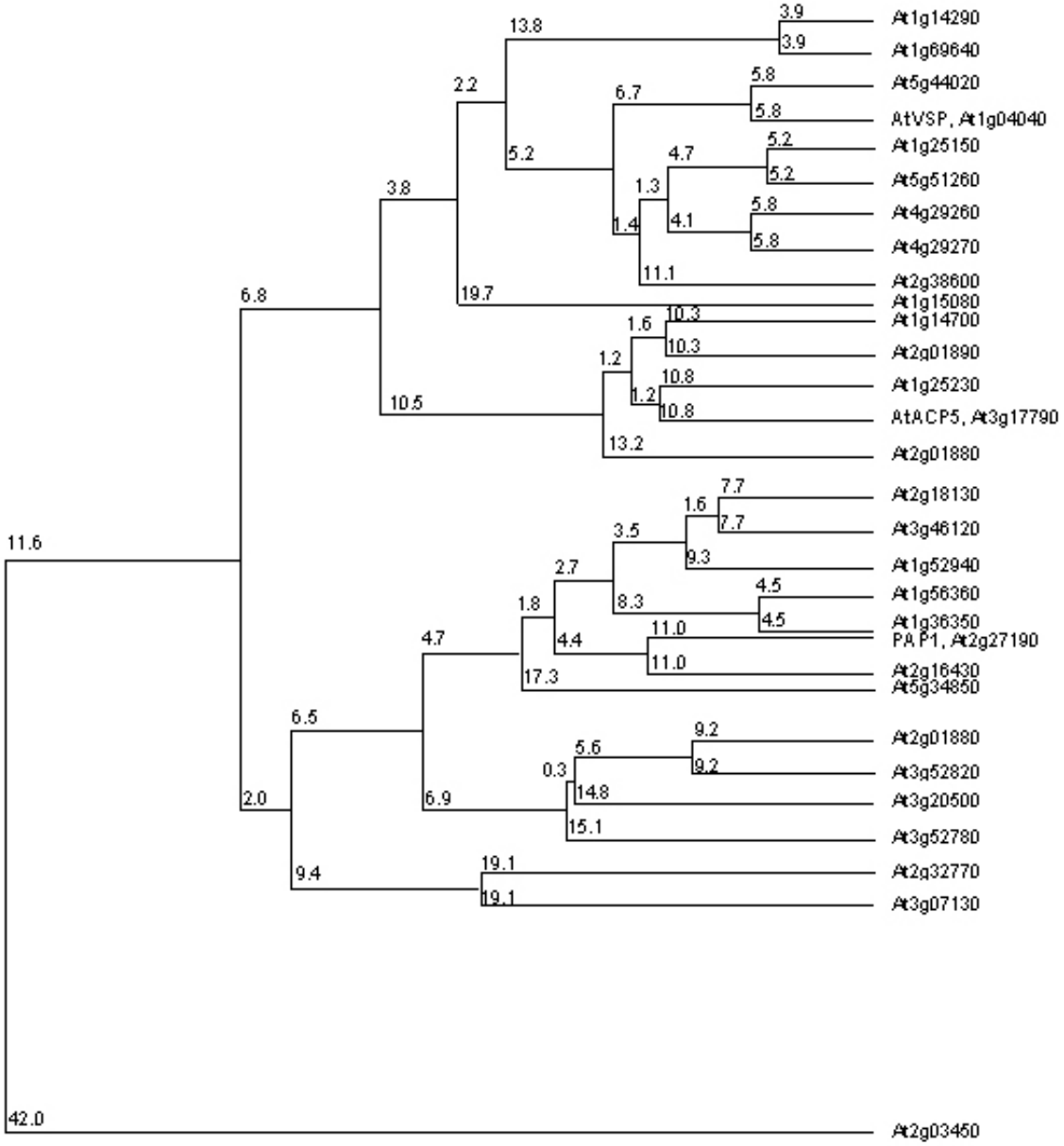

Figure 4.2. Acid phosphatases in *Arabidopsis thaliana*. Predicted proteins from the fully sequenced *Arabidopsis thaliana* genome were searched by homology to known *Arabidopsis* acid phosphatase proteins using BLASTP (www.tair.org). Proteins with significant homology (expected values P<0.01) were aligned using Clustal W from the European Bioinformatics Institute (www.ebi.ac.uk), which returned this average distance phylogenetic tree calculated by PID (a measure of percent identity with a consensus sequence).

