The Arabidopsis Purple Acid Phosphatase AtPAP10 Is Predominantly Associated with the Root Surface and Plays an Important Role in Plant Tolerance to Phosphate Limitation1[W][OA]

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Induction of secreted acid phosphatase (APase) is a universal response of higher plants to phosphate (Pi) limitation. These enzymes are thought to scavenge Pi from organophosphate compounds in the rhizosphere and thus to increase Pi availability to plants when Pi is deficient. The tight association of secreted APase with the root surface may make plants more efficient in the utilization of soil Pi around root tissues, which is present in organophosphate forms. To date, however, no systematic molecular, biochemical, and functional studies have been reported for any of the Pi starvation-induced APases that are associated with the root surface after secretion. In this work, using genetic and molecular approaches, we identified Arabidopsis (Arabidopsis thaliana) Purple Acid Phosphatase10 (AtPAP10) as a Pi starvation-induced APase that is predominantly associated with the root surface. The AtPAP10 protein has phosphatase activity against a variety of substrates. Expression of AtPAP10 is specifically induced by Pi limitation at both transcriptional and posttranscriptional levels. Functional analyses of multiple atpap10 mutant alleles and overexpressing lines indicated that AtPAP10 plays an important role in plant tolerance to Pi limitation. Genetic manipulation of AtPAP10 expression may provide an effective means for engineering new crops with increased tolerance to Pi deprivation.

Phosphorus (P) is an important structural constituent of many macromolecules and participates in a variety of biological processes, such as photosynthesis, energy conservation, regulation of enzyme activities, and control of many cell signaling pathways. Inorganic phosphate (Pi) is the major form of P taken up from soil by plant roots (Bielinski, 1973). However, plants often encounter a scarcity of Pi in soils of both agricultural and natural systems (Raghothama, 2000). To cope with this nutritional stress, higher plants exhibit sophisticated responses that enhance Pi acquisition and utilization for the maintenance of cellular Pi homeostasis (Vance et al., 2003; Yuan and Liu, 2008). These responses include change of root architecture (i.e. inhibition of primary root growth and enhanced production of lateral roots and root hairs), increased expression of Pi transporter genes, induction and secretion of acid phosphatase (APase) and ribonucleases, and accumulation of anthocyanin in the leaves. Among these responses, Pi starvation-induced (PSI) intracellular APases are likely involved in the remobilization and recycling of Pi from intracellular Pi reserves, whereas the secreted APases are believed to release Pi from organophosphate compounds in the rhizosphere (Goldstein et al., 1988; Duff et al., 1991; Tran et al., 2010a). In most arable lands and ecosystems, organic P compounds, mainly in the form of phytate and their derivatives, account for 30% to 80% of total P (Raghothama, 2000; Richardson, 2009). Transgenic Arabidopsis (Arabidopsis thaliana) and soybean (Glycine max) plants overexpressing a secreted phytase or a purple acid phosphatase (PAP) improved Pi acquisition and increased growth on a medium with phytate as the sole P source (Xiao et al., 2006; Ma et al., 2009; Wang et al., 2009). In contrast, an Arabidopsis mutant, phytase under-producer3, that underproduced secreted APase in root tissues accumulated 17% less P in the shoots than wild-type plants when...
grew in a soil matrix with organic P compounds as the major P source (Tomscha et al., 2004).

Although many reports are available about PSI-secreted APase activities in plants, there is still much to be learned about the corresponding genes that encode these APases and their physiological functions during plant growth under both normal and Pi deficiency conditions. Several PSI-secreted APases have been molecularly characterized in higher plants, including those in white lupin (Lupinus albus; Ozawa et al., 1995; Li and Tadano, 1996; Miller et al., 2001), tomato (Solanum lycopersicum; Bozzo et al., 2002, 2006), common bean (Phaseolus vulgaris; Liang et al., 2010), tobacco (Nicotiana tabacum; Lung et al., 2008), and Arabidopsis (Tran et al., 2010b). Some of these secreted enzymes are PAPs, which are purple in water solution because of a Tyr-to-Fe(III) charge transfer transition (Olczak et al., 2003). Arabidopsis has 29 annotated PAPs that are divided into several subgroups based on their sequence similarity (Li et al., 2002). AtPAP17 (AtACP5) was the first putative PSI-secreted PAP characterized in Arabidopsis (del Pozo et al., 1999). However, the existence of AtPAP17 protein in the apoplastic fluid or root exudates has not been reported, and its function in the plant response to Pi limitation is unclear. Haran et al. (2000) fused a GFP with a signal peptide of AtPAP12 and transformed it into Arabidopsis plants under the control of the AtPAP12 gene promoter. In the culture medium of Pi-starved transgenic plants, a green fluorescent signal could be detected, providing indirect evidence that AtPAP12 is a PSI-secreted APase. Recently, AtPAP12 and AtPAP26 have been purified from the culture medium of Arabidopsis cell suspension and seedlings and biochemically characterized (Tran et al., 2010b). The authors concluded that these two enzymes are the predominant forms of PSI-secreted APases in Arabidopsis. Although it has been generally believed for several decades that PSI-secreted APases play important roles in plant adaptation to Pi limitation, and some studies have shown that overexpression of APase could improve plant growth on P− medium with organophosphates as the major P source, definitive genetic evidence supporting this notion has been lacking. It was only recently that Hurley et al. (2010), using a T-DNA knockout line, unambiguously demonstrated for the first time that AtPP26, a PSI-secreted PAP in Arabidopsis, is essential for efficient plant acclimation to Pi deprivation. The functions of other PAPs in plant responses to low-Pi stress remain largely unknown.

The PSI-secreted APases can be classified into two categories. Those in one category are associated with or bound to the root surface, and those in the other are released into the rhizosphere. The PSI root surface-associated APase activities have long been observed in many plant species (McLachlan, 1980; Boutin et al., 1981; Silberbush et al., 1981). These enzymes are tightly bound to the root surface and cannot be easily washed away by moving water. In most soils, Pi is highly immobile. The Pi liberated from organophosphates by the APases at remote distances from roots may not be efficiently utilized by plants. Thus, the production of root surface-bound or associated APases under Pi limitation may make plants more efficient in increasing the availability of free Pi that can be readily absorbed by root tissues. However, many questions regarding PSI root-associated APases remain unanswered. (1) What are the molecular identities of the PSI-secreted APases associated with the root surface? (2) Is any PSI APase predominantly associated with the root surface rather than released into the rhizosphere after it is secreted from root cells? (3) Do root surface-associated APases and the APases that are released into the rhizosphere share the same molecular identity? (4) Is there only one APase or are there multiple APases that are associated with the root surface for a given plant species? (5) Do the root surface-associated APases play an important role in plant tolerance to Pi limitation, as has been generally hypothesized? Although these questions have been recognized for several decades, they have not been experimentally resolved because none of the PSI root-associated APases has been cloned to date.

In this work, we identified and characterized a PSI-secreted APase, AtPAP10, which is predominantly associated with the root surface after its secretion. Through comparative analysis of growth behaviors of multiple atpap10 mutant alleles and overexpressing lines, we provide compelling evidence that AtPAP10 indeed plays an important role in plant tolerance to Pi limitation. Our results also indicate that AtPAP10 is a promising target for the genetic engineering of new crops with increased tolerance to Pi deprivation.

RESULTS

Identification of the nop1 Mutant

To clone the PSI-secreted APases that are bound to the root surface, we performed a large-scale screen for Arabidopsis mutants with altered secreted APase activity. When Arabidopsis seedlings were grown vertically on Murashige and Skoog (MS) P− solid medium, the PSI-secreted APase activity on the root surface could be visualized by applying an agar solution containing 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), which is a substrate of APase (Lloyd et al., 2001). Cleavage of BCIP by APase produces blue precipitates. With this BCIP staining method, 18 mutants with a completely white root phenotype and two with pale-blue staining were identified after screening approximately 120,000 T3 seedlings from two T-DNA-tagging libraries, which represent about 6,000 independent T-DNA lines (Zhang et al., 2005; Koiwa et al., 2006), and 80,000 M2 seedlings from 4,000 ethyl methanesulfonate (EMS)-mutagenized M1 plants generated in this laboratory. The first isolated white-root mutant was named no acid phosphatase activity1 (nop1-1; Fig. 1A).

