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Overexpression of an *Arabidopsis thaliana* high-affinity phosphate transporter gene in tobacco cultured cells enhances cell growth under phosphate-limited conditions

(*PHO84*/phosphate uptake/transgenic cells/biomass production)

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ABSTRACT **A higher plant homologue to the high-affinity phosphate transporter gene of yeast (*Saccharomyces cerevisiae*) *PHO84* was isolated from *Arabidopsis thaliana*. Expression of the *Arabidopsis* gene *PHT1* at high levels in tobacco-cultured cells increased the rate of phosphate uptake. The uptake activity attributable to the transgene was inhibited by protonophores, suggesting an H^+ cotransport mechanism of phosphate uptake, and had a K_m of 3.1 μM which is within limits characteristic of high-affinity transport mechanisms. These results indicate that *PHT1* encodes a high-affinity phosphate transporter. The transgenic cells exhibited increased biomass production when the supply of phosphate was limited, establishing gene engineering of phosphate transport as one approach toward enhancing plant cell growth.**

A major constraint to crop production throughout the world is low availability of the essential macronutrient phosphate in soil (1–3). Higher plants absorb the nutrient from soils as the orthophosphate anion. Much evidence supports an H^+ cotransport mechanism for phosphate uptake in higher plants (4–6). Multiple kinetics for the uptake are observed when examined over an extended range of external phosphate concentrations (from <1 μM to a few tens of millimolars) (7). As most soils are deficient in phosphate (1–10 μM) (8), it is likely that high-affinity phosphate transporters with K_m values of 1–5 μM (9) in roots play a major role in phosphate uptake from soils. Understanding the molecular mechanism of phosphate uptake is thought to be an important step in gene manipulation of crops for high yield, especially from phosphate-deficient soils.

Since the isolation of a high-affinity phosphate transporter gene *PHO84* from yeast *Saccharomyces cerevisiae* using *pho84* mutants (10), homologous genes have been identified in the filamentous fungus *Neurospora crassa* (*PHO-5*) (11) and the mycorrhizal fungus *Glomus versiforme* (*GvPT*) (12). Their characteristic protein structures, especially the 12 membrane-spanning domains, are distinct from that of the *Escherichia coli* phosphate transporter (13, 14). The expansion of expressed sequence tag (EST) databases has led to the identification of plant homologues of microbial phosphate transporters. (The following GenBank accession numbers were identified as showing homology with PHO84. For *A. thaliana*, Z33763, T46507, H76973, Z25636 and H36767; for *Oryza sativa*, D25087, D25132, D39388 and C19700; and for *Brassica nupus*, H07437.) Muchhal *et al* (15) isolated two *Arabidopsis* homologues, named *AtPT1* and *AtPT2*, that shared 32–43% amino acid sequence identity with the microbial phosphate transporters. They reported that the plant genes complemented the phenotypes of the *pho84* mutants, and suggested that these homologues encode plant phosphate transporters. However, we have found no conclusive evidence that an *Arabidopsis* gene (*AtPT1*) complemented the yeast mutants (this study). Thus, in this study we utilized tobacco-cultured cells to prove that the gene product was functional. During the reviewing process of this manuscript, Smith *et al.* (16) also reported that they could not complement the yeast mutation with a nearly identical *Arabidopsis* homologue, *APT1*.

Here we showed that overexpression of the gene alone in tobacco-cultured cells led to increased phosphate uptake, and that the uptake activity attributable to the transgene had a K_m of 3.1 μM, indicating that the plant gene encodes a high-affinity phosphate transporter. We also showed that the transgenic cells exhibited increased biomass production under certain phosphate-limited conditions.

MATERIALS AND METHODS

Gene Isolation. Using the *A. thaliana* sequence found in the expressed sequence tag database (accession no. Z33763), a full-length cDNA clone (1,755 bp) and its genomic clones (5,810 bp) were isolated from DNA libraries of *A. thaliana* (ecotype Columbia) (Stratagene).

