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Pht2;1 Encodes a Low-Affinity Phosphate Transporter from Arabidopsis

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An Arabidopsis genomic sequence was recently shown to share similarity with bacterial and eukaryotic phosphate (Pi) transporters. We have cloned the corresponding cDNA, which we named *Pht2;1*, and subsequently performed gene expression studies and functional analysis of the protein product. The cDNA encodes a 61-kD protein with a putative topology of 12 transmembrane (TM) domains interrupted by a large hydrophilic loop between TM8 and TM9. Two boxes of eight and nine amino acids, located in the N- and C-terminal domains, respectively, are highly conserved among species across all kingdoms (eubacteria, archea, fungi, plants, and animals). The *Pht2;1* gene is predominantly expressed in green tissue, the amount of transcript staying constant in leaves irrespective of the Pi status of the shoot; in roots, however, there is a marginal increase in mRNA amounts in response to Pi deprivation. Although the protein is highly similar to eukaryotic sodium-dependent Pi transporters, functional analysis of the Pht2;1 protein in mutant yeast cells indicates that it is a proton/Pi symporter dependent on the electrochemical gradient across the plasma membrane. Its fairly high apparent K_m for Pi (0.4 mM) and high mRNA content in the shoot, especially in leaves, suggest a role for shoot organs in Pi loading. *Pht2;1* thus differs from members of the recently described plant Pi transporter family in primary structure, affinity for Pi, and presumed function.

INTRODUCTION

Phosphorus, an essential macronutrient for all living organisms, serves various basic biological functions—as a structural element in nucleic acids and phospholipids, as a facilitator in energy metabolism and the activation of metabolic intermediates, and as a component in signal transduction cascades and the regulation of enzymes.

A primary step in the utilization of phosphorus is its uptake into the living cell. In the yeast *Saccharomyces cerevisiae*, two high-affinity phosphate (Pi) permeases, PHO84 and PHO89, mediate Pi transport across the plasma membrane (Bun-Ya et al., 1991; Martinez and Persson, 1998). PHO84 is a H^+/Pi cotransporter, and PHO89 mediates sodium-dependent Pi transport activity. Two related enzymes exist in the fungus *Neurospora crassa*, PHO5 and PHO4, respectively (Versaw and Metzenberg, 1995). Physiological analysis with yeast has indicated the existence of a third system with a low affinity for Pi (K_m = 770 μM) (Tamai et al., 1985). Even though the yeast genome has been entirely sequenced, however, the gene that encodes the corresponding Pi transporter has not been identified.

In vascular plants, several genes and/or cDNAs related to the yeast *Pho84* have recently been isolated (Muchhal et al., 1996; Kai et al., 1997; Leggewie et al., 1997; Smith et al., 1997; Daram et al., 1998; Liu et al., 1998a, 1998b). Computational modeling of the encoded proteins predicted a conserved secondary structure containing 12 transmembrane (TM) domains with a large hydrophilic loop between TM6 and TM7 and both of the hydrophilic N and C termini located in the cytoplasm. Expression of the corresponding genes was generally greater in roots than in shoots and was enhanced in response to Pi deprivation (Leggewie et al., 1997; Liu et al., 1998a). Kinetic measurements and in situ localization of mRNA transcripts in rhizodermal cells indicated a key regulatory role of at least some of the plant PHO84-related proteins in high-affinity Pi uptake at the root–soil interface (Mitsukawa et al., 1997; Daram et al., 1998; Liu et al., 1998a).

In addition to the uptake system at the root–soil interface, three distinct transport systems are expected to participate in Pi translocation within the plant: (1) secretion of Pi into the

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xylem in the root; (2) loading of leaf cells with Pi from the vascular system; and (3) cycling within the plant, for example, during phosphorus deprivation, or senescence. The Arabidopsis mutant *pho1* is defective in Pi loading of the xylem, which suggests that a protein secretes Pi from xylem parenchyma cells into xylem vessels in the wild-type plant (Poirier et al., 1991; Delhaize and Randall, 1995). In contrast, a second mutant, *pho2*, exhibits a defect in phloem loading (Delhaize and Randall, 1995). Whereas the mutation in *pho1* leads to much lower concentrations of Pi in the shoot (Poirier et al., 1991), the *pho2* mutant is characterized by Pi accumulation in leaves even during Pi starvation (Delhaize and Randall, 1995). Cloning of the genes corresponding to the respective phenotypes of the mutants has not been reported.

In vertebrates, a family of sodium-dependent Pi transporters is divided into three unrelated types, of which type I (NaPi-1) and type II (NaPi-2, -3, -4,- 5, and -6) were found by expression cloning in Xenopus oocytes (Biber et al., 1996). The exact role of the type I Na/Pi–cotransporter is currently not clear, but type II transporters are involved in renal and intestinal reabsorption of Pi and thus regulate Pi homeostasis in mammals (Werner et al., 1998). The third transporter type, the type III sodium-dependent Pi cotransporter, is ubiquitous in a wide range of mammalian cells. Two type III proteins, Glvr-1 and Ram-1, have previously been described as cellular receptors for retroviruses in humans and rats, respectively (Miller et al., 1994; van Zeijl et al., 1994). They share ~60% overall sequence identity and are ~25% identical to the yeast PHO89 and *N. crassa* PHO4 Pi permeases. Functional analysis of these proteins has also uncovered sodium-dependent Pi symport activity (Kavanaugh et al., 1994; Olah et al., 1994), which is modulated by retroviral envelope gycoprotein binding to the receptor (Kozak et al., 1995). The role of type III Pi transporters greatly differs from that of the type II transporters, and they probably serve as a housekeeping Na^+/Pi cotransport system; in most cells, that is, the type III enzymes absorb the readily available Pi from interstitial fluid for normal cellular function (Kavanaugh et al., 1994). The sodium dependence of these Pi transporters is in agreement with the general finding that the electrochemical gradient of sodium is the major driving force for Pi uptake across the plasma membrane of animal cells. In contrast, Pi uptake into prokaryotes, plant cells, and mitochondria is driven by a proton gradient (Wehrle and Pedersen, 1989), and both transport mechanisms are found in fungal species (Bun-Ya et al., 1991; Martinez and Persson, 1998).

Here, we report on the characterization of a novel Pi transporter from the vascular plant Arabidopsis. We have designated the gene as *ARAth;Pht2;1* or *Pht2;1*, according to the rules recommended by the Commission on Plant Gene Nomenclature (http://mbclserver.rutgers.edu/CPGN/Guide.html). Pht2;1 shares high similarity with sequences from archaebacteria to humans and with type III mammalian Pi transporters. Its primary functions appear to be low-affinity Pi transport in the shoot and, it is presumed, Pi loading of the leaf.