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Genetic analysis of the other 17 white-root mutants showed that they were all allelic to nop1-1. Later, two pale-blue mutants, nop1-10 and nop1-11, were also found to be new alleles of the NOP1 gene by DNA sequencing. When nop1-1 was backcrossed to the wild-type parent, the roots of the F1 progeny showed pale-blue BCIP staining, and in the F2 progeny, the ratio of white to pale-blue to dark-blue root phenotype was about 1:2:1, indicating that the nop1-1 phenotype was caused by a single semidominant mutation (Supplemental Fig. S1). The nop1-1 mutant was backcrossed to the wild type four times before further characterization. Compared with the wild type, the nop1-1 mutant did not exhibit obvious morphological changes throughout its life cycle under normal growth conditions. When grown under Pi deficiency for 7 d, no clear difference in growth characteristics between nop1-1 and the wild type was observed (Fig. 1A); after 14 d, however, both the root and shoot fresh weight were significantly lower for nop1-1 than the wild type. Detailed comparisons of growth characteristics among the wild type, multiple nop1 mutant alleles, and NOP1 (AtPAP10)-overexpressing lines are reported below.

Cloning of NOP1/AtPAP10

A map-based cloning approach was used to identify the gene responsible for the nop1-1 mutant phenotype. The nop1-1 mutant (Columbia ecotype) was crossed to a plant with the ecotype Landsberg erecta background to establish a mapping population. With a set of simple sequence length polymorphism and cleaved-amplified polymorphic sequences markers, the NOP1 gene was mapped to a 21-kb region around the middle of chromosome 2 (Supplemental Fig. S2). Sequencing of this region revealed a single-nucleotide change of G to A at position 1,776 in the gene AtPAP10 (At2g16430). Under the Pi-deficient condition, the homozygous plants of two AtPAP10 T-DNA insertion lines (SALK_122362 and CS819843, designated nop1-2 and nop1-3) also showed a white-root phenotype when BCIP was applied (Fig. 1B). To confirm that the mutated AtPAP10 gene is responsible for the nop1-1 phenotype, the genomic and cDNA sequences of AtPAP10 were reintroduced into nop1-1 plants under the control of the cauliflower mosaic virus (CaMV) 35S promoter or AtPAP10’s own promoter. All three gene constructs, AtPAP10:AtPAP10 (genomic), 35S:AtPAP10 (cDNA), and 35S:AtPAP10 (genomic), could complement the nop1-1 mutant phenotype (Fig. 1C). As expected, the APase activity of AtPAP10:AtPAP10 (genomic) plants was similar to that of wild-type plants, and 35S:AtPAP10 (cDNA) and 35S:AtPAP10 (genomic) plants had higher APase activities than the wild type, as judged from the intensity of BCIP staining. Among these three types of transgenic plants, the 35S:AtPAP10 (genomic) plants produced the highest level of APase activity under Pi limitation. Interestingly, APase activity was not detectable in most 35S:AtPAP10 (cDNA) lines or was much lower in 35S:AtPAP10 (genomic) lines under the...
Pi-sufficient condition than that under the Pi-deficient condition, although these transgenic plants produced a similar amount of \(\text{AtPAP10}\) mRNA under both Pi conditions (see Fig. 3D below). These results suggested that \(\text{AtPAP10}\) expression was also under posttranscriptional control.

**Molecular Characterization of \(\text{NOP1/AtPAP10}\)**

The \(\text{AtPAP10}\) gene contains a single open reading frame encoding a protein with a molecular mass of 54.2 kD and a pI of 7.39. The point mutation in the \(\text{nop1-1}\) mutant caused an amino acid change from Glu (E) to Lys (K) at position 340 (Fig. 2A). Alignment of \(\text{AtPAP10}\) with the 100 most closely related protein sequences from various organisms indicated that the mutated E340 residue in \(\text{AtPAP10}\) was completely conserved in all 100 sequences (Supplemental Fig. S3). \(\text{AtPAP10}\) was predicted to have a signal peptide of 26 amino acids at its N terminus for targeting to the secretory pathway (Fig. 2A). A conserved Cys residue was found at position 377, which is thought to form a disulfide bridge between two subunits (Olczak et al., 2003) and which suggested that active \(\text{AtPAP10}\) may exist as a dimer or oligomer. Among the 22 \(\text{nop1}\) alleles, three were caused by T-DNA insertion, four by premature termination of the protein sequence, one by alteration of the splicing site, and 14 by single amino acid change (Fig. 2B). Plant PAPs contain a Fe(III)-Zn (II) or Fe(III)-Mn(II) dimetal nuclear center located in five short blocks of conserved amino sequences. Seven invariant residues contained in the five conserved blocks [D\(\times\)G\(\times\)D\(\times\)Y\(\times\)G\(\times\)N\(\times\)H; invariant residues are represented by boldface letters] are determined to be required for metal coordination (Li et al., 2002). Through a BLAST search, a conserved metallophosphatase domain in \(\text{AtPAP10}\) was localized between residues 150 and 450 (Fig. 2B). The premature stop codon introduced into four \(\text{nop1}\) alleles resulted in complete (\(\text{nop1-6}\)) or partial (\(\text{nop1-7}, \text{nop1-8}, \text{and} \text{nop1-9}\)) elimination of the metallophosphatase domain. Molecular modeling of \(\text{AtPAP10}\) based on the crystal structure of a red kidney bean (\(\text{Phaseolus vulgaris}\)) acid phosphatase (Strüter et al., 1995) revealed that most of the mutated residues are located in loops (Fig. 2C). Among the 14 single amino acid mutations, 12 were involved with the charged amino acids. There were six mutations having the change of Gly (G) to positive-charged Arg (R) or negative-charged Asp (D) and Glu (E). Five mutations contained a change of Arg (R) or Glu (E) to positive-charged Lys (K). All mutations, except those in \(\text{nop1-10}, \text{nop1-11}, \text{and} \text{nop1-12}\), occurred within the metallophosphatase domain and mainly at the two ends of the central \(\beta\)-barrel. In fact, \(\text{nop1-10}\) and \(\text{nop1-11}\) are the two alleles with a pale-blue root phenotype, indicating that these two mutations did not cause a complete loss of enzyme activity. The G-to-E mutation at position 27 in \(\text{nop1-10}\) could interfere with the cleavage of the signal peptide, thus decreasing secretion efficiency for \(\text{AtPAP10}\). Two mutated Gly residues (G169 and G195) are located within the conserved short blocks, near the dimetal nuclear center (Fig. 2A). The amino acid D167 is directly
involved in coordinating one of the two metal ions, which are critical for the metallophosphatase activity. Thus, it is not surprising that mutation of the residues D167, G169, and G195 would disrupt the metal binding and concomitantly cause a complete loss of enzyme activity. Besides being involved in the formation of the active site, this group of mutated charged residues, which participated in the packing of one α-helix to the β-sheet and the displacement of this α-helix, may also affect the ability of AtPAP10 to form an active dimer or oligomer. These mutant alleles will be valuable for further study of the structure-function relationships for AtPAP10 and other related PAPs.

Expression of AtPAP10 Is Specifically Induced by Pi Limitation

To understand how the expression of the AtPAP10 gene is regulated, we first examined its mRNA level in different plant parts by real-time reverse transcription (RT)-PCR. The results showed that AtPAP10 mRNA was ubiquitously expressed in all plant parts, including root, leaf, stem, flower, and silique (Supplemental Fig. S4). The expression levels of AtPAP10 were slightly higher in flowers and 20% to 60% lower in stems, leaves, and siliques than in roots. To determine the tissue specificity of AtPAP10 expression, a 2.0-kb AtPAP10 promoter sequence was fused to a GUS reporter gene and transformed into wild-type plants. The expression of GUS activity was examined in 25 independent transgenic lines. Except for two lines that showed no GUS activity, the other 23 lines exhibited similar tissue-specific expression patterns. The level of GUS activity, however, varied from line to line, probably due to the position effect. The GUS expression pattern of a representative line is reported here. When the AtPAP10::GUS plants were grown under the Pi-sufficient condition, GUS activity in aerial parts was detected in the vascular tissues of leaves, stems, sepals, and petals (Fig. 3A, a–e). GUS activity was also observed in pollen grains, pedicles, and ovules in young siliques but not in mature siliques (Fig. 3A, f–h). In both primary and lateral roots, GUS activity was mainly found in the meristematic region and vascular tissues (Fig. 3A, i–l).

