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Phosphate transporters from the higher plant *Arabidopsis thaliana*

(ion uptake/yeast complementation)

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ABSTRACT **Two cDNAs (AtPT1 and AtPT2) encoding plant phosphate transporters have been isolated from a library prepared with mRNA extracted from phosphate-starved *Arabidopsis thaliana* roots. The encoded polypeptides are 78% identical to each other and show high degree of amino acid sequence similarity with high-affinity phosphate transporters of *Saccharomyces cerevisiae*, *Neurospora crassa*, and the mycorrhizal fungus *Glomus versiforme*. The AtPT1 and AtPT2 polypeptides are integral membrane proteins predicted to contain 12 membrane-spanning domains separated into two groups of six by a large charged hydrophilic region. Upon expression, both AtPT1 and AtPT2 were able to complement the *pho84* mutant phenotype of yeast strain NS219 lacking the high-affinity phosphate transport activity. AtPT1 and AtPT2 are representatives of two distinct, small gene families in *A. thaliana*. The transcripts of both genes are expressed in roots and are not detectable in leaves. The steady-state level of their mRNAs increases in response to phosphate starvation.**

Phosphorus is one of the major nutrients required by plants. It is generally accepted that phosphate is acquired in an energy-mediated cotransport process, driven by a proton gradient generated by plasma membrane H^+-ATPases (1, 2). Phosphate absorption is accompanied by H^+ influx with a stoichiometry of 2 to 4 $H^+/H_2PO_4^-$ transported. A dual-mechanism model for uptake of ions, including phosphate, has been proposed (3–5). This is characterized by a high-affinity transport system operating at low (μM) concentration and a low-affinity system functioning at high concentration (mM) of ions. Measurements of phosphate in saturation extracts of a large number of soils showed that phosphate concentration in the majority of samples ranged between 0.65 to 2.5 μM (6). Under these conditions, the high-affinity transport system is considered to be the major mechanism for phosphate acquisition by plants.

An increase in phosphate uptake rate of roots and cultured cells following phosphate starvation has been observed in several plant species (7–9). The enhanced uptake appears to be, in part, due to an increased synthesis of a carrier system in response to phosphate deprivation (10). Similar response to phosphate-limiting conditions has been observed in yeast (11–13) and *Neurospora crassa* (14, 15). Phosphate stress in yeast results in activation/inactivation of several genes associated with the *pho*-regulon, leading to enhanced synthesis of the high-affinity phosphate transporter and phosphatases. The high-affinity phosphate transporter genes have been cloned and characterized from yeast (16), *N. crassa* (17), and the mycorrhizal fungus *Glomus versiforme* (18). All three fungal phosphate transporters are predicted to have a common structure containing 12 membrane-spanning domains separated into two groups of six by a charged hydrophilic region.

Although the control of phosphate uptake in plants is well-documented at the physiological level, information regarding the molecular structure of transport proteins and their genetic regulation is lacking. In this paper, we report the cloning and characterization of two full-length *Arabidopsis* cDNAs encoding phosphate transporters. The expression studies suggest that these genes are root-specific and induced in response to phosphate starvation in *Arabidopsis*.

MATERIALS AND METHODS

Plant Material. *Arabidopsis thaliana* var. Columbia seeds were germinated on a 300-μm mesh nylon screen placed on a petri plate containing solidified agar supplemented with 1/10 Murashige–Skoog salts as described by Poirier *et al.* (19). One week after germination, the nylon filter was removed along with the intact root system and transferred to a sterile floating membrane raft (Lift raft, Sigma). The floating device carrying seedlings was placed in a GA-7 (Sigma) tissue culture box containing 100 ml of half-strength Hoagland II nutrient solution (20). After 1 week, the plants were transferred to fresh half-strength Hoagland II nutrient solution containing either 250 μM phosphate or no phosphate (KH_2PO_4 was replaced with K_2SO_4). The roots and leaves were harvested from these plants after 5 days following the initiation of phosphate starvation.