RESULTS

Cloning of Pht2;1 and Computational Sequence Analysis

The *Pht2;1* cDNA was amplified from an Arabidopsis cDNA library by polymerase chain reaction with oligonucleotide primers based on mRNA sequence information from the EMBL, GenBank, and DDBJ databases (accession number X97484). The respective gene sequence is from a European project, ESSA (European Scientists Sequencing Arabidopsis), in which a 81-kb contig from Arabidopsis chromosome III (Quigley et al., 1996) was among the sequences used. Seventeen novel genes were identified during this work; one of them, *ORF02*, exhibited cross-phylum similarities to genes from *Haemophilus influenza*, yeast, *Caenorhabditis elegans*, and humans. The respective human protein belongs to the gibbon ape leukemia virus receptor family (van Zeijl et al., 1994), members of which have been shown to encode inducible sodium-dependent Pi symporters and share similarity with PHO4, a putative Pi permease from *N. crassa* (Mann et al., 1989; Kavanaugh et al., 1994). The coding sequence of the *Pht2;1* gene is interrupted by two small introns of 129 and 103 bp in length (Figure 1A). The amplified *Pht2;1* cDNA is 1916 bp and encodes a putative protein of 587 amino acids with a predicted molecular mass of 61 kD. The predicted protein sequence consists of >60% hydrophobic amino acids, yielding a calculated isoelectric point of 9.29. Predictions of the number of TM regions and protein orientation were 10 (PSORT II; see Methods), 11 (SOSUI; see Methods), 11 to 12 (PREDICTPROTEIN; see Methods), or 12 (TMpred, Genetics Computer Group program package; Devreux et al., 1984) (Figure 1B), depending on the computer program used. The model strongly preferred by TMpred and PREDICTPROTEIN suggested an extracellular location of the N terminus. A large hydrophilic loop between TM8 and TM9 as well as the C terminus were located either extracellularly or intracellularly, depending on the number of TM segments calculated (Figure 1C).

To determine which sequences from the databases are potentially related to the Pht2;1 polypeptide, a TBLASTN search (Altschul et al., 1990) was performed, and cross-kingdom relationships to sequences from archaea, eubacteria, and eukaryota were found with P values $<10^{-40}$ (P being the probability that the calculated high score of similarity might have occurred by chance). A multiple sequence alignment using representative sequences from evolutionarily distant organisms (Figures 2 and 3) demonstrated high similarities across all kingdoms, especially in the N- and C-terminal regions, whereas poor similarity and large gaps were displayed in the central region of the proteins (data not shown).

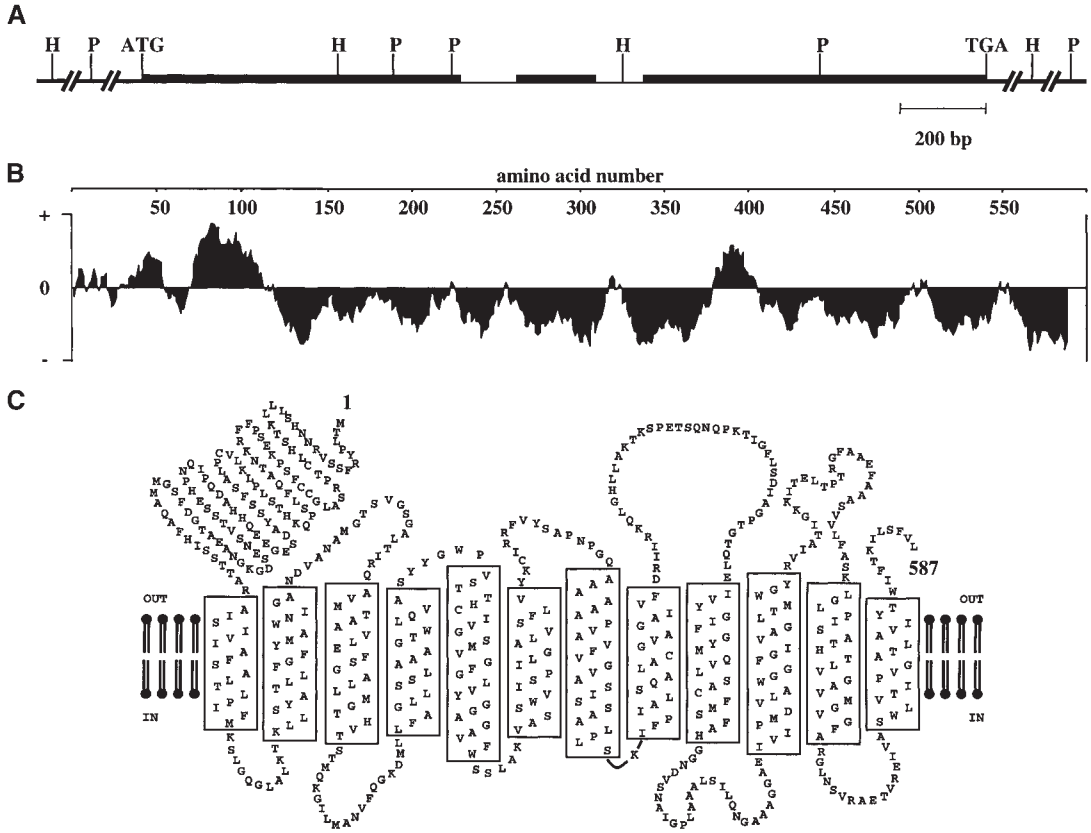

Figure 1. *Pht2;1* Gene Structure and Predicted Topology of the Encoded Protein.

(A) Structure of the *Pht2;1* transcription unit according to the published genomic sequence. The open reading frame is terminated by a TGA in-frame stop codon. The diagram depicts introns (thin lines) and exons (black boxes). Thick lines indicate 5′ and 3′ untranslated regions. Sizes are given in basepairs. H and P denote restriction sites for HindIII or PstI, respectively.

(B) Hydropathy plot of the Pht2;1 protein, performed with the TMpred software. The algorithm is based on the statistical analysis of TMbase (Hofmann and Stoffel, 1993) and allows prediction of TM domains. Hydrophilicity (+) and hydrophobicity (−) are indicated.

(C) Predicted topology of the Pht2;1 polypeptide with 12 TM domains, a long extracellular hydrophilic N terminus, and a hydrophilic loop between TM8 and TM9. Predictions were conducted as described in Methods. OUT and IN denote extracellular and intracellular space, respectively.

Among the predicted TMs within Pht2;1, regions TM2 to TM6, TM10, and TM12 aligned with TM domains from all other sequences compared (Figures 2 and 3). A highly conserved domain in all the aligned sequences was located in TM2 and the adjacent extracellular loop (amino acids 173 to 180) in the sequence GA/GNDV/AA/SNA/S (where A/G indicates the prevalent occurrence of A over G, for example). A similar domain spanning amino acids 435 to 443, H**G**x**ND**V/IA/S**N**A (where x indicates any amino acid and boldface letters indicate the amino acids conserved between the two boxes), was located in the C-terminal half within the intracellular loop between TM9 and TM10. Interestingly, N- and C-terminal regions of Pht2;1 (amino acids 147 to 315 and 476 to 580) exhibited 25% identity to each other. When compared individually with the C-terminal half of the *Archaeoglobus fulgidus* sequence (amino acids 171 to 333), sequence identity was 28 and 51%, respectively, suggesting that a major part of the *Pht2;1* gene may have resulted from the duplication of an ancestral gene during evolution.

