REGULATION OF PHOSPHATE STARVATION RESPONSE

IN *ARABIDOPSIS*

A Thesis

by

BETH ELENE ARMSTRONG THOMAS

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

MASTER OF SCIENCE

December 2006

Major Subject: Biology

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Approved by:

Chair of Committee,	Thomas McKnight
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ABSTRACT

Regulation of Phosphate Starvation Response in *Arabidopsis*.

(December 2006)

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Phosphate is an essential but limited macronutrient for all plants. In response to limited levels of phosphate, plants have developed highly specialized developmental, biochemical, and molecular responses. To further expand our knowledge of the phosphate starvation induced signal transduction pathway in plants, the expression of the phosphate starvation inducible Purple Acid Phosphatase 1 (*PAP1*) gene was studied in transgenic *Arabidopsis*. While few components have been identified regulating gene expression under phosphate starvation conditions in plants, one *cis* regulatory element recognized by the MYB transcriptions factor Phosphate Starvation Response 1 (PHR1) has been identified in many phosphate starvation induced (PSI) genes. *PAP1* and many other genes examined during the course of the mutant characterization contain this *cis* element.

Using the *GUS* reporter gene under control of the *PAP1* promoter, a mutant screen was devised for plants showing abnormal *PAP1* response to phosphate nutrition. Three mutant lines were identified and subsequently characterized for the phosphate starvation-induced signal-transduction pathway in *Arabidopsis*.

Two mutants, *BT1* and *BT2,* both with dominant mutations, showed increased GUS staining. The mutations in *BT1* and *BT2* are tightly linked to the transgene and to each other, but complementation analysis suggested that they are in different genes. Characterization of these mutants indicated that the PSI genes *PAP1* and *At4* (in *BT1* roots), and *RNS1* (in *BT2* leaves) have alternative or additional methods of regulation other than *PHR,* even though these genes all contain PHR1 binding sites.

A third mutant, *BT3*, had a phenotype similar to the *PAP1* null-mutant and did not show *PAP1* phosphatase activity under normal soil-grown conditions. Characterization of *BT3* indicates that *PAP1*, *RNS1,* and *AtIPS1* are not exclusively regulated by *PHR1*.

In an attempt to map the *BT3* mutant in a Columbia background by crossing with Landsberg *erecta* (Ler), it was discovered that the Ler ecotype does not show *PAP1* phosphatase activity under normal soil-grown conditions. The *PAP1* phosphatase regulatory trait, named *BT5*, was mapped to a 15,562 bp-region area containing only two genes between the *GPA1* and *ER* markers on Chromosome 2.

THIS WORK IS DEDICATED TO MY TWO WONDERFUL PARENTS,

RALPH AND LEA ARMSTRONG,

FOR ALL THEIR LOVE AND SUPPORT

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I would also like to thank my parents, Ralph and Lea Armstrong, for all their love, support, encouragement, and help. Thank you for always being there, and for believing in me and loving me regardless.

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Finally, I want to acknowledge the part that God has played in this degree by placing these people here to help me and hold me up when I didn't think that I could keep going, and for allowing me to finish. I can do nothing without him.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

CHAPTER I

GENERAL INTRODUCTION

PHOSPHORUS

Phosphorus is an essential macronutrient for all plants. It is a component of high-energy phosphate compounds, nucleic acids, phospholipids for cell membranes, essential coenzymes, and is also required for a number of important processes in the plant including glycolysis, respiration, photosynthesis, nucleic acid synthesis, membrane synthesis, enzyme activation/inactivation, signaling, carbohydrate metabolism, and nitrogen fixation (Vance et al., 2003; Raghothama, 1999; Schachtman et al., 1998). Consequently, plants are unable to grow without a sufficient supply of this nutrient (Vance et al., 2003; Raghothama, 1999; Schachtman et al., 1998). The form of phosphorus that is preferentially taken up by plants is orthophosphate (Pi) (Schachtman et al., 1998; Raghothama 1999). Even though phosphorus is quite abundant in many soils, more than 80% of it is present in forms that are immobile and thus unavailable for plant uptake (Franco-Zorrilla, et al., 2004; Schachtman, et al., 1998). Of all the elements whose primary reserve is the earth's crust, phosphorus is the element most likely to limit plant growth (Raven et al., 1986; Vance et al., 2003). Pi concentration in soil rarely exceeds 10μM and is only 1 to 2 μM in most soils (Schachtman et al., 1998; Raghothama 1999), which is substantially less than the average content of 5-20mM phosphorus in plant tissues (Raghothama 1999).

The form that Pi exists in solution changes with pH. Phosphorus is freely soluble only under acidic or reducing conditions, and studies show that uptake in higher plants is highest at pHs between 5.0 and 6.0 (Schachtman et al., 1998). In the soil, sometimes half of the phosphorus may be found in organic forms, which must be mineralized before it can be taken up by the plant (Vance et al., 2003). The rest may be either adsorbed to soil particles, clay surfaces, magnesium or calcium carbonates, or precipitated with iron,

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This work follows the format of Plant Physiology.

aluminum, and calcium (Vance et al., 2003; Rausch and Bucher, 2002). Even soluble phosphate fertilizers are rapidly converted to unavailable forms of inorganic phosphate (Schachtman et al., 1998; Vance et al., 2003; Smith, 1980). In addition to the fact that up to 80% of the added fertilizer may become fixed in the soil, there are also other problems associated with the use of added fertilizers. First, the readily available sources of phosphate rocks are non-renewable and are estimated to be depleted in the next 60 to 90 years. Second, the use of added fertilizers can be expensive and not always an option for farmers in poorer regions like the tropics. Third, in areas that do use fertilizers, farmers may have to use so much that the excess Pi not used by the crops can enter the surface water as run-off and cause eutrophication and hypoxia of lakes and marine environments (Vance et al., 2003; Raghothama 1999).

DEVELOPMENTAL OR MORPHOLOGICAL RESPONSES OF PLANTS TO PHOSPHATE STARVATION

To respond to limited levels of phosphate, plants have developed highly specialized mechanisms in two main areas: 1) developmental or morphological responses, and 2) biochemical responses. Developmental responses mainly involve changes in the root system of the plant that allow for enhanced access to and uptake of soil phosphate. These changes in the root system include an increased root-to-shoot growth ratio, increased lateral root formation, and increased length and number of root hairs (Bates and Lynch, 1996; López-Bucio et al., 2002; Ma et al., 2001; Williamson et al., 2001).

Some plants have also developed an additional developmental strategy to increase phosphate uptake by the formation of specialized root clusters or proteoid roots. Proteoid roots not only increase the surface area in which to absorb limited amounts of phosphate, but are also sites where large amounts of organic acids and phosphatases are secreted, aiding in the solubilization of phosphate from inorganic and organic complexes in the surrounding soil (Abel et al., 2002; Vance et al 2003; Raghothama, 1999).

Still other plants increase their uptake of phosphate through establishing symbiotic associations in the root with mycorrhizal fungi. Organic phosphates are either mineralized by soil bacteria into inorganic phosphates, immobilized when they are incorporated into the bodies of microorganism, turned into chemical compounds unavailable to plants, or recycled to plants (Smith, 1980). This recycling to plants is often facilitated through mycorrhizal associations with fungi, which infect the roots of most plants. These fungi absorb carbon from the host plant in return for absorbing and translocating not only phosphorus, but also other minerals needed for nutrition from the soil into the root (Harrison, 1997). This improvement in phosphate acquisition is obtained by increasing the volume of soil that is explored for nutrients (due to the large surface area of the hyphae), the fungi's capacity to store polyphosphates, and its ability to acquire Pi from organic sources that are not available directly to the plant (Smith et al., 2003; Abel et al., 2002; Schachtman et al., 1998). Another possible strategy for acquiring Pi through mycorrhiza has been hypothesized based on transcript levels of a phosphatase increasing with mycorrhizal colonization. The fungi may activate part of the plant system to increase phosphatase secretion, thus improving the overall Pi uptake (Ezawa et al., 2005). Members of the Brassicaceae, which include *Arabidopsis*, however do not form these arbuscular mycorrhizal associations (Harrison, 1997). This is important since the use of *Arabidopsis* as a model system allows for examination of the plant's own mechanisms for obtaining and translocating phosphorus.

BIOCHEMICAL RESPONSES OF PLANTS TO PHOSPHATE STARVATION

Biochemical responses of plants to phosphate starvation are designed to maintain a constant cytoplasmic Pi concentration regardless of external or vacuolar Pi levels (Schachtman et al., 1998). Biochemical responses can be separated into two main categories: 1) responses designed to increase Pi availability or acquisition, and 2) metabolic adaptations to decrease Pi requirements.

Biochemical responses of plants to increase phosphate acquisition or availability

The plants biochemical responses to Pi starvation that are designed to increase Pi availability or acquisition fall into three basic groups: 1) Recycling and movement of Pi pools currently in the plant, 2) Increased uptake and transport of available Pi, and 3) Processes designed to increase the solubilization and thus mobilization of external Pi sources.

Recycling and movement of phosphate pools

Remobilization of Pi from older leaves and vacuolar stores is triggered by Pi starvation. Some RNases were also induced by Pi starvation. The RNases are thought to be active in nutrient remobilization - possibly scavenging phosphate from RNA in the environment (Dodds et al., 1996; Bariola et al., 1994). Some Pi starvation-inducible RNases are excreted well before intracellular Pi concentrations drop when grown in Pi deficient media. The RNases induced under these circumstances are thought to be part of an internal Pi starvation rescue system that may function to liberate Pi from RNA and facilitate its recycling and remobilization from older leaves to growing shoots and from the shoots to the root system (Bariola et al., 1994; Raghothama, 1999). Plants under phosphate starvation conditions also have the ability to synthesize de novo phosphatases that may be used to mobilize internal reserves as well as to release phosphate from organic sources outside the plant (Duff et al., 1991). These intracellular Pi starvation-induced phosphatases function mainly to help recycle Pi from senescing tissue and in alternative metabolic pathways designed to bypass Pi requiring steps or recycle Pi in plant C metabolism (Duff et al., 1989; Duff et al., 1994, Vance et al., 2003).

Transport between intracellular compartments, as well as in and out of the vacuole, also plays an essential role in maintaining the appropriate cytoplasmic Pi concentration in the plant through the movement of Pi pools (Versaw and Harrison, 2002; Schachtman et al 1998). It is generally assumed that low affinity Pi transporters are responsible for the mobilization of Pi within the plant but expression of some high

affinity Pi transporters in areas other than roots may indicate a role in the internal translocation / mobilization of Pi under Pi starvation conditions as well (Karthikeyan, et al., 2002). Also, the phospholipid content of the thylakoid and extraplastidic membranes are reduced under Pi starvation and the phospholipids replaced with non-phosphorous galactolipids and sulfolipids (Ticconi and Abel, 2004; Abel et al 2002).

Uptake and transport of available phosphate

Pi starvation also triggers biochemical responses that increase the uptake and transport of available Pi through the enhanced expression of high affinity Pi transporters. High affinity Pi transporters are membrane-associated proteins that transport Pi from the external environment to the cytoplasm (Raghothama, 1999). While not much is known about the low affinity Pi transport system, it appears to be constitutively expressed in plants, whereas the high affinity Pi transport system is regulated by Pi availability (Raghothama, 1999; Muchhal and Raghothama , 1999; Karthikeyan, et al., 2002).   The transcripts for high affinity Pi transporters in plants are predominantly expressed in the roots and are strongly induced under Pi starvation conditions (Muchhal and Raghothama, 1999; Karthikeyan, et al., 2002). This increased expression of the high affinity transporters under limited Pi is followed by increased number of transport sites and uptake of Pi from the surrounding area (Bariola et al., 1994; Rausch and Bucher, 2002). Thus, it appears that the primary role of high affinity transporters is to acquire Pi from the surrounding area. However, high affinity Pi transporters expressed in other areas of the plant may indicate an additional role in internal translocation / mobilization of Pi under Pi starvation conditions (Karthikeyan, et al., 2002).

Increased solubilization of external phosphate sources

Many different processes designed to increase the solubilization and mobilization of external Pi sources are induced upon Pi starvation. Of the two main pools of phosphorus found in the soil, the organic form generally makes up 20-80% while the

remaining phosphorus is mineralized. While most of these are immobile forms, or otherwise unavailable to the plant in their current state, plants have devised means by which to release some of the phosphate into forms available to the plant (Schachtman et al., 1998).

To mobilize Pi from the external inorganic or mineralized pools in the rhizosphere, plants synthesize and secrete organic acids. The roots of Pi deficient plants exude large amounts of these organic acids - especially citric acid and malic acid. These acids are used by the plant to solubilize Pi from phosphate rock when present. The excretion zone is very specific and targeted to the area of the root in direct contact with phosphate rock (Raghothama, 1999). The release of these organic acids (citric acid, malic acid, and piscidic acid) are also used by plants to chelate Fe^{3+}, Al^{3+}, and Ca^{2+} cations in the soil which then increases the mobilization of Pi from precipitates or other complexes in both acidic and alkaline soils (Vance et al., 2003; Abel et al., 2002; Ticonni and Abel 2004; Raghothama, 1999).   This chelation of the cations by organic acids may also increase the susceptibility of organic phosphates to hydrolysis by acid phosphatases (Vance et al., 2003).

To mobilize Pi from the external organic pool in the surrounding area, phosphohydrolases are induced and secreted. Nucleolytic isoenzymes and other phosphoesterases are induced in both tomato cell cultures and *Arabidopsis* under phosphate starvation (Ticonni and Abel, 2004; Chen et al., 2000). Wild type *Arabidopsis* plants are able to grow on media containing RNA or DNA as the only source of phosphate, while mutants deficient in these Pi starvation-inducible enzymes required Pi supplementation. Under Pi starvation conditions, wild type *Arabidopsis* plants metabolize exogenous DNA and RNA as a phosphate source when grown in liquid media (Ticonni and Abel, 2004; Chen et al., 2000). Tomato cell cultures starved for Pi also secreted extracellular acid phosphatases and other phosphodiesterases that aided in degradation of extracellular RNA substrates and recycling of Pi. (Chen et al., 2000). These results indicate roles for RNases, other nucleases, and phosphoesterases in

mobilization of external phosphorus sources from nucleic acids in addition to internal recycling.

Phosphatases are also used by plants to mobilize Pi from external pools of phosphorus. Phosphatases are a class of enzymes that function to hydrolyze phosphate from orthophosphate monoesters (Duff et al., 1994). Phosphatases are generally classified as either alkaline phosphatases or acid phosphatases based on whether their optimal pH for catalysis is above or below 7.0. Alkaline phosphatases usually show an absolute substrate specificity while most acid phosphatases exhibit little, if any, specificity for substrates (Duff et al.,1994). Some acid phosphatases (APases) do however show clear substrate specificity, and these are thought to have distinct metabolic functions in the plant (Duff et al., 1989; Duff et al., 1994). Many APases are found intracellularly in all areas of the plant including dormant seed, developing seeds, germinating seeds, leaves, stems, roots, storage tubers, flowers, and cultured cells (Duff et al., 1994). Others are extracellular APases and may be localized to the cell wall and/or secreted by the root or suspension cell into the surrounding rhizosphere or media (Duff et al., 1994; Lefebvre et al., 1990). These extracellular root APases are much more stable than the intracellular forms and are more likely to aid in acquiring Pi from the soil (Vance et al., 2003).

Plants under phosphate starvation conditions have the ability to synthesize de novo phosphatases that may be used to either mobilize internal reserves or release phosphate from organic sources outside the plant (Duff et al., 1991). Some of these induced phosphatases are secreted (Dracup et al., 1984; Goldstein et al., 1988; Lefebvre et al., 1990; LeBansky et al., 1992; Haran et al., 2000). *Brassica* suspension cells showed a marked increase in the levels of secreted phosphatase activity upon Pi starvation (Lefebvre et al., 1990). Cultured tobacco cells and tomato suspension cultures secrete APases that are Pi starvation-induced (Duff et al., 1994; Lefebvre et al., 1990). These extracellular / secreted APases have been thought to be involved in hydrolyzing organic phosphates in the soil and mobilizing Pi for the plants (Lefebvre et al., 1990; Duff et al., 1994), especially since an APase would be better suited than an alkaline

phosphatase to the surface of a plant root which is very often acidified (Duff et al., 1994). A number of these phosphatases have now been studied in higher plants including *Arabidopsis* and some have been confirmed to localize to the rhizosphere where they could aid in degradation of soil organophosphates (Chen et al., 2000).

One type of acid phosphatases, the purple acid phosphatases (PAPs), has been isolated from a variety of plant and animal tissues. Purple acid phosphatases (PAPs) are dinuclear metallohydrolases. All plant PAPs contain seven conserved amino acid residues that are involved in coordinating the dimetal nuclear center in their active site. They have a distinctive purple color in solution due to a charge-transfer transition between the metal coordinating tyrosine residue and the Fe (III) ion in the binuclear metal site. In plant PAPs, the other metal ligand in the active site is either zinc or manganese (Olczak et al., 2003; Li et al., 2002).

All known PAPs are N-glycosylated, which is common in plant enzymes that are secreted (Olczak et al., 2003). Also, most known PAPs are soluble, secreted proteins with the exception of the PAP found in *Spirodela oligorhhiza* cell membranes. This PAP is the only one experimentally proven to be glycosyl-phoshatidylinostiol- anchored to the cell membrane (Olczak et al., 2003).

The role of PAPs is not known for certain, but is thought to be similar to that of secreted phosphatases in bacteria and yeast. PAPs may play a role in scavenging forms of phosphate external to the plant such as cleaving of phosphorous from phosphate groups esterified to organic compounds (Delhaize and Randall, 1995; Duff et al., 1991). This role in phosphate acquisition is strongly suggested by both the regulation of some PAPs by Pi levels as well as fact that many PAPs are strongly expressed in the roots and are secreted outside into the extracellular environment of the soil (Olczak et al., 2003). Again as with other phosphatases, some PAPs are localized internally and may play a role in mobilizing internal polyphosphate or phytic acid reserves within the plant or seed (Duff et al., 1991). Since PAPs have been isolated from seeds (Klabunde et al., 1994) they may also be involved in movement of internal stores.

METABOLIC ADAPTATIONS TO DECREASE PLANT Pi REQUIREMENTS

Primary metabolic adaptations

Plants also respond to phosphate starvation by metabolic adaptations designed to decrease Pi requirements. These adaptations affect both primary and secondary metabolism. Some well-documented changes occur in plants undergoing phosphate starvation, which induce alternative pathways in the plant's primary metabolism during glycolysis. These alternative pathways allow the plant to bypass the ATP and phosphate requiring metabolic steps in respiration since Pi is already very limited and concentrations drop under prolonged Pi stress   (Theodorou and Plaxton, 1993). While Pi and adenylate concentrations in the plant decrease upon phosphate starvation, the level of pyrophosphate (PPi) in plant cells does not appear to be affected (Theodorou and Plaxton, 1993). This allows for PPi to serve as an energy donor in certain cases in the plant cytosol (Theodorou and Plaxton, 1993; Plaxton 1996; Vance et al., 2003). One of the well-documented alternative pathways is catalyzed by a PPi dependent phosphofructokinase (PFP) that can bypass the ATP-dependent phosphofructokinase (PFK) to produce fructose 1,6-biphosphate (Vance et al., 2003; Theodorou and Plaxton, 1993; Theodorou and Plaxton, 1996). Other processes might use PPi as a phosphate source are the PPi-dependent sucrose synthase pathway, conversion of UDP glucose to UTP, and the active transport of protons in to vacuoles by a PPi-dependent H$^+$pump in the tonoplasts (Vance et al., 2003; Theodorou and Plaxton, 1993).

Another alternative pathway that bypasses Pi-dependent steps is the non-phosphorylating NADP-dependent glyceraldehyde-3P dehydrogenase (NADP-G3PDH) that bypasses Pi- dependent NAD-G3PDH and phosphoglycerate kinase (Theodorou and Plaxton, 1993; Vance et al., 2003). A third bypass can be catalyzed by combined effort of phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), and NAD-malic acid, or by PEP phosphatase alone, to bypass the use of pyruvate kinase (PK), an enzyme that require both Pi and ADP in converting PEP into pyruvate (Theodorou and Plaxton, 1993). These steps are essential to maintain pathways that can continue to generate the energy needed by the plant to continue to function, and to produce and

excrete large amounts of organic acids under these conditions. While these bypass steps avoid the use of Pi or phosphate compounds, some of them (PFP, PEP phosphate, and PEPC) also fill an additional role in recycling Pi (Theodorou and Plaxton, 1993).

Finally, the electron transport system itself is also dependent on ADP and Pi under normal conditions. In Pi starved plants this is thought to inhibit the cytochrome (Cyt) pathway of electron transport. There are two nonphosphorylative pathways that can bypass energy-requiring steps in electron transport. These are the rotenone-insensitive NADH dehydrogenase and the alternative oxidase (AOX) (Vance et al., 2003). The cyanide - resistant AOX catalyses the oxidation of ubiquinol and the reduction of oxygen to water while bypassing the last two energy producing sites in the Cyt pathway. AOX respiration is induced during P starvation in a number of plants, however it appears that in some plants the inhibition of the CN-sensitive cytochrome pathway of respiration may not be completely compensated by the AOX activity (Vance et al., 3002).

Secondary metabolic adaptations

Increase in secondary metabolism production is a common response of plants that are nutrient limited. Pi-deficient plants typically accumulate secondary metabolites such as flavonoids, and indole alkaloids. There is also generally an increase in the synthesis of anthocyanins in response to Pi limitation, which is thought to alleviate some of the photoinhibitory damage to chloroplasts (Vance et al., 2003). Enzymes in the phenolic and flavonoid pathways are upregulated in some Pi starved plants. Phenolic compounds can be exuded into the rhizosphere in response to Pi starvation, and may act to increase Pi availability by acting as chelators or reductants (Vance et al., 2003). Examples of these are piscidic acid, a phenolic from pigeon pea roots, alfuran from alfalfa, and isoflavanoids from white lupin (Raghothama, 1999; Vance et al., 2003).

Secondary metabolism generally does not use as much Pi as primary metabolism, but it can be important in helping to recycle significant amounts of Pi from phosphate esters. Secondary metabolism however can generate a large excess of reducing

compounds. This resulting cytoplasmic acidification could lead to the activation of the AOX and other pathways that can reduce this accumulation of reducing compounds, as well as aid in releasing protons into the rhizosphere thus acidifying it and again aiding in Pi uptake (Vance et al., 2003).

Pi STARVATION REGULATED GENE EXPRESSION IN PLANTS

Responses to Pi limitation or starvation illicits a coordinated activation of responses in plants. This coordination of responses to Pi limitation leads to the idea that there may be a Pi starvation-inducible rescue system in plants, a PHO regulon, like is found in microorganisms. This idea of a Pi regulatory system was first proposed by Goldstein et al., (1988). However, the lack of yeast PHO regulatory genes homologs in *Arabidopsis* and the scarcity of successful complementation of yeast *pho* mutants with plant cDNAs suggests that the regulatory system controlling Pi starvation in higher plants is different and more complex (Ticconi and Abel, 2004; Franco-Zorrilla et al., 2004).

Studies based on changes in gene expression during Pi starvation generally involved expression analysis of genes presumed to play a role in Pi starvation responses. These RNases, phosphatases, high affinity Pi transporters and other genes isolated in mutant screenings, and Pi starvation-induced promoter**::**reporter gene fusions often show responses that indicate they are controlled transcriptionally (Franco-Zorrilla et al., 2004; Ticconi and Abel, 2004). Recent studies that have analyzed transcriptional changes in *Arabidopsis* shoots have indicated that there are at least two major response categories in terms of transcriptional regulation. The first category is a transient set of responses that occur during the early stages of Pi stress (Franco-Zorrilla et al., 2004; Hammond et al., 2003). These "early response" genes include many genes with a less specific or general stress response. (Franco-Zorrilla et al., 2004; Hammond et al., 2003). Also, the promoters of these "early response" genes were more likely to contain two sequence motifs: the PHO-like (CDHGTGG; D: G, T, or A; H: C, T, or A) and the TATA box like (TATAAATA) elements. These early response elements are different from the *cis*

element found in many of the "late response" genes (Franco-Zorrilla et al., 2004; Hammond et al., 2003).

The second category of genes is most highly induced during the later stages of Pi starvation. These genes were termed the " late response" genes and included genes thought to be specific in their Pi starvation responses. Many of the "late response" genes whose expression increases during Pi starvation share a common *cis* regulatory element, the GNATATNC motif, in their promoter region (Franco-Zorrilla et al., 2004; Hammond et al., 2003). This element is recognized by the MYB transcription factor, Phosphate Starvation Response 1 (PHR1), which is localized to the nucleus regardless of Pi status (Rubio et al., 2001).

In a similar study using an *Arabidopsis thaliana* whole genome Affymetrix gene chip, a total of 80 genes thought to be associated with transcriptional regulation of gene expression had altered expression during Pi starvation. 53 of these genes were up regulated during the course of the studied Pi deprivation. Of these 53 genes, only a few were up regulated during short term Pi starvation, but 47 were induced during long term Pi starvation. This suggests that there are specific groups of transcription factors designed to regulate early and late responses in plants to Pi starvation. This study also found that the " late response" genes were primarily focused on promoting the efficient use of Pi by the plant. Also, most of these genes were specific for either root or shoot, showing that the different plant organs may activate different sets of genes in response to Pi starvation (Misson et al., 2005).

