Phosphatase Under-Producer Mutants Have Altered Phosphorus Relations[1]

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Phosphorus (P) acquisition and partitioning are essential for plant homeostasis. P is available for plant uptake when in its inorganic form ($H_2PO_4^-$, or P_i), but P_i is often limiting in soils. Plants secrete acid phosphatases (APases) into the apoplastic space, which may be important for obtaining P_i from organic P sources; however, the relative importance of these enzymes for plant P nutrition has yet to be determined. We demonstrate that the root-associated APase pool is increased in Arabidopsis when P_i is limiting and document five APase isoforms secreted from Arabidopsis roots. Previously, we presented the identification of the phosphatase under-producer (*pup*) mutants, which have decreased in vivo root APase staining when grown under low P conditions. Here, we present the characterization of one of these, *pup3*, and further studies with *pup1*. *pup3* has 49%, 38%, and 37% less specific APase activity in exudates, roots, and shoots, respectively. Root-associated APase activity is decreased by 16% in *pup1* and 25% in *pup3*, regardless of P treatment. Two APase activity isoforms are reduced in *pup3* exudates, and root and shoot isoforms are also affected. One of the two exudate isoforms is recognized by a polyclonal antibody raised to an Arabidopsis purple APase recombinant protein (AtPAP12); however, *AtPAP12* transcript levels are unaffected in the mutant. The *pup3* mutation was mapped to 68.4 ± 6.0 centimorgans on chromosome 5. Although P concentrations were not altered in *pup1* and *pup3* tissues when grown in nutrient solution in which P_i was the sole source of P, the mutants had 10% (*pup1*) and 17% (*pup3*) lower shoot P concentrations when grown in a peat-vermiculite mix in which the majority of the total P was present as organic P. Therefore, the *pup* defects, which include secreted APases, are functionally important for plant P nutrition.

Phosphorus (P) deficiency is a major limitation to plant growth (Marschner, 1995; Vance et al., 2003). P is taken up by plants in its inorganic form, P_i or orthophosphate ($H_2PO_4^-$). Although a macronutrient for plants, the availability of soil P_i is often below that of micronutrients because P_i is immobilized within soil organic complexes, clay complexes, and precipitated salts (for review, see Marschner, 1995). Sparingly available soil P_i can be ameliorated with P_i fertilizers, and applied fertilizers represent more than 80% of world P_i use (Steen, 1998). Modern agriculture relies on crops that provide maximum yields with these fertilizers; however, world resources of extractable P_i are limited, nonrenewable, and increasingly ecologically hazardous to obtain (Steen, 1998). As extractable world P_i stores become depleted, agriculture will be forced to adjust to a lack of P_i fertilizer while continuing to feed an expanding population. Alternatively, the organic P (P_o) component of agricultural soil is abundant, representing up to 50% of total soil P (for review, see Vance et al., 2003). Plants may mobilize P_o by secreting enzymes into the rhizosphere, including acid phosphatases (APases), ribonucleases, and deoxyribonucleases (for review, see Abel et al., 2002; Vance et al., 2003). Developing plants with enhanced abilities to access the P_o component of soils is important to decrease agriculture's reliance on P_i fertilizers. Toward that goal, our group focused on the potential of plant-derived secreted APases to increase P_o availability for plant nutrition.

APases may be active against a wide array of organic molecules present in soil P_o. These enzymes are nonspecific orthophosphoricmonoester phosphohydrolases (EC 3.1.3.2), cleaving P_i from ester linkage sites. Secreted plant phosphatases preserve >50% activity over a broad pH range (4.0–7.6), maintain >80% activity over a broad temperature range (22°C–48°C), and are stable at temperatures as high as 60°C (LeBansky et al., 1991; Li and Tadano, 1996), making them ideal candidates for active soil enzymes. While most soil enzymes are typically short-lived, APases can be immobilized on or within soil clays and humates that preserve their activity (Burns, 1986). Soil APases are important for P_i acquisition by plant roots (Marschner, 1995), and plants can use P_o as a sole

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source of P_i nutrition in sterile cultures (for review, see Abel et al., 2002). Therefore, plant-derived secreted APases have the potential to facilitate breakdown of soil P_o to P_i for plant uptake.

The significance of plant-derived secreted APases to plant P nutrition needs to be determined because microbes and fungi within the rhizosphere also contribute to the overall pool of soil phosphatases (for review, see Richardson, 2001). The model plant Arabidopsis is convenient for studying this problem because, unlike many crop plants, it does not form mycorrhizal associations. To test whether plant-derived secreted APases are important for P nutrition, we identified Arabidopsis mutants defective in phosphatase secretion, the phosphatase under-producer (*pup*) mutants. Biochemical characterization of the *pup1* mutant, as described previously, showed that *pup1* is missing a low P-inducible APase isoform and lacks low P-inducible APase staining along its roots but is otherwise not defective in P deficiency responses (Trull and Deikman, 1998).

APases are abundant in Arabidopsis and are represented by at least four gene families. A recent survey of the annotated Arabidopsis genome identified sequences for one His APase, four phosphatidic APases, 10 vegetative storage protein APases, and 29 purple APases (PAPs; Li et al., 2002). Twenty PAP cDNAs were detected by PCR amplification of suspension cell culture cDNAs, and a nomenclature for Arabidopsis PAPs was established based on predicted amino acid sequences. Full-length genes from three APases have been cloned from Arabidopsis: a homolog to mammalian type-5 purple APases known previously as *AtACP5* (*AtPAP17*; del Pozo et al., 1999; Li et al., 2002), a homolog to the kidney bean purple acid phosphatase-1 known previously as *PAP1* (*AtPAP12*; GenBank accession no. U48448; Li et al., 2002), and a homolog to the soybean vegetative storage proteins (*AtVSP1*; Berger et al., 1995). Transcript accumulation of *AtPAP17* is induced in both roots and shoots by low P conditions as well as abscisic acid, peroxide, and senescence (del Pozo et al., 1999). *AtVSP* transcripts accumulate to high levels in flower parts but are also expressed at lower levels throughout the rest of the plant. Methyl jasmonate and Suc up-regulate transcripts, while high P_i levels depress *AtVSP* expression (Berger et al., 1995). *AtPAP12* is induced by low P conditions (Haran et al., 2000; Li et al., 2002) and has a signal peptide sufficient for secretion of a marker protein from roots into the surrounding medium (Haran et al., 2000). A secreted APase from Arabidopsis exudates has recently been purified and characterized (Coello, 2002). This APase hydrolyzes a variety of P_o compounds with kinetic properties similar to other APases studied, including tartrate resistance, inhibition by Mo, P_i, F, and V, and activation with Ca. The protein and DNA sequences of this secreted enzyme have not yet been determined. Since APases are such a large group containing multiple gene families, identification of the secreted isoforms for further functional analysis is important for evaluating their importance to plant P nutrition.