A *Mesembryanthemum crystallinum* cDNA with the EMBL, GenBank, and DDBJ accession number U84890 shares high similarity with *Pht2;1* (Figures 2 and 3). Moreover, a BLASTN (Gish and States, 1993) search detected three expressed sequence tags from rice that displayed 69, 74, and 75% identity to regions of the *Pht2;1* cDNA (accession numbers D47102, D47595, and C24895). Thus, Pi permeases similar to Pht2;1 also exist in at least some monocotyledonous species. A unique feature of the two plant polypeptides, compared with all the putative homologs from other kingdoms, is their extended N termini, which harbor both a conserved

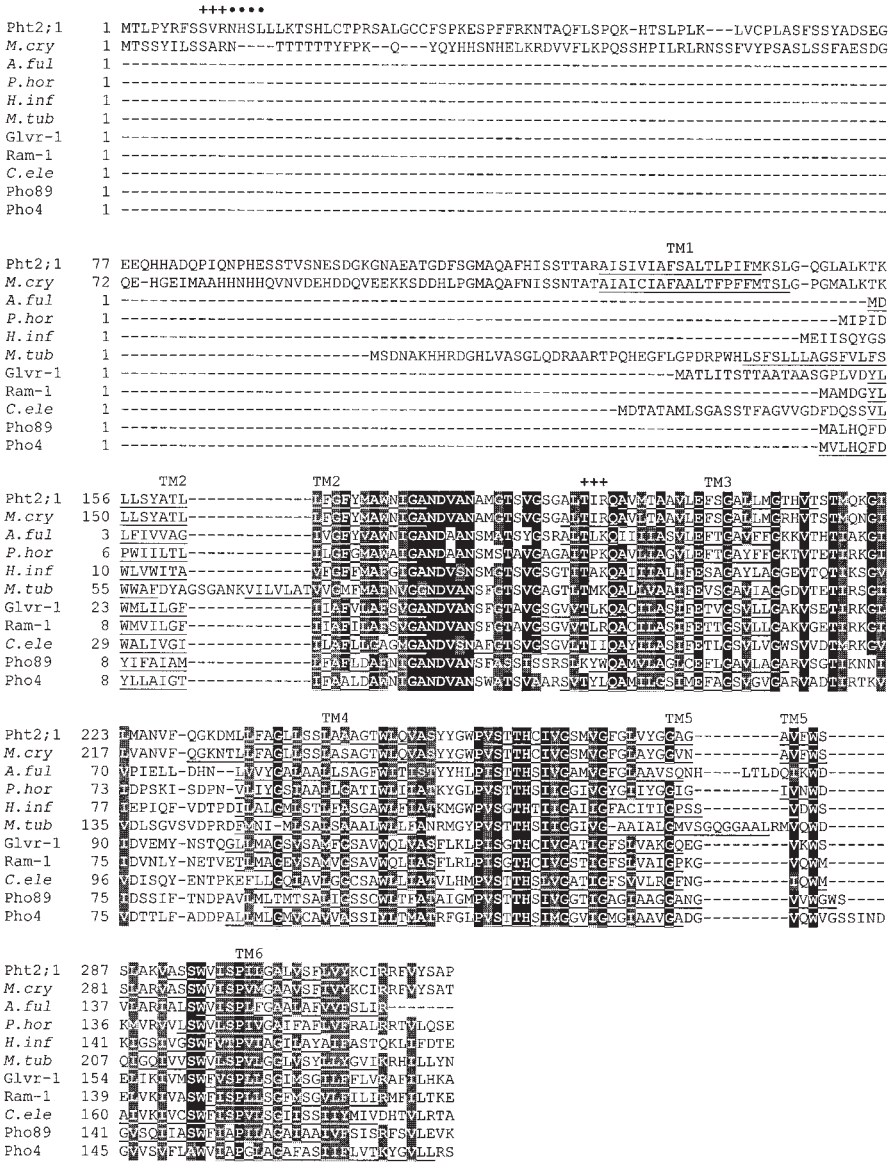

Figure 2. Alignment of the N-Terminal Domains of Type III Pi Transporters.

Alignment of the N-terminal domains of the deduced amino acid sequence of Pht2;1 with that of plant, archaebacterial, eubacterial, and eukaryotic (human, rat, nematode, yeast, and fungal) type III Pi transporters. Identical and similar amino acids are shaded black and gray, respectively. The membrane-spanning domains in the Pht2;1 polypeptide as predicted by TMpred (Hofmann and Stoffel, 1993) are underlined and numbered TM1 to TM6. Dots indicate the consensus site for N-linked glycosylation; (+) denotes sites for phosphorylation by protein kinase C. Polypeptide names are given where protein function has been shown experimentally; otherwise, species initials are given in italics. The following GenBank, EMBL, DDBJ, and SwissProt accession numbers refer to the sequences used for the alignments: *C.ele* (*C. elegans*), U50312; Pht2;1 (Arabidopsis), X98130; *M.cry* (*M. crystallinum*), U84890; *A.ful* (*A. fulgidus*), AE000978; *P.hor* (*P. horikoshii*), AP000003; *H.inf* (*H. influenza*), P45268; Glvr-1 (human), L20859; Ram-1 (*R. norvegicus*), L19931; *M.tub* (*M. tuberculosis*), Z77163; Pho89 (*S. cerevisiae*), P38361; and Pho4 (*N. crassa*), P15710. Dashes indicate artificially introduced gaps to allow optimal alignment. Numbers at left indicate the number of the first amino acid in the line of the respective protein.

N-glycosylation site (amino acids 12 to 15) and a phosphorylation site (amino acids 9 to 11) (Figure 2). A second putative phosphorylation site in the loop between TM2 and TM3 of Pht2;1 (amino acids 191 to 193) is conserved between plants, archaebacteria, eubacteria, and vertebrates.

In a corresponding phylogenetic tree (Figure 4), the plant sequences clustered with archaebacterial and eubacterial sequences. Apparently vascular plants and prokaryotes share a more recent common ancestor of Pht2;1 with each other than with fungal or vertebrate species or with *C. elegans.*

Pht2;1 Expression in Arabidopsis

Hybridization of a radioactively labeled *Pht2;1* cDNA probe to a genomic DNA gel blot showed three hybridizing bands when the genomic DNA was digested with NotI and either HindIII or PstI (Figure 5). This agrees with the restriction map of the 81-kb contig (Figure 1A), except that the 0.14-kb PstI fragment does not appear in Figure 5. No additional bands became visible when the membrane was hybridized and washed at lower stringency (data not shown), from which we conclude that *Pht2;1* is present as a single-copy gene in the Arabidopsis haploid genome.

RNA gel blot analysis (Figure 6A) revealed an abundance of the corresponding mRNA in green tissue, with a strong signal in nonsenescent rosette leaves and weaker signals in cauline and senescent leaves, siliques, and flowers. In contrast, the signal was barely detectable in roots. *RNS1*, which encodes a ribonuclease, was used as a marker of leaf senescence (Bariola et al., 1994).

Enhancement of Pi uptake capacity by Pi deprivation has been observed in all plant species tested and has been attributed, at least partly, to enhanced expression of Pi transporter genes in the root (Marschner, 1995; Leggewie et al., 1997). To determine whether or not *Pht2;1* expression is regulated by Pi concentration, we performed RNA gel blot analysis with total RNA isolated from Pi-starved Arabidopsis plants. In green leaves, the change in transcript amounts was barely detectable during stress from Pi deprivation, whereas in roots, the transcript marginally increased in abundance (Figure 6B). The amounts of *RNS1* mRNA, which we

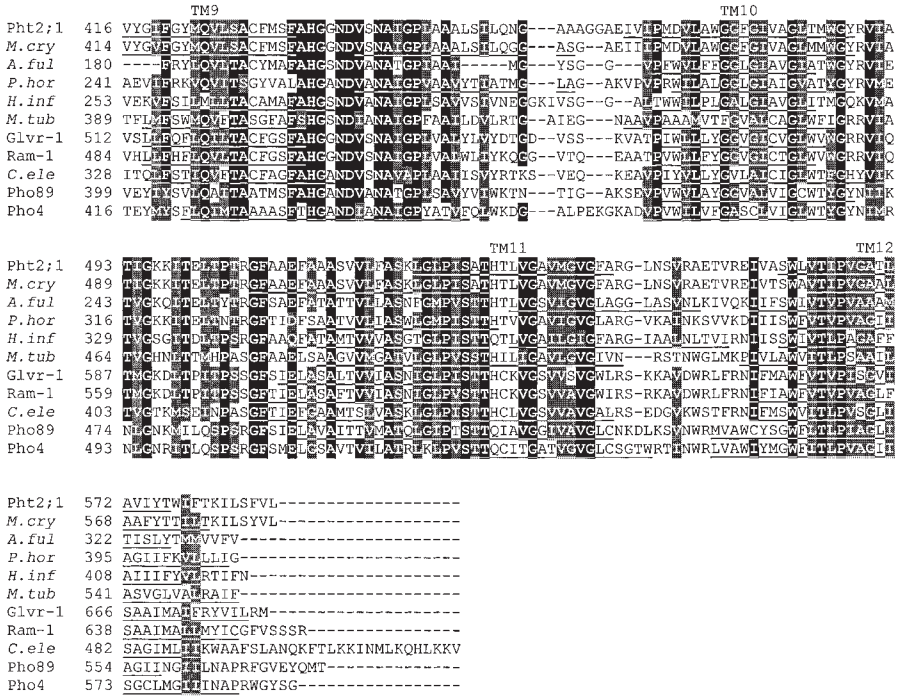

Figure 3. Alignment of the C-Terminal Domains of Type III Pi Transporters.