Complementation Tests of the *pho84* Mutants. The full-length cDNA clone, pPHT1C19, has one nucleotide (adenosine) deletion in the coding sequence resulting in a frame-shift mutation; thus, the *Sac*I–*Bal*I fragment (375 bp) containing the deletion was replaced with a normal fragment (376 bp) from the genomic DNA clone pPHTGZ37 to create pPHT1Crep. To express the cDNA under the *PHO84* promoter in yeast, the *Sac*I–*Xho*I fragment of pPHT1Crep was introduced into the *Eco*RI site of pNE202 (17), and then the *Hin*dIII–*Sac*I fragment was introduced into the *Hin*dIII–*Sac*I site of pNE302. To express the cDNA under the *GAL1* promoter, the *Sac*I–*Xho*I fragment of pPHT1Crep was introduced at the *Xho*I site of pYEUra3 (CLONTECH). These constructs were delivered into yeast cells as described (10).

Overexpression of the *A. thaliana* Phosphate Transporter Gene in Tobacco Cells. The *Sac*I–*Hin*dIII fragment of the genomic clone pPHTGZ37 was placed downstream of the cauliflower mosaic virus 35S promoter (18) by ligating the fragment with the *Bam*HI–*Sac*I fragment of pMi12 to create p35SPHT1. pMi12 was constructed as follows. The *Sst*II–

Abbreviations: fwt, fresh weight; MS medium, Murashige–Skoog medium.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. D86591 and D86608 for *PHT1* cDNA and genomic sequence, respectively).

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*Eco*RI fragment of pBI121 (CLONTECH) was inserted into the *Sst*II and *Eco*RI sites of pGA643 (Pharmacia). From this construct, the *Sal*I fragment carrying the neomycin phosphotransferase (NPT) II gene for selection of transgenic plants was isolated, and inserted into the *Sal*I site of a pUC19 derivative that carries the NPTI gene of pACYC177 (GenBank accession no. X06402).

p35SPHT1 was delivered into tobacco-cultured cell of the BY-2 cell line using a hand-made particle gun as described (19). Transgenic tobacco cells were selected using an antibiotic, kanamycin, on Murashige–Skoog (MS) medium agar plates (20) and then maintained in liquid MS medium.

Measurement of Phosphate Uptake Rates of Tobacco Cells. Transgenic tobacco cells were cultured as described in MS medium (21). Before the phosphate uptake experiments, the cells were cultured in MS medium without kanamycin for 1 week. A portion of the cells (5 g) was transferred to 100 ml of fresh MS medium, cultured for 24 h, and used for the phosphate uptake experiments. Phosphate uptake was measured by the method of Sakano *et al.* (22) with minor modifications. Briefly, the cultured cells were washed four times and resuspended in phosphate-free medium (5 mM Mes-Tris, pH 6.0/20.6 mM NH_4NO_3/18.8 mM KNO_3/3.0 mM $CaCl_2 \cdot 2H_2O$/1.5 mM $MgSO_4 \cdot 7H_2O$/100 μM cycloheximide/3% sucrose) to a final concentration of 0.1 g fresh weight (fwt)/ml. To start the reaction, 0.5 ml of 2 mM phosphate containing 50 μCi of [^{32}P]phosphate (500 mCi/mmol, Amersham; 1 Ci = 37 GBq) was added to 9.5 ml of the cell suspension to a final concentration of 100 μM phosphate. To stop the reaction, 0.25 ml of the cell suspension was transferred into a microcentrifuge tube with a membrane filter (UFC30GV00; Millipore). The filtrate was collected by centrifugation. The remains of radioactivity in the medium was determined by liquid scintillation spectrometry (Beckman).

K_m values of phosphate transporter activity to phosphate in tobacco cultured cells were determined by the depletion-curve method (23).

RESULTS

We isolated an *A. thaliana* full-length cDNA, named *PHT1*, that shares amino acid sequence homology with the yeast high-affinity phosphate transporter *PHO84* (10), and attempted to complement the yeast *pho84* mutant phenotype with the plant gene. [We designate the gene *PHT1* according to the rules recommended by the Commission on Plant Gene Nomenclature (24).] However, our *PHT1* constructs did not restore normal phosphate uptake to yeast *pho84* mutants (see *Discussion*). Thus, to establish that the *PHT1* product functions as a phosphate transporter, we expressed the gene in a heterologous plant cell system (tobacco cultured cells) as shown below.