Two Arabidopsis mutants have been described that are defective in P starvation responses, *pho3* (Zakhleniuk et al., 2001) and *phr1* (Rubio et al., 2001). The *Phr1* gene has been cloned, and it is a transcription factor that likely mediates many low P responses, including the up-regulation of high-affinity transporters and ribonucleases as well as APases (Rubio et al., 2001). By contrast, the *pup* mutants are defective in APase secretion but otherwise respond normally to P deficiency, making them good candidates to test whether plant-derived APases are important for plant P relations. If plant-derived APases are important for P nutrition, then mutants with decreased APase activity may have altered P accumulation. In this study, we present the characterization of a second *pup* mutant, *pup3*. We show that both *pup1* and *pup3* have reduced rhizospheric APase activity and that the *pup3* mutation results in lower APase activity in all tissues tested. When provided P_i in solution, *pup* mutants accumulate P normally, but when grown in a substrate containing P_o, the mutants accumulate less shoot P, demonstrating that the *pup* defects impact plant P nutrition.

RESULTS

Phosphatase In Vivo Activity Staining Is Reduced in *pup3* Roots

The *pup* mutants were identified by screening for reduced secreted APase activity when seedlings were grown in −P medium containing the APase stain 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Trull and Deikman, 1998). Under these conditions, *pup3* exhibits less intense staining than the Columbia (Col) ecotype control (Fig. 1). The *pup3* phenotype is much less dramatic when compared to that of *pup1*, which completely lacks staining for APase activity with this substrate (Trull and Deikman, 1998).

Specific APase Activity Is Reduced in *pup3* But Not *pup1*

As a first step toward characterization of the *pup3* defect, specific APase activity measurements were carried out on concentrated root exudates and protein extracts from root and shoot tissues (Fig. 2A). Relative to Col control plants, the *pup3* mutant had 49%, 38%, and 37% less specific APase activity in exudates, roots, and shoots, respectively. *pup1* exudates showed no change in specific APase activity (data not shown), and APase activity in tissue extracts was shown previously to be normal (Trull and Deikman, 1998).

The Rhizospheric APase Pool Is Reduced in *pup* Mutants

An in vivo root-associated APase activity assay was developed to quantify the reduction in APase activity

Figure 1. In vivo APase staining of *pup3* roots under −P conditions. When cleaved by APases, the substrate BCIP forms a blue precipitate on the root surface. The *pup3* mutant was identified by reduced in vivo APase staining of 5-dpg seedlings when grown with minimal P_i.

staining and determine whether rhizospheric APase is affected in the *pup* mutants (Fig. 2B; Table I). The root-associated APases measured in this assay (and discussed throughout the text) consist of those bound to cell walls at the root surface, those located within the apoplastic space of the root, and those secreted from the root during the course of the assay. Similar assays have been performed on whole root systems of tomato (Boutin et al., 1981), rye, buckwheat, clover, and wheat (McLachlan, 1980a) using an assay buffer containing high levels of sodium acetate. We found that this buffer yielded high levels of APase activity but was inappropriate for the delicate Arabidopsis root system, producing sporadic results with obvious tissue wilting. Instead, we altered the growth medium to serve as the assay buffer by increasing its buffering capacity with MES and balancing it osmotically with Suc.

Using this method, we observed that Arabidopsis, like other plants studied with this technique, increases its root-associated APase activity in response to low P conditions (Fig. 2B). A 2 d −P treatment imposed on adult tissues previously grown under sufficient P conditions increased root-associated APase activity by 22% in Col and 25% in Wassilewskija (WS; Fig. 2B; Table I, $P < 0.0001$). The *pup* mutants responded to the −P treatment with the same relative magnitude as their controls (no genotype × P treatment interaction; Table I). Taking both P treatments together, root-associated APase activity was reduced by 16% in *pup3* and by 25% in *pup1* (Table I, $P < 0.0001$). These decreases in root-associated APase activity indicate that the *pup* mutants are defective in the rhizospheric APase pool.

P concentrations were measured from tissues used in the root-associated APase activity assays (Table I). Although the −P treatment dramatically lowered P tissue concentrations by approximately 25% in roots and more than 30% in shoots (data not shown), mutant plants did not differ from their controls in P accumulation under these conditions (no genotype × P treatment interaction; Table I). This result rules out the possibility that the *pup* mutants' decreased root-associated APase activity is an indirect result of increased P_i accumulation and subsequent repression of APase activity.

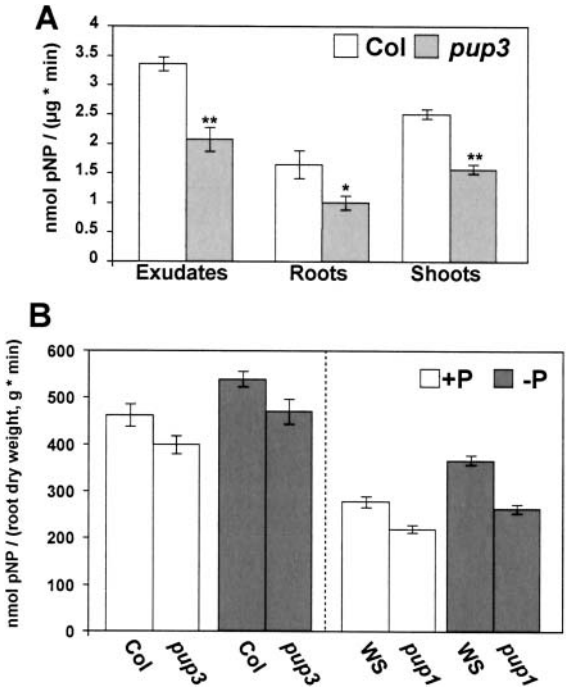

Figure 2. APase activity is decreased in the *pup* mutants. A, Specific APase activity. The hydrolysis of pNPP at 25°C over 10 min was measured for 0.5 µg of protein ($n = 12$ for exudates, $n = 6$ for roots, and $n = 6$ for shoots). For exudates, each protein sample (n) was the concentrated proteins released into liquid culture media over 2 d from 12 plants, and for roots and shoots each protein sample (n) was extracted from pooled tissues representing at least 20 plants. Error bars are SE. Statistical differences between *pup3* and Col for each type of extract were determined with Student's *t* test (*, $P < 0.05$; **, $P < 0.001$). B, Root-associated APase activity. Intact root systems were assayed for APase activity by hydrolysis of pNPP and corrected for root dry weight. One representative replication is shown with $n \geq 8$ for each treatment. Each sample (n) is the activity of three plants grown together in liquid culture. Error bars are SE. Statistical analyses were performed with ANOVA (Table I).