Alignment of the C-terminal domains of the deduced amino acid sequence of *Pht2;1* with that of plant, archaebacterial, eubacterial, and eukaryotic (human, rat, nematode, yeast, and fungal) type III Pi transporters. Identical and similar amino acids are shaded in black and gray, respectively. The membrane-spanning domains in the Pht2;1 polypeptide as predicted by TMpred (Hofmann and Stoffel, 1993) are underlined and numbered TM9 to TM12. Polypeptide names are given where protein function has been shown experimentally; otherwise, species initials are given in italics. The sequences used for the alignments are the same as those in Figure 2, as are the functions of dashes and the numbers at left.

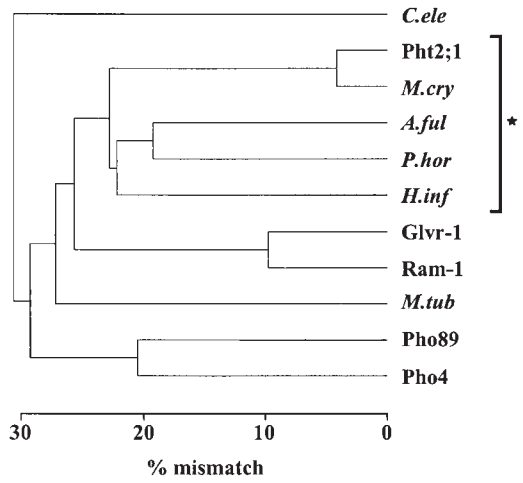

Figure 4. Phylogenetic Relationships of Pht2;1 and Related Proteins or Putative Polypeptides.

The average distance tree was created by using Jalview, a Java multiple alignment editor available on the Baylor College of Medicine Search Launcher. Branch lengths are the percentage of mismatches between two nodes. Polypeptide names are as given in Figures 2 and 3. The cluster formed by plant, archaebacterial, and eubacterial polypeptide sequences is marked with an asterisk.

used as a marker gene for Pi deficiency (Bariola et al., 1994), increased when plants were deprived of Pi. Eukaryotic translation initiation factor 4A (eIF4A; Metz et al., 1992) was used as a marker of constitutive gene expression (Bucher and Kuhlemeier, 1993; Mandel et al., 1995).

Physiological as well as molecular genetic studies have shown that the phosphorus status of the shoot determines Pi uptake capacity as well as the extent of expression of root Pi transporters (e.g., see Liu et al., 1998a). To analyze whether *Pht2;1* expression in leaves responded to a change in the phosphorus status of the shoot, we performed RNA gel blot analysis of samples of total RNA isolated from the two Arabidopsis mutants, *pho1* (Poirier et al., 1991) and *pho2* (Delhaize and Randall, 1995), which exhibit diminished or enhanced phosphorus concentrations in leaves, respectively (Figure 6C). *Pht2;1* mRNA concentrations in the two mutants increased moderately over that of the wild type. Similar changes were observed with the constitutive control *eIF4A*. Thus, we conclude that the slight changes observed in *Pht2;1* mRNA concentrations did not specifically reflect the phosphorus status of the plant but rather correlated with the total mRNA concentrations. In addition, *Pht2;1* expression in leaves of *pho1* and *pho2* is comparable with that in the wild type. In addition, in situ hybridization studies allowed localization of *Pht2;1* mRNA to specific regions in Arabidopsis leaves (Figure 7). *Pht2;1* transcripts were detectable in leaf blade cells and in central vascular cells (Figures 7A and 7B). Use of sense RNA as a hybridizing probe did not lead to purple staining (Figures 7C and 7D). The spatial pattern of total RNA concentrations in acridine orange–stained leaf sections (Figures 7E and 7F) was similar to the pattern for *Pht2;1*, except that the staining in the central vascular cells was slightly stronger with *Pht2;1.* Careful interpretation of this result is required, however, because a constitutive gene will not give an equally strong signal in all cells. In small, densely cytoplasmic cells, the apparent signal will be stronger than in large, vacuolated cells. Greater signal intensity in the smaller cells around the vasculature may therefore simply reflect a difference in cell size rather than give evidence for cell-specific expression of the gene under study. Our data therefore indicate that *Pht2;1* is expressed in all leaf cells. More detailed gene expression analysis will reveal whether or not *Pht2;1* is preferentially expressed in central vascular cells.

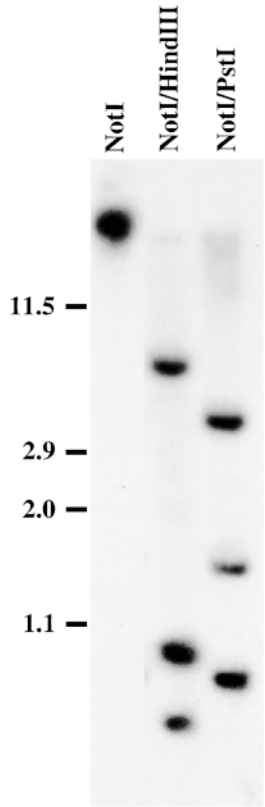

Figure 5. Genomic DNA Gel Blot Analysis.

Genomic DNA was digested with NotI and either HindIII or PstI. The *Pht2;1* cDNA labeled with ^{32}Pi was used as a radioactive probe. The positions of DNA marker fragments and their lengths in kilobases are indicated at left.

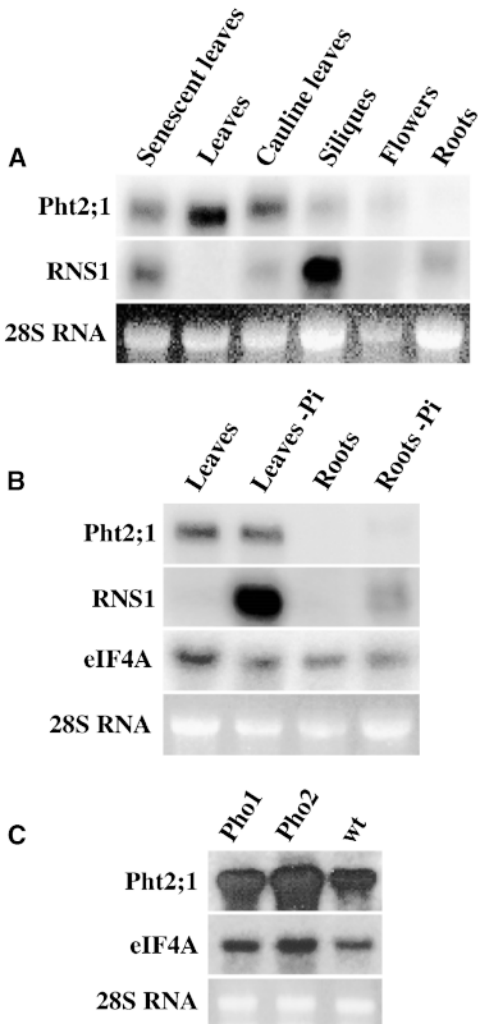

Figure 6. *Pht2;1* Transcript Abundance in Various Plant Organs.