What is known to date about how some of these "late response" genes interact with known transcription factors, as well as other essential components in the Pi starvation pathway can be summarized in Figure 1.1. These interactions will be discussed in the following section.
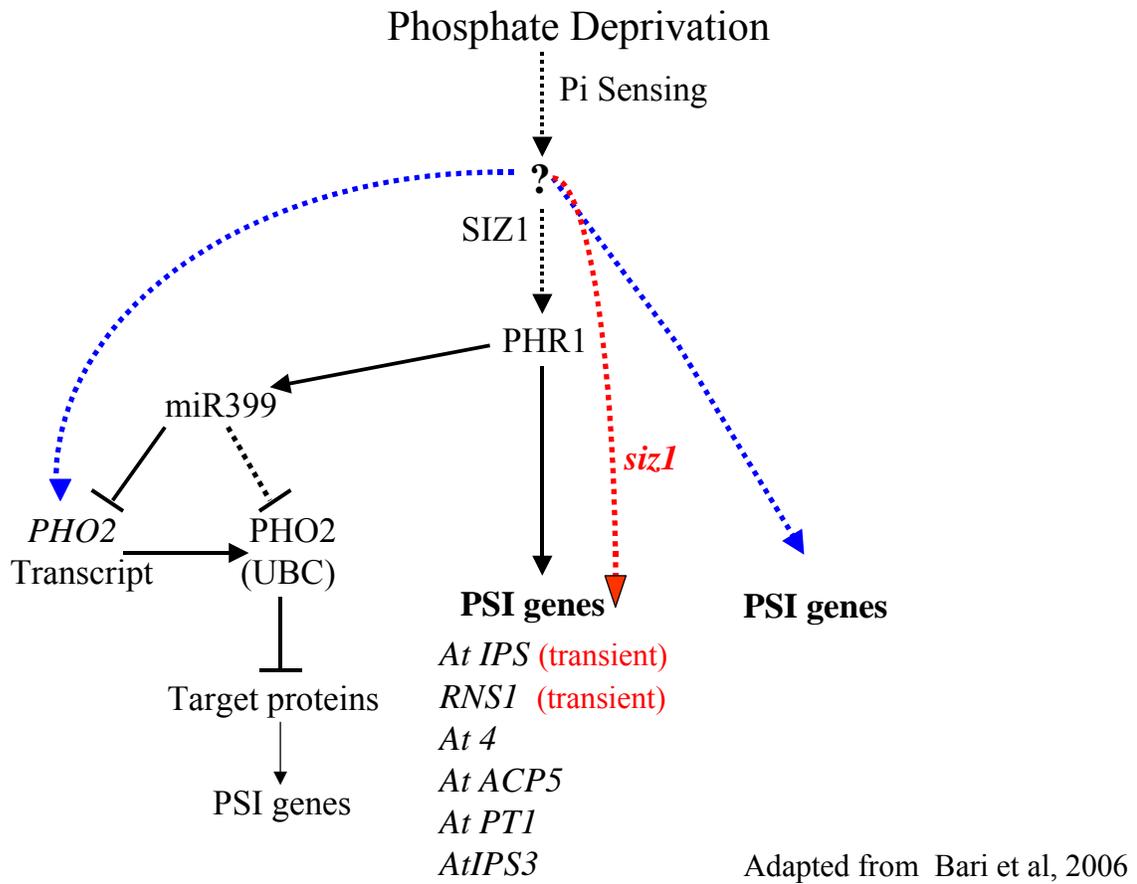
Figure 1.1. Some known components and their relationship in the Pi starvation pathway.

Role of transcription factor PHR1 and its components in plant Pi starvation responses

Transcriptionally controlled expression of genes is due to the interaction of nuclear-localized transcription factors with the *cis* elements of a gene. One transcription factor, *PHR1* appears to be a main transcriptional activator for " late response" genes of the Pi starvation response in *Arabidopsis* (Rubio et al., 2001). In addition to this known transcription factor, there are a number of other components that have been found or are suggested to be involved in the control of some Pi responsive genes both in the form of positive and negative regulation. PHR1 binds as a dimer to the GNATATNC *cis* regulatory element in the promoters of many Pi starvation-induced genes. *phr1* mutants have reduced Pi contents and decreased plant growth under Pi starvation conditions. When six Pi starvation-induced genes were examined on the mRNA level, all six were reduced (Figure 1.1), but the effect was most marked for *RNS1*, *At4,* and *AtIPS1* (Rubio et al., 2001). Since PHR1 protein is localized in the nucleus regardless of Pi conditions, it was suggested that *PHR1* is involved in the regulation of plant Pi status under all Pi conditions. It was also suspected that the protein itself was either post-transcriptionally regulated, or that an additional Pi starvation regulatory protein was required for signaling a Pi starvation response (Rubio et al., 2001).

Another study showed that the transcriptional activator PHR1 is modified by a small ubiquitin-like modifier (SUMO) E3 ligase AtSIZ1. SUMO E3 ligases transfer the SUMO peptide to a substrate and result in increasing the affinity of the conjugating enzyme (E2) subunit for a specific target. This conjugation of the SUMO protein to the substrate affects genes being regulated both positively and negatively by changing protein-protein and protein-DNA interactions and intracellular targeting, as well as controlling the ubiquitination and other covalent modifications of the proteins. (Miura et al., 2005). AtSIZ1 was found to function as a SUMO E3 ligase *in vitro.* In *siz1* mutants, AtSIZ1 appears to functions differently in Pi starvation signaling for two different subsets of genes. *AtPT2*, *AtPS2,* and *AtPS3* are negatively regulated by *AtSIZ1* in Pi sufficient conditions, while *AtIPS1* and *AtRNS1* are positively regulated by *AtSIZ1*

during the initial stages of Pi starvation (Miura et al., 2005). Since *AtIPS1* and *AtRNS1* are known to be positively regulated by *PHR1*, and this transcription factor contains two predicted sumoylation sites, these results indicate that sumoylation of PHR1 positively controls the expression of *AtIPS1* and *AtRNS1*. These data indicate the importance of AtSIZ1 in the regulation of Pi starvation responses in plants by acting upstream of PHR1 as shown in Figure 1.1 (Miura et al., 2005).

Other research has found a microRNA (miRNA), miR399, that is induced by low Pi, but not by low K or low N (Fujii et al., 2005). This miRNA has multiple target sites in the 5' UTR of a gene that encodes for a putative ubiquitin-conjugating enzyme (UBC). Northern analysis showed that the levels of UBC mRNA were reduced under low Pi. Transgenic *Arabidopsis* constitutively expressing this miR399 accumulated more Pi than wild-type *Arabidopsis* (Fujii et al., 2005). After analyzing other Pi starvation-induced genes in these over-expressing miR399 lines, *AtPT1* mRNA was found to be slightly higher than in wild-type plants under normal Pi conditions. Also, those with only the ORF of the UBC (deltaUTR) behind the 35S promoter showed less *AtPT1* mRNA than either the transgenic line containing the 5' UTR and ORF of UBC (with UTR) behind the 35S or wild-type under low Pi conditions (Fujii et al., 2005). No substantial difference was noticed in any other mRNA for other Pi genes tested from these plants using Northern Blot analysis (*AtPT2, RNS1, IPS1*, and *AT4*) but there was a difference in the elongation of primary roots for the deregulated deltaUTR UBC plants. These showed a greater elongation of primary roots than the wild-type when placed on low Pi plates, indicating that the miRNA deregulation altered one of the plant's phenotypic responses to low Pi (Fujii et al., 2005). Taken together, these results show that miR399 functions in the acquisition of Pi, but only affects a certain subset of genes including at least one known Pi transporter.

A related study showed that miR399 accumulation was followed by a decrease in the same putative ubiquitin-conjugating E2 enzyme examined by Fujii et al.(2005) now designated AtUBC24. This E2 enzyme functions in the ubiquitin-dependent protein degradation pathway (Chiou et al., 2006). Upon Pi starvation, miR399 mRNA was

upregulated and was accompanied by a decrease in E2. The idea that this decrease in E2 transcript might be from the direct cleavage of this transcript by miR399 was supported by the fact that cleaved transcripts of E2 were found corresponding to the predicted cleavage target sequences in the 5' UTR. This reduction in E2 levels was followed by an increased uptake of Pi from the soil, thus enabling the plant to cope with Pi limitation. Overexpressing miR399 lines also exhibited greater uptake of ^{33}P from the soil and more allocation of ^{33}P from roots to shoots resulting in a higher shoot-to-root ratio. Overexpression of miR399 limits the remobilization of Pi from older to younger leaves indicating that the interaction of miR399 with E2 is essential to proper Pi plant homeostasis (Chiou et al., 2006).

Another recent study showed by using map-based cloning, that the E2 conjugase that is modulated by miR399 is actually *PHO2* (Figure 1.1). Prior to this study the molecular function of *PHO2* was unknown (Bari et al., 2006). The *pho2* mutant has increased levels of Pi in the leaves of 2 to 4 fold, but is unaltered in Pi content of the roots (Delhaize and Randall, 1995). Sequencing of the clones obtained showed that *pho2* mutants contain a stop codon that deletes the UBC domain of the enzyme inactivating the E2 conjugase. Even though *PHO2* transcripts decrease approximately 8-fold in the early phases of Pi starvation as miR399 transcript levels are increasing, these *PHO2* transcripts remain fairly high and stable in Pi deficient plants even when miR399 levels are high. In addition, the *PHO2* transcript levels decreased after the addition of Pi, in spite of the fact that miR399 transcript also decreased. These results suggest that there is another mechanism for *PHO2* to respond to Pi (Figure 1.1) that is not related to miR399-directed transcript cleavage (Bari et al., 2006). Two Pi starvation-induced genes that were downregulated in the *phr1* mutant (*AtIPS1* and *At4*) were upregulated in the *pho2* mutant under Pi sufficient conditions. This suggests that both PHR1 and PHO2 share some common downstream targets (Bari et al., 2006). Additionally, the information that the Pi starvation-induced miR399 transcripts are substantially less abundant in *phr1* mutants than in wild-type but are not affected in *pho2* mutants, indicates that PHR1 is required for miR399 expression and places PHR1 upstream of

PHO2 in Pi signaling (Figure 1.1). Finally, only a small portion of the Pi starvation-induced genes that were reduced in the *phr1* mutant were reduced in the *pho2* mutant, however all but one of these genes affected in *pho2* were also affected in *phr1*. This information again suggests that PHO2 functions in a branch of the Pi starvation signal pathway downstream of PHR1 (Bari et al., 2006).

Possible role of HD-ZIP proteins and repressors in plant Pi starvation responses

Homeobox-leucine zipper domain (HD-ZIP) proteins and other DNA binding proteins may play a role in the transcriptional control of Pi starvation responses. Based on results obtained from promoter-dissection experiments from the vegetative storage protein B in soybean, it appears that HD-ZIP proteins may be involved in the transcriptional control of some Pi starvation response genes (Franco-Zorilla et al., 2004). A 50 bp fragment lacking the PHR1 binding site but containing two motifs found in soybean and pea was able to confer Pi starvation responses to a CaMV*35S* promoter. One of these two motifs in the promoter was recognized by two soybean HD-ZIP proteins during southwestern experiments, suggesting that HD-ZIP transcription factors may function in Pi starvation responses (Franco-Zorilla et al., 2004).

Also, in experiments using DNA mobility shift assays to examine the interactions of certain Pi starvation-induced promoters (*AtPT1* and *TPSI1*) with any nuclear protein factors, two regions of the *AtPT1* and *TPSI1* promoters bound nuclear protein factors under Pi sufficient conditions. However, this DNA binding activity was lacking in extracts from plants grown in Pi starvation conditions. These results indicate that some Pi starvation-induced genes may be under negative regulation (Mukatira et al., 2001).

Pi signaling

There is not much known about the molecular mechanisms of Pi sensing in plants. However, these mechanisms appear to be much more complex than those found in microorganisms. This is probably due to the fact that not only must the cell itself

maintain the appropriate Pi homeostasis, but also there is a need to communicate information about this status to other parts of the plant in order to coordinate an integrated response to the Pi status on the whole plant level. (Franco-Zorilla et al., 2004). While some Pi starvation responses depend on local Pi status, such as root hair number and length, the majority of the Pi starvation responses are controlled at the whole plant level based on long-distance signaling (Franco-Zorilla et al., 2004).

The idea of long distance signaling regulating Pi responses has been suggested based on split root experiments in *M. truncatula* plants. In these experiments, roots of Pi starved plants were separated into two groups. One group of roots was placed in a high Pi liquid medium while the other remained in a low Pi liquid medium. The Pi starvation responses examined for the *Mt4* gene were systemically suppressed in the roots that were left in the low Pi medium. However, the roots in low Pi did not accumulate high levels of Pi even though there were high levels of Pi in the roots exposed to high-Pi. This suggests that Pi accumulation was not responsible for the systemic down regulation of the *Mt4* gene (Burleigh and Harrison, 1999). In the *Arabidopsis pho1* mutant, the ability to load Pi into the xylem and consequently to translocate the Pi to the shoot is impaired (Poirier et al., 1991). When examining the down regulation of *Mt4*-like genes in *Arabidopsis*, the transcripts that were down-regulated in wild-type plants under high Pi conditions remained at high levels in the *pho1* mutants. This indicates that the Pi status of the shoot controls the Pi starvation responses in the root and that translocation of Pi to the shoot is required to prevent this response (Burleigh and Harrison, 1999).

Effect of hormones on Pi starvation responses

Cytokinins may also be involved in the systemic signaling system since they are able to repress some long-distance controlled Pi responses but not the external controlled Pi responses (Martin et al., 2000). In Pi starved plants, the cytokinin concentration is reduced which could also increase the root-to-shoot growth ratio as well as affect lateral root growth (Franco-Zorilla et al., 2004). In *Arabidopsis* the addition of exogenous cytokinins repress the expression of several Pi starvation-induced genes (*AtPT1*, *ACP5*,

AtIPS1 and *At4*), especially in roots under Pi limited conditions. Cytokinins did not, however, repress responses that are dependent on local Pi concentrations, but rather they repressed those dependant on the whole-plant Pi status (Martin et al., 2002). The Pi starvation-induced genes that these cytokinins repressed were all known to contain the PHR1 binding site indicating that these hormones may play an inhibitory role in the Pi starvation response pathway. Finally, exogenous addition of cytokinins to plants grown on high Pi, inhibit lateral root formation and thus decrease lateral root density (Lopez-Bucio et al., 2002).

These and other results indicate that hormones play a major role in the Pi starvation-induced changes in root development. With respect to other hormones, Pi starvation results in alteration of plant responsiveness to ethlyene and auxin in the root (Lopez-Bucio et al., 2002; Franco-Zorilla et a, 2004). Ethylene decreases root elongation in Pi sufficient plants and increases root elongation in Pi starved plants. Thus ethylene does not promote lateral root formation in plants under Pi starvation (Franco-Zorilla et al., 2004). There has been substantial evidence supporting the role of auxin in lateral root development (Vance et al., 2003). Treatment of P_i sufficient plants with auxin inhibited primary root growth, induced the formation of lateral roots, and increased the later root density (Lopez-Bucio et al., 2002). Also, *Arabidopsis* has an increased sensitivity to auxin under Pi starvation. This might explain the increase of lateral roots observed in response to Pi starvation (Lopez-Bucio et al., 2002). Low Pi may modify the local auxin concentrations in the root through changes in auxin transport instead of auxin synthesis (Nacry et al., 2005). These results indicate that hormones play a major role in the Pi starvation-induced changes in root development.

The role of *PAP1* in plant Pi starvation responses

Our lab has cloned and sequenced what appears to be the full-length cDNA and corresponding genomic sequence encoding a PAP (*PAP1*), At2g27190, from wild type *Arabidopsis*. *PAP1* steady state levels of mRNA increased in response to phosphate

starvation. Even at 6 hours in a phosphate-deficient environment, mRNA levels increased by two fold. After 24 hours, mRNA levels increased by ten fold. Starvation for longer periods of time did not increase the levels of mRNA produced. Since the *PAP1* gene was isolated from a flower cDNA library and then found to be expressed in all organs examined, it is possible that it plays multiple roles in plant phosphate nutrition metabolism.

This *PAP1* gene contains a putative PHR1 binding site of GAATATCC in the promoter located -159bp from the ATG start codon. As mentioned previously, PHR1 binds as a dimer to the GNATATNC *cis* regulatory element in the promoters of many Pi starvation-induced genes (Rubio et al., 2001). This indicates that this gene may be downstream of *PHR1* in the Pi starvation-induced pathway. The expression of the *PAP1* gene was studied in transgenic *Arabidopsis* using constructs containing the *PAP1* promoter fused to the *GUS* reporter gene. The specific aims of this study were to: 1) Identify and characterize mutations in the phosphate starvation signal transduction pathway using this construct and 2) map any mutant genes found to their locations in the *Arabidopsis* genome. The information obtained from this thesis study will help to understand the role of *PAP1* in the Pi starvation signal transduction pathway.

CHAPTER II

ISOLATION AND CHARACTERIZATION OF *BT1* AND *BT2*

INTRODUCTION

As mentioned in the general introduction, members of the Brassicaceae, which includes *Arabidopsis*, do not form arbuscular mycorrhizal associations (Harrison, 1997). The use of *Arabidopsis* as a model system consequently allows for examination of the plant's own mechanisms for obtaining and translocating phosphorus.

A number of genes have been identified in *Arabidopsis* that are Pi starvation-responsive. Some of these Pi starvation-responsive genes are: 1) *RNS1*, an RNase that was determined to be sharply induced during Pi starvation (Bariola et al., 1994), 2) *At4*, an *Mt4*-like gene expressed in *Arabidopsis* roots is also Pi starvation-induced (Burleigh and Harrison, 1999; Shin et al., 2006), 3) *AtIPS1*, another member of the *TPSI1/Mt4* family specifically induced by Pi starvation in all cells of wild-type plants (Martin et al., 2000), 4) *AtACP5*, a type 5 acid phosphatase responsive to Pi starvation as well as some other conditions (del Pozo et al.,1999) , 5) *AtPT1* and *AtPT2*, high affinity phosphate transporters expressed mainly in roots are induced by low Pi (Muchhal et al., 1996) in addition to other Pi transporters (Shin et al., 2004), and 6) *PAP1*, which encodes a purple acid phosphatase (Patel et al., 1997) that was cloned and sequenced in our lab.

One way to further our understanding of any pathway is through analysis of mutants. Mutational analysis is a tool that allows, through the examination of a mutant phenotype, an understanding of the normal functioning of the nonmutant or wild-type from of that gene (Redi et al., 1992; Feldmann et al., 1994; Malmberg, 1993). Through the use of mutagenic agents, thousands of mutants have been obtained. There are now a number of mutants that have been found in *Arabidopsis* that involve phosphate-induced responses. Two mutants in *Arabidopsis* that are of particular importance in terms of phosphate metabolism are *pho1* and *pho2*. *pho1* is a mutant that is always starved for phosphate because it cannot load phosphate from the roots into the xylem of the plant, and thus the aerial portions of the plant are always starving for Pi (Poirier et al.,1991).

pho2 mutants accumulate large excesses of phosphorous in the leaves, to the point of toxicity, when the plants are grown under high transpiration conditions. Under normal growth conditions, *pho2* still accumulates up to 3 times more phosphorus than wild type (Delhaize and Randall, 1995). These mutants were both found in *Arabidopsis* in the Columbia ecotype.

Since one of our main goals was to elucidate part of the phosphate starvation signal transduction pathway, we chose to do this through mutant analysis. We chose to study this pathway by observing the expression of the *PAP1* gene. This gene, as mentioned in the introduction, was found in flower, root and leaf cDNA libraries indicating that it may play multiple roles in phosphate nutritional metabolism. Through the identification and characterization of mutant expression of this *PAP1* gene, we would obtain additional information about the phosphate starvation signal transduction pathway.

The expression of this gene was studied in transgenic *Arabidopsis* using constructs containing the *PAP1* promoter fused to the *GUS* reporter gene and then the *PAP1* terminator (*PAP1::GUS::PAP1*). Plant lines containing the *PAP1::GUS::PAP1* construct were screened for using the GUS histochemical assays. These plants were allowed to self until lines homozygous for the transgene were obtained. These lines were then subjected to mutagenesis using EMS (ethylmethane sulfonate). EMS was chosen because it has a high rate of mutation compared to toxicity. Also, since it is an alkylating agent, it produces a high rate of point muations and substitutes G**:**C and A**:**T in human cell lines (Feldmann et al., 1994).

After EMS mutagenesis, the treated seeds were planted. The M_2 seeds from individual M_1 lines were screened for mutants again using the GUS histochemical assays. A mutant screen was devised to look for any plant that showed an abnormal *PAP1* response to phosphate nutrition. In wild-type plants, the *PAP1* gene is turned off under normal phosphate conditions, and thus the GUS gene would not be activated. Plants expressing the GUS gene in the presence of normal phosphate could indicate a mutation in the phosphate starvation signal transduction pathway either in phosphate

sensing or elsewhere in the signal transduction pathway. Histochemical analysis revealed mutant staining patterns and eventually two mutant lines were isolated.

If mutations are linked to the T-DNA, the plant DNA adjacent to the T-DNA insertion in the mutant line can be analyzed to map the mutation. A number of techniques can be employed to isolate this adjacent DNA: 1) plasmid rescue, using the selectable markers in the T-DNA or other border sequences to isolate the plant-T-DNA junctions (Feldmann et al., 1994); 2) inverse polymerase chain reaction (Inverse PCR) using primers made from the left or right border (Feldmann et al., 1994); 3) adaptor ligation PCR using left and right border primers as well as primers for special adaptors ligated to digested genomic DNA (Siebert et al., 1995; Spertini et al., 1999; Padegimas and Reichert, 1998);   4) thermal asymmetric interlaced polymerase chain reaction (TAIL- PCR) using primers from both the left or right border as well as arbitrary degenerate primers (Liu and Whittier, 1995; Liu et al., 1995). We tried all four methods but were unable to identify the location of the transgene insertion site in either our parental or mutant lines, and thus could not map using these methods. This may be due to what appears to be an insertion of two copies of the transgene construct in the form of a tandem repeat in the initial transformant.

In this chapter, the isolation and characterization of these two mutant lines is described. Upon analysis of these lines, *BT1* and *BT2*, showed varying Pi starvation responses compared to wild-type, to the initial transgenic *PAP1::GUS::PAP1* line, and to each other. These mutants were dominant mutations that are linked to the transgene. However, based on the differential expression of starvation-induced genes found in these two mutants and the analysis of an allelism test, it appears that while *BT1* and *BT2* are tightly linked, they are different mutations in different genes. Finally, using the differential expression found in these two mutant lines, we are able to elucidate more about the order of some of the genes involved in the Pi starvation-induced signal transduction pathway.

MATERIALS AND METHODS

Plant materials

     Arabidopsis (Columbia) plants were vernalized overnight and then grown in soil in growth chambers under 16h light/ 8 h dark and 75 % RH at 21 ^{o}C in the day and 18 ^{o}C at night. GUS histochemical assays were done on plants grown on MS media (Murashige and Skoog, 1962) containing 1% sucrose, and 0.8% agarose (or 0.25% phytogel) at 25 ^{o}C under a 24 hr light cycle. Plants were grown for analysis of root growth on different levels of phosphate media on MS media containing 1% sucrose, and 0.8% agarose with various phosphate contents at 25 ^{o}C under a 24 hr light cycle. Plants grown for northern analysis were grown in +P MS liquid media (1.25mM phosphate) for10-12 days. Next the plants were transferred to new +P MS liquid media for 5 days and finally transferred again to either new +P MS liquid media or -P MS liquid media (0mM phosphate) for 3 days at 25 ^{o}C under a 24 hr light cycle.

DNA construction and *Arabidopsis* transformation

     540 bp of the *PAP1* promoter fragment was isolated from a pGEM plasmid that had been previously constructed in the lab. This promoter fragment had been altered at the end of the *PAP1* promoter to create an Nco I restriction site around the ATG start codon. This promoter sequence was transferred from the resulting plasmid (as Sal I fragments) into the pBSG 19 vector. It was later transferred again (as Sal I - Nco I fragments) into the pNco-*GUS* vector (Burnett et al., 1993) that had 500 bp of *PAP1* terminator fragment (ligated as Sac I - Eco RI fragments) replacing the nopaline synthase terminator.   These manipulations resulted in the *PAP1* promoter being transcriptionally fused to the *GUS* reporter gene followed by the *PAP1* terminator.   The resulting *PAP1* promoter **::** *GUS* **::** *PAP1* terminator constructs were then subcloned into the binary vector pBIN 19 and transferred into *Agrobacterium tumefaciens* GV3101. Plants were transformed using a modified protocol from Pamela Green's lab of Agrobacterium-mediated vacuum infiltration (Bechtold and Pelletier, 1998).

Genetic segregation analysis and further selection

Transformed lines containing only one genetically segregating copy of the transgene were selected for. Seeds from the R_0 plants were harvested. These R_1 seeds were surface- sterilized in 15% bleach with 0.25% Tween detergent for 15 minutes, rinsed with sterile water, and plated out on MS media plates containing 40 mg/ml Kan, 400mg/ml Carbenicillin, 1% sucrose, and 0.8% agarose at 25 $^{\circ}$C under a 24 hr light cycle.   Lines that showed genetic ratios of ~3:1 for Kan resistance were selected to obtain lines with one transgenic locus. The resistant plants from these hemizygous lines were transplanted to soil and allowed to self.

R_2 seeds were harvested from individual R_1 plants. These R_2 seeds obtained from the selected transformant lines were vernalized overnight, and then grown on MS media containing 40 mg/ml Kan, 1% sucrose, and 0.8% agarose at 25 $^{\circ}$C under a 24 hr light cycle. Again the lines were selected that showed genetic ratios of either ~ 3:1 (hemizygous) or 100% (homozygous) for Kan resistance. This process was continued into the R_3 seeds until enough homozygous lines were found.

Once lines homozygous for one genetically segregating copy of the transgene were found, they were grown in soil. These soil-grown plants from the transgenic lines were assayed for GUS histochemical activity. The GUS staining patterns were characterized both in soil, in liquid, and later in MS media for reference as controls.

Subsequent selections on the basis of GUS histochemical activity (including the EMS mutagenized lines) were done on MS media containing 1% sucrose, and 0.8% agarose (or 0.25% phytogel). These lines were occasionally plated on Kan plates to verify that the Kan resistance gene was still functional. When necessary, plants were transferred to soil and grown as mentioned above.