Table I. *Statistical analyses of the root-associated APase activity experiments*

ANOVA was carried out on root-associated APase activity measurements (Fig. 2B) as well as the P concentrations of tissues used in these experiments. Replication effects were not significant for root-associated APase activity after transformation (measurements were expressed as a percentage of that replication's average +P control). Replication effects were insignificant for the P concentration data sets without transformation and as such were eliminated as components of variance. df, degrees of freedom.

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

*$P < 0.0001$.

pup3 Is Defective in the Activity of Some APase Activity Isoforms

APase isoform analysis was carried out to identify the nature of the decreased activity in the *pup* mutants (Fig. 3). Concentrated root exudates (Fig. 3A), shoot protein extracts (Fig. 3B), and root protein extracts (Fig. 3C) were run on native PAGE and stained for APase activity. Heavy and heterogeneous glycosylation (Stahl et al., 1994) of these proteins likely caused the bands to migrate nonuniformly in the gels, giving a smeared appearance. Five major APase activity isoforms were detected in Arabidopsis exudates. Molecular weights cannot be estimated on a native gel because SDS was not present to linearize and uniformly charge the proteins, so APase isoforms are denoted as E1, E2, etc. The slowest-running secreted APase isoform, E1, was −P inducible, while the other four isoforms were not affected by P deprivation. Two bands of constitutive APase activity, E2 and E3, were reduced in *pup3* exudates. Reductions in APase activity isoforms were also observed in *pup3* shoots (Fig. 3B) and roots (Fig. 3C), but these differences were less obvious because multiple APase isoforms migrated together and nonuniformly under the native conditions necessary to retain their maximum activity. It is possible that multiple isoforms may also be affected in *pup3* roots and shoots but could not be detected either because they did not retain activity throughout extraction and electrophoresis or because they comigrated with one of the many other APase isoforms found in these tissues.

When extracts were run on SDS-PAGE under non-reducing and otherwise native conditions, *pup3* shoot proteins were missing a 90-kD APase isoform (Fig. 3D). This is different from the 160-kD isoform shown previously to be missing in *pup1* under these conditions (Trull and Deikman, 1998). APase isoforms from *pup1* exudates were not different from controls (data not shown).

AtPAP12 Is One of the APase Isoforms Defective in *pup3*

Protein and transcript accumulation of a low P-regulated PAP (AtPAP12) was carried out to analyze the effects of the *pup* mutations on this secreted APase. In exudates, one APase isoform, E3, was recognized by the AtPAP12 antibody (Fig. 4A). The decreased APase activity of E3 in *pup3* extracts (Fig. 3A) correlated with reduced reactivity of E3 with the AtPAP12 antibody. The other isoform with reduced *pup3* APase activity, E2, was not recognized by the AtPAP12 antibody.

The AtPAP12 antibody reacted with three isoforms in Col shoots (Fig. 4B) and two isoforms in Col roots (Fig. 4C). The multiple isoforms recognized in extracts from these tissues may have been due to AtPAP12 dimerization (Schenk et al., 2000), cross-reactivity with

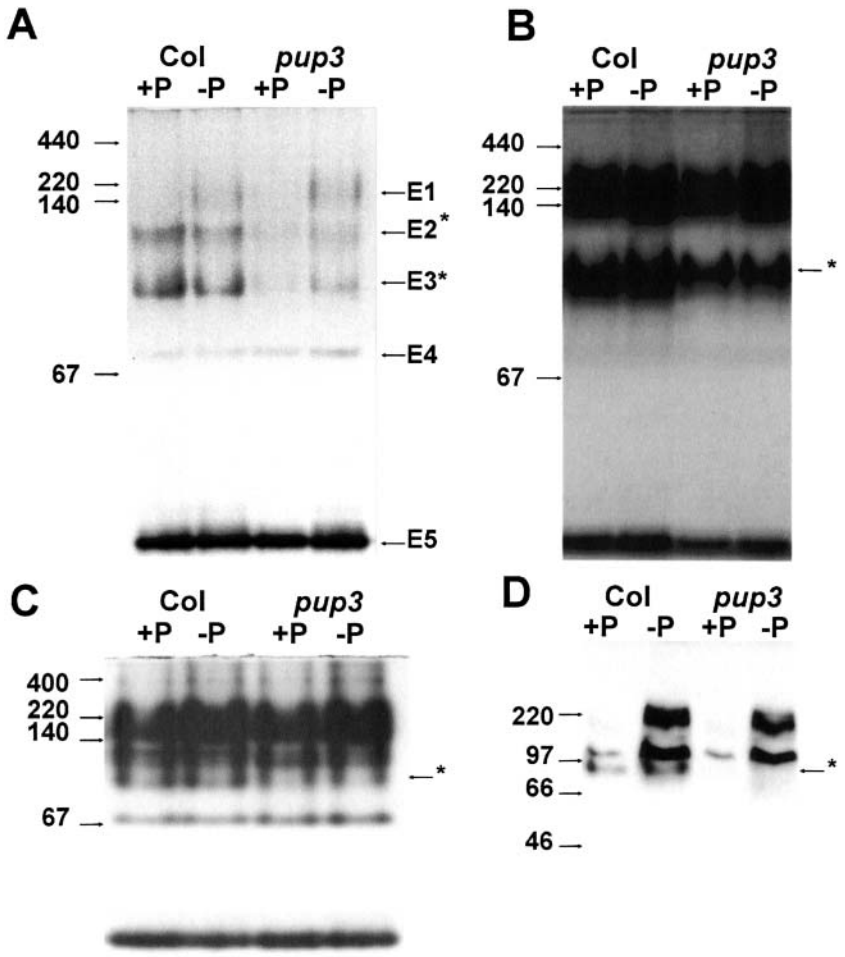

Figure 3. A subset of APase activity isoforms is reduced in *pup3*. A–C, Proteins were run under native discontinuous PAGE conditions and stained for APase activity using Fast Black K and β-naphthyl acid phosphate. Markers are native protein electrophoresis markers (left), and APase isoforms are noted on the right. Isoforms designated with an asterisk have decreased activity in *pup3* exudates. Samples were 7.5 μg of concentrated root exudate proteins (A), 28 μg of shoot proteins (B), and 10 μg of root proteins (C). D, Shoot extracts run under SDS-PAGE but otherwise native conditions and subsequently stained for APase activity also show a reduced *pup3* isoform. Markers for D are Rainbow high M_r markers (Amersham Pharmacia Biotech).

other PAPs (this antibody cross-reacts with a PAP from *Spirodela oligorrhiza*; Nakazato et al., 1998), or cross-reaction with carbohydrates from other glycoproteins (Miller et al., 2001). As in exudates, these patterns were altered in the *pup3* mutant. The AtPAP12 antibody had reduced reactivity with the upper two isoforms in *pup3* shoot extracts (Fig. 4B). Of these two bands, the lower one corresponds to the APase isoform with decreased activity in *pup3* shoot extracts (Fig. 3B). The AtPAP12 antibody did not recognize the lower isoform in *pup3* root extracts (Fig. 4C), and this isoform migrated at the same location as the isoform that had reduced APase activity in *pup3* (Fig. 3C).