RNA Isolation. Total RNA was isolated from the roots and leaves of *Arabidopsis* plants by hot phenol extraction and lithium chloride precipitation (21). Poly(A)$^+$ RNA was isolated by the oligo(dT) cellulose batch binding method (22).

cDNA Library Construction and Screening. A cDNA library representing the mRNA isolated from *Arabidopsis* roots starved for phosphate for 7 days was constructed in the *Eco*RI–*Xho*I site of the Uni-ZAP XR vector according to manufacturer's instructions (Stratagene). The *Arabidopsis* expressed sequence tag clones (stock nos. 134M11T7, 178H14T7, and ATTS2854) showing similarity to yeast PHO84 were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). The inserts from these clones were radiolabeled by random priming (DECAprime II, Ambion, Austin, TX) and used for screening the cDNA library according to standard procedures (22). Final washing of the filters was done with 0.1× SSC and 0.2% SDS at 62°C for 40 min. Two sets of cDNA clones were obtained from this screening based on their hybridization with either 178H14T7 insert or a mixture of ATTS2854 and 134M11T7 inserts. Based on the insert size and restriction mapping, one full-length representative from each of these sets was used for further analysis. The sequence of these two clones was determined on both strands by the

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Abbreviation: SD medium, synthetic defined medium.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base [accession nos. U62330 (AtPT1) and U62331 (AtPT2)].

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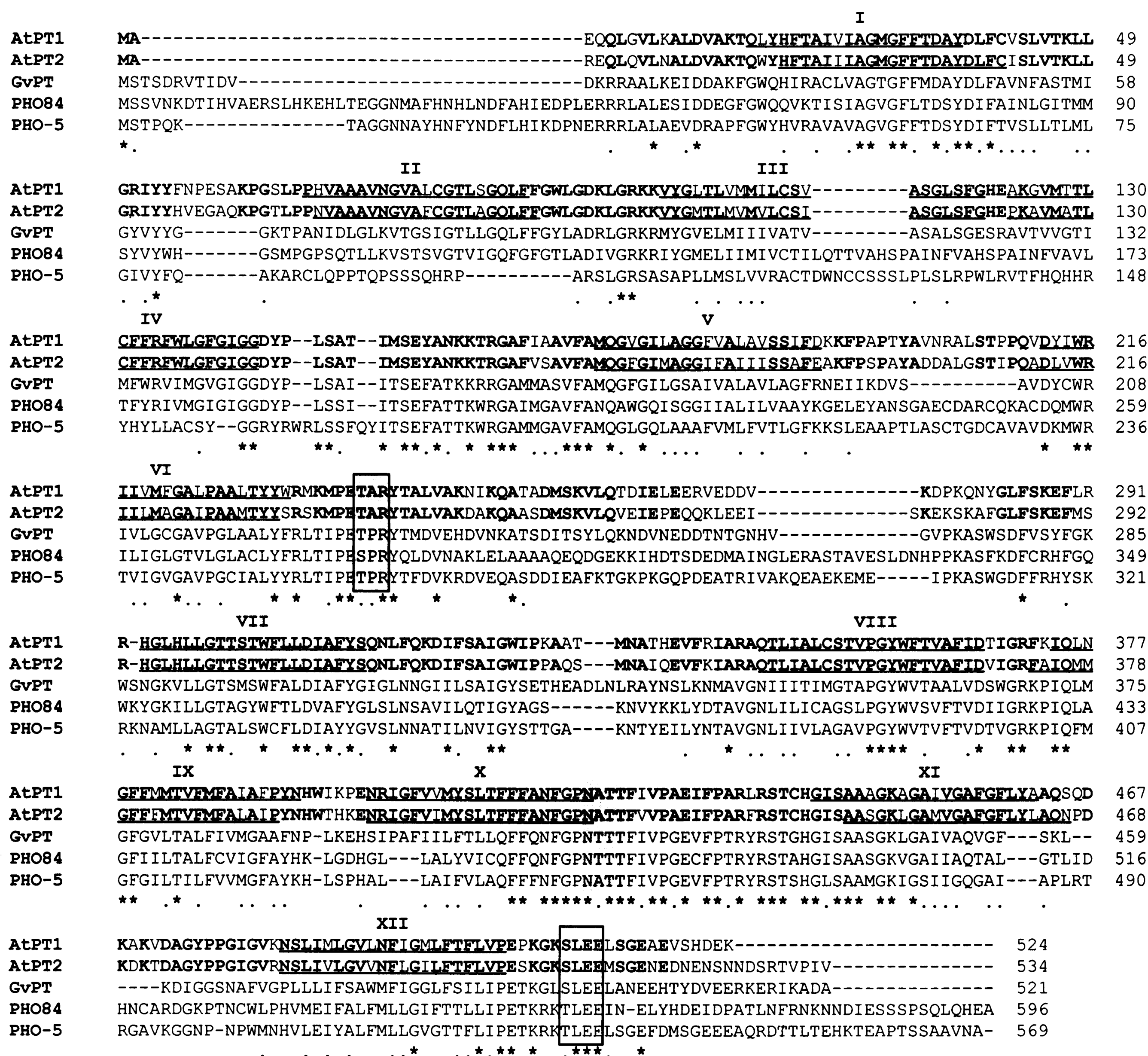