We also examined the AtPAP10::GUS activity during seedling growth. One day after germination, the GUS gene was strongly expressed in whole seedlings regardless of Pi conditions (Fig. 3B). With an increase in plant age on P+ medium, GUS activity decreased in cotyledons and in vascular tissues of roots but did not change in root tips. Seedlings grown on P− medium, however, showed higher GUS expression than those grown on P+ medium starting at day 3 after germination (Fig. 3B), indicating that the expression of AtPAP10 is induced by Pi limitation. Exposure to Pi deficiency not only increased GUS expression but also extended its expression domain from vascular tissue to all the root cells, including epidermal cells and root hairs (Fig. 3C). In addition, we found that AtPAP10::GUS expression was not affected when plants were grown on medium deficient in K or N or were exposed to high salt (150 mM NaCl) or osmotic stress (300 mM mannitol; data not shown).

The induction of AtPAP10 gene expression by Pi limitation in both shoot and root was confirmed by real-time RT-PCR (Fig. 3D). The AtPAP10 mRNA levels were increased 5- and 4-fold in Pi-starved shoots and roots, respectively. In the nop1-1 mutant, the mRNA level of AtPAP10 was only slightly lower than that in the wild type, suggesting that a point mutation in nop1-1 did not significantly reduce the mRNA stability of AtPAP10, although it completely abolished AtPAP10 enzyme activity. The AtPAP10 mRNA level in nop1-2 that carried a T-DNA insertion in its promoter region, however, was dramatically reduced. No AtPAP10 transcript was detected in nop1-3, which has a T-DNA insertion in its fourth intron. The 35S:AtPAP10 line, in contrast, contained a high amount of mRNA regardless of Pi conditions. The mRNA level in nop1-2 and nop1-3 was further examined by RT-PCR. Consistent with real-time PCR results, a trace amount of mRNA was detected in nop1-2, whereas no amplification signal was detected in nop1-3, indicating that nop1-3 was a null allele (Supplemental Fig. S5).

Biochemical Characterization of the AtPAP10 Protein

To study the biochemical properties of AtPAP10, we expressed AtPAP10 in Escherichia coli as a glutathione S-transferase (GST):AtPAP10 fusion protein. This recombinant fusion protein was purified to homogeneity by a combination of affinity chromatography and gel filtration (Supplemental Fig. S6A). The molecular mass of the GST:AtPAP10 fusion protein was estimated by SDS-PAGE to be about 80 kD, a value that was expected given the predicted masses of AtPAP10 (54.2 kD) and GST (26.1 kD).

Metal contents in the purified GST:AtPAP10 fusion protein and the control GST protein were determined using high-resolution inductively coupled plasma-mass spectrometry. Only iron and zinc were found in significant amounts in GST:AtPAP10, and they had a molar ratio of 1:0.75. In contrast, significant amounts of these two elements were not found in the control GST protein. This indicated that the native AtPAP10 protein may contain a bimetal nucleus composed of iron and zinc, as shown by molecular modeling (Fig. 2C). The influence of pH on the activity of GST:AtPAP10 was investigated using p-nitrophenol phosphate (pNPP) as a substrate. The results showed that GST:AtPAP10 could catalyze Pi release from pNPP at pH ranging from 4 to 7, with the optimal values around pH 5 to 6 (Supplemental Fig. S6B). GST:AtPAP10 was enzymatically active at temperatures ranging from 25 °C to 75 °C, with the highest activity at 60 °C (Supplemental Fig. S6C). This indicated that the native AtPAP10 may be a relatively thermostable enzyme. GST:AtPAP10 was also tested for its phosphatase activity on a variety of substrates, with its activity against pNPP taken as
100% (Table I). GST:AtPAP10 was highly active on ATP, ADP, dATP, pyrophosphate, and polyphosphate. It displayed only a low level of activity toward phytate and showed no activity toward AMP. Interestingly, GST:AtPAP10 was highly active on phospho-Ser. Its activity toward phospho-Thr and phospho-Tyr, however, was undetectable.

AtPAP10 Is Predominantly Associated with the Root Surface

The profile of APase activity in Arabidopsis can be determined by in-gel assay (Trull and Deikman, 1998). With this assay, three APase isoforms, designated A1, A2, and A3, were observed in root extracts and culture medium of wild-type plants (Fig. 4, A and B). To determine which APase isoform was encoded by the *AtPAP10* gene, we compared the APase profiles among the wild type, *nop1-1*, *nop1-2*, and a 35S:*AtPAP10* (genomic) transgenic line (hereafter referred to as 35S:*AtPAP10*). In the root extracts (Fig. 4A) and culture medium (Fig. 4B), there were no obvious differences in APase profiles between the wild type and the two *nop1* mutant lines. In the 35S:*AtPAP10* line, however, two new isoforms with molecular mass around 250 kD were observed in both root extracts and culture medium, suggesting that AtPAP10 proteins existed as oligomers; the precise molecular mass of the APase isoforms in these in-gel assays could not be determined because the proteins were extracted and electrophoresed under nonreducing conditions. Notably, the intensity of these new bands was higher in P2 plants than in P+ plants (Fig. 4, A and B), suggesting a posttranscriptional control of this APase activity.

To confirm that the new, high molecular mass bands represented AtPAP10 proteins, we generated transgenic plants that constitutively expressed a hemagglutinin (HA)-tagged AtPAP10 fusion protein under the control of the 35S promoter in the *nop1-1* background. The transformation of the 35S:*AtPAP10-HA* construct could complement the mutant phenotype, indicating that the AtPAP10-HA fusion protein was functional (data not shown). Total proteins were extracted from 14-d-old seedlings under both nonreducing and reducing conditions and were subjected to western blot. When proteins were extracted under the nonreducing condition and separated on a SDS-PAGE gel, a band with molecular mass around 250 kD was detected in both P+ and P2 seedlings by anti-HA antibodies (Fig. 4C, lanes 1 and 2). In P2 seedlings, a very faint band with molecular mass of 70 kD was also detected.
observed. When proteins were extracted under the reducing condition, however, only a 70-kD band was detected by anti-HA antibody (Fig. 7C, lanes 3 and 4). These results confirmed that AtPAP10 exists as an oligomer in vivo. The western-blot results showed that the AtPAP10-HA protein was more abundant in P$^2$ plants than in P$^+$ plants regardless of extraction conditions, further supporting the notion that the expression of AtPAP10 was posttranscriptionally regulated by Pi limitation. Because the predicted molecular mass of the AtPAP10-HA protein was 60 kD, these results also indicated that some posttranscriptional modifications, such as glycosylation, might occur and increase the molecular mass of the AtPAP10 protein to 70 kD. Glycosylation has been reported to occur on AtPAP12 and AtPAP26, two PSI-secreted APases that are closely related to AtPAP10 (Tran et al., 2010b).

Alternatively, the unusual folding or charge distribution on the protein surface may also affect the migration of the AtPAP10 protein.

The results from in-gel assays showed that AtPAP10 could hardly be detected in culture medium and root extracts of wild-type plants. Thus, AtPAP10 might be predominantly associated with the root surface after secretion rather than being released into the rhizosphere. To test this hypothesis, we first quantitatively compared the total APase activity in culture medium among wild-type, nop1-1, nop1-2, and 35S:AtPAP10 lines. One hundred 14-d-old seedlings of uniform size were placed in 100 mL of liquid culture medium and grown for 7 d under both Pi conditions. The culture medium was then collected and concentrated to 500 $\mu$L before APase activity was determined. BCIP and pNPP were used as substrates for quantitative enzyme assays, and similar results were obtained with both substrates. Consistent with the results of the in-gel assays, the mutation of the AtPAP10 gene did not cause a significant reduction in total APase activity in the culture medium of nop1-1 and nop1-2 mutants, and APase activity was only about 10% to 15% greater with the 35S:AtPAP10 line than with the wild type (Fig. 5, A and B).