Accumulation of *AtPAP12* transcripts was analyzed to determine if the *pup3* defects occur at this level of regulation (Fig. 4D). The expressed sequence tag (EST) used as a probe is the last 550 bp of the 3′ end of the *AtPAP12* cDNA, and only one band was recognized on the blot. As shown previously by two other groups (Haran et al., 2000; Li et al., 2002), *AtPAP12* transcript accumulation was increased under −P conditions (Fig. 4D). However, *pup3* did not differ from control tissues in its accumulation of *AtPAP12* mRNA (Fig. 4D). Therefore, the *pup3* mutation affects AtPAP12 protein but not transcript levels. This may mean that PUP3 is involved in regulating the stability of AtPAP12 proteins, and possibly other AtPAPs as well, because there is at least one other isoform with reduced APase activity in *pup3* exudates that is not recognized by the AtPAP12 antibody.

Proteins from the *pup1* mutant were also tested for differences with regards to immunoreactivity with the AtPAP12 antibody and *AtPAP12* transcript accumulation, and they were not different from controls (data not shown).

P Accumulation Is Altered in *pup* Mutants When Organic P Is Present

Although the *pup* mutants accumulated normal P concentrations when grown in a nutrient solution in which P was supplied entirely as P_i (Table I), the *pup* mutants exhibited altered P relations when P was supplied as P_o within a soil substrate (Fig. 5; Table II). The peat-vermiculite soil mix used in this experiment had 2.67 ± 0.14 μg P/g soil; however, only 0.88 ± 0.02 μg P/g soil was available as P_i. Therefore, the majority of P in this substrate was P_o. Plants were also watered with a weak fertilizer solution (Somerville

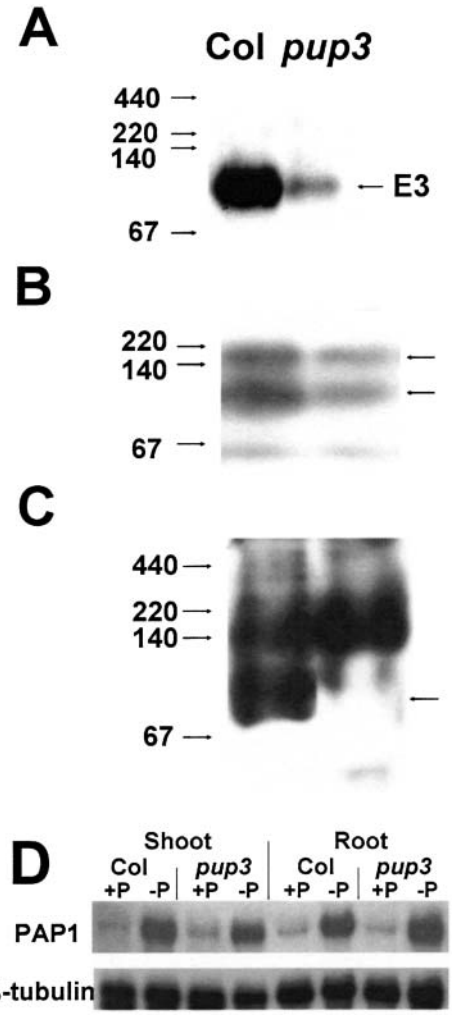

Figure 4. AtPAP12 protein but not transcript accumulation is reduced in *pup3*. Concentrated exudates (A; 7 μg), shoot proteins (B; 7.5 μg), and root proteins (C; 10 μg), were run under native discontinuous PAGE conditions, blotted to a nitrocellulose membrane, and hybridized with the αAtPAP12 antibody. Markers are native protein electrophoresis markers (left). Arrows to the right indicate proteins with reduced reactivity in *pup3*. D, Total RNA (9.25 μg/lane) isolated from 16-dpg plants grown on a continuous treatment of either high or low P was probed sequentially with an EST corresponding to AtPAP12 or β-tubulin (loading control).

and Ogren, 1982) either containing P_i ($+P_i$ fertilizer) or lacking it ($-P_i$ fertilizer).

Shoot P concentrations were lower for both the *pup3* and *pup1* mutants when compared to their controls (Fig. 5A; Table II). In *pup3*, this decrease was 17% lower with the $+P_i$ fertilizer treatment and 13% lower for the $-P_i$ fertilizer treatment. For *pup1*, this decrease was not as pronounced but still significant at 13% for the $+P_i$ fertilizer treatment and 7% for the $-P_i$ fertilizer treatment.

Total P accumulation was also altered in *pup* shoots (Fig. 5B; Table II). For *pup3* the decrease was 28%/$+P_i$ fertilizer and 27%/$-P_i$ fertilizer, while *pup1* accumulated 31% less P in the $+P_i$ fertilizer treatment but the same amount of P in the $-P_i$ fertilizer treatment.

The fertilizer treatment in this study highlights differences between the two ecotypes in their response to applied P_i. While the $+P_i$ fertilizer treatment increased shoot P concentrations in the Col ecotype (Fig. 5A; Table II), the total amount of P accumulated in shoots remained the same (Fig. 5B; Table II). The WS ecotype exhibited the opposite strategy: Shoot P concentrations remained constant with applied P_i fertilizer (Fig. 5A; Table II), while the total amount of P accumulated increased by 30% (Fig. 5B). These results agree with other findings that different Arabidopsis ecotypes demonstrate growth plasticity with regards to P uptake and accumulation (Krannitz et al., 1991). The *pup1* but not the *pup3* mutant was altered in its response to P_i fertilizer. *pup3* was not different from its Col control in this respect (no genotype $\times$ P_i fertilizer treatment interaction; Table II): Shoot P concentrations were increased, while total P accumulation remained constant. However, the *pup1* mutant did not increase its shoot P accumulation when the $+P_i$ fertilizer was supplied (Fig. 5B; Table II, creating the genotype $\times$ P_i fertilizer treatment interaction). Still, both mutants had decreased P concentrations and total P accumulation when compared to their controls at both fertilizer treatments, demonstrating that even the $+P_i$ fertilizer treatment could not compensate for the decreased amount of P_i available to *pup* roots as a consequence of their reduced ability to mobilize P_i from P_o.