(A) *Pht2;1* mRNA concentrations in flowering plants. RNA gel blot analysis was performed with 10 μg of total RNA from the specified organs and hybridized with randomly labeled cDNA probes, as indicated at left.

(B) *Pht2;1* transcript concentrations during Pi deprivation. RNA gel blot analysis was performed with 10 μg of total RNA from rosette leaves and roots derived from plants starved for phosphorus (−Pi) or grown under control conditions and hybridized with randomly labeled cDNA probes as indicated at left.

(C) *Pht2;1* transcript concentrations in Pi transport mutants. RNA gel blot analysis was performed with 5 μg of total RNA from leaves of either *pho1* or *pho2* mutants or wild-type (wt) plants and hybridized with randomly labeled cDNA probes, as indicated at left. The ethidium bromide–stained upper ribosomal band is marked with 28S RNA.

Functional and Biochemical Analyses in Yeast

To obtain biochemical evidence for the function of Pht2;1, we used complementation analysis to study the yeast mutant *PAM2*, which is defective in the two high-affinity Pi transporter genes *PHO84* and *PHO89* (Martinez and Persson, 1998). Transformed cells were plated in aliquots on nutrient agar plates containing different concentrations of Pi and bromocresol purple as a pH indicator. The mutant *PAM2* cells carrying the empty expression vector grew at Pi concentrations of ⩾100 μM, as reflected by medium acidification, but their growth was restricted at 60 μM Pi. This result clearly demonstrates that at micromolar concentrations, Pi was growth limiting (Figure 8). Cells expressing *Pht2;1* (PAM2-Pht2;1) or the tomato Pi transporter *LePT1* (PAM2-LePT1) (Daram et al., 1998) grew well at 60 μM Pi, whereas growth at 20 μM Pi was inhibited in the cells expressing *Pht2;1*. All three strains grew normally at millimolar concentrations of Pi, probably because of the activity of the endogenous low-affinity Pi uptake system (Tamai et al. 1985). We conclude that Pht2;1 is functional as a plasma membrane Pi transporter in yeast cells and mediates Pi uptake at high micromolar concentrations in the medium. Moreover, Pht2;1 has a lower affinity for Pi in the yeast system than does the previously characterized high-affinity Pi transporter LePT1.

In uptake experiments with ^{32}Pi , the rate of net transport was linear with time during the first 5 min of uptake under the conditions applied (data not shown). Uptake velocities at different Pi concentrations indicated that Pi uptake mediated by Pht2;1 followed Michaelis–Menten kinetics (Figure 9A), exhibiting an apparent K_m value of 394 ± 24 μM in four similar experiments. The net Pht2;1-mediated uptake of Pi as a function of pH increased with increased acidity of the medium up to pH 4 but decreased to indistinguishable from background at pH 7 (Figures 9B and 9C), irrespective of whether Na^+ was present in the medium or not. Pi uptake as a function of sodium concentration did not indicate sodium-dependent Pi transport (Figure 9C). This is in contrast to the data obtained with the related yeast protein PHO89 (Martinez and Persson, 1998). Thus, Pht2;1 is a low-affinity Pi transporter that is dependent on the proton gradient across the yeast plasma membrane but independent of the sodium gradient.

To further substantiate this pH dependency of Pi transport, we performed uptake studies using ionophores and inhibitors (Table 1). The transport rate significantly decreased with protonophores in a concentration-dependent manner, compared with the rate in the controls. The H^+-ATPase inhibitor vanadate also reduced the rate of Pi uptake by 36% from that of the controls. In addition, Pi uptake was greatly decreased in the presence of the K^+ ionophore valinomycin. These results indicate that Pht2;1 is a low-affinity H^+/Pi cotransporter and that its function is dependent on the electrochemical gradient across the plasma membrane. Moreover, competition studies performed with different anions

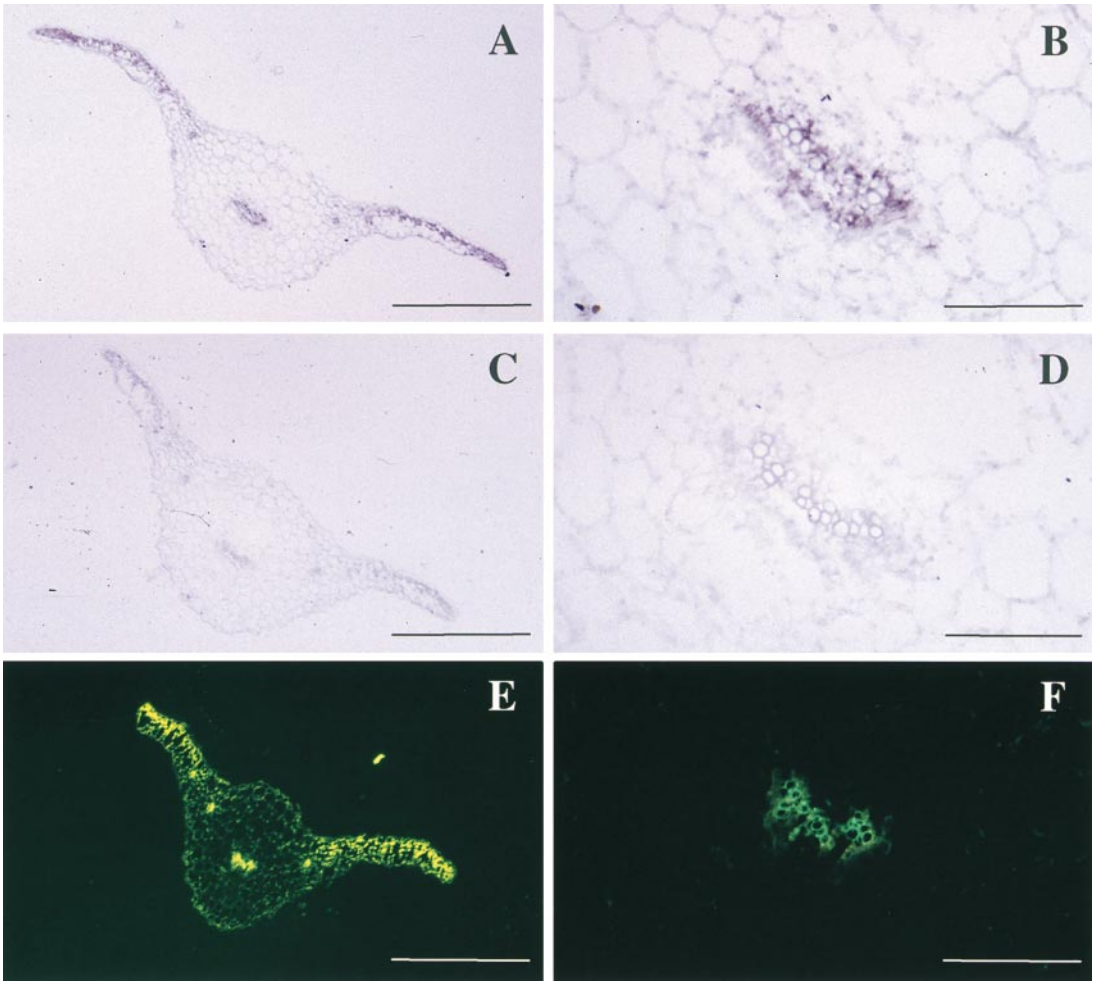

Figure 7. Localization of *Pht2;1* Transcripts in Arabidopsis Leaves.