FIG. 1. Alignment of the deduced amino acid sequence of AtPT1 and AtPT2 with that of *G. versiforme* GvPT (18), yeast PHO84 (16), and *Neurospora* PHO-5 (17). Identical amino acids are indicated by asterisks (*) and conserved substitutions are indicated by dots (·). Boldface letters indicate the identical amino acids between AtPT1 and AtPT2. The membrane-spanning domains of AtPT1 and AtPT2, as predicted by TopPred (25), are underlined and their numbering is indicated by roman numerals (I–XII). The shaded sequences are consensus sites for N-linked glycosylation; boxed sequences are consensus sites for phosphorylation by casein kinase II; and boxed and shaded sequences are sites for phosphorylation by protein kinase C.

dideoxy method using Sequenase (United States Biochemical). The Genetics Computer Group (Madison, WI) software package was used for sequence analysis and data base searches.

Cloning and Expression in *Saccharomyces cerevisiae*. The yeast high-affinity phosphate transporter mutant NS219 (16) was provided by Satoshi Harashima (Osaka University, Japan). The coding regions of AtPT1 and AtPT2 cDNAs were subcloned in yeast expression vector pYES2 (Invitrogen) downstream of the GAL1 promoter. NS219 was transformed by a LiCl/PEG method (23). The procedures and synthetic defined (SD) medium used for growth and selection of transformants were similar to those described earlier (16).

The acid phosphatase activity of the NS219 transformants was detected by a staining method based on the diazo-coupling reaction (16). For growth assays, single colonies of transformants were grown in high phosphate (11 mM) SD medium containing 2% galactose and 0.5% sucrose to an OD_{600} of 1.0. The cells were collected by centrifugation, washed twice with SD medium containing no phosphate, and then resuspended in low phosphate (110 μM) SD medium containing 2% galactose and 0.5% sucrose to an OD_{600} of 0.05. At regular time intervals, aliquots of the culture were removed for OD_{600} measurements.