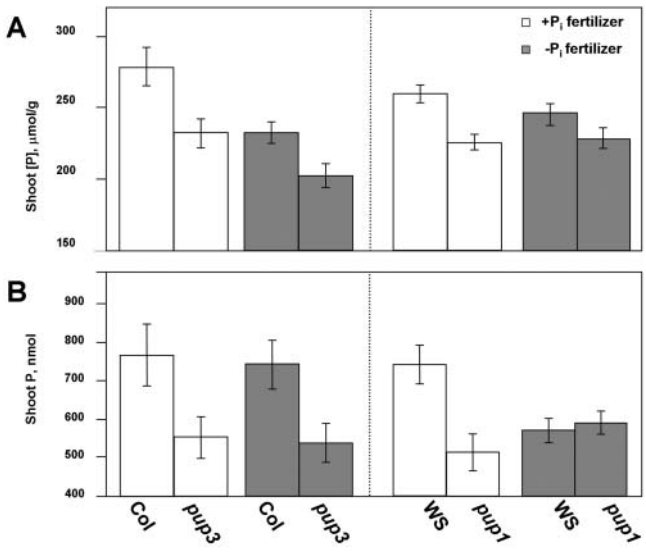

Figure 5. *pup* mutants have decreased shoot P concentration and total P accumulation when grown in P_o soil. *pup* mutants and their respective control plants were grown in a peat-vermiculite soil mix for 3 weeks or until the first sign of the primary reproductive inflorescence, then shoots were harvested for P determinations. Plants were watered with complete fertilizer solution ($+P_i$ fertilizer) or one that lacked the P_i component ($-P_i$ fertilizer). Shoot P concentration (A) is expressed on a per gram dry weight basis, and shoot P accumulation (B) is the total amount of P present in an individual shoot. Each data point represents $n \geq 18$ (pooled data from two replications of $n \geq 9$), and error bars are SE. Statistical analyses were performed with ANOVA (Table II).

Table II. *Statistical analyses of shoot P concentration and total P accumulation between the pup mutants and their respective controls*

ANOVAs were performed with pooled replications. df, degrees of freedom.

Variable	Components of Variance	Error df	*F* Value
P concentration			
pup3 versus Col	Genotype	69	14.3***
	P_i fertilizer treatment		14.1***
	Genotype × P_i treatment		0.7
pup1 versus WS	Genotype	75	14.0***
	P treatment		0.7
	Genotype × P treatment		0.2
Total P accumulation			
pup3 versus Col	Genotype	73	10.6**
	P_i fertilizer		0.1
	Genotype × P_i treatment		<0.1
pup1 versus WS	Genotype	79	5.8*
	P_i fertilizer		1.2
	Genotype × P_i treatment		8.2**

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Genetic Analysis

A mapping population to localize the *pup3* mutation to a chromosomal region within the Arabidopsis genome was developed using an outcross to the WS ecotype. Since APase activity staining was not a reliable method to determine the *pup3* phenotype, the genotype of F_2 progeny was determined based on reduced APase activity from crude shoot extracts (Fig. 6). Of the 110 plants assayed, roughly one-quarter had 70% of the crude shoot APase activity of their control parents, which is comparable to the assays with purified *pup3* shoot proteins (Fig. 2A). The dispersion pattern of this crude assay implies that *pup3* is either a recessive or codominant mutation (Fig. 6). Plants within the lower quartile were confirmed for *pup3* isoform phenotypes by native PAGE before codominant amplified polymorphic sequences (CAPS) mapping (Table III). Three markers on chromosome 5 (NIT4, RBCS-B, and ASB2) are linked to the *pup3* mutation, with RBCS-B demonstrating the tightest linkage to *pup3*. Based on the directionality from the other two markers, the *pup3* mutation is located at 68.4 ± 6.0 cM on chromosome 5. This position places the *pup3* mutation in a different location from both the *pup1* mutation and *AtPAP12*. *pup1* was mapped previously between 34.0 cM and 54.8 cM on chromosome 2 (Trull and Deikman, 1998), and *AtPAP12* is located on BAC T22013 from chromosome 2.

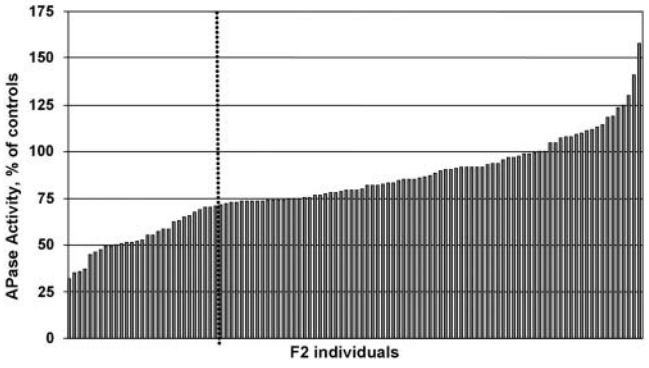

Figure 6. APase activity of the *pup3* mapping population. Crude shoot proteins were isolated from 110 F_2 plants from an outcross to the WS ecotype. APase was assayed and corrected for fresh weight, then compared to the average for Col and WS controls. The dashed line marks the lower quadrant of 29 plants.

DISCUSSION

Plants alter the phosphatase activity of soils. Within the rhizosphere, phosphatase activity is increased and correlates with a zone of P_o depletion around plant roots. Roots, microbes, and mycorrhizal fungi all contribute to rhizospheric phosphatase activity. Free-living soil microbes concentrate around root systems because lysed plant cells and root secretions are sources of carbon and nutrients (Marschner, 1995). While microbes secrete phosphatases that liberate P_i for plants, they also compete with plants for that resource. Mycorrhizal fungi are also a significant source of soil phosphatases, and they can be major providers of P_i to plants when available P is limiting (Miyasaka and Habte, 2001). However, not all plants form mycorrhizal associations, and in nonmycorrhizal roots (such as Arabidopsis), APase activity is associated with the root surface and rhizosphere (McLachlan, 1980a; Dodd et al., 1987). Plants grown in solution culture mobilize a variety of P_o substrates to fulfill their complete P nutritional requirements when deprived of P_i, so plant-derived phosphohydrolases have the capacity to provide for P growth demands (Richardson et al., 2001; Abel et al., 2002). The work presented in this study addresses the importance of

TABLE III. *Genetic location of the pup3 mutation*

CAPS mapping results are shown for markers throughout the Arabidopsis genome. *n* is the number of F_2 plants scored from an outcross to another Arabidopsis ecotype (WS). Significant deviation from the expected segregation ratio of Col/Col:Col/WS:WS/WS (1:2:1; $P < 0.1$) indicates that the marker is linked to the mutation. chr, chromosome.