Ectopic Expression of an *A. thaliana* Phosphate Transporter Gene in Tobacco Cells. *PHT1* was placed under a strong plant promoter, the cauliflower mosaic virus 35S promoter (17), and introduced in cells of the tobacco BY-2 line. A genomic DNA fragment of *PHT1* that contained the coding sequence with the second intron (151 bp) was introduced a plant expression vector pMi12 (Fig. 1). The resultant plasmid DNA was delivered into tobacco suspension-cultured cells (*Nicotiana tabacum*) by particle bombardment. Northern blot analysis of the transgenic lines showed varied levels of gene expression among cell lines. For further analyses, we selected six transgenic lines expressing *PHT1* at high levels and six kanamycin-resistant lines carrying the vector alone as controls.

The tobacco cells expressing the *PHT1* gene at high levels showed increased inorganic phosphate uptake (Fig. 2). The stationary-phased cells (7–9 days cultured) were transferred into fresh medium and cultured for 24 h before the uptake experiments. The phosphate uptake rates of the transgene

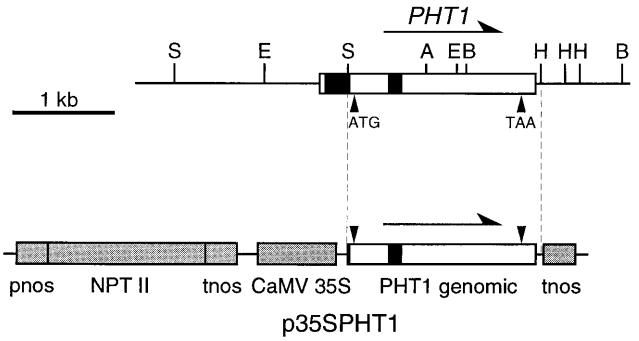

FIG. 1. Structure of the *PHT1* gene and the construct for plant transformation. (*Upper*) The structure of the *PHT1* gene is shown. Open boxes, exon sequences; closed boxes, intron sequences; S, *Sac*I; E, *Eco*RI; A, *Apa*I; B, *Bam*HI; H, *Hin*dIII. The positions of the translation start codon (ATG) and the translation termination codon (TAA) are shown with arrowheads. Note that the first intron (236 bp) locates in the 5′-untranslational region. (*Lower*) To introduce the *PHT1* gene into tobacco cells by particle bombardment, the *Sac*I–*Hin*dIII fragment of *PHT1* was inserted between the cauliflower mosaic virus 35S promoter (CaMV 35S) and the nopalin synthase gene terminator (tnos) in a plant vector pMi12, named p35SPHT1. pMi12 carries the neomycin phosphotransferase II (NPTII) gene, driven by the nopalin synthase gene promoter (pnos), for selection of transgenic cells by kanamycin.

expressing cell lines varied (193–596 nmol/h per g fwt), but correlated well with the amounts of *PHT1* mRNA in each cell line. The control lines carrying the vector alone showed uptake rates of 101–203 nmol/h per g fwt. The stationary phase cells in medium deprived of nutrients show substantially higher uptake activity than the cells in nutrient-rich medium; thus, differences of uptake rates of cells carrying the transgene and the control vector were rather small (490–874 nmol/h per g fwt for the former and 435–658 nmol/h per g fwt for latter). These results indicate that heterologous gene expression alone enhances phosphate uptake in plant cells.

Characterization of the *A. thaliana* Phosphate Transport Activity in Tobacco Cells. To characterize the *A. thaliana*

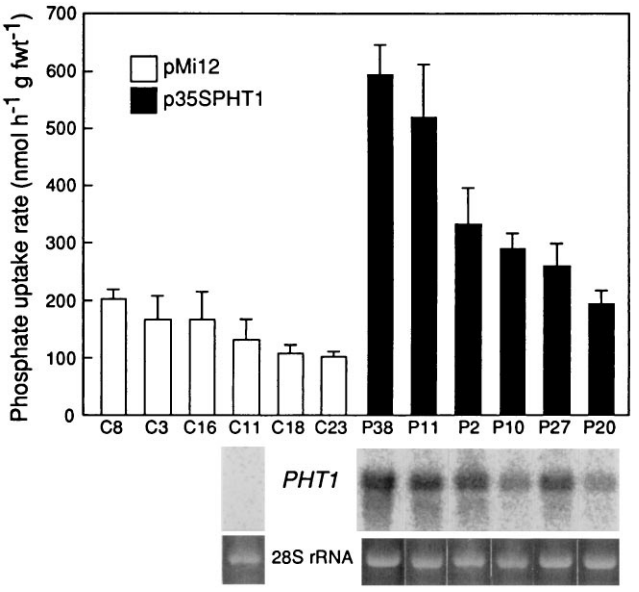

FIG. 2. Phosphate uptake of transgenic tobacco cells. Inorganic phosphate uptake rates of suspension-cultured cells carrying the *A. thaliana* phosphate transporter construct p35SPHT1 (filled bar) and the control construct pMi12 (open bar) were measured. Data are the mean ± SD from nine experiments. The amounts of *PHT1* mRNA and the 28S rRNA on a Northern blot of each cell line (C11, P38, P11, P2, P10, P27, and P20) are shown under the histogram.