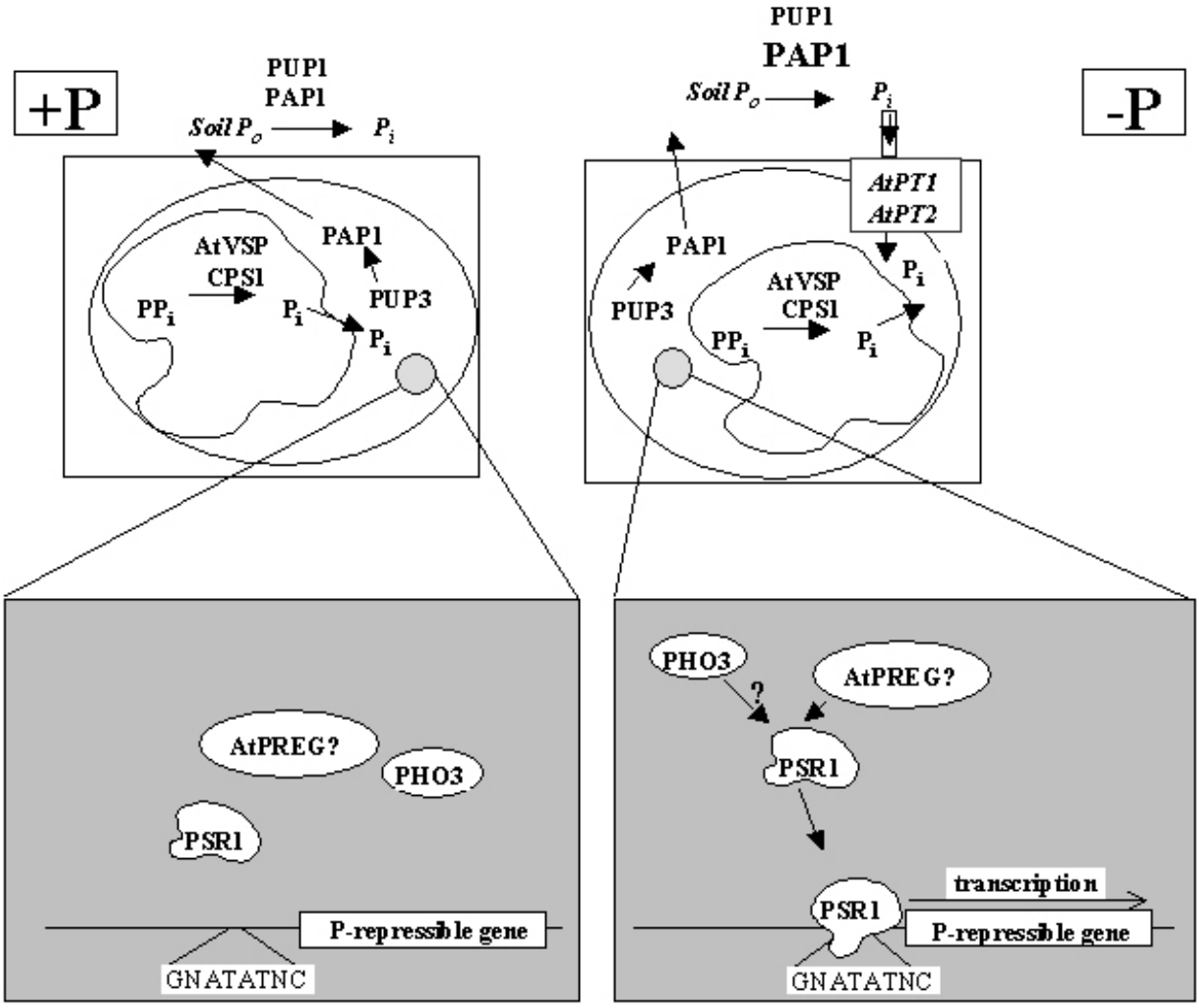

Figure 4.3. Model for a plant PHO regulon. Acid phosphatase regulation is used as a model for the plant PHO regulon. A typical plant cell with cell wall, plasma membrane, vacuole, and nuclear membrane is depicted. The nucleus is shaded, and an insert of the nucleus is pictured below each cell.

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APPENDIX: PCR Conditions for CAPS Mapping Primers

Primer	PCR Recipe	Restriction enzyme	Separation conditions
AsaI	2	BclI	1% agarose
ASB2		HinfI	
BglI	2	RsaI	2% agarose
CAT2	2	DdeI	5% acrylamide
cop1	2	MseI	5% acrylamide
DetI	3	SacI	1% acrylamide
g4539	1	HindIII	2% agarose
GAPB	4	DdeI	2% agarose
m235	2	HindIII	1% agarose
m305	1	HaeIII	2% agarose
NIT4	2	MboII	1% agarose
PhyB	2	XhoI	1% or 2% agarose
PhyC	2	MspI	2% agarose

All reactions were carried out by adding 9.0μL reaction mix to 1.0μL DNA.

Recipe#1: 1X Taq polymerase reaction buffer (Display), 0.2μM each primer (Research Genetics), 0.2mM dNTPs (each), 1U Taq polymerase (Display)

Recipe#2: Recipe #1 + 25mM KCl

Recipe#3: Recipe #1 + 25mM KCl and 2mM $MgCl_2$

Recipe #4: 1X Taq polymerase reaction buffer (Homemade 12/9/98), 50mM KCl, 0.2μM each primer (Research Genetics), 0.2mM dNTPs (each), 1U Taq polymerase (Display)

Restrictions

For each restriction, 5.0μL of restriction mix (1.5X restriction buffer + 2.5U restriction enzyme) was added to each reaction after PCR amplification. Restrictions were carried out for 2 hours in the PCR machine at the optimal temperature for each restriction enzyme.

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J Tomscha, A Dewald, JP Lynch, M Guiltinan, and J Deikman (1999) Constitutive phosphatase secretion mutants in *Arabidopsis thaliana*. *In* Phosphorus in Plant Biology: Regulatory Roles in Molecular, Cellular, Organismic, and Ecosystem Processes. JP Lynch and J Deikman, eds. American Society of Plant Physiologists, pp 376-378.