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

*$P < 0.1$; **$P < 0.01$ (chi square).

plant-derived APases in plant P nutrition using two Arabidopsis mutants that are defective in rhizospheric APase activity.

Previously, the importance of secreted plant APases to plant P nutrition has only been implied. Increased expression of APases under low P conditions is part of an overall P stress response that functions to increase P availability to the plant and is analogous to the microbial PHO regulon (Abel et al., 2002). Proteoid roots are specialized structures whose secretions, including relatively large amounts of APases, increase P adsorption from soils (Vance et al., 2003). When plant-derived APase secretion was compared between efficient and inefficient P genotypes in maize, the results correlated positively (Guame et al., 2001). However, P efficiency is conferred by multiple traits even between recombinant inbred lines of the same species (Yan et al., 2001), which may explain why another study found no significant effect of root-associated APase activity between efficient and inefficient white clover genotypes (Hunter and McManus, 1999). Conversely, a negative correlation between root-associated APase activity and P efficiency was found in a comparison between wheat genotypes domesticated in the low P soils of Australia and their wild progenitors (McLachlan, 1980b). McLachlan reasoned that the domesticated wheat was more successful at obtaining low levels of P and therefore was not inducing low P-responsive APases. The *pup* mutants are good experimental tools for evaluating the relative importance of plant-derived APases because they are only altered from their controls in a single locus, eliminating the genetic variation caused between and among species that may exhibit different strategies for obtaining P.

We establish that Arabidopsis, like other plants previously studied (McLachlan, 1980a; Boutin et al., 1981), increases its root-associated APase pool in response to P deprivation (Fig. 2B). This pool of APases includes apoplastic and cell wall-bound enzymes that would have access to the soil solution and therefore may be important for P acquisition. A major APase isoform has recently been purified from Arabidopsis exudates (Coello, 2002), and we add that at least four more APases are secreted from Arabidopsis roots. The activity of one of these isoforms is increased in response to external P levels (Fig. 3A). Since overexpression of substrate-specific phosphohydrolases such as apyrase (Thomas et al., 1999) and phytase (Richardson et al., 2001) throughout the plant leads to increased ability to take up P_i from those substrates, the overexpression of these secreted APases may lead to an enhanced ability to take up P from a variety of P_o sources. Further analysis of these secreted APases, including their purification, protein sequencing, and genomic sequencing, may be helpful for determining their relative importance to plant P nutrition.

We also present evidence that plant-derived APase activity can be altered by a single mutation, *pup3*. The *pup3* mutant has reduced APase activity as demonstrated by in vivo staining (Fig. 1), root-associated assay (Fig. 2B), and specific activity (Fig. 2A). The amount of reduction in *pup3* APase activity, ranging from 25% to 49% (depending on the localization of the assay within the plant), was unexpected because there are more than 20 actively transcribed APase genes within the Arabidopsis genome (Li et al., 2002). Two secreted APase isoforms are among the APases affected in *pup3* (Fig. 3A). However, only one of these isoforms is recognized by the AtPAP12 antibody (Fig. 3B), and *AtPAP12* transcripts are not altered in the mutant (Fig. 4D). These facts imply that PUP3 may posttranscriptionally modify at least AtPAP12 and one other APase.

The *pup3* mutation maps to chromosome 5 within the Arabidopsis genome between At5g29584 and At5g36210, an area encompassing 2.7 Mb of sequence. This region includes *AtPAP26*, which is closely related to *AtPAP12* (Li et al., 2002), but most genes in this region are annotated as either pseudogenes or genes of unknown function. The AtPAP26 protein is 57% identical and 73% similar to the AtPAP12 protein and of all the AtPAPs is the second most related

sequence. *AtPAP26* cDNAs have been isolated (Li et al., 2002), so it is an active gene. If *pup3* is defective in the *AtPAP26* structural gene, then AtPAP26 would have to affect the accumulation of AtPAP12. This phenomenon may be possible if APase enzymes stabilized each other, but this has not been reported for APases despite extensive isozyme and protein purification studies. *pup3* is distinct from another mutant reported with decreased APase activity, *pho3* (Zakhleniuk et al., 2001). Although genetic mapping was not reported for *pho3*, it is defective in various P deficiency responses. By contrast, our findings show that *pup3* responds normally to low P conditions by inducing P-sensitive root-associated APase activity, APase isoforms, and transcripts (Figs. 2B, 3, A and B, and 4D).

The *pup1* mutant was described previously (Trull and Deikman, 1998), and we add to its characterization here. In the earlier work, *pup1* was shown to be missing an isoform in root and shoot extracts but only when separated in gel systems that preserve disulfide bonds (Trull and Deikman, 1998). Therefore, PUP1 may be required for dimerization of an APase. PAPs dimerize through disulfide bonds, and disruption of these bonds can lead to decreased activity and stability of the protein (Cashikar and Rao, 1996). Previous work also showed that *pup1* lacks APase activity staining along the root surface; however, root and shoot protein extracts have normal specific APase activity (Trull and Deikman, 1998). We add that *pup1* has reduced root-associated phosphatase activity (Fig. 2B) but that the *pup1* isoform is not detected in exudates. One explanation for this data is that PUP1 activity may be restricted to the apoplastic space. The PUP1 isoform may be bound to cell walls, perhaps similar to the glycosylphosphatidylinositol-anchored phosphatase from *S. oligorrhiza* (Nishikoori et al., 2001). Ultimately, the cloning of the *pup* mutations will add to our understanding of this important enzyme family.