(A) to **(D)** Bright-field microscopy of leaf sections. Shown in **(A)** and **(B)** are sections hybridized with the *Pht2;1* antisense probe. The purple dye reflects *Pht2;1* mRNA. **(C)** and **(D)** show sections hybridized with the *Pht2;1* sense probe as a negative control.

(E) and **(F)** Acridine orange staining of total RNA and detection by epifluorescence. Orange staining reflects total RNA. Xylem vessels exhibit autofluorescence.

(A), **(C)**, and **(E)** show a whole-leaf section; **(B)**, **(D)**, and **(F)** show the magnified region around the central vascular bundle. Bars in **(A)** and **(C)** = 0.5 mm; bars in **(B)** and **(D)** = 0.1 mm; bar in **(E)** = 0.66 mm; bar in **(F)** = 0.13 mm.

did not reduce the Pi uptake rate, thus demonstrating high specificity of Pht2;1 for Pi.

DISCUSSION

Within the past few years, several Pi transporters from vascular plants sharing high similarity with each other and with the yeast PHO84 protein have been characterized (Muchhal et al., 1996; Leggewie et al., 1997; Smith et al., 1997). According to the rules recommended by the Commission on Plant Gene Nomenclature, this plant gene family is named the Pht1 family (C. Price, personal communication). This family, together with the similar yeast and fungal permeases, was recently given the name Pi:H^+ symporter (PHS) family and designated as belonging to the major facilitator superfamily (Pao et al., 1998). The Pht1 family members are predominantly expressed in roots, whereas the amount of mRNA measured in shoots is low or undetectable. Functional analysis of one member, LePT1 from tomato, has shown that it is a high-affinity Pi transporter (Daram et al., 1998) and that it is expressed in the root. Because *LePT1* mRNA is located in peripheral root cells of tomato, the pro-

tein is likely to play an important role in Pi uptake at the root–soil interface.

Pht2;1 encodes a novel Pi translocator in vascular plants, which, according to its primary structure, is unrelated to the PHS family. The gene thus belongs to a new plant gene family, which we have named the *Pht2* family. The encoded protein shares high sequence similarity with proteins from archaebacterial, eubacterial, and eukaryotic organisms. Its structure is similar to that of specific retroviral receptor proteins from vertebrates and to yeast and fungal transporters of the anion:cation symporter family (Pao et al., 1998), proteins known to possess Na^+-dependent Pi transport activity (Kavanaugh et al., 1994; Versaw and Metzenberg, 1995; Martinez and Persson, 1998).

Two similar domains exist in both the N- and C-terminal regions of Pht2;1 and all related sequences (Figures 2 and 3). Conserved amino acids in the sequences of evolutionary distant organisms are likely to represent structurally or functionally important domains and residues. Within these two domains, the two polar amino acids asparagine and aspartate are fully conserved and may therefore be particularly relevant for Pi binding and transport activity. Fully conserved residues, such as glycine, proline, and alanine, probably have structural functions (Pao et al., 1998). Further studies, including site-directed mutagenesis, should give insight into the structure–function relationships within Pht2;1. The predicted extracellular N terminus of the plant polypeptides is distinctive for plants and may be involved in Pi binding or regulation of transporter activity. The finding of conserved putative phosphorylation sites suggests a regulatory function of phosphorylation/dephosphorylation in controlling Pht2;1 activity. Increasing evidence indicates that nutrient and water transport across membranes is regulated through such mechanisms in vascular plants (Maurel et al., 1995; Desbrosses-Fonrouge et al., 1998).

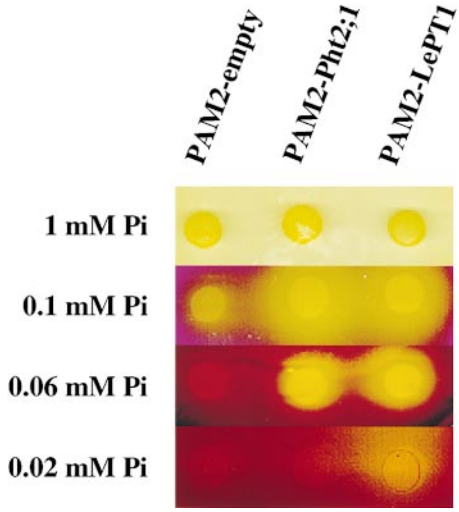

Figure 8. Complementation of Pi Uptake–Deficient Yeast.

The yeast PAM2 mutant was transformed with the expression vector 181A1NE harboring no insert (PAM2-empty), *Pht2;1* cDNA (PAM2-Pht2;1), or *LePT1* cDNA (PAM2-LePT1), and aliquots were plated on plates containing 0.02, 0.06, 0.1, or 1 mM Pi. A color shift on the plate from dark red to yellow reflects medium acidification, which correlates with growth of the yeast cells.

The *Pht2;1* gene occurs as a single-copy gene in the Arabidopsis genome, as was shown by DNA gel blot analysis at high (Figure 5) and low stringency. This conclusion is supported by the absence of similar sequences in the dbEST expressed sequence tag database, which has ~38,000 Arabidopsis sequences. The expression pattern of *Pht2;1* in Arabidopsis plants, as determined by RNA gel blot analysis, indicates that expression is predominantly in green tissues (Figure 6), which suggests a possible role of Pht2;1 in the loading of the leaf with Pi. In addition, the pattern of expression, as determined by in situ hybridization experiments (Figure 7), is in agreement with a possible role of Pht2;1 as a housekeeping H^+/Pi cotransport system, which may play a role in the absorption of Pi from the extracellular space required for normal cellular function. If this is true, the function of type III Pi transporters, such as the mammalian type III Na^+/Pi contransporter (Kavanaugh et al., 1994), and Pht2;1 has apparently been conserved in multicellular organisms during evolution.

Enhancement of the Pi uptake capacity by Pi deprivation has been observed in all plant species tested (Marschner, 1995). Activation and deactivation of the Pi uptake systems are hypothesized to be mediated by allosteric regulation of the transporter (Cogliatti and Clarkson, 1983) or by regulation of the number of transport sites (Lee, 1982), which at least can be explained in part by an increased expression of genes encoding Pi transporters in roots (Leggewie et al., 1997; Smith et al., 1997; Liu et al., 1998a). *Pht2;1* expression thus differs from that of the PHS family in plants in that mRNA concentrations are predominant in the shoot and are not regulated by the Pi concentration in leaves (Figures 5A and 5B). Further support for this conclusion is provided by the RNA gel blot analysis with *pho1* and *pho2* Arabidopsis mutants (Figure 6C). Whereas *pho1* leaves are Pi deficient, the *pho2* mutant exhibits greater Pi concentrations in the shoot than does the wild type (Poirier et al., 1991; Delhaize and Randall, 1995). No substantial differences in *Pht2;1* mRNA abundance were observed in the two mutants, indicating that *Pht2;1* expression is not affected by Pi content in the tissue.