We then compared the root surface-associated APase activities among wild-type, nop1-1, nop1-2, and 35S:AtPAP10 lines. For such comparison, two 12-d-old seedlings grown on P$^+$ solid medium were
transferred to 2-mL Eppendorf tubes that contained 0.6 mL of P+ or P− liquid medium. After growing for another 3 d, the APase activity in the culture medium and the root-associated APase activity of the seedlings were determined. Once again, the total APase activities in the culture medium of all plants at the time examined were very low and did not statistically differ among the four types of seedlings, regardless of P+ or P− conditions (Fig. 5C). For all plants, however, the total root-associated APase activity was much higher than the APase activity in the culture medium. This indicated that most PSI APases were associated with the root surface rather than being released into the rhizosphere. Furthermore, the root-associated APase activities were 20% to 30% lower in nop1-1 and nop1-2 than in the wild type regardless of growth conditions (Fig. 5C). In contrast, the root-associated APase activity was two times greater in 35S:AtPAP10 plants than in wild-type plants. The total root-associated APase activities were also compared among wild-type, nop1-2, and 35S:AtPAP10 lines using BCIP and the naturally occurring organophosphate compounds ADP, ATP, phytate, and Fru-6-P as substrates. Similar results were obtained (i.e. total root-associated APase activity against all the substrates tested was lower in the nop1-2 mutant and higher in the 35S:AtPAP10 line; Fig. 5D).

The remaining APase activity in nop1-1 and nop1-2 may represent other APase isoforms that are associated with root surfaces. This seems to be in conflict with the result that a single AtPAP10 gene mutation resulted in a complete loss of BCIP staining on root surfaces (Fig. 1, A and B). We reasoned that the remaining APase activities may consist of several different APase isoforms and that each of them may make only a small contribution to the total root-associated APase activity or that each has a low affinity to BCIP. Thus, elimination of any isoform of these APases would not cause an obvious reduction in total APase activity that could be detected by the BCIP staining method. In other words, the concentration of BCIP used for our mutant screening could detect the change in AtPAP10 enzyme activity but was not sufficient to detect the changes in the activities of other root-associated APases. This inference is supported by the fact that, when the concentration of BCIP used for staining was increased from 0.01% (the concentration used for our mutant screen) to 0.16%, the roots of nop1-1 and nop1-2 were stained as blue as those of the wild type (Fig. 5E).

Next, we generated transgenic lines that expressed AtPAP10-GFP fusion proteins under the control of CaMV plants against different substrates. The names of the substrates are indicated at the bottom of the chart. The experiments in A through D were repeated three times with similar results. Values represent means with SE of six replicates. Asterisks indicate significant differences compared with the wild type (P < 0.05, t test). E, Detection of APase activities by BCIP staining on the root surfaces of wild-type, nop1-1, and nop1-2 seedlings grown on P− medium. The concentrations of BCIP used are indicated at the left bottom corner.
35S or its own promoter. These two gene constructs were transformed into nop1-1, and the mutant phenotype could be rescued, indicating that the AtPAP10-GFP proteins were functional (data not shown). As a positive control, the GFP signal in the 35S:GFP line was uniformly distributed in cells of all types of root tissues (Fig. 6A). Eight lines of 35S:AtPAP10-GFP and seven lines of AtPAP10:AtPAP10-GFP were examined for expression patterns of AtPAP10-GFP. The results from one representative line of each construct are presented here. As a positive control, the GFP protein expressed from the 35S promoter was observed everywhere in the root cells (Fig. 6A). For the 35S: AtPAP10-GFP line, a layer of strong green fluorescence on the root surface was observed under both P+ and P− conditions (Fig. 6B; data not shown). The strong GFP signal was also observed on the surface of root hairs (Fig. 6D). In individual root cells of the 35S: AtPAP10-GFP line, the green fluorescence signal was observed in a punctate pattern. In the AtPAP10: AtPAP10-GFP line, the GFP signal was also detected in all root cells and on the root surface, including root hairs, but overall, the signal intensity was much lower and was more enriched in vascular tissues relative to the 35S:AtPAP10-GFP line (Fig. 6, C and E). The low GFP signal in nonvascular tissues of the AtPAP10:AtPAP10-GFP line (Fig. 6C) was consistent with the expression patterns of AtPAP10-GUS (Fig. 3C). Based on these results, we conclude that AtPAP10 is also an intracellular APase.

**Growth of nop1 Mutants Is Reduced under Pi Limitation**

To elucidate the physiological functions of AtPAP10 in tolerance to Pi limitation, we compared the growth characteristics of wild-type plants with that of four strong nop1 alleles (i.e. nop1-1, nop1-2, nop1-3, and nop1-6, whose roots did not exhibit BCIP staining) or one weak allele (i.e. nop1-10, a line with roots that exhibited pale-blue BCIP staining). The seeds of the wild-type and various nop1 lines were sown directly on the P+ and P− media and on P− medium supplemented with 10, 50, and 150 μM ADP. After 14 d, fresh shoot and root weights were determined for each line. On all the media except P+, shoot and root fresh weights of four strong nop1 alleles were significantly lower than the wild type (Fig. 7, A and B). The reductions in shoot and root fresh weights ranged from 15% to 30%. For the weak nop1-10 allele, differences in growth relative to the wild type varied depending on the medium conditions, but overall, growth of the nop1-10 line was similar to that of the wild type. The wild type and the strong nop1 alleles also differed morphologically when grown on P− medium (Fig. 7C). On P+ medium or in soil, however, no obvious difference was observed between the wild type and five nop1 alleles (data not shown).

In addition, in the experiments on P− medium with ADP, there was a direct correlation between plant growth and APase activity on the root surface, as revealed by BCIP staining (Fig. 7C). Taken together, our results provide compelling genetic evidence that AtPAP10 plays an important role in plant tolerance to Pi limitation.

**Growth of AtPAP10-Overexpressing Plants Is Enhanced under Pi Limitation**

Because the disruption of AtPAP10 function reduced plant tolerance to Pi limitation, we wondered whether the overexpression of AtPAP10 would have the opposite effect. The seeds of the wild type, nop1-2, and four independent homozygous 35S:AtPAP10 lines were sown directly on P+ medium, P− medium, and P− medium supplemented with 10, 50, and 150 μM ADP. On P+ medium, there were no obvious morphological differences among the wild type, nop1-2, and four 35S:AtPAP10 transgenic lines, although the transgenic line constitutively showed high APase activity on the root surface (Supplemental Fig. S7). Quantitative analysis indicated that the four 35S:AtPAP10 lines produced more shoot biomass than the wild type on the P− medium supplemented with various amounts of ADP, but not on P+ or P− medium (Fig. 8B). Morphological differences between the wild type and the four 35S:AtPAP10 lines were evident when
plants were grown on P− medium supplemented with various amounts of ADP (Fig. 8A; data not shown). However, the differences in root biomass (Fig. 8C) among the wild type, *nop1-2*, and the four 35S:AtPAP10 lines were greater than the differences in shoot biomass (Fig. 8B). Root fresh weight significantly differed among the wild type, *nop1-2*, and the four 35S:AtPAP10 lines on all types of medium tested, except for P+ (Fig. 8C). On P− medium with 150 μM ADP, root fresh weight was nearly four times greater for the four 35S:AtPAP10 lines than for the *nop1-2* mutant. However, cellular Pi and total Pi contents per unit fresh weight did not significantly differ among the wild type, *nop1-2*, and the four 35S:AtPAP10 lines (Supplemental Fig. S8), regardless of medium. These results suggested that, although AtPAP10 plays an important role in Arabidopsis tolerance to Pi deprivation, it may not participate in maintaining plant Pi homeostasis under Pi-deficient conditions.