Although the rhizospheric APase pool is affected by the *pup* mutations, APase activity in other parts of the plant may be important as well. The effects of PUP3 are not limited to APases secreted into the rhizosphere because the APase activity pool and APase activity isoforms are affected in both root and shoot tissues. Similarly, the PUP1 isoform is present in shoots as well as roots (Trull and Deikman, 1998). Other groups have also reported that cell wall-bound or secreted APases are not exclusive to plant roots but occur in shoots as well. For example, the *AtPAP12* gene has a signal peptide that causes a marker protein to be secreted from roots into the surrounding medium, but studies with its promoter have shown that this gene is transcribed first in shoots in response to P deficiency (Haran et al., 2000). Similarly, a cell wall-localized APase has been isolated from leaf-petiole cell suspension cultures from *Brassica nigra* (Duff et al., 1991), and in soybean APase activity has been localized to shoot vascular tissues and the lower epidermis of leaves (Staswick et al., 1994). In plants, the role for APases throughout the organism in P partitioning has not been established, but the situation may be analogous to the roles for these enzymes in animal systems. For example, PAPs in mice regulate calcium phosphate deposition and mobilization and are essential for proper osteoclastic bone turnover (Hayman et al., 1996). However, not all shoot APases are important for P recycling and regulation; for instance, the segregation of a major leaf APase in common bean recombinant inbred lines did not correlate with increased P use efficiency (Yan et al., 2001). Further uptake and partitioning studies with the *pup* mutants may define the role of these enzymes in P cycling throughout the plant.

The *pup* mutants demonstrate that secreted and/or cell wall-bound APases are important for P nutrition. When grown in a soil mix containing P_o as a major source of P, *pup1* and *pup3* have lower shoot P concentrations (Fig. 5). However, P concentrations are not decreased when P_i is the sole source of P (Table I). Therefore, the *pup1* and *pup3* phenotypes are functionally important when P_o can be utilized as a P source. This implies that proteins affected by the *pup* mutants are important for obtaining and/or maintaining shoot P concentrations when grown in soil. Even when P_i is added to the soil mix, the *pup* mutants cannot concentrate as much P_i in their shoots as their wild-type counterparts. The hydrolysis of P_o into P_i adds to the total amount of P_i available for uptake, and in soils P_i uptake is a diffusion-limited process resulting in a zone of P_i depletion around plant roots (Marschner, 1995). Therefore, decreased hydrolysis of P_o by the *pup* mutants may decrease the total amount of P_i available, regardless of the amount of P_i added to the system by fertilization. The peat used as a soil substrate in this study, though naturally occurring and high in P_o, is not necessarily analogous to agricultural soils with established microbial populations. Therefore, further field studies with these mutants will reveal whether plant-secreted APases are important for plant P nutrition in the context of these microbial populations that also compete for P_o.

MATERIALS AND METHODS

Plant Materials, In Vivo Activity Staining, and Growth Conditions

Identification of the *pup* mutants was based on decreased staining with the APase substrate BCIP or XP (Sigma, St. Louis; Trull and Deikman, 1998). The *pup1* mutant originated from Arabidopsis T-DNA insertion lines in the WS ecotype background but was unlinked to an insertion, while the *pup3* mutant was identified from ethyl methanesulfonate mutagenized Arabidopsis seeds from the Col ecotype background (Trull and Deikman, 1998). *pup3* was backcrossed at least three generations prior to this study to remove unrelated ethyl methanesulfonate mutations.

For the in vivo APase staining, seedlings were grown to 5 d postgermination (dpg) in solid medium (0.5× modified Hoagland salts [Johnson et al., 1957], 0.2% P_i-purified phytagel, 1% Suc, and 0.05% MES, pH 5.7) either +P (1 mM) or −P (0 mM) containing 0.008% BCIP (Trull and Deikman, 1998). When P_i was reduced or eliminated from the growth medium, P_i salts were replaced with appropriate sulfate salts, such that the conjugate cation remained constant. Unless otherwise stated, plants were grown in a growth chamber

under the following conditions: 22°C, ambient relative humidity, white light at 100 μmol m^{-2} s^{-1}, and 16-h-light/8-h-dark photoperiod.

Root-Associated Phosphatase Activity Assays

Seedlings were grown to 7 dpg on +P solid medium (0.5× modified Hoagland salts [Johnson et al., 1957], 0.2% phytagel, 1% Suc, and 0.05% MES, pH 5.7). Plants of uniform size were moved to 10 mL of liquid medium (0.5× modified Hoagland salts with 0.5 mM P_i, 3% Suc, and 2.6 mM MES, pH 5.7), three plants per 150-mL flask. Flasks were shaken at 150 rpm in the dark. After 10 d, plants were rinsed in −P medium, transferred to fresh medium (either +P or −P), and allowed to grow for an additional 48 h.

For the assay, plants were removed from the shaking flasks, briefly rinsed in −P medium, and transferred in a time-dependent manner to rocking Magenta boxes containing 10 mL of reaction buffer (−P medium with 19.1 mM MES, 1.3% Suc, and 5 mM *p*-nitrophenol phosphate, or pNPP). Reactions proceeded for 30 to 50 min at approximately 22°C, then 185 μL of the reaction buffer was removed to 832 μL of 1 N NaOH. *p*-Nitrophenol (pNP) accumulation was read as A_{410} and converted to nanomoles by plotting values against a pNP standard curve generated with assay reagents. Roots and shoots were dissected, transferred to preweighed aluminum foil envelopes, and dried for 2 d at 65°C. Root-associated phosphatase activity was calculated as nanomoles pNP liberated by the root system per min per root dry weight (grams).

Because the two mutants were generated from different ecotype backgrounds, separate experiments were performed with *pup1* and *pup3* with their respective controls. Completely randomized designs were used, and each of these experiments was replicated at least twice.

Tissue Protein Extraction

Plants used as material for protein extractions were grown as described (Muchhal et al., 1996) with the following exceptions. Seeds were plated on 3 × 3-cm, 300-μm mesh nylon filters (Spectra/Mesh; Spectrum Laboratories, Los Angeles) at a density of nine seeds per filter on 0.1× Murashige and Skoog medium, 1% Suc, and 1% agar. After a 2-d 4°C cold treatment, plants were grown under continuous light until 7 dpg, then the nylon mesh filters were transferred to floating membrane rafts (LifeRafts; Sigma) over 100 mL of liquid 0.5× modified Hoagland medium with 1% Suc. Plants were grown for another 7 d, transferred to fresh +P (1 mM) or −P (0 mM) 0.5× modified Hoagland medium with 1% Suc, and grown for an additional 5 d.

For the protein extraction, roots and shoots were ground in liquid N, then ice-cold extraction buffer (0.1 M K-acetate, pH 5.5, 20 mM $CaCl_2$, 2 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride) added to 4 mL/g tissue. Polyvinylpolypyrrolidone was added at 60 mg/g tissue, and the samples were gently agitated at 4°C for 1 h. Samples were centrifuged at 27,000*g* and 4°C for 30 min, the supernatant removed to a fresh tube, and glycerol added to 20% (v/v). Proteins were quantified with the Bradford method using Coomassie Plus protein assay reagent (Pierce, Rockford, IL) and stored at −80°C. Proteins were isolated from three separate plantings.