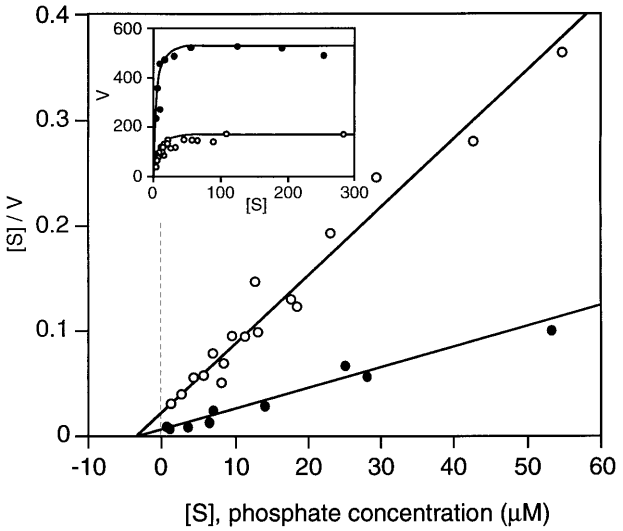

FIG. 3. Phosphate uptake in transgenic tobacco cultured cells carrying the *Arabidopsis* gene *PHT1*. Phosphate uptake rates (V; n mol/h per g fwt) in a transgenic cell line carrying p35SPHT1 (P11) (●) and a control cell line carrying pMi12 (C11) (○) were determined by the depletion-curve method (23). [S], phosphate concentration (μM).

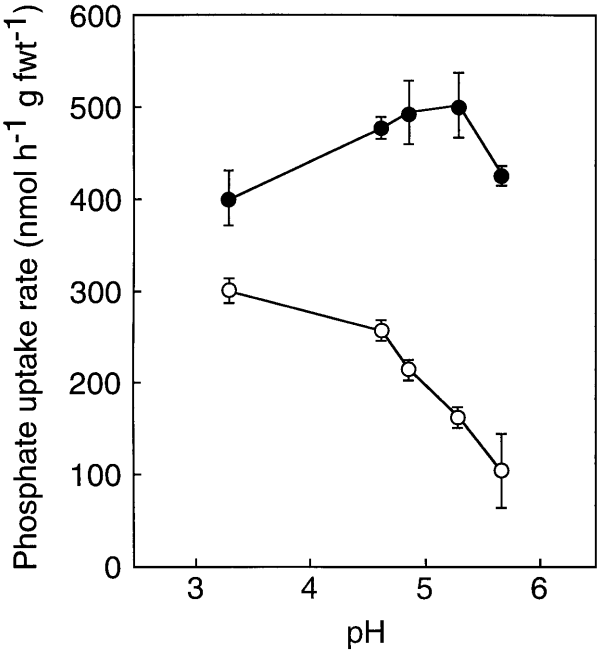

FIG. 4. pH-dependence of phosphate uptake of transgenic tobacco cells. Cultured cells carrying either p35SPHT1 (●) or the vector pMi12 alone (○) were washed four times with phosphate-free medium (see *Materials and Methods*) of different pH (adjusted by Tris). Just before the phosphate uptake experiments, pH in each medium was measured. Data are the mean ± SD from three experiments.

phosphate transport activity, we analyzed the P11 cell line, which had the highest level of expression of the *A. thaliana* gene among the cell lines examined, in comparison with a control line, C3. Phosphate uptake of these cells was measured 24 h after culturing in fresh medium. The phosphate uptake activities in the two cell lines showed a similarity in their K_m values (3.1 μM for P11 and 3.4 μM for C11) (Fig. 3), indicating that they are a high-affinity type. The K_m values are consistent with those of other plants with high-affinity phosphate transport activity (5, 9, 22, 25, 26). In addition, phosphate uptake inhibition by protonophores 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone, and the plasma membrane H^+-ATPase inhibitor diethylstilbestorol (27) (Table 1) suggests that the *A. thaliana* transport activity is based on an H^+ cotransport mechanism. Inhibition by arsenate, a phosphate analogue (28), and competition by phosphate were seen in both plant cells (Table 1).