**The Effects of AtPAP10 Mutation and Overexpression on Root Architecture**

A major developmental response of plants to Pi limitation is change in root architecture, which includes the inhibition of primary root growth and the formation of more and longer lateral roots (Williamson et al., 2001; López-Bucio et al., 2003). These morphological changes together with the enhanced production of root hairs are thought to increase the surface area for Pi absorption. Because growth characteristics were similar among all *nop1* mutants and among all 35S:AtPAP10 lines, we chose one line from each group as a representative for detailed analyses of root growth. The seeds of the wild type, *nop1-2*, and a 35S:AtPAP10 line (line 18) were sown directly on the various media. Fourteen days after seed germination, each seedling was removed from the agar and the length of the primary root and the number and total length of lateral roots were determined. At the same time, the APase activities on the root surface were monitored by BCIP staining to ensure that the lines tested had the root surface-associated APase activity consistent with their genotype. As shown in Figure 9A–B, the 35S:AtPAP10 line developed a larger root system than the wild type or the *nop1-2* mutant on all types of Pi-deficient media, especially on the P− medium supplemented with 150 μM ADP (Fig. 9, A and B). On all types of P− media, primary roots of the wild type were significantly longer than those of *nop1-2* but significantly shorter than those of the 35S:AtPAP10 line (Fig. 9C). The *nop1-2* mutant also showed significant reductions in the number and total length of lateral roots compared with the wild type when grown on all types of P− media (Fig. 9, D and E), whereas the 35S:AtPAP10 line produced more and longer lateral roots than the wild type, especially on the P− medium supplemented with 150 μM ADP. On this medium, the number of lateral roots was about 30% greater for the 35S:AtPAP10 line than for the wild type (Fig. 9E) and the total length of lateral roots was nearly two times greater than that of the wild type (Fig. 9D). When grown on Pi-sufficient medium or in soil, there was no obvious morphological difference among the wild type, *nop1-2*, and the 35S:AtPAP10 transgenic lines (Supplemental Fig. S7). This indicated that AtPAP10 is not essential for plant growth and development under normal nutritional conditions. Also, overexpression of the AtPAP10 gene did not seem to cause any detrimental effects on plant growth and development.
DISCUSSION

AtPAP10 Is Predominantly Associated with the Root Surface

Arabidopsis contains 44 APase genes. To identify novel PSI-secreted APases that play important roles in plant adaptation to Pi limitation, we took a genetic approach to search for candidate genes. Using the BCIP staining method developed by Lloyd et al. (2001), we recovered a group of Arabidopsis mutants with complete or partial loss of BCIP staining on their root surfaces. Molecular cloning indicated that the APase deficiency phenotype of these mutants is caused by a mutation on the AtPAP10 gene.

PSI APases can be associated with root surfaces or can be released into the rhizosphere after they are secreted from root epidermal cells. Until now, however, no root surface-associated APase had been molecularly and functionally characterized. In this work, we provide the following lines of evidence that AtPAP10 is predominantly associated with the root surface instead of being released into the rhizosphere after secretion. (1) Loss-of-function mutation on the AtPAP10 gene did not cause a significant change of the total APase activity in the root tissues and culture medium (Figs. 4, A and B, and 5, A and B). (2) Changes in root surface-associated APase activities in the AtPAP10 mutant lines and the overexpressing line were well correlated with the level of AtPAP10 expression (Fig. 5, C and D). (3) GUS staining of the AtPAP10:GUS transgenic lines confirmed that AtPAP10 is expressed in root epidermal cells and root hairs (Fig. 3C). (4) Confocal image analysis of transgenic plants showed that the AtPAP10-GFP fusion protein was expressed on the surface of root epidermal cells and root hairs (Fig. 6). (5) Consistent with our results, Tran et al. (2010b) did not find AtPAP10 in their efforts to biochemically purify APases from Arabidopsis culture medium. Three previous studies on the cell wall proteome of Arabidopsis suspension cells have indicated that AtPAP10 is a cell wall-bound protein (Robertson et al., 1997; Chivasa et al., 2002; Bayer et al., 2006). So, AtPAP10 is probably tightly bound to the cell walls of root epidermal cells and root hairs after its secretion. These proteomic studies, however, did not show whether AtPAP10 is also released into the culture medium or whether it accumulated in root tissues. Our results clearly indicated that the amount of AtPAP10 protein released into the rhizosphere is negligible. Tran et al. (2010b) demonstrated that AtPAP12 and AtPAP26 are two APases that predominantly accumulated in the culture medium, but they did not report whether these two PAPs were also associated with the root surface. Thus, this report, to our knowledge, is the first to molecularly identify a PSI-secreted APase that is predominantly associated with the root surface after its secretion.

Our analyses of transgenic lines with AtPAP10:GUS and AtPAP10:AtPAP10-GFP constructs indicated that AtPAP10 also exists in plant internal parts, mostly in the vascular tissues of shoots and roots (Figs. 3, A–C, and 6). Although the absolute abundance of AtPAP10 protein may be higher in root internal tissues than on the root surface, our in-gel assay showed that knock-out of the AtPAP10 gene did not cause an obvious reduction of total APase activity in root extracts. This
indicated that AtPAP10 is not a major form of intracellular APase within root tissue.

Biochemical Properties of AtPAP10 and Regulation of Its Expression

Using E. coli-produced recombinant AtPAP10 proteins, we showed that AtPAP10 has the properties of a typical acid phosphatase. In Arabidopsis, AtPAP12, AtPAP26, AtPAP15, and AtPAP23 have been biochemically characterized using either plant cell- or E. coli-produced proteins (Zhu et al., 2005; Kuang et al., 2009; Tran et al., 2010b). All these PAPs exhibit broad substrate specificity, but their substrate spectra do not completely overlap. For example, AtPAP12 and AtPAP26 do not have any phytase activity, whereas AtPAP10, AtPAP15, and AtPAP23 have moderate phytase activity. The relative enzyme activities of these PAPs against a given organophosphate are also different. In addition, Tran et al. (2010b) showed that in culture medium, AtPAP12 exists as a monomer and AtPAP26 as a homodimer. Our results suggested that AtPAP10 probably exists as an oligomer in vivo (Fig. 4C). Alignment of protein sequences among these three closely related members indicated that they contain different numbers of Cys residues, which are located in different positions (data not shown). These differences may explain why these three APases exist in different forms. These subtle biochemical differences also suggest that these APases may have different physiological functions during plant adaptation to Pi deprivation.

Expression of the AtPAP10 gene is specifically induced by Pi limitation but not by nitrogen or potassium deprivation or by salt and osmotic stress. This indicates that AtPAP10 does not function in the responses to these conditions.
other abiotic stresses. Strong expression of AtPAP10 in germinating seeds and cotyledons indicated that AtPAP10 may play some roles during seed germination in mobilizing Pi from phytate, which is a major reservoir of Pi in seeds. This is consistent with the fact that AtPAP10 has phytase activity, although it is not high (Table I). Another interesting observation is that AtPAP10-GUS was expressed in the meristematic region and vascular tissues of roots when plants were grown under Pi-sufficient conditions. Exposure to Pi deficiency not only increases the expression level of the GUS gene but also extends its expression domain from the root vasculature to all types of root cells, including root epidermal cells and root hairs. The AtPAP10:-GUS expression patterns suggest that there is a basal-level expression of AtPAP10 that occurs mainly in vascular tissues when plants are grown under normal growth conditions. Under Pi deficiency, however, all the root cells can produce AtPAP10 and can thus facilitate its secretion. Additional evidence to support this notion is that, for AtPAP10:AtPAP10-GFP lines grown under low-Pi conditions, the GFP signal was detected in all layers of root cells, including root epidermal cells and root hairs, although its expression was stronger in vascular tissues (Fig. 6). Recently, the preferred expression of APase activity in the epidermis and vascular tissues was reported in the roots of Pi-starved white lupin (Wasaki et al., 2008). High quantities of various organophosphate compounds, such as hexose monophosphate, ATP, other nucleotide triphosphates and diphosphates, and phosphocholine, have been detected in the vascular tissues of apple (Malus domestica), celery (Apium graveolens), and Chinese cabbage (Brassica campestris ssp. pekinensis; Bieleski, 1966; Sheldrake and Northcote, 1968). These compounds are thought to be part of a transport form of Pi during Pi recycling in plant bodies. Thus, it is possible that AtPAP10 also functions in vascular tissues of both leaves and roots to unload Pi from these organophosphate compounds.