Northern Blots. Total RNA (10 μg) was electrophoretically separated on 1% denaturing formaldehyde agarose gels and blotted onto BA-S (Schleicher & Schuell) nitrocellulose membrane (22). The nitrocellulose filters were hybridized overnight with ^{32}P-labeled DNA probe (10^6 cpm/ml) in a solution containing 50% formamide, 5× Denhardt's solution, 0.1% SDS, 6× SSPE, and 100 μg/ml denatured salmon sperm DNA at 42°C. Filters were washed twice in 2× SSC and 0.2% SDS at room temperature for 10 min, twice in 1× SSC and 0.2%

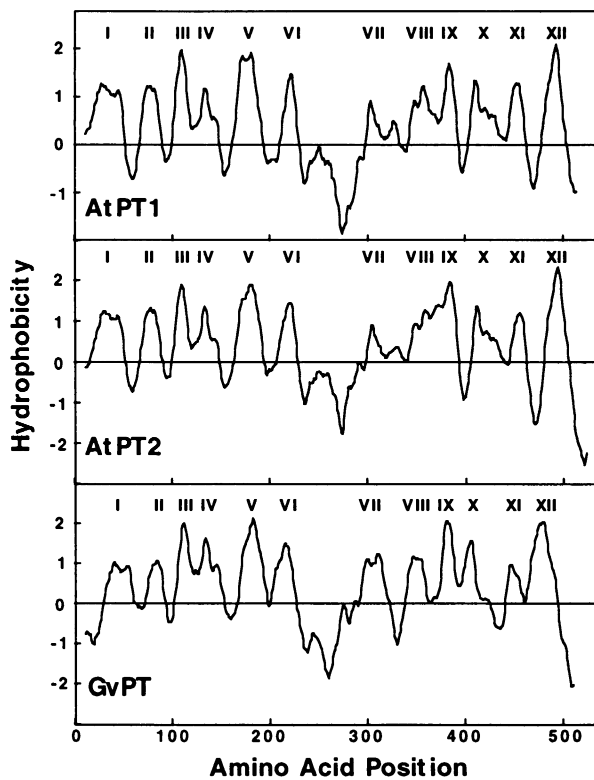

FIG. 2. Hydrophobicity profiles of the *Arabidopsis* AtPT1 and AtPT2 compared with *G. versiforme* GvPT (18). Hydropathy values for a window of 21 residues were calculated by TopPred (25) using algorithms of Kyte and Doolittle (26). Hydrophobic regions correspond to the positive index numbers. The roman numerals refer to putative membrane-spanning domains.

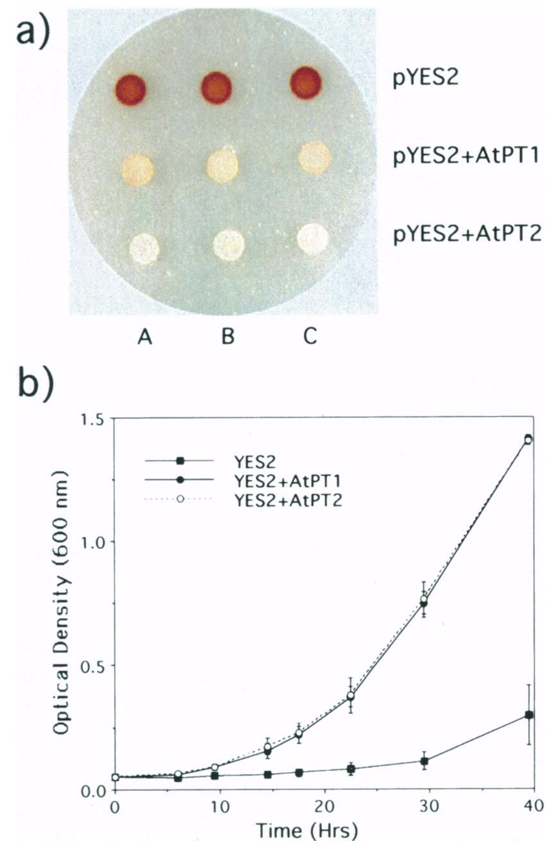

FIG. 3. Complementation of yeast high-affinity phosphate transporter mutant NS219. (*a*) Acid phosphatase activity in NS219 transformants containing either the vector (pYES2) or the vector containing AtPT1 (pYES2 + AtPT1)/AtPT2 (pYES2 + AtPT2) coding regions. The cells were grown on SD medium–high-phosphate (11 mM) plates containing 3% glycerol and 0.5% galactose for 4 days before staining for acid phosphatase activity (16). The red color indicates presence of acid phosphatase activity. Three independent transformants (*A–C*) for each construct are shown. (*b*) Growth of NS219 transformants in SD medium–low phosphate (110 μM) medium containing 2% galactose and 0.5% sucrose. Values are the averages from two experiments using three independent transformants for each construct. Error bars indicate the standard deviation.