Histochemical localization and characterization of GUS activity

Histochemical localization and characterization of GUS activity in plant tissues was determined using a modified protocol (Jefferson et al., 1987). Either the entire plate

was flooded, or individual tissues were incubated with 1mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide acid in either 100mM MOPS buffer or sodium phosphate buffer. After an overnight incubation at 37°C, tissues were either photographed directly or cleared with 70% to 90% ethanol and then photographed. Photographs were either video-captured by Snappy video snapshots (Play Incorporated) using an Olympus SZH10 stereomicroscope with a mounted JVC color video camera KY-F30 (Victor Company of Japan), or captured on a compact flash card (Sandisk) using a Canon EOS Digital Rebel.

EMS mutagenesis and mutant selection

Lines homozygous for one genetically segregating copy of the transgene were now in hand and were subsequently used for mutagenesis. Homozygous lines were chemically mutagenized with EMS using a protocol obtained online from the Meyerowitz lab. These M_0 seeds were sown in soil and grown in growth chambers as previously described.

The M_1 seeds were harvested individually from the M_0 plants.   20 to 30 of the M_1 seeds from each plant were surface sterilized, plated on MS plates and selected on the basis of upregulated GUS activity as compared to the non-mutagenized control line. When a plant line was found that had a minimum of two to four M_1 plants showing abnormal GUS activity, more seeds from that M_0 plant line were plated out and transferred to soil.

These soil-grown M_1 plants from putative mutant lines had leaves removed and tested for abnormal histochemical GUS activity. Any plants showing upregulated GUS activity from this line were allowed to self-pollinate and seeds again collected from the individual plants. 20 to 30 of the seeds from these M_1 plants (showing upregulated GUS activity) were again plated on MS media and selected on the basis of histochemical GUS activity.

M_1 plant lines with M_2 seed showing genetic ratios of $\sim$ 3:1 staining pattern (3 plants showing upregulation of the *GUS* reporter gene: 1 plant showing non-

upregualtion) were selected for during these GUS histochemical assays. Once these lines heterozygous for the mutations were found, additional M_2 seeds from these heterozygous M_1 lines were plated out and transferred to soil in hopes of eventually obtaining a line homozygous for these mutations.

These soil-grown M_2 plants were tested for upregulated GUS activity. Any M2 plants showing the upregulated GUS activity were allowed to self, and again seeds were collected individually.   The M_3 seeds from these M_2 plants were again plated out and selected histochemically for either a heterozygous line showing a 3:1 staining pattern or a line that showed homozygous upregulation of the *GUS* reporter gene. The process was continued as needed until obtaining a number of homozygous candidate mutant lines. Once candidate lines homozygous for the mutations controlling the increased GUS staining had been found, further characterization of these lines was begun for correlation with the GUS data.

Promoter nucleotide sequencing and analysis

The *BT1* and *BT2* transgene promoter sequences were amplified by setting up an automated sequencing reaction using Perkin Elmer ABI Big Dye Reaction Mix (1/4 reaction) in a MJ Research PTC-150 mini-thermocycler. The nucleotide sequences of these reactions were then determined with the dye-terminator cycle sequencing method using an ABI Prism 3100 sequencer machine. Analysis of the sequences was performed with Sequencher software 4.2. The determined sequences were compared against the endogenous wild-type *PAP1* sequences listed in the database using BLAST and no differences were found.

Genetic analysis for dominance and linkage of *BT1* and *BT2* mutants

Both mutant lines were crossed to Columbia wild-type plants to determine the dominance of the traits. Crosses were done using Columbia wild-type plants for the female parent and the mutant line as the male parent (Col ♀ X Mutant ♂). The female

plants were emasculated by removing all anthers prior to dehiscence without damaging the pistil. Then flowers or anthers from the mutant male parent were used to transfer an excess of pollen to the emasculated Columbia wild-type female pistil. The resulting crossed buds were labeled and seeds harvested in approximately 2-3 weeks later.

The F1 seeds from the Col X Mutant crosses were plated out on MS media plates containing 1% sucrose, 0.8% agarose, 40 mg/ml Kan. The seeds were screened for Kan resistance and categorized according to GUS histochemical activity as either wild-type phenotype or mutant phenotype.

Genetic analysis for allelism test of *BT1* and *BT2* mutants

An allelism test for *BT1* and *BT2* mutant lines was performed to determine if they were alleles of the same gene or represented two independent genes (different loci). The two lines were crossed both directions so that each were used as male and female donors (*BT1* ♀ X *BT2* ♂, and *BT2* ♀ X *BT1* ♂). The flowers were emasculated, and then pollinated with the donor male pollen.   The resulting crosses were labeled and seeds were harvested 2-3 weeks later.

The F1 plants from the crosses were grown in soil and allowed to self-pollinate. F2 seeds were collected from the individual F1 plants. These F2 seeds were examined as individual lines for complementation. The seeds were plated on MS containing 1% sucrose, 0.8% agarose, 40 mg/ml Kan, and screened for Kan resistance and categorized according to GUS histochemical activity as either mutant phenotype or wild-type phenotype.

Attempted mapping of *BT1* and *BT2*

BT1 and *BT2* had also been crossed to Landsberg *erecta* ecotype for mapping purposes, but the crosses did not yield enough plants with the mutant phenotype to allow for mapping. Since the mutations were linked to the T-DNA, we attempted to isolate the plant DNA adjacent to the T-DNA insertion for the two mutant lines *BT1* and *BT2* in

order to map the mutations. A number of techniques were employed in hopes of isolating this adjacent DNA. Plasmid rescue was attempted using a modified protocol obtained online by Friedrich J. Behringer and June I. Medford, Department of Biology, Pennsylvania State University. Inverse polymerase chain reaction (Inverse PCR) was attempted using modified protocols (Ochman et al., 1990 and Silver, 1991). Adaptor ligation PCR was attempted using the basic protocol (Siebert et al., 1995). Thermal asymmetric interlaced polymerase chain reaction (TAIL- PCR) was attempted using a combined modified protocol (Liu et al., 1995; Michiels et al., 2003). We tried all four methods in an attempt to identify the location of the transgene insertion in either our parental or mutant lines and thus map the mutation.

mRNA expression analysis

To correlate the GUS data with the endogenous mRNA activity, northern blots were run. RNA was isolated from both roots and leaves of *BT1*, *BT2*, and control line plants grown in both +P MS liquid media and -P MS liquid media using a modified LiCl RNA extraction protocol.   RNA (15µg/lane) was loaded and run on 1.2% RNase-free formaldehyde gels. The gels were rinsed 4X with RNase-free water and then transferred to S&S Nytran N nylon membrane according to the manufacturer's instructions by capillary action overnight. The blots were rinsed and then crosslinked with the autosetting of a UV crosslinker and allowed to dry. Hybridization was performed overnight at 65ºC in hybridization solution (1% BSA, 1mM EDTA, 0.5M $NaHPO_4$ pH 7.2, 7% SDS). Appropriate fragments were α- ^{32}P radiolabeled and used to probe the blots. The blots were washed with 2X SSPE + 0.1%SDS 15-20 min 1 or 2 times, then washed with 0.2XSSPE + 0.1%SDS 20 min 1or 2 times at 65ºC depending on signal strength. The blots were placed on a FugiFilm Imaging Plates and after overnight exposure at room temperature, the plates were developed in a FugiFilm BAS-500 Bio-Imaging Analyzer.

Analysis of *PAP1* phosphatase activity

      BT1 and *BT2* mutant lines were examined for any changes in the levels of *PAP1* phosphatase activity in leaves for correlation to the GUS results using *PAP1* acid phosphatase activity stain gels assays. Leaf tissue was ground in an extraction buffer of 25mM Tris HCl pH 8.0, 2.5mM EDTA, 10mM NaCl, and 10% glycerol in an Eppendorf tube and then spun for 5 min in the coldroom. The supernatant was loaded onto a 10% non-denaturing PAGE gel and run at 20m Amps for approximately 3 hours.   The gels were then removed from the plates and rinsed 10 min in a 0.1mM Na Acetate, 5mM NaCl solution. The gels were then developed in an activity substrate solution (0.1mM Na Acetate, 5mM NaCl, 0.02% Fast Garnet, 0.02% Sodium Napthyl Phosphate) overnight at room temperature, rinsed and photographed.

Analysis of root growth at varying levels of phosphate

      BT1 and *BT2* plants were grown on MS media plates containing 1% sucrose, and 0.8% agarose with varying phosphate contents to determine if there was any difference between them and the Columbia wild-type root growth patterns or the root growth patterns of the transgenic control lines.   Plants were plated out on MS plates containing 1.25mM, 0.25mM, 0.1mM, 0.05mM, or 0.025mM phosphate. The source of phosphate in the media was KPO_4. To make sure any differences seen were only from the difference in the phosphate content of the media, the levels of potassium in the media were supplemented by other compounds. To accomplish this, half the molar amount of K_2SO_4 was used to replace any KPO_4 that had been omitted from the media.

      The seeds were surface-sterilized and plated out on various phosphate contents. These plates were then placed upright at a slight angle to allow the roots to grow down along the surface of the media and grown at 25 ^{o}C under a 24 hr light cycle. Root differences were observed daily and analyzed at 2 weeks for any differences in overall growth patterns. Pictures were captured on a compact flash card (Sandisk) using a Canon EOS Digital Rebel and the lengths were measured using Image J.

RESULTS

Isolation of *BT1* and *BT2* by Histochemical staining

To obtain information about the phosphate starvation pathway in plants, we designed a screen that would search for mutants in the phosphate starvation signal transduction pathway. Constructs of the *PAP1* promoter fused to GUS were used to reflect any changes in phosphate responsive gene expression.

Lines homozygous for the transgene were obtained and mutagenized with EMS. These EMS treated lines were then screened under normal phosphate conditions for any altered GUS expression as compared to the non-mutagenized lines.

Histochemical staining was used to detect lines that had increased GUS expression as compared to the non-mutagenized control line (Figure 2.1) These lines were further self-pollinated and histochemically screened until lines homozygous for mutations controlling the increased GUS staining were obtained.

The lines homozygous for increased GUS staining were named *BT1* and *BT2*. *BT1* and *BT2* both showed an increase in the staining of the leaf petiole, main vein of leaf, and leaf blade as well as an increase in the staining of the hypocotyl and roots (Figure 2.2).

Verification of intact transgene promoter in *BT1* and *BT2* mutants

The GUS expression patterns found in *BT1* and *BT2* were consistent regardless of whether the plants were grown in solid MS media, in liquid MS, or in the soil. These results showed an increase in GUS expression under normal phosphate conditions. Since the *PAP1* promoter fused to GUS was used to reflect any changes in phosphate responsive gene expression, this increase in GUS expression under normal phosphate conditions had two potential explanations. There could be a mutation in the phosphate starvation pathway of the plant, or the *PAP1* promoter in the transgene could have been mutated.

PGP24
Parental Control Line
(non-EMS treated)
1.25mM Phosphate
MS media

Mutant (EMS treated) *PGP24* lines showing increased
GUS staining on 1.25mM Phosphate MS Media
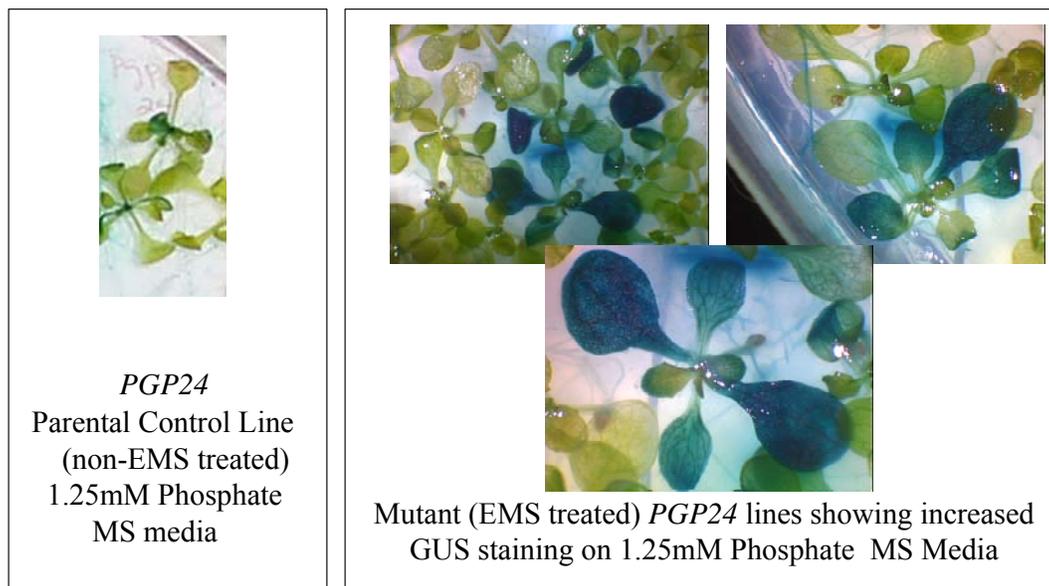
Figure 2.1. GUS histochemical mutant screen identified plant lines having increased
GUS staining under +P conditions.

BT1 mutant line

PGP24 control line
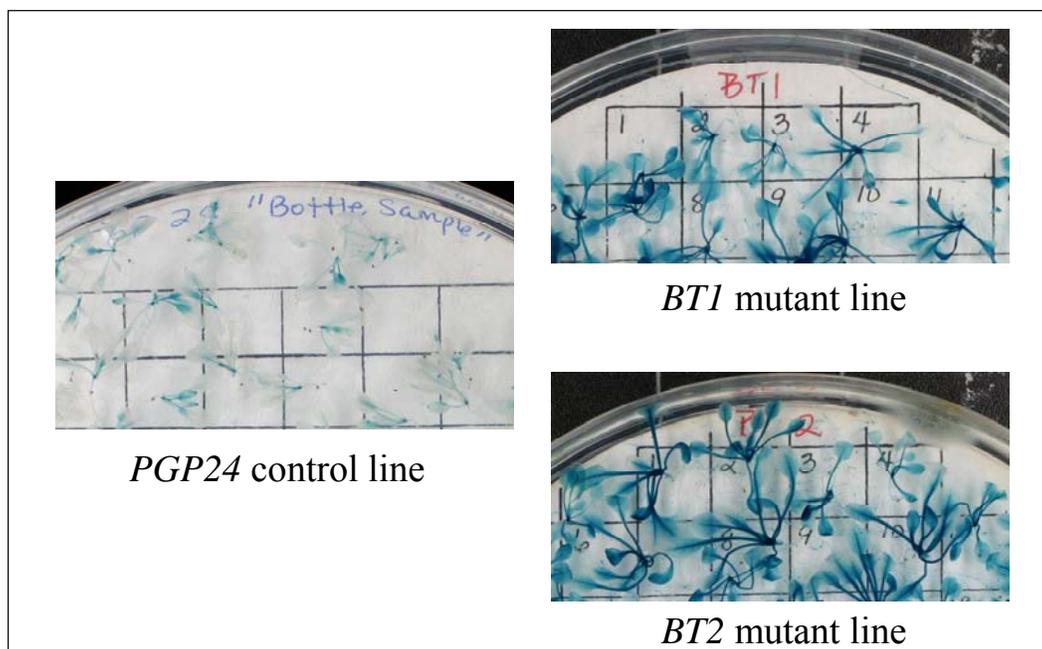
BT2 mutant line

Figure 2.2. *BT1* and *BT2* mutant lines show increased histochemical staining in +P
MS media (1.25mM PO$_4$) compared to control line.

The 540bp transgene *PAP1* promoter was amplified by PCR and sequenced to verify that the transgene promoter itself was not mutated and causing the increase in GUS expression. No differences were found between the promoters sequenced from *BT1* and *BT2* and the endogenous wild-type *PAP1* sequences listed in the database. Since no mutation was found in the transgene promoter, the best explanation for the increased levels of GUS expression in the mutant lines was a mutation somewhere in the phosphate starvation pathway of the plant.

BT1 and BT2 are dominant mutations linked to the transgene

Both mutant lines were crossed to Columbia wild-type plants to determine whether the staining phenotype was dominant or recessive. Col x *BT1* F1 showed 23/23 plants with increased or dark staining and Col x *BT2* F1 showed 15/15 plants with increased or dark staining indicating that both of the mutations are dominant.

The mutant lines were then checked for linkage to the transgene itself using the Kan R as a marker. The linkage analysis was done using F2 progeny from the above Col x *BT1* and Col x *BT2* crosses. Absolute linkage of the mutations to the T-DNA would yield a 12:0:0:4 ratio (12 Kan R/Staining : 0 Kan R/Nonstain : 0 Kan Sen/Stain : 4 Kan Sen/Nonstain) while absolute non-linkage would yield a 9:3:3:1 ratio (9 Kan R/Staining : 3 Kan R/Nonstain : 3 Kan Sen/Stain : 1 Kan Sen/Nonstain). Chi-squared analysis was done on the results shown in Table 2.1. This analysis rejects non-linkage and does not disprove linkage of the genes to the T-DNA indicating that both *BT1* and *BT2* are tightly linked to the T-DNA.

Attempted mapping of BT1 and BT2

BT1 and *BT2* had also been crossed to Landsberg *erecta* ecotype for mapping purposes, but the crosses did not yield enough plants with the mutant phenotype to allow for mapping. Since the mutations were linked to the T-DNA, we attempted to isolate the plant DNA adjacent to the T-DNA insertion for the two mutant lines *BT1* and *BT2* in

Table 2.1.   *BT1* and *BT2* are both tightly linked to T-DNA as indicated by chi square analysis.

Ratios for Absolute Linkage of genes to T-DNA in F2   12:0:0:4
(12 Kan R/Staining : 0 Kan R/Nonstain : 0 Kan Sen/Stain : 4 Kan Sen/Nonstain)

 Col X *BT1*   F2   (total of 800 plants tested)
  Expected outcome if linked   600 :   0   : 0 :   200   = 800
  Observed outcome   580 :   0   : 1 :   217   = 800

 Col X *BT2*   F2   (total of 699 plants tested)
  Expected outcome if linked   523.25 :   0   : 0 : 173.75   = 699
  Observed outcome   502   :   8   : 1 : 158   = 699

Chi Square Analysis of Data Does Not Disprove Linkage of Mutant Genes to T-DNA.

Ratios for Absolute Non-linkage of genes to T-DNA in F2   9:3:3:1
(9 Kan R/Staining : 3 Kan R/Nonstain : 3 Kan Sen/Stain : 1 Kan Sen/Nonstain)

Col X *BT1*   F2   (total of 800 plants tested)
  Expected outcome if not linked   450 : 150 : 150 :   50   = 800
  Observed outcome   580 :   0   : 1 :   217   = 800

Col X *BT2*   F2   (total of 699 plants tested)
  Expected outcome if not linked   393 : 131 : 131 : 43.7   = 699
  Observed outcome   502 :   8   : 1 : 158   = 699

Chi Square Analysis of Data Rejects Non-Linkage of Mutant Genes to T-DNA.

order to map the mutations. A number of techniques were employed in order to isolate this adjacent DNA. Plasmid rescue, inverse PCR, adaptor ligation PCR, and TAIL- PCR all failed to yield results that would identify the location of the transgene insertion in either mutant line and thus we could map these mutants using these methods either.

Characterization of *BT1* and *BT2* by histochemical staining

In an effort to assay the levels of endogenous *PAP1* mRNA, *BT1* and *BT2* lines were grown in liquid MS media under normal phosphate (1.25mM; +P) and then transferred again to either new +P media or to -P media (without phosphate). The plants were harvested and the leaves and roots were analyzed separately. As the plants were harvested, one plant from each flask was taken and histochemically stained using X- Gluc. Both the *BT1* and *BT2* lines showed increase in the staining of the leaf petiole, main vein of leaf, and leaf blade as well as an increase in the staining of the hypocotyl and roots under +P conditions. In -P media, both of the plant lines stained darkly everywhere in the shoot and root (Figure 2.3).

Analysis of *BT1* and *BT2* root growth at varying levels of phosphate

BT1 and *BT2* plants were grown on MS media plates with varying phosphate contents to determine if there was any difference between them and the Columbia wild-type root growth patterns or the root growth patterns of the transgenic control lines. Plants were plated out on MS plates containing 1.25mM, 0.25mM, 0.1mM, 0.05mM, or 0.025mM phosphate. The plates were then placed upright at a slight angle to allow the roots to grow down along the surface of the media. The root plates were photographed at 2 weeks and analyzed for any differences in overall growth patterns.   Measurements were taken for overall root growth of both mutant lines and compared to both Columbia wild-type plants and to the transgenic control lines. No overall difference in the length of roots or pattern of root growth was found between the mutants and the wild-type or the transgenic controls.
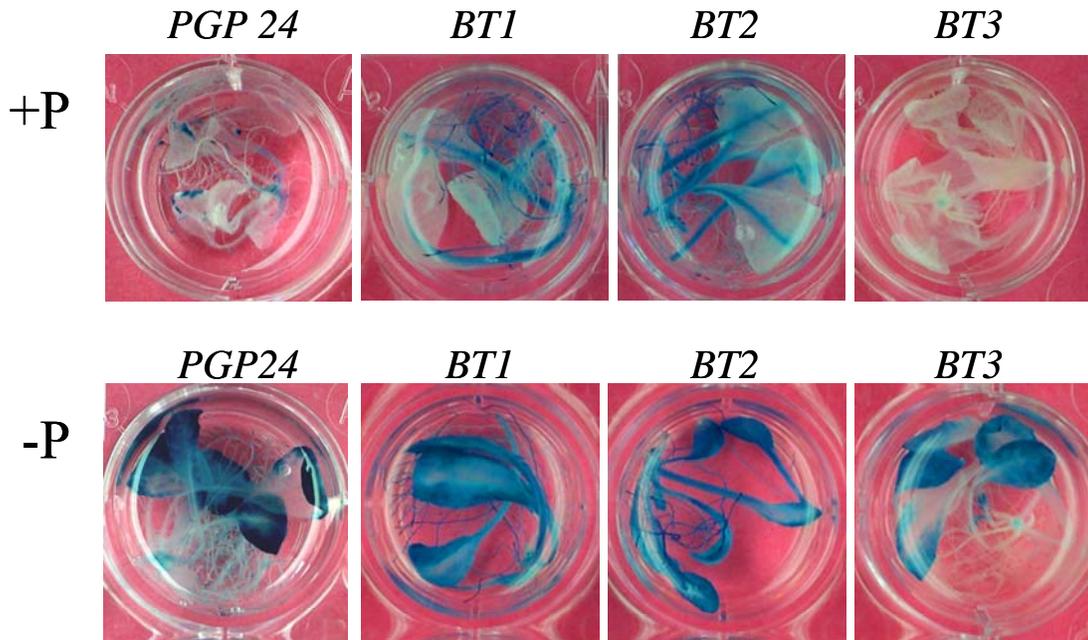
Figure 2.3. *BT1* and *BT2* show increased level of histochemical staining in +P and –P liquid media.
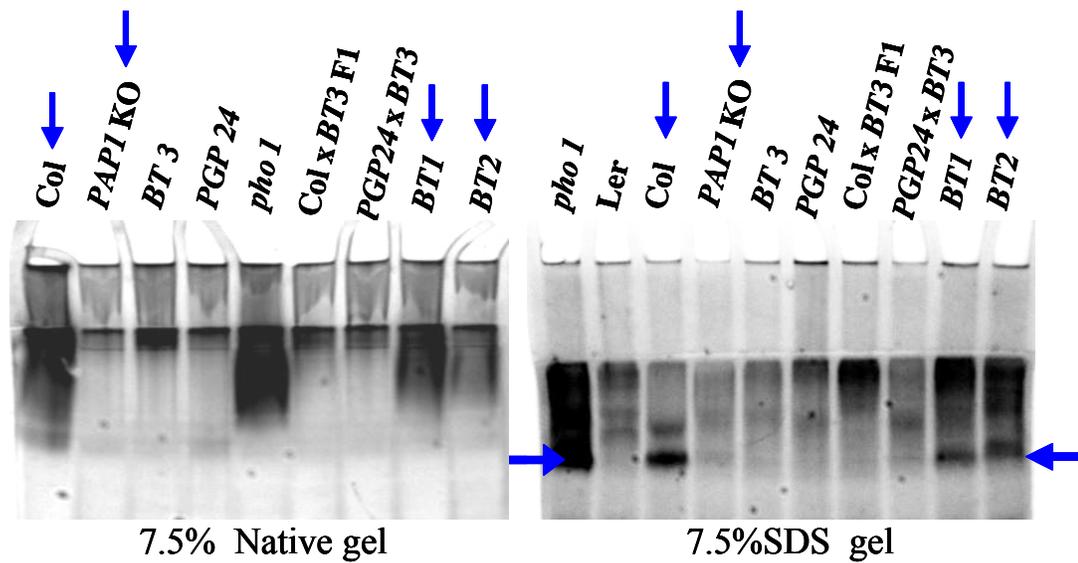
Figure 2.4. *BT1* and *BT2* mutant lines show *PAP1* phosphatase activity similar to Columbia wild-type.

Analysis of *PAP1* phosphatase activity and mRNA levels in *BT1* and *BT2*

       BT1 and *BT2* mutant lines were then examined for any changes in the levels of *PAP1* phosphatase activity in leaves and the endogenous *PAP1* mRNA for correlation to the GUS results using *PAP1* acid phosphatase activity stain gels assays and Northern analysis respectively. Next mRNA levels from other phosphate-starvation induced genes such as the phosphate-starvation inducible RNase gene *RNS1* (Bariola et al., 1994), the phosphate induced phosphate transporter *At4* (Burleigh et al., 1999), *AtACP5* (del Pozo et al., 1999), and *AtISP1* (Martin et al., 2000) were also examined to see if there were any changes in the expression of these genes.

       BT1 and *BT2* leaves both exhibited *PAP1* phosphatase activity as expected under normal phosphate conditions for soil grown plants. The *PAP1* phosphatase activity was expected to be at least somewhat higher than the normal wild-type based on the GUS histochemical results we had obtained in comparing *BT1* and *BT2* to the non-mutagenized control line.   However, the levels of *PAP1* phosphatase activity in these soil grown *BT1* and *BT2* were found to be similar to or lower than the levels in Columbia wild-type (Figure 2.4) under normal phosphate conditions.