The Roles of AtPAP10 in Plant Tolerance to Pi Limitation

An important aspect of this work is our clear demonstration of the causal relationship between the AtPAP10 phosphatase activity and plant tolerance to Pi limitation. Our results show that on the various P-deficient media, the overall growth of four strong nop1 alleles is significantly reduced compared with the wild type, whereas the growth of a line with a weak allele is less affected (Fig. 7). In contrast, four AtPAP10-overexpressing lines are relatively tolerant to Pi limitation (Figs. 8 and 9). Our genetic and molecular studies provide direct experimental evidence that a Pi starvation-induced root surface-associated APase plays an important role in plant tolerance to Pi limitation. The differences in tolerance to Pi limitation among the wild type, nop1, and the 35S:AtPAP10 lines may be simply due to their different capacities to scavenge the Pi group from the organophosphate compounds in the culture medium. At the same time, we cannot exclude the possibility that AtPAP10 may also participate in the control of Pi starvation-induced root architecture changes. Our results showed that overexpression of AtPAP10 can cause plants to develop a more robust root system; in contrast to AtPAP10-overexpressing lines, nop1 mutants have fewer and shorter lateral roots than the wild type. Biochemical analyses have indicated that phospho-Ser is an effective substrate for AtPAP10 (Table I), suggesting that some proteins residing on walls of root epidermal cells may be its natural targets. NtPAP12 is a cell wall-bound PAP in tobacco with a protein sequence very similar to that of AtPAP10. Studies have shown that NtPAP12 could dephosphorylate and activate some cell wall-bound enzymes, such as α-xilosidase and β-glucosidase, which are involved in the biosynthesis of cell walls (Kaida et al., 2009, 2010). It is possible, therefore, that AtPAP10, like NtPAP12, may function in regulating the growth of root cells through the control of cell wall biosynthesis when plants are stressed by Pi deficiency. More experimental work is needed to support this hypothesis.

In Arabidopsis, AtPAP10, AtPAP12, and AtPAP26 are three closely related genes that form a subgroup in the family of 29 PAPs (Li et al., 2002). The products encoded by these three genes have all been demonstrated to be Pi-secreted APases (Tran et al., 2010b; this work). Transcription of the AtPAP26 gene is constitutive, but its APase activity is up-regulated by Pi deprivation. Under Pi deficiency, the atpap26 mutant had a shorter primary root and less root biomass and accumulated more anthocyanin than the wild type (Tran et al., 2010a); in addition, cellular Pi and total P concentrations were 35% and 50% lower in the leaves of Pi-starved atpap26 plants than in the leaves of Pi-starved wild-type plants, respectively. In contrast, elimination of AtPAP10 did not cause a reduction of cellular Pi and total P concentrations in leaves or roots of Pi-starved plants. Besides, expression of AtPAP10 is induced by Pi limitation at both transcriptional and posttranscriptional levels. It seems that AtPAP10 and AtPAP26 may have both overlapping and distinct functions during plant adaptation to Pi limitation. So far, the function of AtPAP12 remains elusive. Thus, it will be interesting to examine the growth phenotypes of the atpap10/26, atpap10/12, and atpap12/26 double mutants and the atpap10/12/26 triple mutant to determine whether these genes interact in response to Pi limitation.

Another interesting phenomenon documented in this work was the absence of a correlation between plant root architecture and Pi homeostasis. It has been generally thought that increased formation of lateral roots and root hairs could help plants maintain Pi homeostasis under Pi starvation. Our results, however, clearly showed that, although 35S:AtPAP10 lines developed more root laterals than the nop1 mutants on Pi-deficient media, both genotypes had similar cellular Pi and total P contents. The causes behind this phenomenon need further investigation.
AtPAP10 as a Target for Plant Biotechnology

This work has clearly demonstrated that overexpression of the AtPAP10 gene improves plant growth on a Pi-deficient medium supplemented with the organophosphate ADP. We suspect that similar results would have been obtained with the organophosphate phytate, because AtPAP10 has moderate phytase activity. In most natural and arable lands, about 30 to 80% of the total P in soils is in the form of organophosphates. Previous research has shown that overexpression of a phytase gene (either from a fungus or plant) or a plant PAP gene could improve the growth of transgenic plants on a medium with phytate as the sole P source (Xiao et al., 2006; Ma et al., 2009; Wang et al., 2009). Our results show, to our knowledge for the first time, that overexpression of a plant PAP gene (AtPAP10) improved plant growth on a P-deficient medium supplemented with an organophosphate other than phytate. Thus, AtPAP10 could be a promising target for genetic engineering of new crops with high P efficiency. In addition, the counterparts of the AtPAP10 gene have been found in a wide range of plant species (Supplemental Fig. S3), including rice (Oryza sativa; Zhang et al., 2011) and tobacco (Kaida et al., 2010). These genes may also represent useful targets for engineering crops with high P nutrition.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The Arabidopsis (Arabidopsis thaliana) ecotype for all plants used in this study was Columbia, except that the Landsberg erecta ecotype was used for molecular mapping. Surface-sterilized seeds were placed on plates of solidified full-strength MS medium (Murashige and Skoog, 1962) with 1% Suc and 0.55% (w/v) agar (Sigma; catalog no. A1269). The pH of the medium was adjusted to 5.8. This Pi-sufficient medium was referred to as P+ medium. For Pi-deficient medium (referred as P−), KH2PO4 in P+ medium was replaced by K2SO4. After seeds were stratified at 4°C for 2 d, the plates were placed horizontally in a growth room with a 16-h-light/8-h-dark photoperiod (100 mmol m−2 s−1) at 23°C. For mutant screening or examination of root surface APase, culture plates were placed vertically in the same growth room and the plates were grown vertically for 7 to 8 d were overlaid with a 0.5% agar solution containing 0.01% BCIP for 12 to 24 h at 23°C. The plates were placed horizontally in a growth room with a 16-h-light/8-h-dark photoperiod (100 mmol m−2 s−1) and further confirmed by their resistance to kanamycin or hygromycin and their growth on a Pi-deficient medium supplemented with the organophosphate ADP. We suspect that similar results would have been obtained with the organophosphate phytate, because AtPAP10 has moderate phytase activity. In most natural and arable lands, about 30 to 80% of the total P in soils is in the form of organophosphates. Previous research has shown that overexpression of a phytase gene (either from a fungus or plant) or a plant PAP gene could improve the growth of transgenic plants on a medium with phytate as the sole P source (Xiao et al., 2006; Ma et al., 2009; Wang et al., 2009). Our results show, to our knowledge for the first time, that overexpression of a plant PAP gene (AtPAP10) improved plant growth on a P-deficient medium supplemented with an organophosphate other than phytate. Thus, AtPAP10 could be a promising target for genetic engineering of new crops with high P efficiency. In addition, the counterparts of the AtPAP10 gene have been found in a wide range of plant species (Supplemental Fig. S3), including rice (Oryza sativa; Zhang et al., 2011) and tobacco (Kaida et al., 2010). These genes may also represent useful targets for engineering crops with high P nutrition.

Mutant Screening

Two sets of T-DNA-mutagenized T3 seeds (Zhang et al., 2005; Koizumi et al., 2006) and one set of EMS-mutagenized M2 seeds were used for mutant screening. The EMS-mutagenized lines were generated in this laboratory according to Weigel and Glazebrook (2002). The roots of seedlings that had grown vertically for 7 to 8 d were overlaid with a 0.5% agar solution containing 0.01% BCIP for 12 to 24 h at 23°C. The seedlings with decreased BCIP staining were identified as putative mutants and transferred to soil. The plants were self-pollinated, and the mutant phenotypes were confirmed in the next generation.

Genetic Mapping of the NOP1 Gene

The mapping population was generated by crossing the mutant nop1-1 to a plant of the Landsberg erecta ecotype. The 1,400 F2 progeny that displayed the mutant phenotype were selected, and DNAs from these seedlings were isolated for molecular mapping. A set of simple sequence length polymorphism and cleaved- amplified polymorphic sequence markers was used to map the NOP1 gene. The sequences of the molecular markers and marker positions on the chromosome are listed in Supplemental Table S1.