A striking difference between the two lines was in pH dependence of phosphate uptake (Fig. 4); the phosphate uptake activity of the P11 cells showed a preference for higher pH than that of the host cell. Three factors may affect the optimum pH of phosphate uptake in higher plants (29): (*i*) the intrinsic sensitivity of a transporter to pH; (*ii*) the relative concentration of the possible transported form $H_2PO_4^-$, which corresponds well with the effects of pH on phosphate uptake; and (*iii*) a thermodynamic control by pH, since an H^+ cotransport mechanism has been suggested. It is possible that the *A. thaliana* phosphate transporter itself has an intrinsic pH response or enhancement of the transporter alone affects the overall response of the cells to pH.

Table 1. Effects of inhibitors and competitors on phosphate uptake

Substance*	Relative uptake of phosphate, %	
	pMi12	p35SPHT1
None	100	100
2,4-DNP, 100 μM	0	17
CCCP, 10 μM	15	12
Diethylstilbestrol, 100 μM	24	18
Phosphate, 100 μM	39	39
Phosphate, 2 mM	10	9
Arsenate, 100 μM	63	61
Arsenate, 2 mM	21	20

*In the assay medium, the phosphate concentration was 100 μM for the phosphate competition experiments, in which cold phosphate was added to 100 μM or 2 mM. 2,4-DNP; 2,4-dinitrophenol, CCCP; carbonyl cyanide *m*-chlorophenylhydrazone.

Increased Cell Growth Under Phosphate-Limited Conditions. The growth rates of the tobacco cells expressing *PHT1* were higher than those of the control cells when the supply of phosphate was limited (Table 2). The stationary-phased cultured cells (0.5 g) of the lines P11, P38, and C3 were cultured in fresh MS medium (30) (100 ml, 2.5 mM phosphate) for 24 h and then allowed to grow in phosphate free MS medium for 4 days. The fresh weights of these cells after culturing for 24 h were almost the same. However, the 4-day-cultured transgenic cells showed 42% higher fresh weights or 55% higher dry weights, on average, than the control.

Chromosomal Location of the Phosphate Transporter Gene. Chromosomal mapping of the *A. thaliana* transporter gene *PHT1* using recombinant inbred lines (31, 32) showed that the gene located on chromosome 5 (Fig. 5). It has been suggested that the mutant locus for phosphate loading to xylem of *A. thaliana pho1* (33) is a phosphate transporter. Another mutation for phosphate accumulation in leaf, *pho2*, was also found in *A. thaliana* (34). However, the mapping data indicate that *PHT1* is allelic with neither *pho1* (mapped on chromo-

Table 2. Cell growth of tobacco lines expressing the *A. thaliana* phosphate transporter

Cell line	Initial weight (a), g	After 4 days (b), g	Ratio (b/a)	Dry weight, g
P11	0.70 ± 0.05	1.52 ± 0.01	2.19	0.15 ± 0.00
P38	0.80 ± 0.08	1.65 ± 0.05	2.07	0.16 ± 0.00
C3	0.73 ± 0.04	1.09 ± 0.01	1.51	0.10 ± 0.01

Cells were cultured in fresh medium (2.5 mM phosphate) for 24 h and then grown in phosphate-free MS medium for 4 days. Data are the mean ± SD from three experiments.