       The levels of endogenous *PAP1* mRNA were examined from plants grown in liquid MS media. The plants were germinated and grown in normal +P MS media (1.25mM phosphate) and then transferred again to either new +P media or to -P media (without phosphate). The plants were harvested and the leaves and roots were analyzed separately.

       The levels of *PAP1* mRNA found in the leaves under +P and -P conditions showed levels comparable to the Columbia wild-type plants grown under both +P and -P conditions (Figure 2.5). mRNA levels from other phosphate-starvation induced genes were also analyzed in *BT1* and *BT2* leaves under +P and -P conditions. The phosphate-starvation induced gene *RNS1* showed a decreased level of induction in the *BT2* mutant in -P conditions.   No other genes analyzed showed any change in induction level under either +P or -P conditions when compared to the Columbia wild-type plants (Figure 2.5).
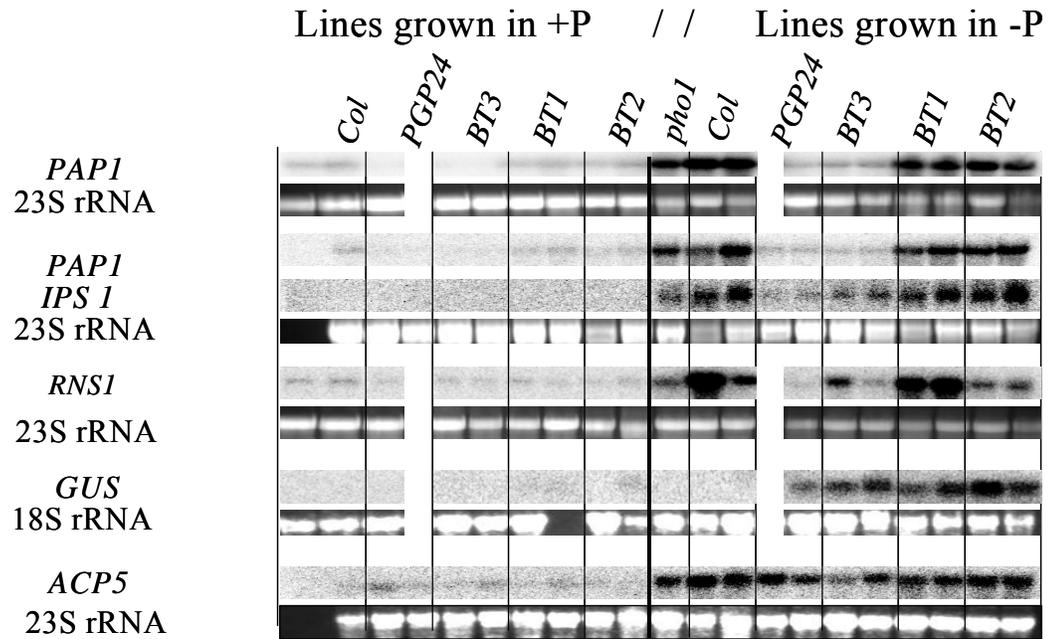
Figure 2.5. *BT2* leaf mRNA levels show altered expression for *RNS1* under Pi deprivation.
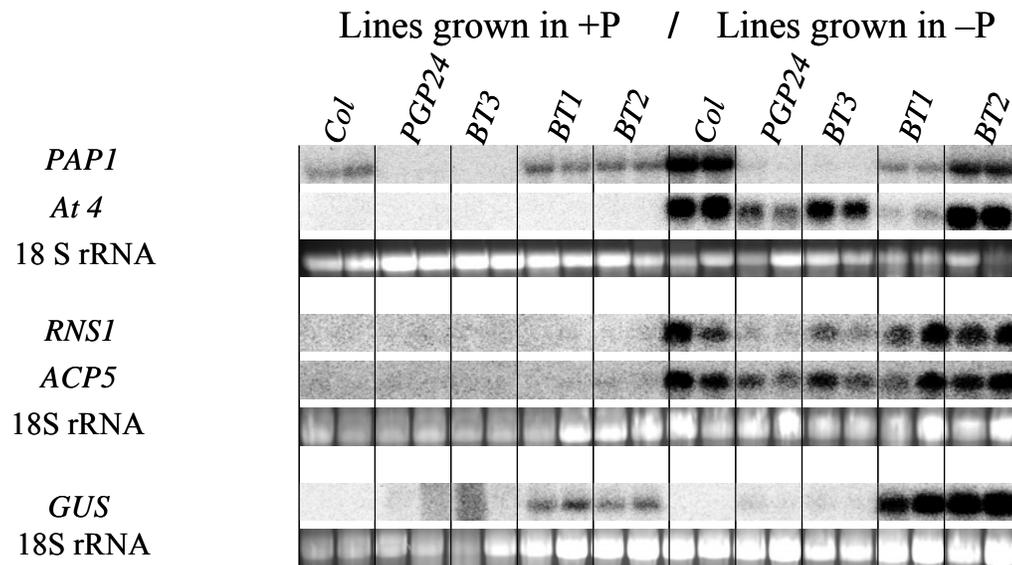
Figure 2.6. *BT1* root mRNA levels show altered expression for *PAP1* and *At4* under Pi deprivation.

Next the levels of endogenous *PAP1* mRNA levels were examined in the roots from the plants grown in liquid MS media under +P and -P conditions (Figure 2.6). The *BT1* roots showed a definite decrease in levels of *PAP1* under -P conditions as compared to the Columbia wild-type plants. *BT2* roots showed levels that were slightly decreased in *PAP1* mRNA.

Levels for other Pi starvation-induced genes were examined. The only other differences in mRNA induction levels were observed in *BT1* where *At4* mRNA levels were markedly decreased under -P conditions, and *AtACP5* mRNA levels showed a slight decrease in one biological sample. *BT2* showed levels comparable to Columbia wild-type plants in every other gene tested (*At 4, RNS1, ACP5,* and *GUS*) under both +P and -P conditions (Figure 2.6).

Allelism analysis for *BT1* and *BT2* mutants

Both *BT1* and *BT2* mutants exhibited similar dominant phenotypes (staining darkly in +P media or liquid). To determine if these mutants were alleles of the same gene or represented two independent genes (different loci), an allelism test was performed. By crossing these two homozygous dominant mutant lines together in an allelism test, we could look for the appearance of the wild-type phenotype, which indicates that the two mutants complement each other. Complementation of the mutant phenotype would further indicate that the mutations were not allelic, but rather at two independent loci.

However, since both mutations were dominant and had the same phenotype, the F1 plants from the double mutant cross would not yield any information. The F1 plants were self-pollinated, and then the F2 examined for complementation of the mutant phenotype. According to Koornneef et al. (1998), "Very close linkage of two loci with a dominant allele for gene 1 in one parent and a dominant allele for gene 2 in the second parent cannot be distinguished from allelism until a recombinant is found". When the F2 from the crosses were examined, 86/5756 plants or 1.5% showed a wild-type phenotype (Table 2.2.) Chi square analysis of the double mutant data was performed and the

Table 2.2. Complementation analysis results.   1.5% of F2 from *BT1x BT2* and
BT2 x *BT1* double mutant crosses have wild-type staining patterns.

Expected Recombination (wild type) frequencies
            Absolute Linkage of genes to each other in F2
                            Recombination %   <<<< 1% or   0%

Observed Outcome *BT2* x *BT1*	F2	2/2924	No stain except meristem
		7/2924	No stain except cotyledons
		33/2924	Partial or light(wt) stain
Observed Outcome *BT1* x *BT2*	F2	1/2832	No stain except meristem
		10/2832	No stain except cotyledons
		33/2832	Partial or light(wt) stain
Total # of wild type staining phenotype		86/5756	

                        = **1.5%**   plants exhibiting wild type staining phenotype

Table 2.3.   *BT1* and *BT2* are tightly linked to the T-DNA, but appear to be mutations in
different genes as shown through complementation analysis.

Absolute Linkage of genes to each other in F2
            Expected Recombination %   <<<< 1% or   0%

Non Linkage of genes to each other in F2
            Recombination %   1/16 or   6.25%

	# of Recombinants	# of Mutant Phenotypes	Total # of plants
Expected outcome for Linkage (1% recombination)	57.56	5698.44	5756
Observed outcome	86	5670	5756
Expected outcome for Non Linkage (6.25% recombination)	359.75	5396.25	5756
Observed outcome	86	5670	5756

   Chi square analysis of Data   –   Rejects both Absolute linkage and Non- Linkage

results rejected both absolute linkage and absolute non-linkage of the two mutations (Table 2.3). This analysis indicates that *BT1* and *BT2* are tightly linked but appear to be different mutations in different genes. Tight linkage between *BT1* and *BT2* is also consistent with the previous linkage analysis showing that both mutations were linked to the T-DNA.

DISCUSSION

Our understanding of the genes involved in the Pi starvation-induced signal transduction pathway as well as the regulation of that pathway is limited. One way to further our understanding of this pathway is through analysis of mutants. Using a mutant screen devised to look for any plant that showed an abnormal *PAP1* response to phosphate nutrition, two mutant lines were isolated based on their histochemical mutant staining patterns.

These two mutant lines, *BT1* and *BT2*, exhibited the same phenotype based on the mutant screen. They both exhibited a dark GUS staining pattern throughout the plant under normal phosphate conditions. What was surprising was not only the fact that they both were dominant mutations, but also that they both appeared to be linked to the transgene itself upon analysis.

However, it appears that *BT1* and *BT2* are not the same mutation for a number of reasons. First, the northern results show that levels of gene expression for the same gene differs from these two lines in both root (*PAP1, At4,* and *AtACP5* -slightly in one sample) and in the leaves (*RNS1*). Also, this difference in gene expression is specific to roots in *BT1* and specific to leaves in *BT2*. *BT1* shows a definite altered or decreased Pi starvation-induced gene expression in only the roots (*PAP1* and *At4),* while *BT2* has altered or decreased Pi starvation gene expression in the shoot (*RNS1*).

Second, while the data from the linkage analysis shows both of the mutations to be linked to the T-DNA, the F2 from the double mutant crosses of these two lines showed a wild-type phenotype in 86/5756 plants, or 1.5%, indicating complementation had occurred. In addition to this, the results from the allelism test's chi square analysis

of these double mutants rejected both absolute linkage and absolute non-linkage of the two mutations. This chi square analysis indicates that *BT1* and *BT2* are tightly linked but appear to be different mutations in different genes. These results of tight linkage between *BT1* and *BT2*, and the fact that both mutations are linked to the T-DNA are consistent with the each other.

The results from these mutants may also give us an indication of a possible role that *PAP1* could play in plants. *At4* is an ortholog of *TPSI1* in tomato that is negatively regulated. Also, *At4* shares some of the same *cis* regulatory factors that are found in *TPSI1* such as a NIT 2 element and helix-loop-helix (Pho box-like domain) element that are similar to the Pho4 regulator binding domain of the PHO regulon in yeast (Mukatira et al., 2001). This similarity in *cis*-regulatory regions suggests that *At4* could also be negatively regulated by some protein factor that is bound to the DNA similar to *TPSI1* under Pi sufficient conditions. Other studies show that *At4* is downregulated by what appears to be a translocatable shoot factor dependent on the translocation of Pi to the shoot (Burleigh and Harrison, 1999). This downregulation could be due to negative regulatory protein binding. *At4* is regulated at both the level of transcription and post-transcriptionally by a miRNA. *At4* appears to function in the control of Pi allocation between shoots and roots influencing Pi retranslocation to the roots (Shin et al., 2006). Since the *At4* is downregulated in *BT1* roots along with *PAP1* it is possible that *PAP1* may also play a role in Pi translocation within the plant.

As mentioned previously in the introduction, *PAP1* contains a putative PHR1 binding site of GAATATCC in the promoter at -159bp from the ATG start codon. PHR1 binds as a dimer to the GNATATNC *cis* regulatory element in the promoters of many Pi starvation-induced genes (Rubio et al., 2001). This site is found in all the Pi starvation-induced genes examined for changes in expression during these experiments. *BT1* and *BT2* mutants both show differential expression of only some of the Pi-starvation induced genes examined containing PHR1 binding sites as compared to wild type. These results indicate that the Pi starvation sensing mechanism and *PHR1* gene are intact in both lines.

At4, AtIPS1, AtPT1, AtACP5, AtIPS3, and *RNS1* genes not only have a PHR1 binding site, but to also have reduced Pi starvation-inducibility in the *phr1* mutant lines, the effect being most marked in *AtIPS1* followed by *RNS1* and *At4*. (Rubio et al., 2001). This indicates that these genes are located downstream of *PHR1* in the Pi starvation-induced pathway (Rubio et al., 2001). *AtIPS1* and *RNS1* are positively regulated in *Arabidopsis* through SIZ1 interaction with PHR1 during the initial stages of Pi limitation (Miura et al., 2005). Pi starvation induction of *AtIPS1* and *RNS1* in *siz1* mutants occurred more slowly than in wild-type plants, but did eventually increase to levels equivalent to wild-type after an extended period of Pi limitation from 48 to 72hrs indicating that SIZ1 has a transient function in the sumoylation of PHR1 and positive regulation of these genes. It may also be that PHR1 function in *Arabidopsis* is redundant. (Miura et al., 2005)

All our - P samples were grown in -P MS for 3 days (72hrs) allowing for any transient effect of SIZ1 interaction with PHR1 to be dismissed. In *BT2,* mRNA levels (72 hrs) of *RNS1* were decreased as compared to wild-type but *AtIPS1* levels were not, nor were any of the other Pi starvation-induced genes examined containing a PHR1 binding site.   In *BT1,* mRNA levels (72hrs) of *PAP1* and *At4* both show reduced Pi starvation-inducibility as compared to wild-type while all the other genes tested, again containing PHR1 binding sites, do not. This suggests that there is yet another level of regulation either in addition to SIZ1 interaction with *PHR1*, additional regulation upstream or downstream of this interaction, or there may be alternative pathways controlling the expression of these genes.

While PHR1 may be one factor in the initiation of some Pi starvation-induced genes, there are alternative pathways known to regulate some of these genes. Cytokinins activate the *CRE1* gene which downregulates *IPS1*, *At4*, *AtACP5*, and *AtPT1* in the roots (Franco-Zorilla et al., 2002; Martin et al., 2000). *At4* was substantially downregulated in *BT1* while *AtACP5* only had one biological sample that was very slightly downregulated in *BT1*. This differential response of these two genes, along with the controlled levels of hormones in the media, indicates that the downregulation of the *At4* gene in *BT1* is not

due to the cytokinin pathway, but rather something else. Since this difference was only apparent under Pi limiting conditions, the data indicates something in the Pi starvation pathway downstream of sensing, possibly another branch of the Pi starvation pathway than *PHR1* or involving additional regulation upstream or downstream of SIZ1 interaction with *PHR1*.

Finally, *PAP1* expression is unaltered in the *phr1* mutant (Patel, unpublished data). Examination of the *PAP1* promoter showed no Pi regulatory sites in the region containing the PHR1 binding site at -159bp from the ATG start codon. A Pi responsive element was actually delineated to the region upstream of this area between -482 and 452bp from the ATG start codon (Patel unpublished data). This also indicates an additional method of regulation for this Pi starvation-induced gene downstream of the Pi sensing mechanisms other than *PHR1* (Figure 2.7). The differences between *BT1* and *BT2* expression of the Pi starvation-induced genes *PAP1, At4,* and *RNS1*, all containing PHR1 binding sites, also supports the idea of additional methods of regulation for these genes (Figure 2.7). This could be either alternative branches in the Pi starvation-induced pathway prior to *PHR1,* or that there may be additional activators, repressors, or modifiers involved upstream, as well as downstream, of *PHR1* in the Pi starvation-induced pathway.
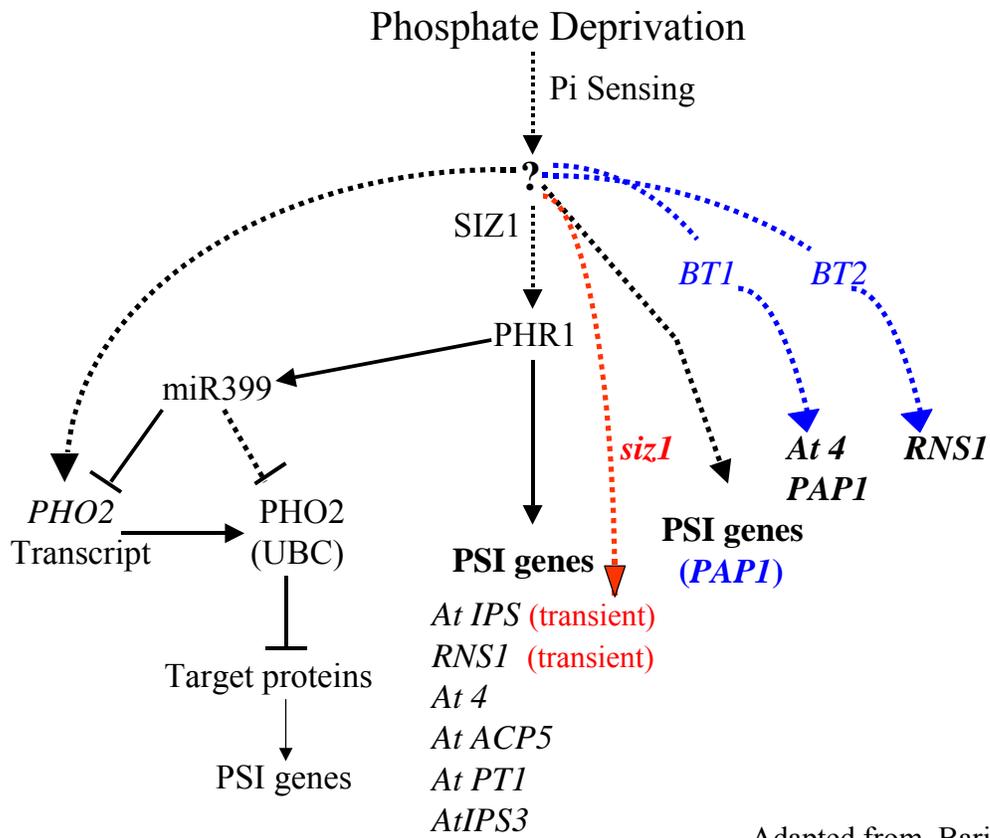
Figure 2.7. Additional methods of regulation found for *PAP1, At4,* and *RNS1* in *BT1* and *BT2* mutants.

CHAPTER III

ISOLATION AND CHARACTERIZATION OF *BT3*

INTRODUCTION

As previously mentioned, one of our main goals was to elucidate part of the phosphate starvation signal transduction pathway in *Arabidopsis.* We chose to study this pathway by observing the expression of the *PAP1* gene, a gene that may play multiple roles in phosphate nutritional metabolism. Through the identification and characterization of mutant expression of this *PAP1* gene, we would obtain additional information about the phosphate starvation signal transduction pathway.

The expression of this gene was studied in transgenic *Arabidopsis* using constructs containing the *PAP1* promoter fused to the *GUS* reporter gene and then the *PAP1* terminator (*PAP1::GUS::PAP1*). Plant lines containing the *PAP1::GUS::PAP1* construct were screened for using GUS histochemical assays . Lines homozygous for one segregating copy of the transgene construct were obtained and mutagenized with EMS. These EMS treated lines were then screened under normal phosphate conditions for any altered GUS expression as compared to the non-mutagenized lines.

During the process of screening, it appeared that the control line might have either become contaminated, or was somehow being affected by the environmental conditions. Seeds from older control-line seed stock were germinated and grown. These plants were used as controls along with lines re-isolated from current stock. Later, after analysis of all the data, another possible explanation was considered. It also was possible that either the initial transformant harbored / generated a spontaneous mutation, or that insertion of the T-DNA itself had caused a mutation.

Insertional mutagenesis occurs when the insertion of a DNA fragment inactivates a gene. Two elements used in plants for mutagenesis are T-DNA and transposons (Feldmann et al., 1994). The insertion of a T-DNA, or transfer DNA, from *Agrobacterium tumefaciens* to a plant genome is random. The T-DNA can sometimes insert into the middle of an important gene and inactivate the gene. The mutants that are

created by disrupting the function of a gene are most likely to be a loss-of-function, but some insertions have generated a gain-of-function mutant. Depending on where this T-DNA is inserted, a mutation may occur such that the T-DNA and a mutation may cosegregate (Feldmann et al., 1994; Malmberg 1993). However, in screening for T-DNA mutants, some mutants obtained have actually been due to spontaneous nucleotide changes and not insertions since the frequency of T-DNA mutagenesis is low (Malmberg 1993).

While the frequency of T- DNA mutagenesis is low, it is possible that the initial transgenic line harbored a mutation caused by T- DNA insertion that affected the Pi starvation signal transduction pathway. If the initial insertion of T-DNA were to result in insertional mutagenesis, then the use of EMS as a mutagenizing agent could act as a secondary mutagenesis. This secondary mutagenesis may end up creating second site revertants / and or suppressors of the original mutation. These revertants and suppressors may be dominant (Malmberg 1993).

To isolate the plant DNA disrupted by an insertion, a number of techniques can be employed: 1) generation of genomic libraries from the mutant and subsequent screening with sequences homologous to the right or left border of the T-DNA (Feldmann et al., 1994), 2) plasmid rescue, using the selectable markers in the T-DNA or other border sequences to isolate the plant-T-DNA junctions (Feldmann et al., 1994), 3) Inverse polymerase chain reaction (Inverse PCR) using primers made from the left or right border (Feldmann et al., 1994), 4) Thermal asymmetric interlaced polymerase chain reaction (TAIL- PCR) using primers from both the left or right border as well as arbitrary degenerate primers (Liu and Whittier, 1995; Liu et al., 1995). We tried the later three methods in addition to adaptor ligation PCR which uses T-DNA left and right border primers as well as primers for special adaptors ligated to digested genomic DNA (Spertini et al., 1999; Padegimas and Reichert, 1998) to isolate the T-DNA. However, none of these methods were successful in identifying the location of the transgene insertion in either our parental or mutant lines. This may be due to the initial

transformant inserting what appears to be two copies of the transgene construct in the form of a tandem repeat.

In this chapter, the isolation of lines mutant in the Pi starvation signal transduction pathway from the initial transgenic *PAP1::GUS::PAP1* line that was used as the parental control line is described. These lines (*PGP24* and *BT3*) lack *PAP1* phosphatase activity like the *PAP1* knockout line. The results for the Col x *BT3* F1 were the same as the *PAP1* knockout line, *BT3*, and *PGP24* lines, showing no *PAP1* phosphatase activity at all, indicating *BT3* is a dominant mutation. Analysis of these lines, *PGP24* and *BT3*, showed varying Pi starvation responses compared to wild-type, and to both the mutant lines, *BT1* and *BT2*, described in Chapter II. Analysis of the *PGP24* and *BT3* lines show that both lines appear to be the same, exhibiting the same overall responses although these varied in intensity at times (such as in histochemical staining) and were renamed *BT3*. Using the information obtained, we were able to elucidate more about some of the genes involved in the Pi starvation-induced signal transduction pathway. Finally, using the difference in expression of the *BT3* lines as compared to wild-type and the *BT1* and *BT2* mutant lines, we were able to separate the Pi starvation-induced genes examined in this chapter into two main groups of genes containing PHR1 binding sites. These groups react differently to Pi starvation and may indicate a difference in response to interactions of SIZ1 with the PHR1 binding site, additional regulation of *PHR1*, or alternative routes in the Pi starvation response pathway.

MATERIALS AND METHODS

Plant materials, DNA construction and *Arabidopsis* transformation, and genetic segregation analysis and further selection

Construction, growth, analysis, and selection of transgenic plants were performed as described in Chapter II.

Characterization of *PGP24* and *BT3* by histochemical staining

 PGP24 and *BT3* were grown under various conditions to determine if there was a correlation of GUS patterns of staining with levels of environmental free phosphate. Levels of free phosphate in the media were determined using a spectrophotometer. Plants were grown on +P (1.25mM phosphate, 1% sucrose) MS plates (Murashige and Skoog, 1962) and on 2x +P MS plates (2.5mM phosphate, 1% sucrose) and checked for staining. The plants were also grown in +P MS liquid media (1.25mM phosphate, 1% sucrose) and 2x +P MS liquid media (2.5mM phosphate, 1% sucrose) and placed either in eppendorf tubes, or in culture plates for staining.

 Histochemical localization and characterization of GUS Activity in plant tissues was determined as described in Chapter II.

Promoter nucleotide sequencing and analysis

 The *PGP24 PAP1* transgene promoter sequence was determined as described in Chapter II. DNA analysis of the sequences was performed with Sequencher software 4.2. The determined sequence was compared against the endogenous wild-type *PAP1* sequence listed in the database using BLAST, and no differences were found.

Southern blot analysis

 DNA was isolated from leaves of mutant, controls, and Columbia wild-type *Arabidopsis* leaves using a modified CTAB protocol from Steven Jacobsen's Lab. DNA (2μg/lane) was digested with restriction enzymes, separated on an 0.8% agarose gel by electrophoresis and then transferred to Hybond N+ paper according to the manufacturer's instructions and blotted by capillary action overnight. The blot was rinsed and then crosslinked with the autosetting of a UV crosslinker and then allowed to dry. Hybridization was performed overnight at 65ºC in hybridization solution (1% crystalline BSA, 1mM EDTA, 0.5M $NaHPO_4$ pH 7.2, 7% SDS). A 1.8kb Nco I/Sac I fragment containing GUS from the transgene construct was α P 32 radiolabeled and used

to probe the blot. The blot was washed with 2X SSPE + 0.1% SDS for 20 min, repeated
for 30 min, then washed with 0.2XSSPE + 0.1%SDS 30 min twice at 65ºC. The blot
was placed on a FugiFilm Imaging Plate, and then after overnight exposure at room
temperature, the plate was developed in a FugiFilm BAS-500 Bio-Imaging Analyzer.