The *pho2* locus is linked to *as1* on chromosome 2 (Delhaize and Randall, 1995), indicating that *Pht2;1*, located on chromosome 3, is not the mutated target gene in the *pho2* mutant. Dong et al. (1998) have suggested that in the *pho2* mutant an uncontrolled uptake of Pi by shoot cells could result from (1) a specific impairment of phloem transport of Pi between shoots and roots, possibly the result of a mutation in a gene encoding a Pi transporter in these cells, or (2) a defect in a gene encoding either a shoot-specific Pi transporter

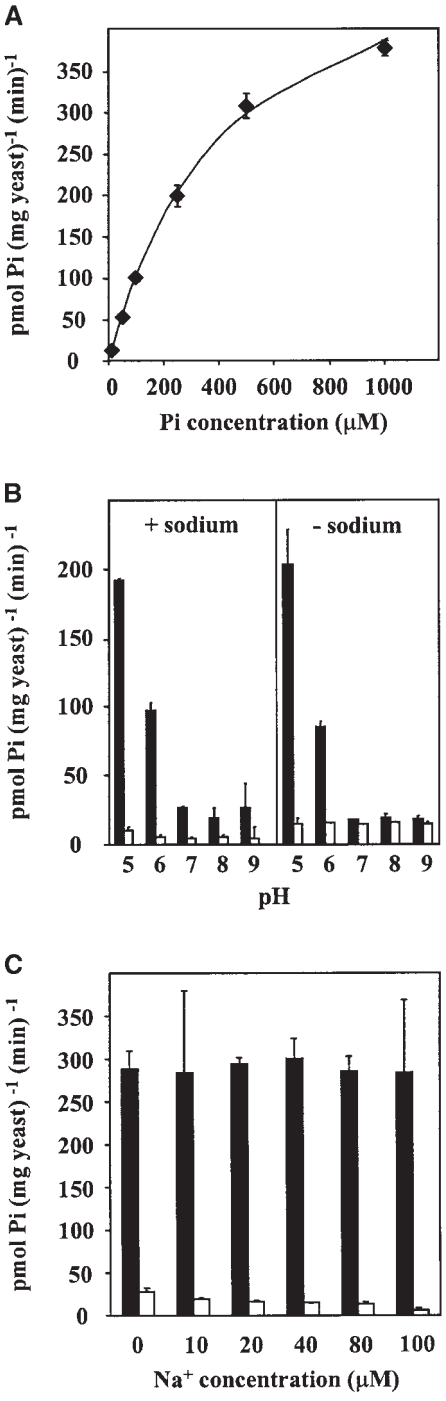

Figure 9. Kinetics of Pi Uptake.

(A) Nonlinear regression of Pi uptake of strain PAM-Pht2;1 versus external Pi concentration at pH 4 was used to estimate the K_m value. Values for one of four independent experiments are shown. Error bars reflect standard errors for three replicates.

(B) Pi uptake into complemented yeast as a function of pH. Uptake rate of Pi by strain PAM2-empty (white columns) and PAM2-Pht2;1 (black columns) at different pH values of the medium at 250 μM Pi in the presence (+ sodium) or absence (− sodium) of 100 μM Na^+. Error bars reflect standard errors from one of three independent experiments (three replicates each).

(C) Pi uptake as a function of external sodium concentration. Uptake rate of Pi by strain PAM2-empty (white columns) and PAM2-Pht2;1 (black columns) at different sodium concentrations of the medium at 250 μM Pi, pH 4. Error bars reflect standard errors from one of three independent experiments (five replicates each). For descriptions of the strains, see the legend to Figure 8.

or a regulator of the activity or expression of this transporter. Accordingly, the *PHO2* gene could encode a regulator protein of Pht2;1 activity.

Despite the sequence similarity to members of the anion:cation symporter family, we were unable to demonstrate Na^+ dependence of Pht2;1-mediated Pi transport. Biochemical analysis of complemented yeast cells showed that Pht2;1 is most probably an H^+/Pi cotransporter, dependent on the electrochemical gradient across the yeast plasma membrane (Table 1). Therefore, whereas the animal, yeast, and fungal proteins are sodium symporters, the plant Pht2;1 and presumably also the corresponding archaebacterial and eubacterial proteins appear to be proton symporters (Pao et al., 1998).

The data presented here indicate that Pht2;1 is a Pi transporter differing from the published Pht1 family of plant Pi transporters in structure, affinity for Pi, and function. We suggest that the Pht1 transporters predominantly mediate high-affinity Pi acquisition at the root–soil interface, whereas Pi transport in the shoot is at least in part performed by the low-affinity Pht2;1 proton symporter described here.

METHODS

Strains and Plasmids

Plant material was from *Arabidopsis thaliana* ecotype Columbia. The Arabidopsis mutants *pho1-2* (Poirier et al., 1991; Delhaize and Randall, 1995) and *pho2-1* (Delhaize and Randall, 1995) were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). The yeast strain PAM2 (Δpho89::*TRP1* Δpho84::*HIS3 ade2 leu2 his3 trp1 ura3*; Martinez et al., 1998) is devoid of high-affinity inorganic phosphate (Pi) transport but harbors a single low-affinity Pi uptake system operative under Pi-rich conditions (reviewed in Borst-Pauwels, 1981). This strain was used for complementation studies with the yeast expression vector 181A1NE, which is 112A1NE (Riesmeier et al., 1992) with the *trp1* marker gene replaced with *leu2. Escherichia coli*, DH5α (*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relAl*; Bethesda Research Labo-

Table 1. Pharmacology of Pi Uptake in Yeast Strain PAM2-Pht2;1

Inhibitors[a]	^{32}Pi Uptake % of Control[b]
CCCP[c] (10 μM)	75
CCCP (100 μM)	57
DNP[d] (10 μM)	92
DNP (100 μM)	66
Valinomycin (10 μM)	41
Valinomycin (100 μM)	6
Vanadate (10 μM)	64
$MgSO_4$ (100 mM)	98
$MgCl_2$ (100 mM)	100
KNO_3 (100 mM)	99
KCl (100 mM)	98

[a] Inhibitors were added to cells in 25 mM Mes, pH 4, 30 sec prior to the start of the uptake experiment. The values given were derived from three independent measurements per treatment; standard errors were <12%.

[b] The uptake rate of the controls (210 pmol [mg yeast]$^{-1}$ [min]$^{-1}$) was set as 100%.

[c] CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

[d] DNP, 2,4-dinitrophenol.

ratories, Bethesda, MD), and XL1-Blue (Stratagene, La Jolla, CA) were cultured according to standard techniques.

Oligonucleotide primers based on the ATORF02 sequences (GenBank accession number X97484) 5′-CCTCCTCCTCATTTTC-3′ and 5′-GGGTTTTTTCAAACTCG-3′ were used to amplify the corresponding cDNA from an Arabidopsis cDNA library (kindly provided by Thomas Altmann, Max-Planck-Institute for Molecular Plant Physiology, Golm, Germany) with Pfu DNA polymerase (Stratagene). A unique polymerase chain reaction fragment of ~2 kb was cloned into the pCR-Script SK+ plasmid by using the pCR-Script Cam SK+ cloning kit (Stratagene). The double-stranded template was sequenced on both strands to verify sequence identity with the published ATORF02 sequence. This cDNA, which we named *Pht2;1*, was subsequently cloned into the NotI-BamHI sites of the shuttle vector 181A1NE. *eIF4A*, a partial cDNA encoding Arabidopsis translation initiation factor eIF-4A from amino acid 197 (nucleotide 738) to amino acid 325 (nucleotide 1122) (Metz et al., 1992) was used as a control on RNA gel blots (see Mandel et al. [1995] for a detailed analysis of the use of *eIF4A* as a constitutive probe). *RNS1*, encoding an Arabidopsis ribonuclease (Bariola et al., 1994), was used as a marker of leaf senescence and Pi deficiency.