Exudate Protein Concentration

Growth medium from the 2-d induction period of the root-associated phosphatase study was passed through a 0.2-μm filter to remove debris, then concentrated >200× with Centriplus-10 centrifugal concentrators (Millipore, Bedford, MA) at 4°C according to manufacturer's instructions. Growth media from four separate flasks were bulked together per exudate sample (therefore, each sample, *n*, consisted of exudates from 12 plants). Buffer exchange was carried out with protein extraction buffer during the concentration, and glycerol added to 20% (v/v) after concentration was complete. Exuded proteins were stored at −80°C. Exudates were collected from three separate experiments.

Specific APase Activity

Extracted proteins were dialyzed overnight at 4°C in dialysis tubing (6,000–8,000 M_r cutoff; Spectra/Por; Spectrum Laboratories, Los Angeles) against protein extraction buffer with 20% glycerol to remove P_i and other possible APase inhibitors. To measure specific APase activity, 0.5 μg of protein (<40 μL) was added to 300 μL of prewarmed 10 mM pNPP in 50 mM sodium acetate, pH 5.5. Reactions proceeded for 10 min at 25°C, were stopped with 600 μL of 1 N NaOH, and were quantified as for the root-associated APase assay. Samples from each of three protein harvests were assayed simultaneously.

APase Activity Isoforms

Protein electrophoresis was carried out on 5% stacking/10% resolving (w/v) native acrylamide gels at 30 to 60 V and 4°C. Equal amounts of concentrated exuded proteins (7.5 μg), roots (10 μg), or shoots (28 μg) were loaded for comparison between samples. APase staining was carried out with Fast Black K salt and β-naphthyl acid phosphate as described (Trull et al., 1997). High M_r native electrophoresis markers (Amersham Pharmacia Biotech, Piscataway, NJ) were used to estimate isoform migration. Proteins run under SDS-PAGE but otherwise native conditions and subsequently stained for APase activity were isolated and run as described previously (Trull and Deikman, 1998).

αAtPAP12 Immunodetection

αAtPAP12 polyclonal antibody, generated from recombinant Arabidopsis purple acid phosphatase (*AtPAP12*) gene product (GenBank accession no. U48448), was obtained from Dr. Thomas McKnight (Department of Biology, Texas A&M University). Equal amounts of exuded protein (7 μg) were separated by native PAGE on a Bio-Rad MiniGel apparatus (Hercules, CA) and blotted to a polyvinylidene difluoride membrane with a semidry electroblotter (Panther; Owl Scientific, Woburn, MA). Detection of the antibody/antigen interaction was carried out with the ECL western-blotting analysis system (Amersham Pharmacia Biotech) using a 1:2,500 dilution of αAtPAP12 1° antibody and a 1:1,000 dilution of peroxidase-linked anti-rabbit 2° antibody. Washes and detection were conducted according to manufacturer's instructions using 1× Tris-buffered saline plus Tween 20 (20 mM Tris base, 137 mM NaCl, and 0.1% Tween 20, pH 7.6) as the wash buffer.

RNA Accumulation

Plants for RNA isolation were grown vertically until 16 dpg on either +P (1.2 mM) or −P (9 μM) 0.5× modified Hoagland medium with P-purified phytagel (Trull et al., 1997). Total RNA was isolated using chloroform/phenol/isoamyl alcohol with subsequent LiCl precipitations. Equal amounts of total RNA (9.25 μg) were run on a formaldehyde agarose gel, blotted, hybridized, and washed using standard conditions. Probes were radioactively labeled with the Random Primer DNA labeling system using [^{32}P]dATP (Gibco-BRL, Carlsbad, CA), then run over a Sephadex G-50 spin column to remove unincorporated radioactivity. Blots were hybridized sequentially with 100 ng of a ^{32}P-labeled EST corresponding to *AtPAP12* (EST 155C5; Arabidopsis Biological Resource Center, Ohio State University), then 100 ng of ^{32}P-labeled soybean β-1 tubulin cDNA (Guiltinan et al., 1987) was hybridized to the blot as a loading control after stripping the *AtPAP12* signal. The *AtPAP12* EST 155C5 was excised from the lambda-Ziplox vector (Invitrogen, Carlsbad, CA) using *Not*I and *Sal*I restriction enzymes, gel purified, and confirmed by sequencing. This experiment was carried out three times with identical results.

Plant Growth Conditions in Peat-Vermiculite Soil Mix

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

P Determinations

Total P determinations were carried out on dried tissues using the molybdate method (Murphy and Riley, 1962). For available soil P_i, 0.3 g of soil was suspended in 10 mL of deionized water and the eluate removed for P determination. To determine the total amount of P in the soil, 0.1 g was converted to ash by baking in a 490°C oven for 12 h prior to P determination. Soil tests were repeated three times each.

Statistical Analysis

Statistical analysis was performed with StatView version 5.0.1 (SAS Institute, Cary, NC). The cutoff was $P > 0.1$ for values termed not significant.

Genetic Mapping

A *pup3* mapping population was generated by outcross to the WS ecotype. F_2 mutant segregants were identified first by their decreased APase activity in crude shoot protein extracts. Crude proteins were isolated by grinding individual small leaves from 14 dpg seedlings (grown in peat-vermiculite soil mix) in 500 μL of 50 mM Tris, pH. 7.5, pelleting debris by centrifugation (14,000*g*, 4°C, 5 min), and collecting the supernatant. APase activity measurements were carried out on these extracts by adding 200 μL of extract to 2 mL of 2.5 mM pNPP in 50 mM sodium acetate, pH 4.8, and the reaction carried out at 37°C for 30 min. Reactions were stopped with the addition of 4 mL of 1 N NaOH. A_{420} was read with the Brinkmann PC-800 colorimeter (Westbury, NY). APase activity was calculated as A_{420} per shoot fresh weight per minute and expressed as a percentage of the average between the two control genotypes. Candidate *pup3* protein samples with <75% APase activity compared to Col and WS control samples were quantified using the Bradford method (Bradford, 1976) and run on native activity gels to confirm the *pup3* phenotype by a slight reduction in a major APase isoform when compared to control extracts (data not shown).

The *pup3* mutation was mapped using CAPS as markers (Konieczny and Ausubel, 1993). Chi square analysis was used to determine marker linkage, and the Kosambi method was used to calculate genetic distances from linked markers along a chromosome (Koornneef and Stam, 1992).

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