Northern blot analysis

Northern analysis was performed as described in Chapter II using RNA isolated
from both roots and leaves of *PGP24*, *BT3*, and Columbia wild-type control line plants
grown in both +P MS liquid media and -P MS liquid media.

Attempted Mapping of *PGP24* and *BT3*

In an attempt to isolate the plant DNA adjacent to the T-DNA insertion site
numerous techniques were employed. As described in Chapter II for *BT1* and *BT2*
mapping, we tried using plasmid rescue, inverse PCR, adaptor ligation PCR, and TAIL-
PCR.

Analysis of *PAP1* phosphatase activity

PGP24 and *BT3* were analyzed to show levels of *PAP1* phosphatase activity in
leaves for correlation to the GUS results. *PAP1* acid phosphatase activity stain gel
assays were performed as described in Chapter II.

Analysis of root growth at varying levels of phosphate

PGP24 and *BT3* plants were grown on MS media plates containing 1% sucrose,
and 0.8% agarose with varying phosphate contents to determine if there was any
difference between the Columbia wild-type root growth patterns and the root growth
patterns of the transgenic control lines.   Setup, growth, and analysis of root plates was
performed as described in Chapter II.

RESULTS

Isolation and characterization of *PGP24* and *BT3* by histochemical staining

To obtain information about the phosphate starvation pathway in plants we used constructs of the *PAP1* promoter fused to *GUS* to identify mutants in the Pi starvation response pathway. Lines homozygous for one segregating copy of the transgene construct were obtained and mutagenized with EMS. These EMS treated lines were then screened under normal phosphate conditions for any altered GUS expression as compared to the non-mutagenized lines. Two homozygous lines were eventually mutagenized and screened for altered GUS expression. The lines *PGP6* and *PGP24* showed slightly different patterns and levels of histochemical staining (Figure 3.1).

As the mutant lines were identified using histochemical screening for altered GUS expression they were compared to the non-mutagenized control lines. *PGP24* was the control line that yielded the isolated mutant lines *BT1* and *BT2*. During the course of these continued screenings staining patterns appeared in the *PGP24* line that were identical to the mutant lines. These increased histochemical staining patterns indicated that the line had possibly become contaminated or was somehow being affected by environmental conditions (Figure 3.2).

The *PGP24* control lines were grown on +P (1.25mM phosphate) MS plates and on 2x +P MS plates (2.5mM phosphate) and checked by staining. In contrast to previous results with this line, there was no apparent correlation of the increased histochemical staining to levels of phosphate in the media (Table 3.1).

The plants were also grown in +P MS liquid media (1.25mM phosphate) and 2x +P MS liquid media (2.5mM phosphate). Every day, a few individual seedlings were checked from each flask for increased histochemical staining over a period of 8 days. Levels of phosphate were also measured to determine if a reduced phosphate level was responsible for triggering the GUS expression in the plants. Again, no correlation of the increased histochemical staining to level of phosphate in the media was found (Table 3.2). After analyzing the combined data from the MS plates and liquid media results, we
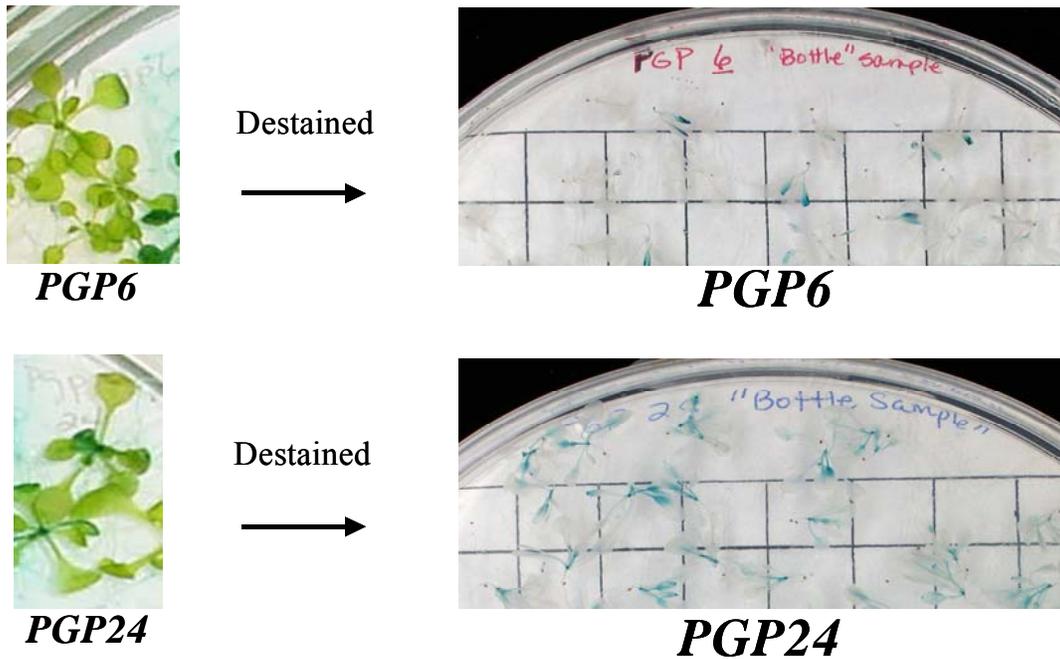
Figure 3.1. The two homozygous control lines used for EMS mutagenesis. Each was derived from unique transformation events and showed different levels of histochemical staining.
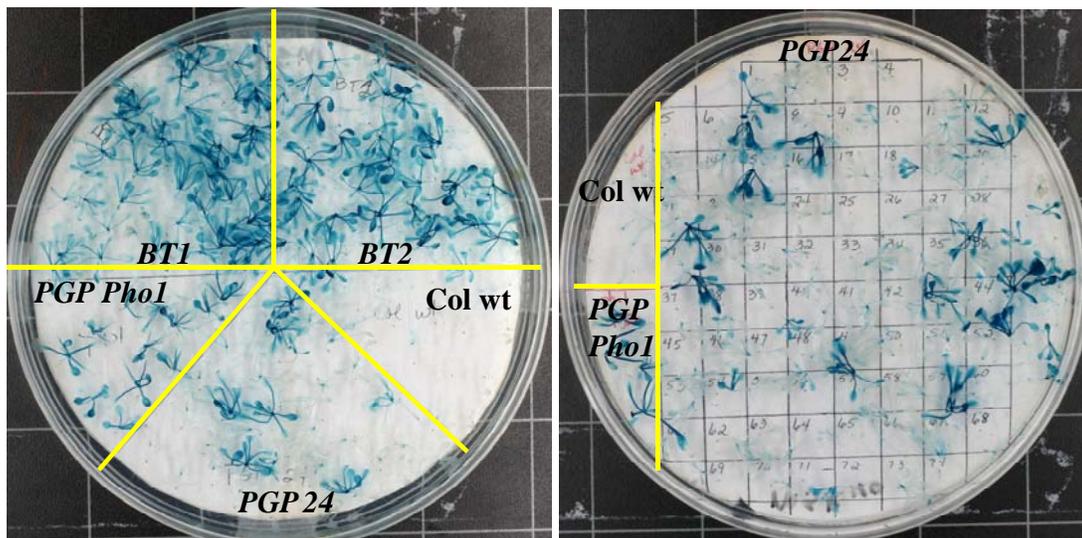
Figure 3.2. *PGP24* control line showed abnormal staining during the screening process

Table 3.1. *PGP24* abnormal staining pattern shows no relationship to levels of phosphate in MS media plates.

Line plated	# of plants staining in 1.25 mM phosphate	# of plants staining in 2.5 mM phosphate
PGP24 plate #1	15/74 stain darkly	27/74 stain darkly
" " plate #2	25/74 stain darkly	23/74 stain darkly
Columbia wild-type	0/47 none stain	0/74 none stain
pho1 PGP6	74/74 stain med. dark	74/74 stain (lighter than 1.25mM plate)
BT1	74/74 stain darkly	74/74 stain darkly
BT2	74/74 stain darkly	74/74 stain darkly

Table 3.2. *PGP24* abnormal staining pattern shows no relationship to levels of phosphate in liquid MS media.

Line grown in liquid MS	# of plants staining in 1.25 mM phosphate	# of plants staining in 2.5 mM phosphate
PGP24	9/40 stain darkly	9/40 stain darkly
BT1	21/21 stain darkly	20/20 stain darkly
BT2	21/21 stain darkly	20/20 stain darkly

concluded that the control line had been contaminated - very probably with one of the previously isolated mutant lines.

An accurate control line had to be obtained in order to analyze the *BT1* and *BT2* mutants. Older seed stocks of *PGP24* were examined to see if there was an uncontaminated control line that could be used. The newer seed stock examined repeatedly showed some plants with the mutant staining pattern (increased histochemical staining), while in the older seed stock, the seeds failed to germinate. After repeated efforts, a line with the original *PGP24* staining pattern was recovered from older seed stock. The uncontaminated seed lines were eventually bulked and again histochemically stained to verify their accuracy as a control line (Figure 3.3). This line was eventually used as a new *PGP24* control line.

While accurate *PGP24* control lines were being recovered from older lines, the lines containing plants with mutant-like staining pattern of increased histochemical staining were also planted. Plants were easily identified as either having mutant-like staining patterns, or the original *PGP24* GUS staining patterns. Plants from these lines showing original *PGP24* staining patterns were selected for use as a control line in case the older seed stock failed to produce an accurate control line.

Different plants were independently examined to make sure the *PGP24* staining pattern was genetically heritable. Within the lines exhibiting the original *PGP24* staining pattern, there was some variation as to the degree of staining as in the original stock but unlike the mutant-like staining which stained dark blue throughout (Figure 3.2). One line was found during the course of this selection that showed almost no GUS staining except at the apical meristematic region of the plant. This line (later named *BT3*) was also selected for use as a new control line since it had the least amount of histochemical staining and was the easiest to distinguish from the lines with increased histochemical staining (Figure 3.3).

PGP24 line
isolated from older
seed stock

BT3 line
isolated from
PGP24 line with
irregular staining
patterns
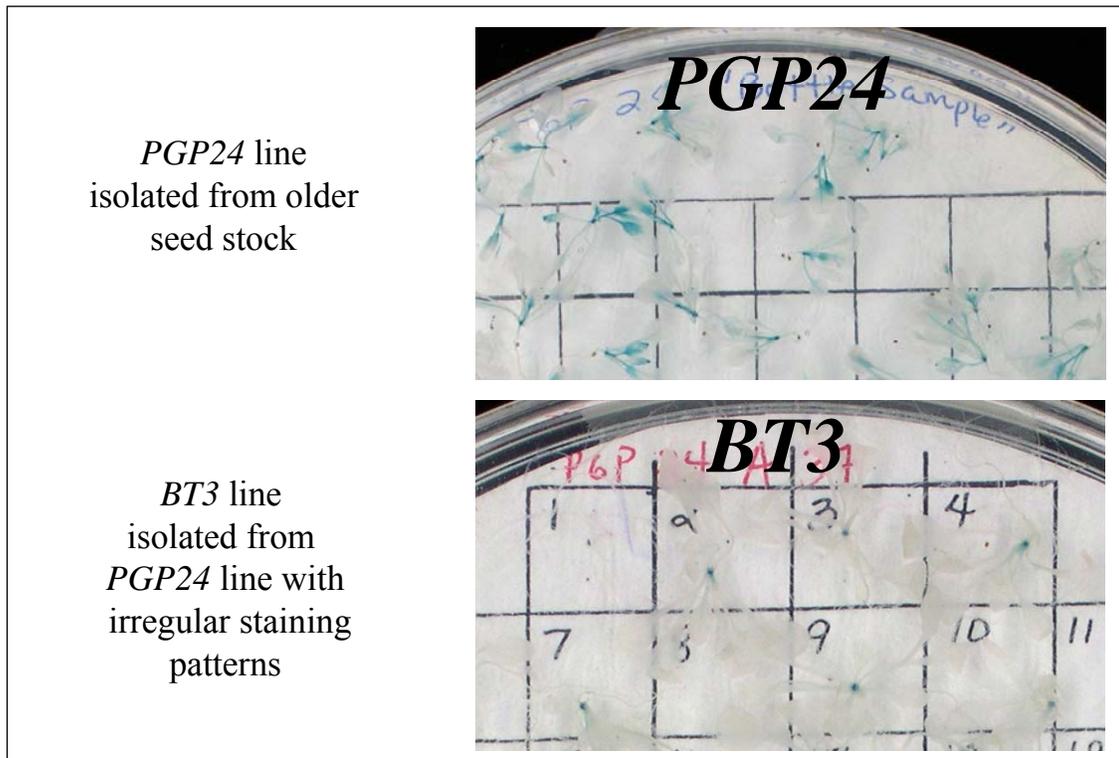
Figure 3.3. *PGP24* control lines were re-isolated from both older stock seed and from the line with irregular staining.
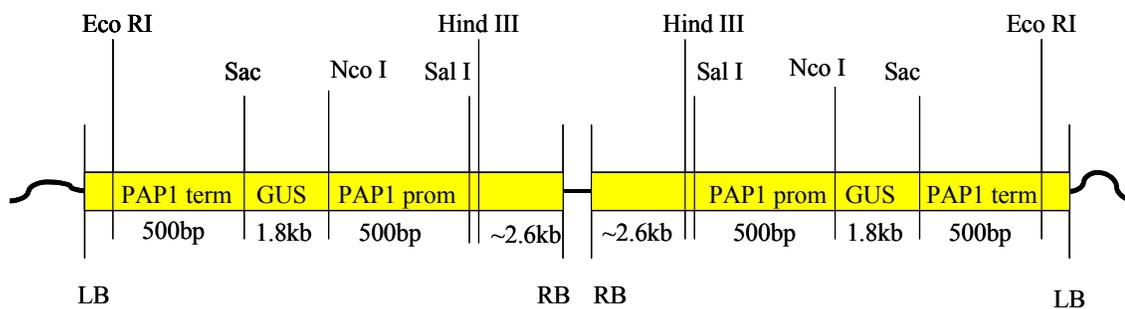
Cut with Hind III - get 2 fragments of unknown size when probe with GUS

Cut with Nco I   - get 2 fragments of unknown size when probe with GUS

Cut with Sac I   - get 1 fragment   ~ 2600 bp   between HindIII and RB
                                          500   bp   Pap promoter
                                         ~1800 bp   GUS
                                         ~4898 bp x 2 inserts   =   ~ 9.8 kb

Cut with Eco RI - get 1 fragment   ~ 2600 bp   between HindIII and RB
                                          500   bp   Pap promoter
                                         ~1800 bp   GUS
                                          500   bp   PAP1 Terminator
                                         ~5398 bp x 2 inserts   =   ~ 10.8 kb

Figure 3.4. Digest patterns suggest a possible tandem T-DNA insertion orientation.

Verification of control lines by Southern analysis

The sudden appearance of unexpected staining patterns in the control line could have been due to contamination from other GUS expressing lines in the laboratory. To assess this possibility, we analyzed the new control lines *PGP24* and *BT3* with Southern blots. DNA from each line was extracted, digested with different enzymes, and run on a gel overnight, and blotted. The resulting patterns of fragments containing the GUS portions of the construct were examined after probing the blot with a α-P 32 radiolabeled GUS containing probe.

The pattern of fragments found in the digestions suggested a possible tandem orientation of the T-DNA construct in both the *PGP24* and *PGP6* lines with the right borders placed internally (Figure 3.4). Even though there was more than one copy of the transgene inserted, it would still segregate as one copy when analyzed genetically. If this orientation was present in the control lines, then the digests would result in the fragments that were actually found on the Southern blot of : 1) ~ 9.8kb when cut with Sac I and 2) ~10.8 kb when cut with EcoRI. The other digest with Hind III and Nco I would both yield two fragments of unknown sizes.

When the Southern blots were analyzed, we detected the expected size bands for a tandem repeat insertion for both the Sac I and the Eco RI digestions. There were also some additional matching bands found on the blots for *PGP24* and *BT3* that possibly came from extra partial T-DNA insertion sites. Using just these two digestions it was impossible to determine if we had an accurate control line. However, based on the Hind III digestion pattern, the mutant lines could only have come from *PGP24* parent line. The Hind III pattern found in *PGP6* was completely different and did not contain either of the 2 distinct fragments that were found in all of the other lines (Figure 3.5). The Nco I digest was inconclusive.
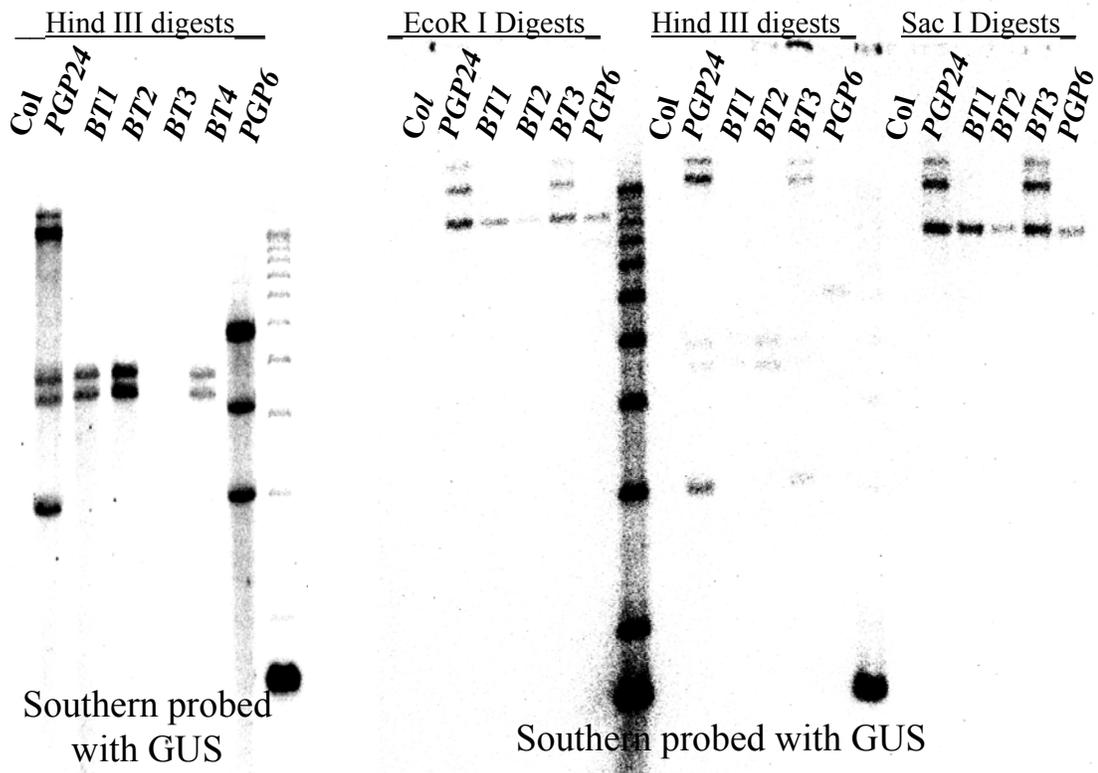
Figure 3.5. Two original control lines *PGP24* and *PGP6* were EMS treated and screened for mutants.   Based on Hind III digest pattern alone, the resulting mutant lines found could only have come from *PGP24 (BT3)* parent line.

Verification of intact transgene promoter in *PGP24*

Since the *PAP1* promoter was used to drive the GUS reporter gene, any change in GUS expression should also indicate a change in the endogenous *PAP1* expression. The 540bp transgene *PAP1* promoter from the *PGP24* line was also sequenced to verify that the transgene promoter itself was not mutated and causing any change in GUS expression. No differences were found between the promoter sequenced from *PGP24* and the endogenous wild-type *PAP1* sequences listed in the database.

Re-characterization of *PGP24* and *BT3* by histochemical staining

In an effort to assay the levels of endogenous *PAP1* mRNA, the new *PGP24* control lines (*PGP24* and *BT3*) were grown in +P liquid MS media and then transferred again to either new +P media or to -P media. The plants were harvested and the leaves and roots were analyzed separately. One plant from each flask was taken and histochemically stained using X-Gluc (Figure 3.6).

The new *PGP24* control line isolated from the older seed stock showed staining in the shoot meristematic region, some in the leaf petioles, and occasionally in the main vein region of some leaves under +P conditions. The roots also showed staining at the root tips under +P conditions. This staining pattern seen in the new *PGP24* control line was fairly consistent with the original staining pattern seen when grown on +P media. In -P conditions the *PGP24* line showed staining again in the shoot meristematic region, some in the leaf petiole, a little in the main vein regions of a few leaves, but did show dark staining in all of the leaf blade perimeters. There was also more staining in the roots further in from the tips but it was not complete like in the *BT1* and *BT2* mutant lines.

The *BT3* line, isolated from seed stock lines containing plants with mutant-like staining pattern of increased histochemical staining, failed to induce GUS anywhere in the entire plant except a medium staining in the meristematic region of the shoot under +P conditions. The staining pattern seen in the *BT3* lines under -P conditions showed
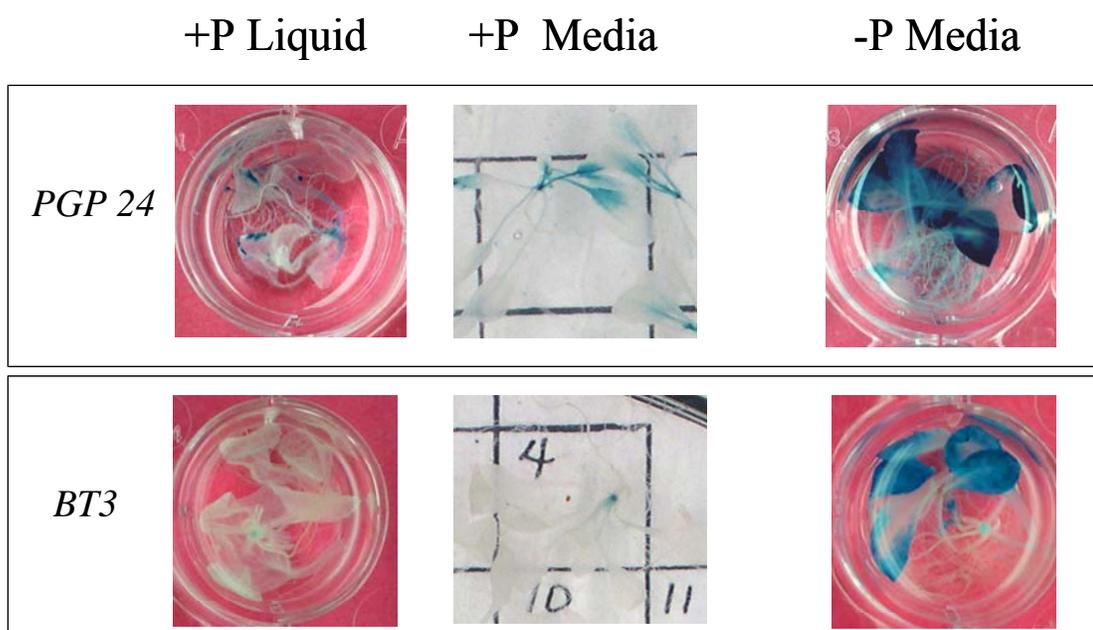
Figure 3.6. Histochemical staining patterns for *PGP24* and *BT3* under +P and -P conditions. The two lines showed similar staining patterns but differed in intensity.

medium staining again in the shoot meristematic region and in the leaf blade perimeters. There was almost no staining (only at a very few root tips) seen in the roots in -P media.

Analysis of mRNA levels for phosphate starvation induced genes in *PGP24* and *BT3*

The levels of endogenous *PAP1* mRNA were examined from *PGP24* and *BT3* plants grown in liquid MS media for correlation to the GUS results. These plants were to be used as control lines along with Columbia wild-type to compare with the isolated mutant lines. The new control plants were germinated and grown in normal +P MS media and then transferred again to either new +P media or to -P media. The plants were harvested and the leaves and roots were analyzed separately for mRNA levels.

The levels of *PAP1* mRNA found in the *PGP24* and *BT3* leaves (Figure 3.7) were markedly decreased compared to the Columbia wild-type plants grown under both +P and -P conditions. In the leaves the *PAP1* mRNA levels were non-detectable in +P, and the levels of *PAP1* mRNA in -P media for both *PGP24* and *BT3* were only induced to a level comparable to +P levels in Columbia wild-type.

The mRNA levels from other phosphate-starvation induced genes were also analyzed in *PGP24* and *BT3* leaves under +P and -P conditions (Figure 3.7). The phosphate-starvation induced gene *RNS1* showed a markedly decreased level of induction in all but one of the biological replicates for *PGP24* and *BT3* under -P conditions. In addition, the phosphate-starvation induced gene *AtIPS1* showed substantially decreased levels of induction under -P conditions for both the *PGP24* and *BT3* lines. *AtACP5* also showed a slight decrease in the level of induction under -P conditions when compared to the Columbia wild-type plants.