SDS at 50°C for 15 min, and twice in 0.1× SSC and 0.2% SDS at 62°C for 20 min before autoradiography.

Southern Blots. High molecular weight genomic DNA was isolated from young leaves of *Arabidopsis* as described by Dellaporta *et al.* (24). Genomic DNA (10 μg) was digested with restriction enzymes, electrophoretically separated through 0.8% agarose gels, denatured, and transferred to a supported nitrocellulose membrane (22). The hybridization and washing conditions were the same as those described above for Northern blots.

RESULTS

Cloning of *Arabidopsis* Phosphate Transporter cDNAs. Two full-length cDNA clones, AtPT1 and AtPT2, encoding the *A. thaliana* phosphate transporters were isolated from a phosphate-starved root library using the inserts from three *Arabidopsis* expressed sequence tags as probe. AtPT1 is 1754-bp long and contains an open reading frame encoding a 524 amino acid polypeptide (molecular mass of 57.6 kDa), whereas AtPT2 is 1897 bp long and encodes a 534 amino acid polypeptide (molecular mass of 58.6 kDa). The open reading frames of AtPT1 and AtPT2 are flanked by 47 and 151 bp of untranslated sequence at the 5′ end, and by 135 and 144 bp of untranslated sequence including the poly(A) tail at the 3′ end, respectively. These two clones are 70% similar in their nucleotide sequence within the coding region. The two polypeptides are 78% identical in their amino acid sequence. A homology search of the sequence data bases with both of these clones revealed the highest degree of similarity with three recently characterized fungal high-affinity phosphate transporters. AtPT1 shares 43.3%, 36.5%, and 37.1% amino acid identity with high-affinity phosphate transporters from *G. versiforme* (GvPT), yeast (PHO84), and *N. crassa* (PHO-5), respectively (Fig. 1). Similarly, AtPT2 shows 41.9%, 37.0%, and 32.3% amino acid sequence identity with GvPT, PHO84, and PHO-5 respectively. The *Arabidopsis* and fungal phosphate transporters are 57–64% similar to each other based on the conservative substitutions in their amino acid sequence.

Structure of the *Arabidopsis* Phosphate Transporters. Hydropathy plots of the deduced polypeptides suggest that both are integral membrane proteins consisting of 12 membrane-spanning regions (Figs. 1 and 2), a common feature shared by other membrane-associated cotransporters (27–29). The position and spacing of these membrane-spanning regions are very similar in all five phosphate transporters. Based on secondary structure analyses (25), both N and C termini of the polypeptides are predicted to be on the cytoplasmic side of the plasma membrane. Several amino acid domains are highly conserved between these five transporters and include sites for protein kinase C and casein kinase II-mediated phosphorylation, as well as N-linked glycosylation (Fig. 1). An N-terminal signal sequence is absent in all of them.