Construction of Plant Transformation Vectors

To generate plant transformation vectors for genetic complementation, genomic and cDNA sequences of the AtPAP10 gene were amplified from genomic DNA or cDNAs from shoot tissues using primers 5′-GGGAGATCTGGTGTGCTT-3′ and 5′-GGGACCTGCGGACAAAGACT-3′. The AtPAP10 cDNA was cloned into the site after the CaMV 35S promoter of the plant expression vector pZH01, which carries a hygromycin-resistant gene. The AtPAP10 genomic DNA was cloned into the pBI121 vector after the CaMV 35S promoter. The pBI121 vector carries a kanamycin-resistant gene. The AtPAP10 promoter (2.0-kb sequence upstream of the start codon of the AtPAP10 coding region) was amplified from genomic DNA using primers 5′-GCCAGCTCTTGAACTGGAAG-3′ and 5′-GGGATCCGAGAATACCTAAAGAAG-3′. The AtPAP10 promoter-GUS fusion construct was generated by replacing the CaMV 35S promoter with the AtPAP10 promoter on the pBI121 vector. To generate the AtPAP10 promoter:AtPAP10 (genomic) gene construct, the AtPAP10 promoter was used to replace the CaMV 35S promoter already contained in the AtPAP10 genomic sequence. To make the CaMV 35S:AtPAP10-GFP plant transformation vector, the coding sequence of AtPAP10 (without a stop codon) was amplified using primers 5′-TCCATGGTGTGCTGAAAATCAG-3′ and 5′-GAGATTTCTCAGAAATCAAAGAGGAG-3′ and was inserted into the Nol/BamHI site within the CaMV 35S promoter and the GFP gene on the vector pCK-GFP. The fused CaMV 35S:AtPAP10-GFP cassette was excised and replaced the CaMV 35S promoter on the plant transformation vector pBI121 using HindIII/Xhol enzymes. To generate the AtPAP10:AtPAP10-GFP vector construct, the CaMV 35S promoter in the CaMV 35S promoter:AtPAP10-GFP construct was replaced by the AtPAP10 promoter, which was used for the AtPAP10:GUS construct. For the 35S:AtPAP10-HA construct, the AtPAP10 gene was amplified with primers 5′-GGCTCAGAAGTGAGTGTGTTGGAA-3′ and 5′-GGCACTAGTGGAGGAAAGA-3′ and cloned into the Xhol and SpeI cloning sites of the binary vector pCAMBIA1300, which has been modified to include HA tag sequences.

Plant Transformation

All the gene constructs were transferred into Agrobacterium tumefaciens strain GV3101 and transformed into Arabidopsis plants by the floral dip method (Clough and Bent, 1998). The AtPAP10:GUS construct was transformed into wild-type plants, and transgenic plants were selected on kanamycin MS medium. All other constructs were transformed into the nop1-1 mutant. The resultant transgenic plants were first selected by BCIP staining and further confirmed by their resistance to kanamycin or hygromycin and PCR analysis.

Analysis of GUS Activity in Transgenic Plants

Histochemical staining of GUS activity was performed according to the method described (Jefferson et al., 1987). Plant tissues were incubated at 37°C overnight in GUS staining buffer (2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 50 mM sodium Pi buffer, pH 7.2) containing 0.1% Triton X-100, 2 mM K3Fe(CN)6, 2 mM K4Fe(CN)6, and 10 mM EDTA. The stained seedlings were sequentially transferred to 50%, 70%, and 100% (v/v) ethanol to remove chlorophyll. The stained materials were examined with a light microscope (Olympus BX51) and a dissecting microscope (Olympus SZ61). The results were recorded with a digital camera.

Analysis by Quantitative Real-Time RT-PCR and Semiquantitative RT-PCR

Total RNA was extracted with the Qiagen RNAeasy kit. DNase-treated RNA (1 μg) was reverse transcribed in a 20-μl reaction using reverse transcriptase (Toyobo) according to the manufacturer’s manual. cDNA was amplified with SYBR Premix Ex Taq (Takara) on the Bio-Rad CFX96 real-time PCR detection system. Actin mRNA was used as an internal control, and the relative expression level of each gene was calculated by the 2−ΔΔCt method.
The primer sequences for amplification of AtPAP10 are 5′-ATCTGTGTTG-ATGATTCCTCCTCTGG-3′ and 5′-ATCTCATTTATGATGGTGCTCA-3′, and those for Actin are 5′-GACCTGTCCGACGCTACATC-3′ and 5′-GTA-GTCAACGACAAGAAGAGGACG-3′. The sizes of the amplification products are 97 bp for AtPAP10 gene and 135 bp for the Actin gene. For RT-PCR analysis, the primers used for amplification of AtPAP10 are 5′-CCCTTGTGATGAACTCCAGCCTTG-3′, and those for internal control Actin are 5′-TCTCATTCACGACGTC-3′ and 5′-CTCAGCAAGACGGTTAC-3′. The sizes of amplification products are 1,500 bp for AtPAP10 and 356 bp for Actin.

Expression and Purification of the GST:AtPAP10 Fusion Protein

The expression and purification of the GST:AtPAP10 fusion protein in *E. coli* cells was performed as described for the production of the GST:AtPAP23 fusion protein (Zhu et al., 2005). Basically, the coding sequence of AtPAP10 was cloned downstream of the GST coding sequence in the bacterial expression vector pGEX-KG. The *E. coli* strain XA90 harboring pKG-GST:AtPAP10 was used to express the fusion protein. All the buffer and experimental conditions were the same as used for expression and purification of the GST:AtPAP23 fusion protein, except that, after gel filtration, one more purification step was employed using a Resource Q column. The purity of the protein was verified using SDS-PAGE. The same protocol was also used for purifying the GST protein from XA90 cells.

Metal Analysis and Biochemical Analysis of GST:AtPAP10

Metal contents of the GST:AtPAP10 fusion protein were determined using inductively coupled plasma-mass spectroscopy in the Key Laboratory of Orogenic Belts and Crustal Evolution of Peking University. The effects of pH and temperature on phosphatase activity of the GST:AtPAP10 fusion protein were examined as described by Zhu et al. (2005). To test the substrate specificity of the GST:AtPAP10 fusion protein, phosphatase activity assays were conducted on a 96-well microtiter plate in 50 mM acetate buffer (pH 5) containing 5 mM MgCl and 5 mM of the appropriate substrates (in a volume of 50 μL). The reaction was initiated by adding 10 ng of the purified GST:AtPAP10 to each microtiter plate well and allowed to proceed for 15 min at room temperature. Pi released from the substrate was estimated by the malachite green-molybdate reagent.

In-Gel Assays of APase Profile

The protein extraction and in-gel assay for APase profiles were performed essentially as described (Trull and Deikman, 1998). The roots of Arabidopsis seedlings were ground to a fine powder in liquid nitrogen. A 2-mL volume of ice-cold extraction buffer (0.1 M potassium acetate, 20 mM CaCl₂, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 20% [v/v] glycerol, pH 5.4) was added to 1 g of the powder. Samples were gently agitated on ice for 0.5 h and then centrifuged at 13,400g for 4°C for 15 min. The supernatant was transferred to a fresh tube before use. Plant liquid culture media were collected and lyophilized at −50°C. The lyophilized samples were resuspended in buffer and dialyzed against buffer (50 mM sodium acetate) for 24 h and then against distilled, deionized water for 8 h. After dialysis, the samples were centrifuged at 10,000 rpm for 5 min, and the supernatants were transferred to a fresh tube and relyophilized at −50°C. The relyophilized samples were resuspended in 200 μL of ice-cold extraction buffer. The proteins were quantified and separated on a 10% nonreducing SDS-PAGE gel at 4°C. The gels were gently washed in cold distilled water (10 min per wash and a total of six washes) to remove the SDS. The gels were then gently shaken in the buffer containing 50 mM sodium acetate (pH 4.9) and 10 mM MgCl₂ (two times, 15 min each time) to allow the proteins to renature. After equilibrium with the buffer, the gels were stained for APase activity in the buffer containing 0.5 mM MgL⁻¹ Fast Black potassium salt and 0.3 mg mL⁻¹ β-naphthyl acid phosphate or 0.08% BCIP.