The levels of endogenous *PAP1* mRNA levels were examined in the roots from *PGP24* and *BT3* plants grown in liquid MS media under +P and -P conditions (Figure 3.8). There was no apparent induction of *PAP1* at all in the root under either condition in either *PGP24* or *BT3*. The +P levels of *PAP1* mRNA were not detectable in either *PGP24* or *BT3* roots - the same results as had been observed in the leaves. However, as
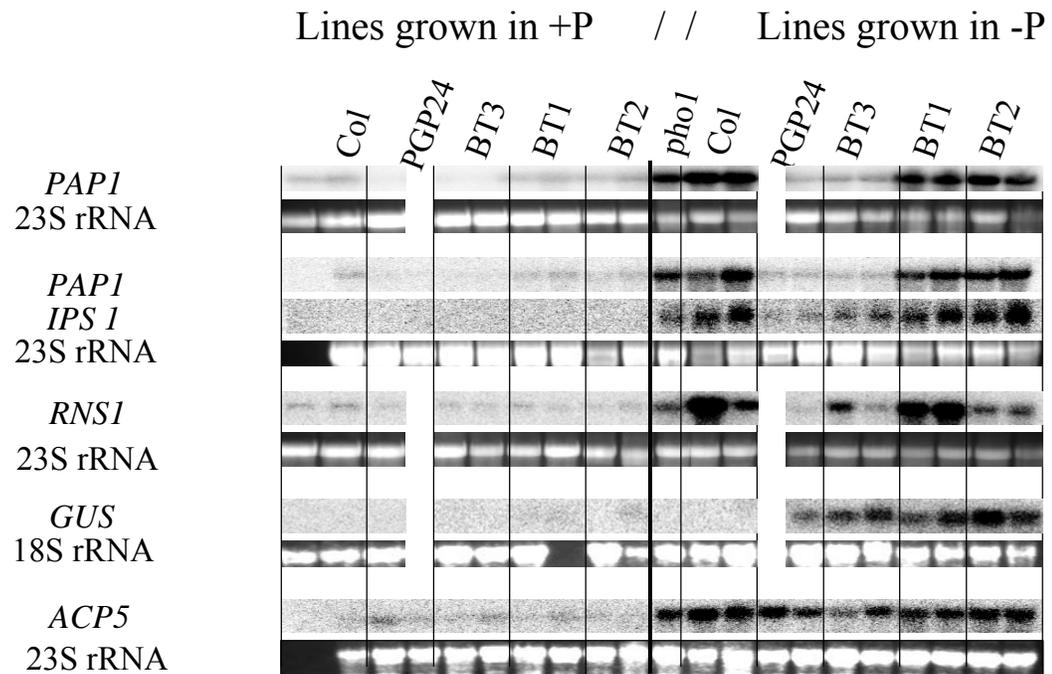
Figure 3.7. *PGP24* and *BT3* leaf mRNA shows decreased levels of induction for *PAP1, IPS1,* and *RNS1*.
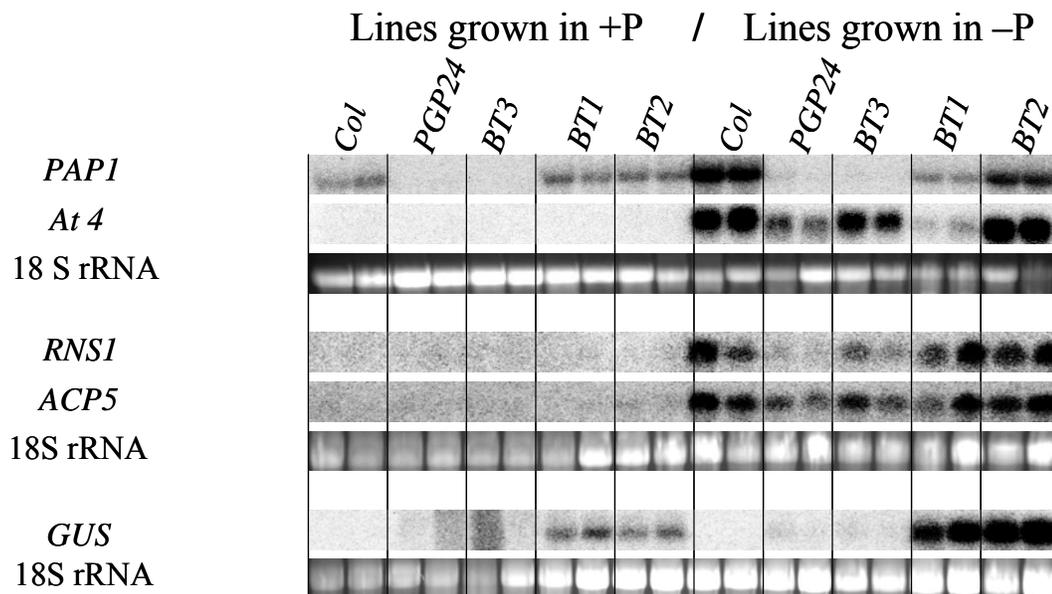
Figure 3.8. *PGP24* and *BT3* root mRNA shows decreased levels of induction for *PAP1* and *RNS1*.

opposed to the leaves where *PGP24* and *BT3* showed levels of induction comparable to the wild-type under -P conditions, *PGP24* and *BT3* roots showed no apparent induction at all of *PAP1* under -P conditions.

PGP24 and *BT3* roots (Figure 3.8) showed levels comparable to Columbia wild-type for all of the other phosphate-starvation induced genes analyzed, *At4, AtACP5*, and *RNS1* under +P conditions. Under -P conditions, *RNS1* showed substantially reduced induction for all of the biological replicates as compared to the Columbia wild-type plants, except one of the replicates which showed only a slight decrease in the level of induction. This was the same sample that showed a higher level of *RNS1* in the leaves as compared to the other biological replicates. Under -P conditions, *At4 and AtACP5* also showed a slight decrease in levels of induction (again with the exception of the one biological replicate).

Analysis of *PAP1* phosphatase activity in *PGP24* and *BT3*

PGP24 and *BT3* lines were also examined for any changes in the levels of *PAP1* phosphatase activity in leaves for correlation to the GUS results using *PAP1* acid phosphatase activity stain gels assays. The *PAP1* phosphatase activity in *PGP24* and *BT3* was expected to be at least the normal Columbia wild-type level based on the GUS histochemical results we had obtained in comparing *PGP24* and *BT3* to the mutant lines *BT1* and *BT2*.

PGP24 and *BT3* however, both failed to exhibit *PAP1* phosphatase activity in leaves under normal phosphate conditions for soil grown plants. The levels of *PAP1* phosphatase activity in both *PGP24* and *BT3* leaves were found to be similar to the levels in the *PAP1* knock out line (in the Columbia background) under normal phosphate conditions as opposed to the Columbia wild-type (Figure 3.9). These findings correlated with the reduced induction of PAP1 on the northern blots.
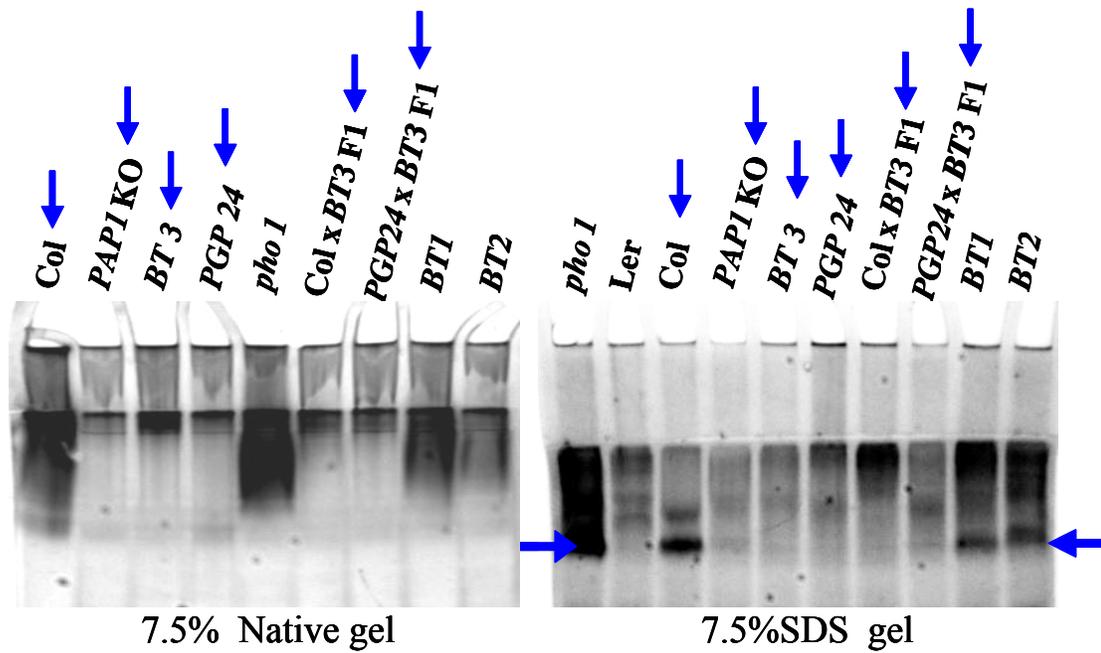
Figure 3.9. *PGP24* and *BT3* both fail to exhibit *PAP1* phosphatase activity in leaves under normal phosphate conditions. F1 crosses between Col x *BT3* and *PGP24* x *BT3* also fail to show *PAP1* phosphatase activity indicating the *BT3* trait is dominant.

Analysis of *PGP24* and *BT3* root growth at varying levels of phosphate

  PGP24 and *BT3* plants were grown on MS media plates with varying phosphate contents to determine if there was any difference between them and the Columbia wild-type root growth patterns or the root growth patterns of either of the mutant lines *BT1* and *BT2*. Plants were plated out on MS plates containing 1.25mM, 0.25mM, 0.1mM, 0.05mM, or 0.025mM phosphate. The plates were then placed upright at a slight angle to allow the roots to grow down along the surface of the media. The root plates were photographed at 2 weeks and analyzed for any differences in overall growth patterns. Measurements were taken for overall root growth of both transgenic control lines and compared to both Columbia wild-type plants and to both the mutant lines. No overall difference in the length of roots or pattern of root growth was found between the *PGP24* and *BT3* lines, and either the mutants or the wild-type controls.

***BT3* is a dominant trait**

  Analysis of mRNA and phosphatase activity was initially done using only the *BT3* line as a control. When the reduced level of PAP1 expression was noticed in *BT3*, this line was crossed with Columbia wild-type plants to determine whether this trait was dominant or recessive. Col x *BT3* F1 seeds were germinated on MS plates with kanamycin and then transferred to soil. Leaves stained histochemically showed 8/8 plants with decreased staining somewhere between the *PGP24* and *BT3* phenotypes. *BT3* was also crossed to the *PGP24* line. The F1 were also germinated on MS kanamycin plates and then transferred to soil. The results from the *PGP24* x *BT3* F1 showed 6/6 plants grown in soil with a similar staining pattern to *BT3* with basically no staining in the leaves, indicating that the expression of *PAP1* in *BT3* is a dominant trait.

  The soil-grown leaves from these crosses were also analyzed for *PAP1* phosphatase activity (Figure 3.9). The results for the Col x *BT3* F1 were the same as the *PAP1* knockout line, *BT3*, and *PGP24* lines showing no *PAP1* phosphatase activity at all. The Columbia wild-type line was also grown and assayed under the same

conditions. The Columbia wild-type plants showed *PAP1* phosphatase activity. *BT3* mutation or trait was again determined to be dominant based on the lack of *PAP1* phosphatase activity in the F1 plants, which also correlated with the reduced histochemical staining pattern.   *PGP24* x *BT3* F1 plants were also analyzed for *PAP1* phosphatase activity. The leaves from the F1 plants showed no *PAP1* phosphatase activity under normal soil grown conditions. These results were the same as both the *PGP24* and *BT3* lines used in the cross.

Attempted mapping of *PGP24* and *BT3*

While the frequency of T- DNA mutagenesis is low, it is possible that the initial transgenic line harbored a mutation caused by T- DNA insertion that affected the Pi starvation signal transduction pathway. To isolate the plant DNA surrounding the transgene insertion site numerous techniques were employed. As mentioned in Chapter II for *BT1* and *BT2* mapping, we tried using plasmid rescue, inverse PCR, adaptor ligation PCR, and TAIL- PCR but were unable to identify the location of the transgene insertion in either our *PGP24/BT3* lines or the *BT1* and *BT2* mutant lines. This may be due to the apparent insertion of two copies of the transgene construct in the form of a tandem repeat in the initial transformant.

DISCUSSION

During the process of characterizing mutants *BT1* and *BT2* (described in the previous chapter) in the Pi starvation pathway, we were surprised to find that the parental control line itself appeared to be itself a mutant. This new mutant (found in both older seed stock "*PGP24*" and re-isolated lines " *BT3*") now needed to be characterized as well.

While there was variation in the GUS histochemical staining between the older seed stock "*PGP24*" and the re-isolated lines " *BT3*", the other analysis on these two lines was basically the same. The DNA banding patterns in the Southern were identical,

the *PAP1* phosphatase staining patterns were the same, and there is no difference seen in either the *PAP1* or *GUS* mRNA levels of expression between *PGP24* and *BT3* in the northern analysis. Also, all levels of mRNA for other Pi starvation-induced genes analyzed by northern are identical except in one biological replicate. The difference found in the one northern sample with *RNS1* mRNA levels appears to be due to naturally occurring differences in the biological replicates since this was the only sample out of over 8 tested in the course of these experiments that varied in *RNS1* results. Based then on the analysis of these two lines we have come to the conclusion that *PGP24* and *BT3* are actually the same mutant. The fact that *PGP24* and *BT3* are apparently the same suggests the possibility that either the initial transformant harbored / generated a spontaneous mutation, or that insertion of the T-DNA itself caused a mutation. These lines, *PGP24* and *BT3,* are apparently the same and were eventually renamed *BT3*.

We attempted to isolate the plant DNA adjacent to the T-DNA on the chance that the mutations were linked to the T-DNA and would allow us to map the mutation. While the frequency of T- DNA mutagenesis is low, it is possible that the initial transgenic line harbored a mutation caused by T- DNA insertion that affected the Pi starvation signal transduction pathway. We attempted to isolate the plant DNA surrounding the transgene insertion site using numerous techniques. As mentioned in Chapter II for *BT1* and *BT2* mapping, we tried using plasmid rescue, inverse PCR, adaptor ligation PCR, and TAIL-PCR but were unable to identify the location of the transgene insertion in either our *PGP24* or *BT3* lines. This could possibly be due to the apparent insertion of two copies of the transgene construct in the form of a tandem repeat in the initial transformant as was suggested by the Southern analysis.

Characterization of the mutant lines was carried out. Northern analysis, using multiple biological replicates of *PGP24* and *BT3*, showed *PAP1* was always downregulated under both +P and -P conditions in both root and leaves. There was no apparent induction of *PAP1* at all in the root under +P or -P conditions. In the leaves the *PAP1* mRNA levels were again undetectable in +P, and in -P the levels were just

equivalent to wild-type under +P conditions. The mutation affected *PAP1* levels throughout the entire plant.

A number of other Pi starvation-induced genes were also affected in these plants. In the roots, *PAP1 and RNS1* were obviously downregulated in *PGP24/BT3* lines compared to wild-type, while there was only a slight downregulation of *At4, and AtACP5* (again with the exception of the one biological line). In the leaves, *PAP1, RNS1,* and *AtIPS1* were all substantially downregulated under -P conditions as compared to wild-type, while *AtACP5* levels were basically unchanged.

It did not appear that these responses were being regulated by changes in cytokinin levels or long distance controlled Pi responses. Cytokinins appear to completely repress *AtIPS1, AtACP5,* and *AtPT1* transcript levels and also greatly reduce the expression of another Pi starvation-induced gene *At4* under Pi starvation conditions in roots but not shoot (Martin et al., 2000). Cytokinins thus cannot be a possible explanation for the downregulation of *AtIPS1* in leaves for two reasons. First, cytokinins do not affect *AtIPS1, At4, AtACP5, and AtPT1* in shoots, which is where the *PGP24/BT3* lines show the *AtIPS1* repression (Martin et al., 2000). Second, if cytokinins were responsible for the downregulation of *AtIPS1*, there should also be a marked downregulation of *At4* and *AtACP5* in the roots as well which does not exist.

If these changes are not due to cytokinin levels, then there must be something else affecting the regulation of these genes. *AtIPS1* is induced specifically in response to Pi starvation and no other stress or hormone has been found to induce its expression (Martin et al., 2000). Since these plants were under Pi starvation conditions, *AtIPS1* should be induced as in wild-type, unless something is blocking either the Pi sensing or something else downstream in the Pi starvation-induced response pathway.

Since other Pi starvation-induced genes are upregulated in this mutant as in wild-type, it appears that the Pi sensing mechanism is intact. Some of the genes we analyzed are upregulated in wild-type under Pi starvation (*AtIPS1, AtACP5, At4,* and *RNS1*) and have PHR1 binding sites (Rubio et al., 2001). As mentioned previously, our Pi starvation-induced *PAP1* gene was also found to contain a putative PHR1 binding site.

PHR1 binds as a dimer to the GNATATNC *cis* regulatory element in the promoters of many Pi starvation-induced genes (Rubio et al., 2001), including all the Pi starvation-induced genes examined for changes in expression during these experiments. *AtIPS1, AtACP5, At4, and RNS1* as well as two other Pi starvation-induced genes not only have a PHR1 binding site, but to also have reduced Pi starvation-inducibility in the *phr1* mutant lines, the effect being most marked in *AtIPS1* followed by *RNS1* and *At4*. (Rubio et al., 2001). This suggests that *AtIPS1, AtACP5, At4,* and *RNS1* genes are downstream of *PHR1* in the Pi starvation-induced pathway.

PGP24/*BT3* mutants show differential expression of only some of these genes examined as compared to wild type in both root and shoot. The induction of at least some of these genes suggests that the *PHR1* gene itself is intact. The differential response of these known Pi starvation-induced genes again suggests the possibility that there may either be multiple branches to this pathway prior to or beyond *PHR1,* or that there may be additional activators, repressors, or modifiers involved upstream or downstream of *PHR1* in the Pi starvation-induced pathway similar to the SIZ1 ligase or miRNAs (Miura et al., 2005; Fujii et al., 2005).

AtIPS1, and *RNS1* are positively regulated through SIZ1 interaction with PHR1 during the initial stages of Pi limitation, while another group of genes (including *AtPT2*) are negatively regulated under normal Pi conditions (Miura et al., 2005). Pi starvation-induction of *AtIPS1* and *RNS1* in *siz1*mutants occurred more slowly than in wild-type plants, but did eventually increase to levels equivalent to wild-type after an extended period of Pi limitation from 48 to 72hrs indicating that SIZ1 has a transient function in the sumoylation of PHR1 and positive regulation of these genes. It may also be that PHR1 function in *Arabidopsis* is redundant. (Miura et al., 2005) This information suggests that there is yet another level of regulation either in addition to SIZ1 interaction with *PHR1*, additional regulation upstream or downstream of this interaction, or there may be alternative pathways controlling the expression of these genes.

All our - P samples were grown in -P MS for 3 days (72hrs) allowing for any transient effect of SIZ1 interaction with PHR1 to be dismissed. *PGP24/BT3* lines

showed basically no induction of *PAP1* and *RNS1* mRNA (72hrs) in roots while there is only a slight decrease in the level of induction for *At4,* and *AtACP5* compared to wild-type (again with the exception of the one biological line). In *PGP24/BT3* leaves, mRNA levels (72hrs) for *PAP1, RNS1,* and *AtIPS1* all show a substantially decrease in the level of induction under -P conditions as compared to wild-type, while *AtACP5* levels are basically unchanged as compared to wild-type.

Results from *PGP24/BT3* lines*,* now renamed *BT3,* show two main groups of Pi starvation-induced genes containing PHR1 binding sites. The first group containing *PAP1, RNS1,* and *AtIPS1*, has mRNA levels greatly reduced or repressed in both leaves and roots under Pi starvation as compared to wild-type. The second group containing *At4* and *AtACP5,* showed only a slight reduction in mRNA levels under Pi starvation as compared to wild-type.

BT3 (PGP24/BT3) lines, unlike the *siz1* mutant, never exhibited levels of induction for *RNS1* and *AtIPS1* comparable to wild type after 48 to 72 hours (Miura et al., 2005). This suggests that there is an alternative regulation for these genes apart from SIZ1 interaction with *PHR1*, and that some part of this control mechanism is damaged in these lines. As mentioned in the previous chapter, *PAP1* expression is unaltered in the *phr1* mutant, and no Pi regulatory site was found in the region of the promoter that contains the PHR1 binding site. Taken together, these data indicate that at least the genes *RNS1, AtIPS1,* and *PAP1* are not exclusively regulated by *PHR1* (Figure 3.10). There is some alternative mechanism for regulating Pi starvation-induced responses in these genes downstream of Pi sensing.

At4 and AtACP5, however, showed only a slight reduction in levels of mRNA in response to Pi starvation as compared to wild-type. *At4*and *AtACP5* appear to respond differently indicating that they may respond to yet another factor or combination of factors. Taken in its entirety, all these data suggest that the Pi starvation sensing and signal control in *Arabidopsis* is complex and includes many different molecular components, many of which may not be part of the "PHR1 regulon" and are still yet to be discovered.
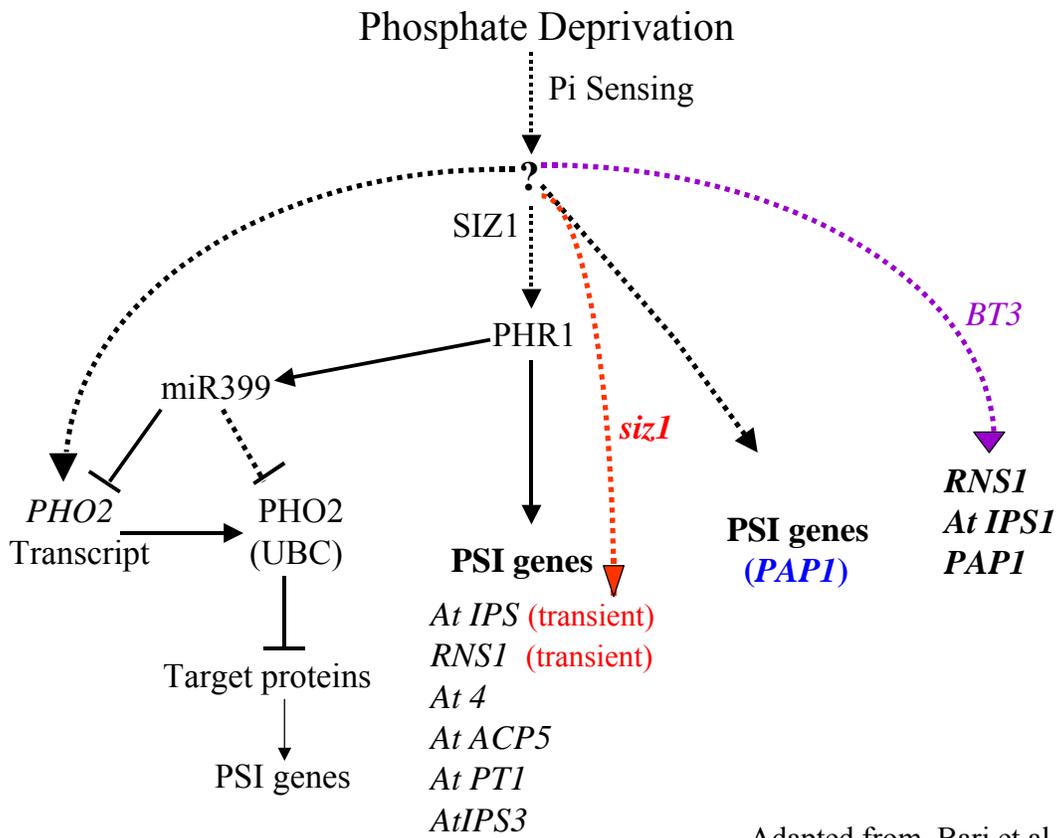
Figure 3.10. Additional methods of regulation found for *PAP1*, *AtIPS1,* and *RNS1* in *BT3* mutants.

CHAPTER IV

MAPPING DIFFERENCES IN THE *PAP1* PHOSPHATASE EXPRESSION BETWEEN COLUMBIA AND LANDSBERG *ERECTA* ECOTYPES

INTRODUCTION

The previous chapter showed that the control lines *PGP24*/*BT3* actually contain a mutation in the Pi starvation response pathway. It is important to map any mutation that is found to uncover the number of genes that influence a trait, the locations of these genes on the chromosomes, and the effect that these genes play in differences in phenotype. While genetic mapping has its limitations, it also provides a starting point for the cloning of genes whose functions are only known from mutant phenotypes. Genetic linkage can be used to delineate a gene to a region of a genome and then physical mapping can be used to eventually clone stretches of DNA including the gene. The location of a mutation on the chromosome is determined by the identification of nearby genetic markers that are also transmitted from the parent to offspring along with the mutant phenotype (Paterson, 1996).

Sometimes this trait or marker is a visible one, but more are molecular in nature. One way to map these molecular markers is with the use of recombinant inbred lines. Recombinant inbred lines are especially good for many mapping purposes since they have permanent population where the segregation is fixed. Using this permanent set of fixed genotypes along with molecular markers allows for locating almost any part of the genome (Reiter et al., 1992). In *Arabidopsis*, Recombinant Inbred (RI) lines were generated from a cross between the *Arabidopsis* ecotypes Columbia and Landsberg *erecta* (Ler) (Lister and Dean, 1993) (with Columbia as the male parent). If a trait is polymorphic between Columbia and Landsberg *erecta* it can be directly mapped using these lines. Since observed *PAP1* phosphatase activity is different in these two lines it can be scored as either Columbia or Landsberg *erecta*, and thus mapped using these lines.

Eventually, the sequences from this region could be isolated and ultimately the target gene identified by mutant complementation (Paterson, 1996). In cases of monogenic traits, when the correct gene has been isolated it can be re-introduced into the mutant as a wild-type sequence to confirm its identity. In cases of recessive genes, this will result in the mutant phenotype being reverted to the wild type (Feldmann et al., 1994). In the case of a dominant mutation, transforming a wild-type plant with the mutant allele will result in the mutant phenotype (Meyer et al., 1996).

As mentioned previously, one of our main goals was to elucidate part of the phosphate-starvation signal transduction pathway, which we chose to do through mutant analysis. Once those mutants were identified, we were able to begin working towards our second goal to map the mutant genes to their location in the *Arabidopsis* genome.

As was mentioned in chapters II and III, we tried to isolate the plant DNA adjacent to the T-DNA insertion for the two mutant lines *BT1* and *BT2* as well as the control line *PGP24*/*BT3*. We were unable to identify the location of the transgene insertion in either our parental or mutant lines and thus could not map using these methods.