Expression of AtPT1 and AtPT2 in Yeast Phosphate Uptake Mutant. The yeast strain NS219 contains a mutation in the *PHO84* gene and thereby lacks the high-affinity phosphate transport system (16). As a result of this mutation, NS219 cells

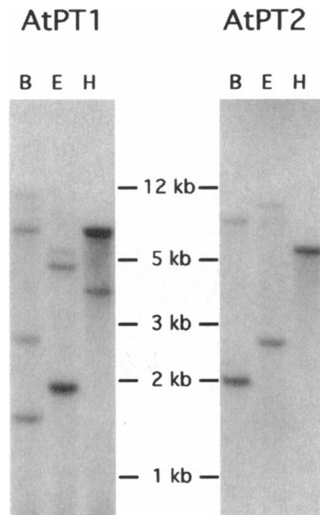

FIG. 4. Southern blot analysis of *Arabidopsis* genomic DNA digested with *Bam*HI (B), *Eco*RI (E), or *Hin*dIII (H). The blots were hybridized with labeled cDNA inserts from AtPT1 or AtPT2.

exhibit reduced rates of phosphate uptake and growth in low-phosphate medium. In addition, the mutant cells continue to produce an acid phosphatase when grown in high-phosphate medium (30), whereas this activity is repressed in wild-type cells under these conditions. To test the ability of AtPT1-and AtPT2-encoded polypeptides to complement this mutation, the coding regions of the cDNAs were ligated into a yeast expression vector pYES2 and transformed into NS219. The cells transformed with only the pYES2 vector exhibited the acid phosphatase activity on high-phosphate medium as seen from their red color after staining (Fig. 3*a*). The NS219 transformants expressing either AtPT1 or AtPT2 mimicked wild-type cells, showing no acid phosphatase activity. NS219 cells expressing AtPT1 or AtPT2 were also able to grow much faster in low-phosphate (110 μM) medium than the cells containing pYES2 (Fig. 3*b*). The average generation time of these cells was ≈2.5 times higher than the control cells during the initial phases of their growth in low-phosphate medium. These results suggests that expression of AtPT1 or AtPT2 was able to complement the *pho84* mutant phenotype of NS219.

Organization of *Arabidopsis* Phosphate Transporter Genes. The full-length AtPT1 and AtPT2 cDNA probes hybridized

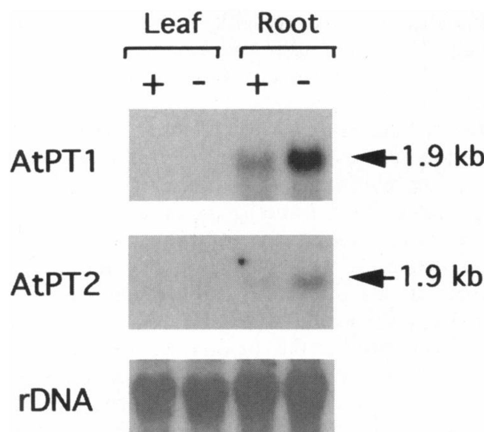

FIG. 5. Northern blot analysis of the expression of phosphate transporter genes in *Arabidopsis*. Total RNA from roots or leaves of *Arabidopsis* plants grown hydroponically in a solution containing 250 μM (+) or no (−) phosphate was hybridized with labeled probe from either AtPT1, AtPT2, or rDNA. The blots were exposed to x-ray film for 2 days (AtPT1), 5 days (AtPT2), or 5 hr (rDNA).

with two or three distinct bands on the Southern blot (Fig. 4) of *Arabidopsis* genomic DNA digested with different restriction enzymes, indicating the presence of a small gene family.

AtPT1 and AtPT2 Transcripts Are Induced Under Phosphate Starvation in Roots. Expression of AtPT1 and AtPT2 transcripts was compared by Northern blot analysis of total RNA isolated from roots and leaves of *Arabidopsis* plants grown either in the presence of 250 μM phosphate or no phosphate. A 1.9-kb transcript was detected for both of these genes in roots (Fig. 5). Their expression was markedly increased in the roots of plants subjected to phosphate starvation. The AtPT1 message was more abundant compared with that of AtPT2 in phosphate-starved roots. There was no detectable message for either of the genes in leaves even under phosphate starvation.