Plant Material and Growth Conditions

Arabidopsis plants were grown in a conventional greenhouse with a light/dark cycle of 14 and 10 hr, respectively, and temperature settings of 17 to 20°C and 13 to 16°C, respectively; relative humidity ranged from 40 to 80%. Various organs from soil-grown plants were harvested for RNA gel blot analysis. For nutrient starvation studies, plants were grown in quartz sand culture as described previously (Daram et al., 1998). Pi starvation conditions were applied during the 8 days before harvesting, the nutrient solution being replaced with fresh solution every 3 to 4 days.

Computational Analysis

Sequence analysis was performed by using the Genetics Computer Group software package (Madison, WI; Devreux et al., 1984). Transmembrane regions and protein orientation were also predicted by using the Genetics Computer Group package (Windows set to 18 residues) as well as the TMpred tool (Hofmann and Stoffel, 1993; http://www.ch.embnet.org/software/TMPRED_form.html; ISREC, Lausanne, Switzerland); PSORT II (http://psort.nibb.ac.jp) and SOSUI (http://www.tuat.ac.jp/~mitaku/adv_sosui/), both available on the GenomNet WWW server (http://www.genome.ad.jp/; Institute for Chemical Research, Kyoto University, Japan, and Human Genome Center, Institute of Medical Science, University of Tokyo, Japan); and PREDICTPROTEIN (Rost et al., 1995; http://www.embl-heidelberg.de/predictprotein/predictprotein.html; European Molecular Biology Laboratory—EMBL, Heidelberg, Germany).

To scan the protein sequences for the occurrence of patterns stored in the PROSITE database, we used ScanProsite (Appel et al., 1994). The TMpred and ScanProsite tools are available on the ExPASy Molecular Biology server of the Geneva University Hospital and the University of Geneva (Switzerland) (http://expasy.hcuge.ch/www/tool.html).

Multiple sequence alignment was performed by using ClustalW (Thompson et al., 1994), which is accessible on the BCM Search Launcher (Human Genome Center, Baylor College of Medicine, Houston TX; http://kiwi.bcm.tmc.edu:8088/search-launcher/launcher.html), and Jalview, a Java multiple alignment editor also available on the BCM Search Launcher. Printing and shading of multiple-alignment files were performed with Boxshade 3.21 from ISREC Bioinformatics Group (Lausanne, Switzerland) (http://ulrec3.unil.ch/software/BOX_form.html) and using a shading strategy of 0.8. The average distance tree was determined by using Jalview, with branch lengths indicating the percentage of mismatches between two nodes. Pairwise alignment of amino acid sequences and identity calculations were also performed with Jalview.

Scientific names of the sources of the genes compared are *Caenorhabditis elegans* for *C. elegans*, *Mesembryanthemum crystallinum* for *M. crystallinum*, *Archaeoglobus fulgidus* for *A. fulgidus*, *Pyrococcus horikoshii* for *P. horikoshii*, *Haemophilus influenza* for *H. influenza*, *Rattus norvegicus* for *R. norvegicus*, *Mycobacterium tuberculosis* for *M. tuberculosis*, *Saccharomyces cerevisiae* for *S. cerevisiae*, and *Neurospora crassa* for *N. crassa. ARAth;Pht2;1* designates the first member of the second gene family of genes that encode proton/Pi cotransporters in Arabidopsis.

Extraction of Nucleic Acids and Gel Blot Analysis

Genomic DNA was isolated by using a Nucleon PhytoPure kit (Amersham Pharmacia Biotech, Zurich, Switzerland). Twenty micrograms of DNA was digested overnight with NotI and either PstI or HindIII. The digested DNA was separated on an agarose gel, blotted to Hybond-NX nylon membrane (Amersham Pharmacia Biotech), and hybridized with the QuickHyb hybridization solution (Stratagene) at 68°C with a final wash at 50°C in 0.1 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) containing 0.1% SDS. RNA was extracted by using the hot phenol extraction method (Verwoerd et

al., 1989). RNA gel blot analysis was performed as described previously (Hull, 1985; Bucher et al., 1997), each sample consisting of 5 to 10 μg of total RNA. Hybridization with QuikHyb was performed at 68°C; the final wash with 0.1 × SSC and 0.1% SDS was at 65°C for Figures 5A and 5B or at 42 and 55°C for *Pht2;1* and *eIF4A*, respectively, in Figure 6C.

In Situ Hybridization

Localization of mRNA in 8-μm-thick leaf sections was performed by in situ hybridization, essentially as described previously (Jackson, 1991). Digoxigenin-labeled antisense and sense RNAs were generated from the pCR-Script Cam SK+ plasmid (Stratagene) containing *Pht2;1* cDNA by using the Riboprobe combination system from Promega. Dye formation by reaction with the anti-digoxigenin antibody coupled to alkaline phosphatase in the presence of substrate 5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt, and the oxident nitro blue tetrazolium chloride occurred at room temperature overnight. Stained sections were analyzed by light microscopy (model AZ70; Olympus Optical Schweiz AG, Volketswil, Switzerland). Control sections were stained with a 0.1% (w/v) solution of acridine orange in 50 mM sodium phosphate buffer at pH 6.3, which labels nucleic acids. Orange staining reflects RNA distribution in the sections and was viewed by epifluorescence microscopy with appropriate filters (excitation at 470 to 490 nm). Preincubation with DNase (25 units per slide) before the staining procedure did not affect the staining pattern.

Yeast Manipulations

Pht2;1 was subcloned into the NotI-BamHI site of the yeast shuttle vector 181A1NE. *LePT1*, encoding a tomato H^+/Pi contransporter, was used as a control (Daram et al., 1998). Competent yeast cells were prepared and transformed according to Dohmen et al. (1991), after which they were grown to the logarithmic phase in YNB medium (Difco, Chemie Brunschwig AG, Basel, Switzerland), harvested, washed in Pi-free medium (YNB medium containing an equivalent concentration of potassium chloride rather than potassium phosphate), resuspended in the same medium, and incubated at 30°C for 4 hr. For growth experiments, cells were plated onto YNB agar plates containing 0.02, 0.06, 0.1, or 1 mM Pi and 0.08 μM bromocresol purple.

For experiments on ^{32}Pi uptake, washed and Pi-starved cells were resuspended and activated in a 20% glucose solution to ensure optimal energization of the plasma membrane (Cirillo, 1989). Ten microliters of the cell suspension was incubated at 30°C in the presence of 14.8 kBq of ^{32}Pi at a final concentration of 250 μM Pi (0.37 MBq mL^{-1}; final specific activity in the medium, 1.8 TBq mol^{-1}) in the absence or presence of 100 μM Na^+ for 5 min. Uptake was stopped by adding 10 mL of ice-cold water followed by filtration on glass fiber filters (Schleicher and Schuell, Riehen, Switzerland). After another washing, the radioactivity incorporated into the cells was determined with a liquid scintillation counter (Packard, Zurich, Switzerland).

Inhibition and competition studies were performed at 250 μM Pi, pH 4. The reagents, as indicated in Table 1, were added 30 sec before the addition of ^{32}Pi. Data on the uptake velocity for calculating the K_m values and uptake values in the presence of inhibitors and putative competitors were determined 5 min after ^{32}Pi addition. The kinetic data were analyzed by using SigmaPlot (Jandel Scientific Software, San Rafael, CA) applications for nonlinear regression, calculated as $f(S) = V_{max} \times S/(K_m + S)$, where f defines function and S and V define substrate concentration and uptake velocity, respectively. Mes buffer at a final concentration of 25 mM was used to determine transport activity at different pH values and 250 μM Pi.

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