During the process of characterizing *BT1* and *BT2*, reduced levels of *PAP1* mRNA and *PAP1* phosphatase activity were found in *BT3*. It was thought that this reduction in *PAP1* phosphatase activity could be used to map the location of *BT3*. *BT3* was crossed to the Landsberg *erecta* ecotype for mapping using molecular markers. We were surprised when Landsberg *erecta* showed the same phenotype as *BT3* for the *PAP1* phosphatase activity trait that was to be mapped. Consequently this cross between *BT3* and Landsberg *erecta* could not be used to map the trait. The phenotype for observed *PAP1* phosphatase activity trait was different, however, between the Columbia wild-type control and the Landsberg *erecta*, with Columbia showing *PAP1* phosphatase activity. This trait could be scored as Columbia or Landsberg *erecta*, and thus mapped using the Lister and Dean recombinant inbred lines.

Upon analysis of the trait using the initial Lister and Dean markers, it was determined that the *PAP1* phosphatase regulatory trait mapped to a region on

Chromosome 2. Using additional molecular markers for that specific region of Chromosome 2, the *PAP1* phosphatase regulatory trait was mapped to an area between the *GPA1* and *ER*, a span of only 15,562 bp. There were only two open reading frames that were found between these two genes. One of these is an expressed protein of unknown function. The other is a MADS-box protein (AGL33), containing a Pfam profile PF00319: SRF-type transcription factor (DNA-binding and dimerization domain).

In this chapter, the mapping of the difference in *PAP1* phosphatase activity between the Landsberg *erecta* and Columbia ecotypes using recombinant inbred lines from Lister and Dean (1993) is described.

MATERIALS AND METHODS

Plant materials

Arabidopsis seeds were vernalized overnight and then allowed to grown in soil in growth chambers under conditions of 16h light/ 8 h dark and 75 % RH at 21 ^{o}C in the day and 18 ^{o}C at night.

PAP1 phosphatase expression in Columbia and Landsberg *erecta* ecotypes

BT3 was crossed to the Landsberg *erecta* (Ler) ecotype for mapping using molecular markers after observing reduced levels of *PAP1* mRNA and *PAP1* phosphatase activity in *BT3*. Crosses were done using Landsberg *erecta* plants for the female parent and the mutant line as the male parent (Ler ♀ X *BT3* ♂). Flowers on the female parent were emasculated by removing all anthers prior to dehiscence without damaging the pistil. Then flowers or anthers from the *BT3* male parent were used to transfer an excess of pollen to the emasculated Landsberg *erecta* female pistil. The resulting crossed buds were labeled and seeds harvested in approximately 2-3 weeks later.

The F1 seeds from the Col X Mutant crosses were plated out on MS media plates containing 1% sucrose, 0.8% agarose, 40 mg/ml Kan. The seeds were screened for Kan resistance. All the plants were Kan resistance and were transferred to soil.

While these plants were growing, activity gels were run to determine the *PAP1* phosphatase activity levels in Landsberg *erecta* to compare with the *BT3* parental line and Columbia wild-type. Leaf tissue was ground in an extraction buffer of 25mM Tris HCl pH 8.0, 2.5mM EDTA, 10mMNaCl, and 10%glycerol in an eppendorf tube and then spun for 5 min in the coldroom. The supernatant was loaded onto a 10% non-denaturing PAGE gel and run at 20mAmps for approximately 3 hours. The gels were then removed from the plates and rinsed 10 min in a 0.1mM Na Acetate, 5mM NaCl solution. The gels were then developed in an activity substrate solution (0.1mM Na Acetate, 5mM NaCl , 0.02% Fast Garnet, 0.02% Na Napthyl Phosphate) overnight at room temperature, rinsed and photographed.

Analysis of *PAP1* phosphatase activity in recombinant inbred lines

The first set of 100 recombinant inbred lines (RILs) from Drs. Clare Lister and Caroline Dean (1993) were ordered from the stock center and grown in soil. Leaves from each individual plant in the set of RILs were examined along with the parental Columbia wild-type and Landsberg *erecta* control lines for *PAP1* phosphatase activity. *PAP1* acid phosphatase activity gel assays described previously were used to determine the presence or absence of *PAP1* acid phosphatase activity. Plants having *PAP1* Activity were scored as "+" or "A" = like Columbia parent, and plants that were lacking *PAP1* Activity were scored as "-" or "B" = like Landsberg *erecta* parent.

***PAP1* phosphatase regulatory trait mapped to Chromosome 2**

All like scoring plants were grouped together in an Excel worksheet and the 67 original Lister and Dean markers were compared to find any linkage. Using only the 67 original markers, an area on Chromosome 2 was found to show linkage to this trait.

Now that the trait was narrowed down to a specific area on Chromosome 2, additional molecular markers were found for that area that would more finely map the trait by searching more databases online. As these additional molecular markers were found, they were added to new Excel worksheets to further delineate the region of Chromosome 2 where the *PAP1* phosphatase regulatory trait is located.

Marker locations were determined in relationship to each other as well as base pair location on the chromosome when possible by using the information found at the TAIR website, www.Arabidopsis.org, as well as the SIGnAL website, www.signal.salk.edu/cgi-bin/atta? . After correlating this information, it was determined that the trait mapped to a region on Chromosome 2 very close to the *ER* marker near *PAP1*, but separate from *PAP1*.

After it was determined that the trait was closely linked to the *ER* marker, a visual inspection of all the plants was done for the *ER* trait (*erecta* phenotype). Not all of the lines listed in the database had information listed for each marker. Many of them had no results listed for certain markers including *ER*. A visual inspection for the *ER* trait was done on the 95 plant lines that had germinated and were analyzed for *PAP1* phosphatase activity. Through this visual inspection, three plants were found to show crossovers with the *ER* marker suggesting that despite the tight linkage, the *ER* gene is not responsible for the *PAP1* staining polymorphism.

Data for one final RIL marker found in this region, *EIL*, was obtained and analyzed. This additional marker was added to a new Excel worksheet to further delineate the region of Chromosome 2 near the *ER* gene where the *PAP1* phosphatase regulatory trait is located. Again, all like scoring plants were grouped together in the Excel worksheet and all the markers were compared to find any linkage. Using information from the RIL genetic map and the *Arabidopsis* genomic sequence posted on the TAIR website, www.Arabidopsis.org, the trait was finely mapped to a region on Chromosome 2.

RESULTS

Difference in *PAP1* phosphatase expression between Columbia and Landsberg *erecta* ecotypes

As shown in Chapter III, the *BT3* mutant line showed a marked decrease in the levels of *PAP1* mRNA found in the leaves (Figure 3.7) as compared to the Columbia wild-type plants grown under both +P and -P conditions. The *BT3* line also failed to induce GUS anywhere in the entire plant under +P conditions except a medium staining in the meristematic region of the shoot.

Again, as shown in Chapter III, *BT3* lines were also examined for any changes in the levels of *PAP1* phosphatase activity in leaves for correlation to the GUS and mRNA results using *PAP1* acid phosphatase activity stain gels assays. *BT3* failed to exhibit *PAP1* phosphatase activity in leaves under normal phosphate conditions for soil grown plants. The levels of *PAP1* phosphatase activity in *BT3* leaves were similar to the levels in the *PAP1* knock out line (in the Columbia background) under normal phosphate conditions as opposed to the Columbia wild-type (Figure 3.9). These results correlated with the reduced induction of *PAP1* mRNA on the northern blots, as well as GUS histochemical staining results.

After obtaining these results of reduced levels of *PAP1* mRNA and *PAP1* phosphatase activity in *BT3*, the line was crossed to the Landsberg *erecta* ecotype for mapping using molecular markers. The plants would be separated into two phenotypic groups for mapping based on the differences in *PAP1* phosphatase activity. It was known that *BT3* plants did not have activity and assumed that Landsberg *erecta* plants would have activity. The F1 seeds were harvested and grown on MS kanamycin plates. All the seeds were kanamycin resistant and were transferred to soil.

While these plants were growing, test gels were run to determine the *PAP1* phosphatase activity levels in Landsberg *erecta* to compare with the *BT3* parental line and Columbia wild-type. It was discovered that the Landsberg *erecta* ecotype had basically the same low phosphate phenotype as *BT3*. Grown in soil under normal conditions, Landsberg *erecta* and *BT3* leaves showed little or no *PAP1* phosphatase
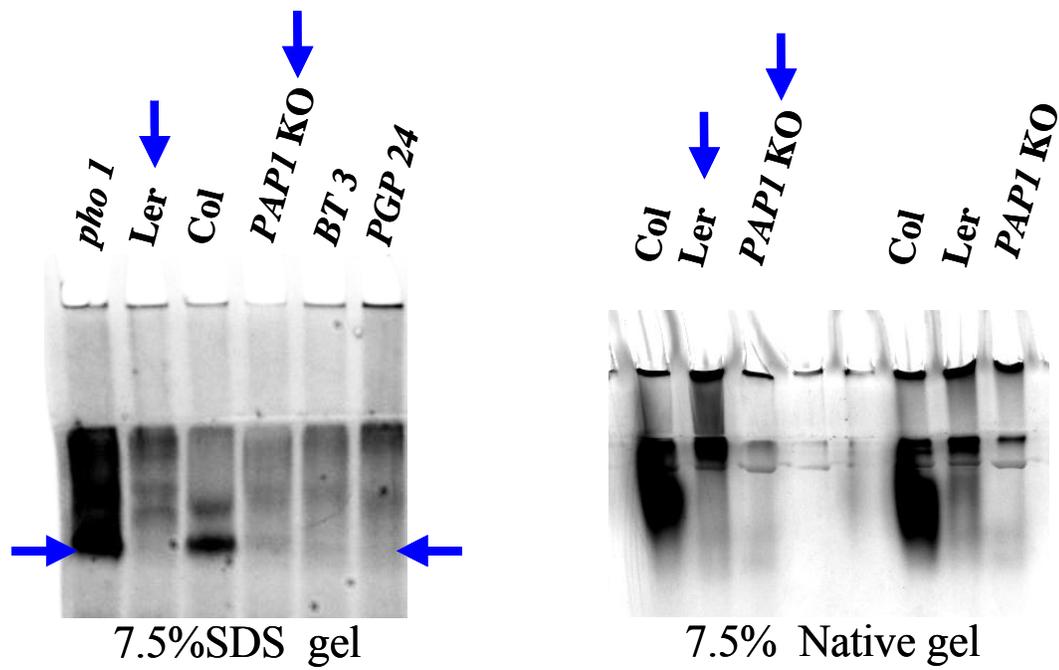
Figure 4.1. Landsberg ecotype shows no *PAP1* phosphatase activity in leaves under normal soil grown conditions.

activity, while Columbia leaves showed normal levels of *PAP1* phosphatase activity (Figure 4.1).

Analysis of *PAP1* phosphatase activity in recombinant inbred lines

Since the *BT3* mutant line and Landsberg *erecta* both showed the same low phosphate phenotype, this cross could not be used to map the trait. Instead, we decided to map the difference in *PAP1* phosphatase activity between the Landsberg *erecta* and Columbia ecotypes using recombinant inbred lines from Lister and Dean (1993).

The first set of 100 recombinant inbred lines from Lister and Dean were ordered and grown in soil. Leaves from each individual line were analyzed for phosphatase activity and scored when compared to the Columbia and Landsberg *erecta* parental lines. Plants having *PAP1* Activity were scored as "+" or "A" = like Columbia parent, and plants that were lacking *PAP1* Activity were scored as "-" or "B" = like Landsberg *erecta* parent (Figure 4.2).

***PAP1* phosphatase regulatory trait mapped to Chromosome 2**

All like-scoring plants were grouped together (Table 4.1) and the 67 original Lister and Dean markers were compared to find any linkage. Most of the original 67 Lister and Dean markers were randomly distributed among the high- and low-phosphate expression groups. The only exception to this random distribution was for markers near *Erecta* (*ER*) on Chromosome 2 where the Landsberg markers were strongly correlated with low *PAP1* phosphatase expression and the Columbia markers were strongly correlated with high *PAP1* phosphatase expression (Table 4.2). Now that the trait was narrowed down to a specific area on Chromosome 2, additional molecular markers were found for that region that would help to more finely map the trait. These additional markers were added to the original 67 and used to further delineate the region of Chromosome 2 where the *PAP1* phosphatase regulatory trait is located. Marker locations were determined in relationship to each other as well as base pair location on the
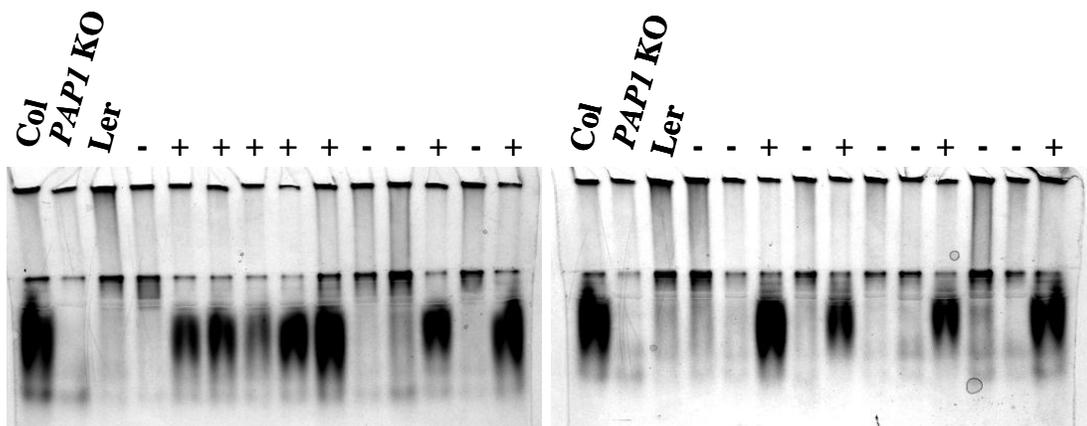
**Figure 4.2.** RIL leaves analyzed for *PAP1* phosphatase activity. "+" = like Columbia parent and "-" = like Landsberg parent.

Table 4.1. Expression of *PAP1* phosphatase activity in RIL lines. Number of plants identified from total of 95 plant lines that germinated.

	PAP1 Phosphatase Activity	
	Like Columbia parent	Like Landsberg *ER* parent
# of plants identified	32	63

Table 4.2. Linkage of *PAP1* phosphatase trait to region on Chromosome 2. Previously determined molecular markers: "A" = molecular marker like Columbia parent, "B" = molecular marker like Landsberg *erecta* parent, "-" = molecular marker unknown.

A.

Chromosome 2	
Markers	Columbia-like RIL lines
*m251	A A A A A A B A A A B B A A - A B - A - A A B B A A A A A A A A
*O802F	A A A A A A B A A A B B A A - A B A A A A A B B A A A A A A A A
*g6842	A A A A A A A A A A A A A - A B - A - A A B B A A A A A A A A
*GPA1	A - A A A A A A A A A A A - A B A A A A A B B A A A A A A A A
*B68	A A A A A A A A A A A A A - A A A A A A A - - - - - - - - - -
*er	A A A A A A A A A A A A A A A A A A A A A - - A A A A - A A A -
*pGC2	- - - - - A - A A - - - - - - - - B - - A A A - A A - A B A A -
*mi54	A A A A A A A A A A A A A A A A A A B A A A A A A A A A B A A -
*CDs9	A A A A A A A A - A A A A A A A A B A A A A A A A A A B A A -
*ve014	A A A A A A A A A A A A A A A A A A B A A A A A A A A A B A A -
*m220	A A A A A A A A A A A A A A A A - B - A A A A A A A A B A A A

B.

Chromosome 2	
Markers	Landsberg-like RIL lines
*m251	B B B B B B B B A B B B B B B A B B - B A B B B A B B B B B B B
*O802F	B B B B B B B B A B B B B - B A B B B B A B B B A B B B B B B B
*g6842	B B B B B B B B A B B B B B B B B B - A B B B B B B B B B B B B
*GPA1	B B B B B B B B B B B B B B B B B B B B B B B B B B B B B A B B
*B68	- B B B - B - B B B B B B B B B B B B B B B B B B B B B B B B B
*er	B B B B B B B B - B - B B B B B B B B B B B - B B B B B B B B B
*pGC2	- - B B - - B B - B A B B - - - - - B B - - B B - B A - B - - -
*mi54	B B B B B A B B A B A B B B B B B B B B B A B B B B A B B B B B
*CDs9	B B B B B A B B - B - B B B B B B B B B - B B B B A B B B B B
*ve014	B B B B B A B B A B A B A B B B B B B B B A B B B B A B B B B B
*m220	B B B B B A B B A B A B A B B B B B - B B B B B B A B B B B B B

Table 4.2. continued

B.

| Chromosome 2	
Markers	Landsberg-like RIL lines (cont.)
*m251	B B A B B B B B B B B B A A B B B B A B B B A B B B A B B
*O802F	B B A B B B - B B B B B A A B B B B A B B B A B B B A B B
*g6842	B B B B B B B B B B B B A B B B B - B B B A B B B B - B
*GPA1	B B B B B B B B B B B B B B B B B B B B B B A B B B B B B
*B68	B B B B -
*er	B B B B B B B B B B B B B B B B B B B B B B B A B B B B B B
*pGC2	- - B - B - B B B B B B - B - - - - - - B A - - B B B A
*mi54	A B B B B A B B B B B B B B B B B B B B B B - B B B B B A
*CDs9	A B B B B A B B B B B B B B B B B B B B B B - B B B B B A
*ve014	A B B B B A B B B B B B B B B B B B B B B A B B B A B B B B A
*m220	A B B B B A B B B B B B B B B B B B B B B B A B B B A B B B B A
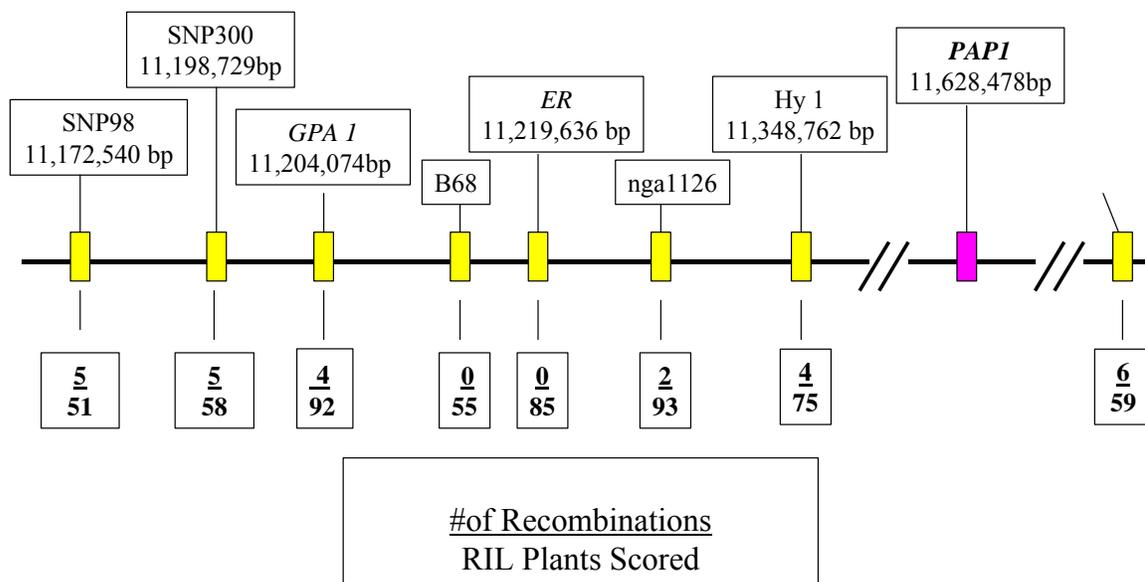
Figure 4.3. *PAP1* phosphatase activity trait (*BT5*) maps to a region on Chromosome 2 close to the *ER* marker.

chromosome when possible by using the information found in the databases. After correlating this information, it was determined that the trait mapped to a region on Chromosome 2 very close to the *ER* marker near *PAP1*, but separate from *PAP1* (Figure 4.3). This *PAP1* phosphatase activity trait was renamed *BT5*.

After it was determined that the trait was closely linked to the *ER* marker, a visual inspection of all the plants was done for the *ER* trait. This was done because not all of the lines listed in the database had information for each marker. Many of them had no results listed for certain markers including *ER*. This visual inspection for the *erecta* phenotype identified three plants that showed crossovers between the *ER* marker and the *PAP1* phosphatase activity (Table 4.3). Now, in addition to the data that had been collected from the online databases, we also identified two plants with the Columbia phenotype that failed to exhibit *PAP1* phosphatase activity and one plant with the Landsberg *erecta* phenotype that showed *PAP1* phosphatase activity. These results suggested that despite the tight linkage, the *ER* gene was not responsible for the *PAP1* phosphatase staining polymorphism.

After visual inspection of the plants for the *ER* marker, data for one final RIL marker found in this region, *EIL*, was obtained and analyzed. Based on recombination frequencies, it appears that *BT5* maps closest to the molecular markers B68 (mapping on the RIL genetic map at 50.59cM) with 0 recombinations out of 55 plants, and *EIL* (mapping on the RIL genetic map at 50.63cM) with 1 recombination out of 85 plants. However, both of these markers are only genetic markers and have not been assigned a physical location at this time. Both the RIL genetic maps and our recombination frequencies place these two markers between *GPA1* (which is located at 11,204,074 bp and maps on the RIL genetic map at 48.90cM) and *ER* (which is located at 11,219,636 bp and maps on the RIL genetic map at 50.64cM) (Figure 4.4).

Using information from the RIL genetic map and the *Arabidopsis* genomic sequence posted on the TAIR website, www.Arabidopsis.org, *BT5* was now finely mapped to a region between the *GPA1* and *ER* markers, a span of only 15,562 bp. There are only two open reading frames between the genes containing these markers.   One of

Table 4.3. Expression of *PAP1* phosphatase activity in lines showing visible crossovers for *ER* trait. Previously determined molecular markers: "A" = molecular marker like Columbia parent, "B" = molecular marker like Landsberg *erecta* parent, "-" = molecular marker unknown.

|	Lines with crossovers for *ER* trait			
	Phenotype			
	Landsberg-like *PAP1* activity with *ER* phenotype		Columbia-like *PAP1* activity with *er* phenotype	
Markers	CS/N1942	CS/N1989	CS/N1968	
*m251	B	A	B	
*O802F	B	A	B	
*g6842	B	A	B	
*SGCSNP98	B	-	B	
*SGCSNP300	B	A	B	
*GPA1	B	A	B	
*B68	B	-	-	
EIL 1	B	-	B	
*er	-	A	-	
*nga1126	B	A	A	
*HY1	B	-	B	
*MIT257A	-	-	A	
*SGCSNP135	B	A	A	
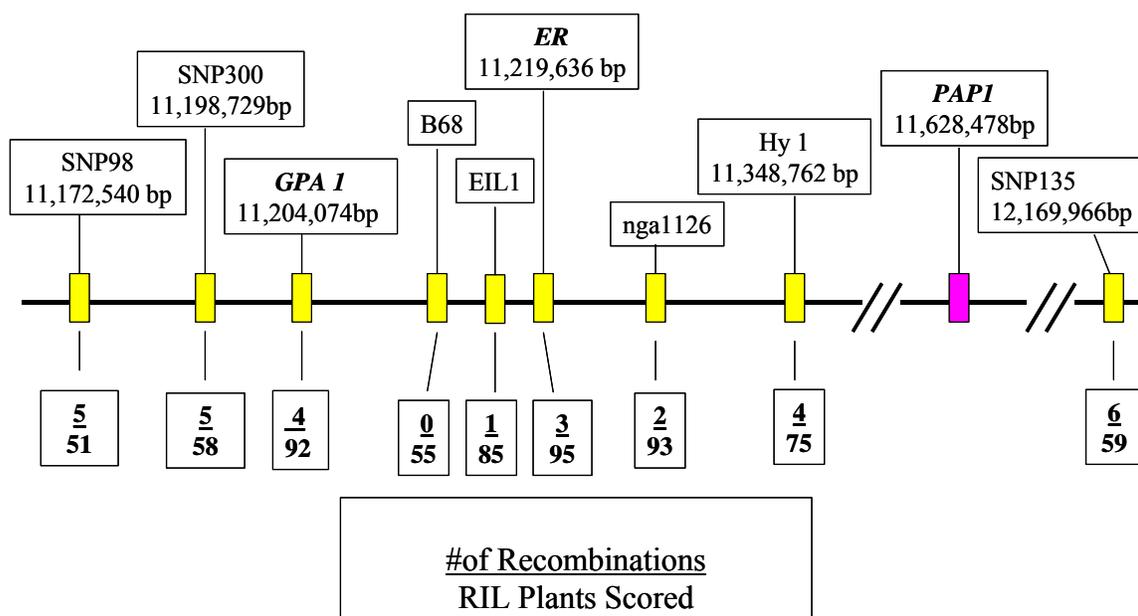
Figure 4.4. *BT5* trait, regulating *PAP1* phosphatase activity, maps to a region of only 15,562 bp on Chromosome 2 between the *GPA1* and *ER* markers.
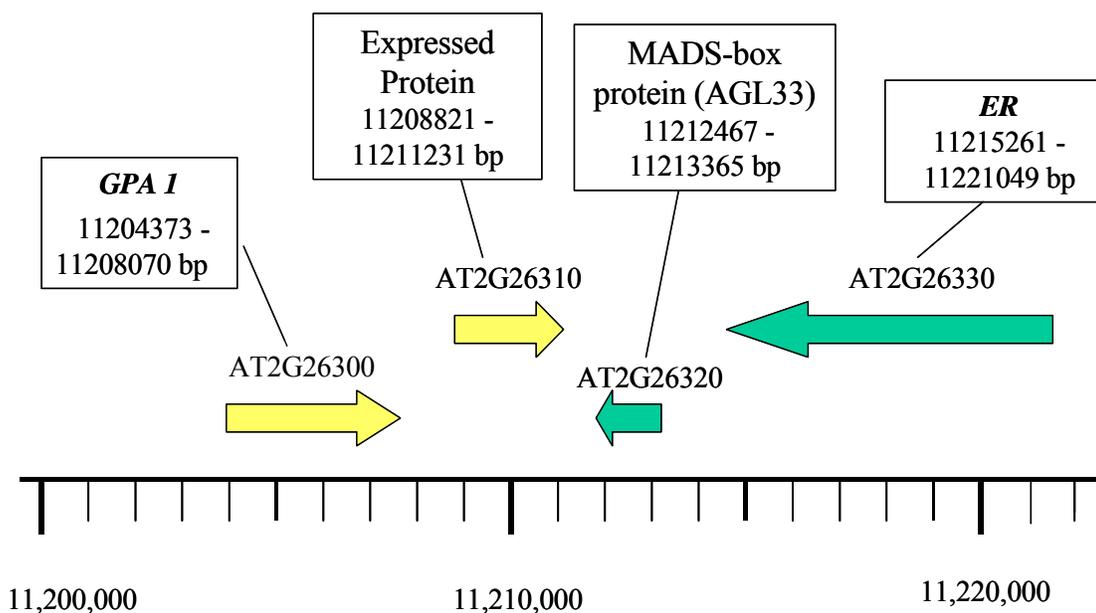

Figure 4.5. *BT5* was mapped to a region between *GPA1* and *ER* markers containing two open reading frames.

these, AT2G26310.1 is an expressed protein of unknown function. The other is AT2G26320.1 a MADS-box protein (AGL33), containing a Pfam profile PF00319: SRF-type transcription factor (DNA-binding and dimerization domain) (Figure 4.5). These data suggest that one of these two genes is the *BT5* trait regulating *PAP1* phosphatase activity.