Quantitative Analysis of Medium APase Activities

For quantitative analysis of total APase activities in root tissues and culture medium, 10 μL of proteins extracted from roots or collected from culture media was mixed with 470 μL of MES buffer (15 mM MES, pH 5.5, and 0.5 mM CaCl₂) and 20 μL of pNPP (Sigma N-2770). After incubation at 27°C for 30 min, the reaction was terminated with 500 μL of 0.25 M NaOH. The developed color was determined spectrophotometrically at 410 nm. The absorbance was determined spectrophotometrically at 635 nm. To measure the APase activity against phytate, Fru-6-P, ADP, or ATP, 200 μL of reaction buffer containing 0.5 mM substrate was added to the tubes and incubated at 37°C for 1 h. The Pi released was measured using the method described by Ames (1966). These experiments were repeated three times with six replicates for each sample.

Quantitative Analysis of Root-Associated APase Activity

The quantitative analysis of root-associated APase activity was performed according to Boutin et al. (1981) and McLachlan (1980) with some modifications. Two 12-d-old seedlings of uniform size were transferred to a 2-mL Eppendorf tube containing 0.6 mL of P₀ or Pₐ − liquid medium. After growing for another 3 d with gentle shaking at 23°C, seedlings were briefly rinsed in distilled, deionized water and transferred to a new tube containing 0.6 mL of P₀ or Pₐ − liquid MS medium. When pNPP was used as a substrate, 80 μL of pNPP (1.0 mg mL⁻¹) was added to the tubes and incubated at 37°C for 1 h. Then, 120 μL of 0.4 M NaOH was added to terminate the reaction, and absorbance was determined spectrophotometrically at 410 nm. When BCIP was used as a substrate, 600 μL of reaction buffer containing 0.4 μM BCIP was added to allow the reaction to proceed at 37°C for 1 h. Then, the reaction was terminated by adding 1 mL of 1 M HCl. After sitting at room temperature for 25 min, the samples were centrifuged for 5 min at 10,000g, and the precipitates were dissolved in 1 mL of dimethyl sulfide. The absorbance was determined spectrophotometrically at 635 nm.

Quantitative Analysis of Total Shoot and Root APase Activities

For quantitative analysis of total APase activities in root tissues and culture medium, 10 μL of proteins extracted from roots or collected from culture media was mixed with 470 μL of MES buffer (15 mM MES, pH 5.5, and 0.5 mM CaCl₂) and 20 μL of pNPP (Sigma N-2770). After incubation at 27°C for 30 min, the reaction was terminated with 500 μL of 0.25 M NaOH. The developed color was determined spectrophotometrically at 410 nm, and APase activity was expressed as A410 mg⁻¹ protein. To measure root-associated APase activity against substrate pNPP, two 12-d-old seedlings of uniform size were transferred to a 2-mL Eppendorf tube containing 0.6 mL of P₀ or Pₐ − liquid medium. After growing for another 3 d with gentle shaking at 23°C, seedlings were briefly rinsed in distilled, deionized water and transferred to a new tube containing 0.6 mL of P₀ or Pₐ − liquid MS medium. A 50-μL volume of 1.0 mg mL⁻¹ pNPP was added to the tubes, which were incubated at 37°C. After incubation for 30 min, 150 μL of 0.4 M NaOH was added to terminate the reaction, and the light A₄₁₀ was measured.

Western-Blot Analysis of AtPAP10-HA Proteins

Total proteins were isolated from 14-d-old whole seedlings grown on P₀ and Pₐ − medium as described before, separated on 10% SDS-polyacrylamide gels, and transferred to a polyvinylidene difluoride membrane in transfer buffer (25 mM Tris, 43 mM Gly, and 20% methanol). The membrane was blocked with 5% milk in phosphate-buffered saline (PBS; 0.27 mM NaCl, 20 mM Na₂HPO₄, 5 mM KCl, and 3.5 mM KH₂PO₄) at room temperature for 1 h and then incubated with HA antibodies (1:3,000; Kangweishiji) at room temperature for 1 h in TBBS (PBS + 0.05% Tween 20) containing 5% milk. After reaction with HA antibodies, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Kangweishiji) at 14,000 dilution for 1 h. Between transfers, the membrane was washed with TPBS five times with each wash for 6 min. After completion of all reactions, the membrane was washed three times with TPBS (6 min each) and incubated for 1 min at room temperature in a mixture (1:1) of the two enhanced...
chemiluminescence solutions. The excess liquid was dried out, the membrane was wrapped in Saran wrap, and the result was detected with film (Kodak).

Quantitative Analyses of Cellular Pi, Total P, and Anthocyanin

The cellular Pi contents were determined using the method described by Ames (1966). Basically, the preweighed fresh shoot and root tissues were submerged in 1 mL of 1% glacial acetic acid and frozen/thawed eight times. One hundred micromolars of extract was mixed with 200 μL of water and 700 μL of Pi reaction buffer containing a mixture of 0.48% NH₄H₂MoO₄, 2.85% (v/v) H₂SO₄, and 10% (v/v) ascorbic acid in a ratio of 6:1. The reaction was allowed to proceed at 37°C for 1 h. The Pi content was determined as A₅₆₀ according to a premed standard curve and was expressed as μmol g⁻¹ fresh weight. To determine the total P content, about 50 mg of fresh tissues was oven dried at 50°C for 3 h and flamed to ash. The ashes were dissolved in 100 μL of 30% (v/v) HCl and 10% (v/v) HNO₃. Ten microliters of dissolved sample was mixed with 200 μL of H₂SO₄, and 10% (w/v) ascorbic acid in a ratio of 6:1. The reaction was allowed to proceed for 1 h. The Pi contents of plant tissues were determined and expressed as Pi contents (v/v) HCl and 10% (v/v) HNO₃. Ten micromolars of dissolved sample was mixed with 200 μL of distilled, deionized water and 700 μL of Pi reaction buffer, and the Pi was quantified by the method of Ames (1966). The total P contents of plant tissues were determined and expressed as Pi contents extracted from flamed ashes. For the quantification of anthocyanin, 50 mg of shoot tissue from 2-week-old seedlings grown on P⁺ or P⁻ medium was ground in liquid N₂ and extracted with 1% HCl in methanol (v/v) overnight. Distilled, deionized water (0.5 volume) and chloroform (1.0 volume) were added to remove the chlorophyll. After centrifugation for 5 min at 13,000 rpm, the upper aqueous phase was used for spectrophotometric quantification at 530 nm. Relative anthocyanin levels were expressed as A₅₃₀ R₂⁻¹ fresh weight.

The Arabidopsis Genome Initiative locus numbers for the major genes discussed in this article are as follows: AtPAP10 (AT1G16430), AtPAP12 (AT2G22790), AtPAP15 (At3g07130), AtPAP17 (At3g17790), and AtPAP26 (At5g34850).

Supplemental Data

The following data are available in the online version of this article.

Supplemental Figure S1. Genetic analysis of the nop1-1 mutant.

Supplemental Figure S2. The strategy for fine-mapping of the NOP1 gene.

Supplemental Figure S3. Alignment of part of the protein sequences between AtPAP10 and the 100 most related genes.

Supplemental Figure S4. RT-PCR analysis of AtPAP10 mRNA in different plant parts.

Supplemental Figure S5. RT-PCR analysis of AtPAP10 mRNA of the wild type, nop1-2, and nop1-3 grown on P⁻ and P⁻ medium.

Supplemental Figure S6. Biochemical properties of recombinant GST-AtPAP10 fusion protein.

Supplemental Figure S7. Root architecture of seedlings of wild type, nop1-2, and 5SS/AtPAP10 line grown on P⁺ medium.

Supplemental Figure S8. Cellular Pi and total P contents of wild type, nop1-2, and four 5SS/AtPAP10 lines grown on various medium conditions.

Supplemental Table S1. Sequences of the primers used for map-based cloning of the NOP1 gene.

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