DISCUSSION

Ion transport in plants consists of high-affinity and low-affinity components (4). The recent cloning and characterization of potassium (31–33) and sulfate transporters (34) supports the existence of both types of ion transport mechanisms in plants. The deduced amino acid sequences of the *Arabidopsis* cDNA clones AtPT1 and AtPT2 show significant similarity with those of known high-affinity phosphate transporters from yeast, *Neurospora*, and the mycorrhizal fungus *G. versiforme*. They all have 12 membrane-spanning domains with a large hydrophilic, charged region separating them into two groups of six. This structural feature is common to a large family of proteins responsible for transport of substrates as diverse as sugars, ions, antibiotics, and amino acids (27–29). The C-terminal halves of transporters that recognize structurally similar substrates are more homologous than their N-terminal halves, suggesting that substrate specificity is determined by sequence motifs present in the C-terminal halves (27). Consistent with this hypothesis, inhibitor and photoaffinity labeling studies have located the substrate binding sites for homologous sugar transporter proteins in their C-terminal halves (35). A highly conserved domain of 38 amino acids is present between and including the 10th and 11th transmembrane domains of the plant and fungal phosphate transporters. A search of nucleotide data bases also identified a similar domain in two *Schizosaccharomyces pombe* cosmid clone sequences (GenBank accession nos. Z64354 and U33010) predicted to code for phosphate transporters. It is tempting to speculate on the potential involvement of such a highly conserved domain in determining substrate specificity for phosphate transport. Phylogenetically, AtPT1, AtPT2, GvPT, PHO84, and PHO-5 all fall into a distinct group. However, AtPT1 and AtPT2 are more closely related to GvPT (data not shown).

The absence of a high-affinity phosphate transporter function in the yeast mutant NS219 results in its reduced growth in low-phosphate medium, and constitutive production of a repressible acid phosphatase in high-phosphate medium (16, 30). Expression of AtPT1 and AtPT2 in NS219 cells complemented both these phenotypes. Similar results were obtained in complementation experiments using the high-affinity phosphate transporter clones of yeast (16) and the mycorrhizal fungus (18). Enhanced ability of the yeast mutant expressing the two plant cDNAs to grow in the presence of μM concentrations of phosphate further supports the role of AtPT1 and AtPT2 as phosphate transporters. Our preliminary studies suggest that these cells expressing AtPT1/AtPT2 are also capable of enhanced phosphate uptake from low-phosphate medium (data not shown).

Based on the exposure times required, the transcript for AtPT1 appears to be more abundant than that of AtPT2. Both AtPT1 and AtPT2 transcripts are present in roots and are not detectable in leaves of *Arabidopsis*. Roots are the organs involved in nutrient acquisition, and the expression pattern of

these two genes in the roots correlates well with their function in phosphate uptake. Members of the high-affinity sulfate transporters of *Stylosanthes humata* are also expressed specifically in roots and their expression is highly induced by sulfate starvation (34). After uptake by roots, phosphate must be translocated to other plant organs. Recently, two *Arabidopsis* mutants impaired in loading of phosphate into xylem and accumulation in leaves have been reported (19, 36). Since the Southern blot analysis showed that AtPT1 and AtPT2 are members of two distinct and small gene families in *Arabidopsis*, it is likely that other isoforms of phosphate transporters involved in phosphate allocation exist in plants.

The induction of AtPT1 and AtPT2 in response to phosphate starvation is of particular interest. Phosphate starvation increases the rate of phosphate uptake by plants (7–9). Kinetic studies of phosphate uptake in suspension cultures have shown that the low-affinity system is expressed constitutively, whereas the high-affinity system is induced at low phosphate concentrations (9), a process that may require *de novo* protein synthesis (10). Enhanced transcription of phosphate transporter genes in response to phosphate limiting conditions has been well-documented in microorganisms (37). Our results provide the first evidence showing an increase in the transcript levels of phosphate transporters under phosphate starvation in plants. These results, together with the ability of AtPT1 and AtPT2 to complement a *pho84* yeast mutant, suggest that AtPT1 and AtPT2 may be high-affinity phosphate transporters.

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