DISCUSSION

During the process of characterizing the mutant lines *BT1* and *BT2* described previously, reduced levels of *PAP1* mRNA and *PAP1* phosphatase activity were found in *BT3*. It was thought that this reduction in *PAP1* phosphatase activity could be used to map the location of the *BT3* mutation. *BT3* was crossed to the Landsberg *erecta* ecotype for mapping using molecular markers. We were surprised, however, when Landsberg *erecta* showed the same phenotype as *BT3* for the *PAP1* phosphatase activity trait that was to be mapped. Since Landsberg *erecta* failed to have a different phenotype in terms of *PAP1* phosphatase activity, this cross between *BT3* and Landsberg *erecta* could not be used to map the trait.

The phenotype for observed *PAP1* phosphatase activity was different between the Columbia wild-type control and the Landsberg *erecta*, with Columbia showing *PAP1* phosphatase activity and Landsberg *erecta* showing the absence of *PAP1* phosphatase activity. This trait could be scored as Columbia or Landsberg *erecta*, and thus mapped using the Lister and Dean recombinant inbred lines.

Recombinant inbred lines from Lister and Dean (1993) were used to map the difference in *PAP1* phosphatase activity between the Landsberg *erecta* and Columbia ecotypes. Using the 67 initial Lister and Dean molecular markers, it was determined that the *PAP1* phosphatase regulatory trait, renamed *BT5,* mapped to a region of Chromosome 2, very close to the *ER* marker near *PAP1*, but separate from *PAP1*. After a visual inspection for the *ER* marker and analysis of additional molecular markers for that specific region of Chromosome 2, the trait *BT5*, regulating *PAP1* phosphatase

activity, was mapped to an area between *GPA1* and *ER* markers, a span of only 15,562 bp.

These results indicate that the region between the *GPA1* gene and the *ER* gene is a region that should contain the *BT5* trait.   There were only two open reading frames that were found between these two genes. One of these, AT2G26310.1, is an expressed protein of unknown function. The other is AT2G26320.1 a MADS-box protein (AGL33), containing a Pfam profile PF00319: SRF-type transcription factor (DNA-binding and dimerization domain). These findings suggest that one of these is the *BT5* trait.

Since one of these genes, AT2G26310.1, is an expressed protein of unknown function we can make no suppositions about it. The other AT2G26320.1 is a SRT-type or type I MADS-box protein (AGL33). The fact that it is a transcription factor makes it a plausible candidate for the regulation of *PAP1* phosphatase activity.   Also, since this gene is a MADS-box protein (AGL33), containing a Pfam profile PF00319: SRF-type transcription factor (DNA-binding and dimerization domain), or type I MADS-box protein, it is of special interest. Since there have been no mutants found in type I MADS-box proteins, and no known functions in *Arabidopsis* for these genes, it would be very informative if the AGL33 gene was found to be the gene determining the *BT5* trait.

MADS-box proteins are transcription factors that often recruit other transcription factors/proteins into multi-component regulatory complexes involved in many developmental processes (Shore et al., 1995; Messenguy and Dubois 2003). There are two categories of MADS-box proteins. Type I genes differ from type II in that: 1) they contain a different binding site in the MADS domain similar to the SRF protein as opposed to the binding site of type II or MEF2-like proteins (Messenguy and Dubois 2003), 2) they are missing the keratin-like or K box found in the C-terminal domain of type II proteins (De Bodt et al., 2003; Parenicova et al., 2003), 3) most type I proteins have one or two exons as opposed to an average of seven for type II proteins (De Bodt et al., 2003), and 4) most are expressed at very low levels (Parenicova et al., 2003, De Bodt et al., 2003).

While MADS-box proteins are known to interact with other proteins, plant MADS proteins appear to combine mostly with each other (Messenguy and Dubois 2003). While some require other factors or multiple proteins to dimerize, many have been detected with two-hybrid systems. Specifically, it appears that type II proteins preferentially bind to other type II proteins, but can interact with type I proteins. Type I proteins also can form dimers with other type I proteins (Folter et al., 2005).

Almost nothing is known about the function of type I MADS-box genes. All of the known mutants for MADS-box genes in *Arabidopsis* are type II genes (De Bodt et al., 2003; Parencicova et al., 2003). While it has been proposed that these type I MADS-box genes may actually be pseudogenes, most are expressed in different tissues, but at lower levels (Parenicova et al., 2003). This may be that the genes are only expressed under very specific conditions (De Bodt et al., 2003), or they may control much more subtle functions (Parenicova et al., 2003). There has also been no evidence of transposable elements flanking these genes (De Bodt et al., 2003).

Using RT-PCR, Parenicova et al (2003) analyzed the expression levels of the 107 known MADS-box proteins in *Arabidopsis*. In 101 of the 107 MADS-box genes expression of these genes was detected under normal conditions for one or more of the four tissues tested (root, leaf, inflorescence, siliques). AGL33 showed expression in leaf, inflorescence, and siliques under normal conditions. If AGL33 is responsible for regulating the *PAP1* phosphatase trait, it would be the first *Arabidopsis* type I MADS-box protein whose function was known. Also, any other protein/factor(s) associated with or dimerizing with AGL33 to determine its specificity, could hopefully be determined using yeast two-hybrid system or other methods. This would add not only to our understanding of the *PAP1* phosphatase regulation in *Arabidopsis,* but also to our knowledge of type I MADS-box genes and their functions. With these candidate genes, the sequences from this region of the chromosome can now be isolated from Columbia and checked through molecular complementation in Landsberg *erecta* to determine if one of these is indeed the *BT5* trait responsible for *PAP1* phosphatase activity.

CHAPTER V

CONCLUSIONS AND FUTURE PERSPECTIVES

Responses to Pi limitation / starvation illicits a coordinated activation of responses in plants. This coordination of responses to Pi limitation leads to the idea that there may be a Pi starvation-inducible rescue system in plants, a PHO regulon, similar to that found in microorganisms. However, there is not much known about the molecular mechanisms of Pi sensing in plants, or the pathways downstream of this Pi sensing.

BT1 AND *BT2* MUTANT LINES

One of our main goals for this project was to elucidate part of the phosphate-starvation signal transduction pathway, and we chose to do this through mutant analysis. We studied this pathway by observing the expression of the *PAP1* gene. Using a mutant screen devised to look for any plant that showed an abnormal *PAP1* response to phosphate nutrition, two mutant lines were isolated based on their histochemical mutant staining patterns. Once isolated, these two lines were characterized. Analysis of these lines, *BT1* and *BT2*, showed altered Pi starvation-responses to wild-type, the initial transgenic *PAP1::GUS::PAP1* line, and each other. These mutants were dominant mutations linked to the transgene. However, based on the differential expression of starvation-induced genes found in these two mutants and the analysis of an allelism test, it appears that while *BT1* and *BT2* are tightly linked, they are different mutations in different genes.

Using the differential expression found in these two mutant lines, we are able to elucidate more about the Pi starvation-induced signal transduction pathway. These results suggest that the *PHR1* gene, as well as the Pi-sensing mechanism, is intact. *At4, AtIPS1, AtPT1, AtACP5, AtIPS3,* and *RNS1* genes are located downstream of *PHR1* in the Pi starvation-induced pathway (Rubio et al., 2001). *AtIPS1* and *RNS1* are positively regulated in *Arabidopsis* through SIZ1 interaction with PHR1 during the initial stages of

Pi limitation (Miura et al., 2005). *siz1* mutants eventually induce *AtIPS1* and *RNS1* mRNA to levels equivalent to wild-type after an extended period of Pi limitation from 48 to 72hrs indicating that SIZ1 has a transient function in the sumoylation of PHR1 and positive regulation of these genes. (Miura et al., 2005).

After 72 hrs or Pi- starvation, *BT2* mRNA levels of *RNS1* were decreased as compared to wild-type but *AtIPS1* levels were not, nor were any of the other Pi starvation-induced genes examined containing a PHR1 binding site.   In *BT1,* mRNA levels of *PAP1* and *At4* both show reduced Pi starvation-inducibility at 72 hrs as compared to wild-type while all the other genes tested, again containing PHR1 binding sites, do not. This suggests another level of regulation either in addition to SIZ1 interaction with *PHR1*, additional regulation upstream or downstream of this interaction, or alternative pathways controlling the expression of the genes *RNS1* in *BT1*, and *PAP1,* and *At4* in *BT2* that contain PHR1 binding sites (Figure 5.1).

The differential response of the two genes, *At4* and *AtACP5,* known to be downregulated by cytokinins in the roots, suggests another method of control. *At4* was substantially downregulated in *BT1* while *AtACP5* only had one biological sample that was very slightly downregulated in *BT1*. This difference in expression level, along with the controlled levels of hormones in the media, indicated that the downregulation of the *At4* gene in *BT1* is not due to the cytokinin pathway, but rather something else. Since this difference was only apparent under Pi limiting conditions, the data indicated something in the Pi starvation pathway downstream of sensing. The *phr1* mutant showed decreased levels in both of these genes. The downregulation of only one of these genes suggest that the *PHR1* gene is intact in the *BT1* mutant, and that again some additional regulation upstream or downstream of this SIZ1 interaction with *PHR1*, or some other branch of the Pi starvation pathway other than *PHR1* is responsible for this difference in expression.

Finally, *PAP1* expression is unaltered in the *phr1* mutant, as mentioned in Chapter II, with a Pi regulatory site being located in the *PAP1* promoter in a region different than the one containing the PHR1 binding site (Patel unpublished data). This
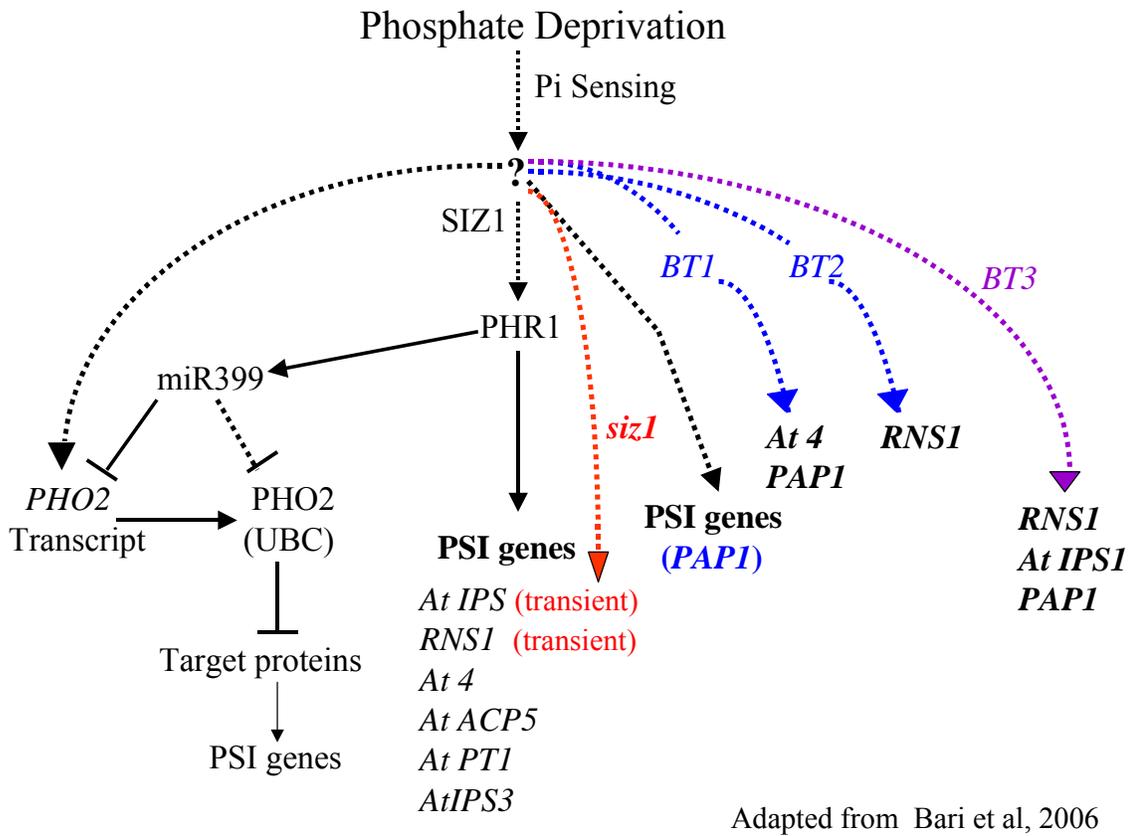
Figure 5.1. *BT1, BT2,* and *BT3* mutants and their relationship in the Pi starvation response pathway.

indicates an additional method of regulation for this Pi starvation-induced gene downstream of the Pi sensing mechanisms other than *PHR1* (Figure 5.1). The differences between *BT1* and *BT2* expression of the Pi starvation-induced genes *PAP1, At4,* and *RNS1*, all containing PHR1 binding sites, also supports the idea of additional methods of regulation for these genes downstream of Pi sensing. Taken together, the data indicate that at least the genes, *RNS1, At4,* and *PAP1,* are not regulated by *PHR1* alone. There is some alternative mechanism for regulating Pi starvation-induced responses in these genes downstream of Pi sensing.

In order to obtain more information, more Pi starvation-induced genes could be examined. Both PHR1 and PHO2 share some common downstream targets (Bari et al., 2006). Also, the information that the Pi starvation-induced miR399 transcripts are substantially less abundant in *phr1* mutants than in wild-type but are not affected in *pho2* mutants, indicates that PHR1 is required for miR399 expression and places *PHR1* upstream of *PHO2* in Pi signaling.   *PHO2* expression could be examined to learn more about the location of the *BT1* and *BT2* mutations in the Pi starvation pathway in relationship to *PHR1* and *PHO2*. Finally, since the mutations are linked to the T-DNA, if we could isolate the plant DNA adjacent to the T-DNA insertion for the mutant line, we should be able to map the mutation.

BT3 MUTANT LINE

During the process of characterizing mutants *BT1* and *BT2* in the Pi starvation pathway, we found that the parental control line showed abnormal staining patterns. Two control lines, *PGP24* and *BT3,* were isolated and characterized. After analyzing these two lines we came to the conclusion that *PGP24* and *BT3* are actually the same mutant and were eventually renamed *BT3*. Northern analysis, using multiple biological replicates of *PGP24*/*BT3*, or *BT3* showed *PAP1* was always downregulated under both +P and -P conditions in both root and leaves.

As mentioned previously, this *PAP1* gene contains a putative PHR1 binding site of GAATATCC in the promoter at -159bp from the ATG start codon. As these *BT3*

mutant lines were examined, a number of other Pi starvation-induced genes were also affected in these plants. Since other Pi starvation-induced genes were upregulated in this mutant as in wild-type, it appears that the Pi sensing mechanism is intact. Some of the genes we analyzed that are upregulated in wild-type under Pi starvation (*AtIPS1, AtACP5, At4,* and *RNS1*) not only have a PHR1 binding site, but to also have reduced Pi starvation-inducibility in the *phr1* mutant lines, the effect being most marked in *AtIPS1* followed by *RNS1* and *At4*. (Rubio et al., 2001). This suggests that *AtIPS1, AtACP5, At4,* and *RNS1* genes are downstream of *PHR1* in the Pi starvation-induced pathway. The *BT3* mutants show differential expression of only some of these genes examined as compared to wild type in both root and shoot. These results again suggest that that the *PHR1* gene itself is intact. Our results also suggest two main groups of genes containing PHR1 binding sites.

The first group containing *PAP1, RNS1,* and *AtIPS1* are greatly reduced or repressed in terms of induction in both leaves and roots under Pi starvation. *AtIPS1* and *RNS1* are both positively regulated through SIZ1 interaction with PHR1 during the initial stages of Pi limitation, while other genes (including *AtPT2*) are negatively regulated (Miura et al., 2005). *PGP24/BT3* lines, unlike the *siz1* mutant, never exhibited levels of induction for *RNS1* and *AtIPS1* comparable to wild type after 48 to 72 hours (Miura et al., 2005). These data suggest that there is an alternative regulation for some of these genes apart from SIZ1 interaction with PHR1 (Figure 5.1). As mentioned previously, *PAP1* expression is unaltered in the *phr1* mutant, and no Pi regulatory site was found in the region of the promoter that contains the PHR1 binding site. Again, this indicates that at least the genes, *RNS1* and *AtIPS1,* as well as *PAP1* are not exclusively regulated by *PHR1* (Figure 5.1). There is some alternative mechanism for regulating Pi starvation induced responses in these genes downstream of Pi sensing.

At4 and AtACP5, the second group of genes, showed only a slight reduction in levels of mRNA in response to Pi starvation as compared to wild-type. *At4* and *AtACP5* appear to respond differently indicating that they may respond to yet another factor or combination of factors.

Due to the differential response of these known Pi starvation-induced genes, it is again indicative of the possibility that there may either be multiple branches to this pathway prior to *PHR1,* or that there may be additional activators or repressors involved with *PHR1* in the Pi starvation-induced pathway. Taken in its entirety, all the data suggest that the Pi starvation sensing and signal control in *Arabidopsis* is complex and includes many different molecular components, many of which may not be part of the "PHR1 regulon" and are still yet to be discovered.

In order to obtain more information about these other components, more Pi starvation-induced genes (including the SIZ1 negatively regulated *AtPT2*) could be examined in the Northern analysis. Again, *PHO2* activity could be examined to learn more about the location of the *BT3* mutation in the Pi starvation pathway in relationship to *PHR1* and *PHO2*.

MAPPING OF *PAP1* PHOSPHATASE REGULATORY TRAIT, *BT5*

Once these mutants were identified, we were able to begin working towards our second goal to map the mutant genes to their location in the *Arabidopsis* genome. As mentioned above, we were unable to map the transgene location and thus locate the *BT1* and *BT2* mutations. Next, in the process of trying to map the mutation in *BT3*, it was discovered that the Landsberg *erecta* phenotype also shared the lack of *PAP1* phosphatase activity while Columbia wild-type showed the *PAP1* phosphatase activity. Since Landsberg *erecta* failed to have a different phenotype in terms of *PAP1* phosphatase activity, this cross between *BT3* and Landsberg *erecta* could not be used to map the trait. Recombinant inbred lines from Lister and Dean (1993) were used to instead map the difference in *PAP1* phosphatase activity between the Landsberg *erecta* and Columbia ecotypes. To be able to map the *BT3* mutation in the Columbia background, it could be necessary to cross *BT3* to another ecotype with a differing phenotype for the *PAP1* phosphatase activity and enough difference in molecular markers to allow for mapping.

Using molecular markers, the *PAP1* phosphatase regulatory trait, renamed *BT5,* was mapped to a region of Chromosome 2 very close to the ER marker near *PAP1*, but separate from *PAP1*. After a visual inspection for the *ER* marker and analysis of additional molecular markers for that specific region of Chromosome 2, the *BT5* trait regulating *PAP1* phosphatase activity was mapped to an area between *GPA1* and *ER*, a span of only 15,562 bp. The mapping data indicated that this region between the *GPA1* gene and the *ER* gene is the region that should contain the *BT5* trait regulating *PAP1* phosphatase activity. There were only two open reading frames that were found between these two genes. One of these, AT2G26310.1, is an expressed protein of unknown function. The other is AT2G26320.1 a MADS-box protein (AGL33), containing a Pfam profile PF00319: SRF-type transcription factor (DNA-binding and dimerization domain). These results suggest that one of these should be the *BT5* gene controlling *PAP1* phosphatase activity.

MADS-box proteins are transcription factors that often recruit other transcription factors/proteins into multi-component regulatory complexes involved in many developmental processes (Shore et al., 1995; Messenguy and Dubois 2003). There are two categories of MADS-box proteins. Type I genes differ from type II in that: 1) they contain a different binding site in the MADS domain similar to the SRF protein as opposed to the binding site of type II or MEF2-like proteins (Messenguy and Dubois 2003), 2) they are missing the keratin-like or K box found in the C-terminal domain of type II proteins (De Bodt et al., 2003; Parenicova et al., 2003), 3) most type I proteins have one or two exons as opposed to an average of seven for type II proteins (De Bodt et al., 2003), and 4) most are expressed at very low levels (Parenicova et al., 2003, De Bodt et al., 2003).

While MADS-box proteins interact with other proteins, plant MADS proteins appear to combine mostly with each other (Messenguy and Dubois 2003). While some require other factors or multiple proteins to dimerize, many have been detected with two-hybrid systems. Specifically, it appears that type II proteins preferentially bind to

other type II proteins, but can interact with type I proteins. Type I proteins also can form dimers with other type I proteins (Folter et al., 2005).

  Almost nothing is known about the function of type I MADS-box genes. All of the known mutants for MADS-box genes in *Arabidopsis* are type II genes (De Bodt et al., 2003; Parencicova et al., 2003). While it has been proposed that these type I MADS-box genes may actually be pseudogenes, most are expressed in different tissues, but at lower levels (Parenicova et al., 2003). This may be that the genes are only expressed under very specific conditions (De Bodt et al., 2003), or they may control much more subtle functions (Parenicova et al., 2003). There has also been no evidence of transposable elements flanking these genes (De Bodt et al., 2003).

  With these two candidate genes, an expressed protein of unknown function and a SRT-type or type I MADS-box protein, the sequences from this region of the chromosome can now be isolated and checked through molecular complementation to determine if one of these is responsible for the *BT5* trait controlling *PAP1* phosphatase activity. Dominance of the trait would need to be determined through the examination of F_1 from a cross between Columbia and Landsberg *erecta* ecotypes.   Also further analysis of the differences, examination of the levels of *PAP1* phosphatase activity in different tissues of Columbia and Landsberg *erecta* ecotypes as well as possibly *BT3* could be correlated with; 1) northern analysis showing the effects on both *PAP1* and other Pi starvation-induced genes for each line, and 2) microarray analysis to determine if any other factors are seen to be differentially induced between the lines under both normal and Pi starvation-induced conditions.

   Since the T-DNA insertion lines for both of the genes mapped to the region between *GPA1* and *ER*, (AT2G26310.1, an expressed protein of unknown function, and AT2G26320.1, a MADS-box protein "AGL33") are unavailable, they cannot be used to verify the identity of the gene responsible for the *BT5* trait through molecular complementation. Alternative approaches will be necessary to determine the identity of this gene.

One alternative approach allowing for positive identification of the *BT5* trait would be to sequence the region of DNA between the *GPA1* and *ER* markers (or at least the two open reading frames) in Col, Ler, and *BT3* lines. Once this has been done, the sequences could be analyzed for any differences that might indicate one of the genes being responsible for the *BT5* trait. Constructs could be designed to over-express this gene (or both genes)using both Col and Ler DNA sequences. The construct over-expressing the Col genomic DNA sequence for the gene could be transformed into Ler plants and checked to see if *PAP1* phosphatase activity is restored. It is however possible that the Ler form of the gene is dominant. In this case, activity would most likely not be restored even if the correct gene was over-expressed in the Ler background. This is why a construct over-expressing Ler genomic DNA form of the gene must also be designed and transformed back into the Col background. In this case, complementation of the gene would be indicated by a loss or absence of *PAP1* phosphatase activity in the Col line.

Comparison between the sequences around the *ER* marker in Ler and *BT3* (with the same *PAP1* phosphatase phenotype under +P conditions) would give additional information since *BT3* did not show induction of *PAP1* mRNA under –P conditions but Ler did induce *PAP1* mRNA under –P conditions (Patel, unpublished data). *PAP1* phosphatase activity gel assays could be performed using plants (leaves) that had been grown in –P conditions.

mRNA levels for the gene(s) could also be checked for Col, Ler, and *BT3* lines. Since the sequences for both genes are known, probes could be designed. The results obtained this analysis could determine whether or not the mRNA is truncated or has a conformational change.

If AGL33 is responsible for regulating the *PAP1* phosphatase trait, it would be the first *Arabidopsis* type I MADS-box protein whose function was known. Other proteins that bind to this MADS-box protein could hopefully then be identified through the yeast two-hybrid systems. However additional methods might be required since some factors that require multi-components to function or stabilize the dimerization

cannot be identified using the yeast two-hybrid system nor can it identify homodimers (de Folter et al., 2005). This would add not only to our understanding of the *PAP1* phosphatase regulation in *Arabidopsis,* but also to our knowledge of type I MADS-box genes and their functions.

Information from this study has expanded our knowledge about the Pi starvation-induced pathway as well as identifying the location of a *PAP1* phosphatase regulatory trait in the *Arabidopsis* genome. This information can be expanded on to increase our understanding of the Pi starvation-induced signal transduction pathway in plants through further analysis, as well as identifying some of the regulatory factors controlling it.

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