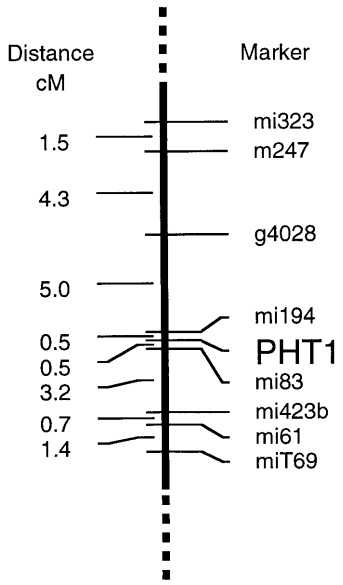

FIG. 5. The chromosomal position of the *PHT1* gene. The recombinant inbred lines of ecotypes Columbia and Landsberg *erecta* (31) and restriction fragment length polymorphism markers (31, 32) were used for mapping of the gene. Segregation data from 100 chromosomes were analyzed with the computer program MAPMAKER (version 2.0).

some 3) or *pho2* (mapped on chromosome 2). Root-specific expression of *AtPT1* (*PHT1*) was reported by Muchhal *et al.* (15) and in this study (data not shown); thus, the phosphate transporter seems to be the machinery of phosphate uptake in roots. Allelic tests of the *PHT* gene family (to be published elsewhere) with *pho1* and *pho2* are under way in our laboratory.

DISCUSSION

In this study we showed that the *A. thaliana* gene *PHT1* encodes a high-affinity phosphate transporter. Ectopic expression of *PHT1* alone at high levels in tobacco suspension-cultured cells enhanced phosphate uptake activity (Fig. 2). The uptake activity attributable to the gene expression shows a high affinity to phosphate (K_m = 3.1 μM) (Fig. 3) and responds to the chemical inhibitors in a similar manner to the endogenous phosphate uptake activity of the host cells (Table 1), but differs in its pH dependence from that of the host cell (Fig. 4). Taken together with the high cell growth attributable to the transgene under certain phosphate-limited conditions (Table 2), these results indicate that the plant gene product homologous to the yeast PHO84 functions to uptake phosphate in plant cells.

This study indicates that a major control point for cell growth can be regulated by gene manipulation of the high-affinity phosphate transporter (Table 2). Phosphate acquisition in cultured cells is complicated by adaptive changes of the uptake activity (V_{max}) dependent on the status of phosphate starvation (5, 9). The tobacco BY-2 cell line used in this study also shows such adaptive response (26); for example, the activity of the starved control cells C3 (471 ± 56 nmol/h per g fwt) decreased to 168 ± 39 nmol/h per g fwt 24 h after transferal to fresh medium. However, the transgenic cells have higher phosphate uptake activity even in phosphate-sufficient medium due to constitutive expression of *PHT1*; for example, the activity of the P11 cells was 874 ± 150 nmol/h per g fwt in phosphate-starved medium and 520 ± 93 nmol/h per g fwt 24 h after transferal to fresh medium. As starved cultured cells of tobacco scavenge phosphate for 1–2 days after transferal to fresh medium (35), higher phosphate uptake activity may result in higher amounts of phosphate accumulation in transgenic cells compared with control cells. As rapidly absorbed phosphate is deposited in vacuoles (36) and thought to be released gradually during cell growth (22), the initial phosphate uptake is a major control point for the subsequent biomass production.

In our study *PHT1*, the plant homolog of *PHO84* failed to complement yeast strains with either of two *pho84* mutations. The plant gene was placed under the control of either the *PHO84* promoter or the inducible *GAL1* promoter in an *E. coli*/yeast shuttle vector and introduced into yeast *pho84* mutants. All transformants examined for each construct showed high level expression of the *PHT1* mRNA (data not shown), yet failed to take up phosphate normally when measured as described (37). PHO84 has been suggested to interact with other components for phosphate uptake in yeast (38, 39), and so one potential explanation for our results is that the plant homologue lacks some residue(s) needed for proper interaction with these components. In contrast to our results, Muchhal *et al.* (15) reported that either of two *A. thaliana* phosphate transporter cDNAs, *AtPT1* (*PHT1*) or *AtPT2* complemented a *pho84* mutation. They used a high-copy plasmid vector, pYES2 (20–50 copies in yeast), while we used a low-copy number plasmid vector, pYEUra3 (1–2 copies per plasmid-bearing cell). While this difference could explain our discrepant results, recently, Smith *et al.* (16) also reported inability to complement *pho84* yeast strains with another homolog 99% identical to *PHT1* despite using the high-copy pYES2 vector.

Many soils throughout the world are phosphate-deficient (40). Even after fertilization, the added phosphate is immobilized rapidly in soils as insoluble phosphate salts or in organic form, or locked into the crystal lattice of the mica surfaces (41). In this context, the phosphate uptake ability of plants is a major growth-limiting factor. Our results suggest that crops can be improved by enhancing phosphate uptake activity.

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