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Identification and Characterization of the Arabidopsis *PHO1* Gene Involved in Phosphate Loading to the Xylem

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The Arabidopsis mutant *pho1* is deficient in the transfer of Pi from root epidermal and cortical cells to the xylem. The *PHO1* gene was identified by a map-based cloning strategy. The N-terminal half of PHO1 is mainly hydrophilic, whereas the C-terminal half has six potential membrane-spanning domains. PHO1 shows no homology with any characterized solute transporter, including the family of H^+-Pi cotransporters identified in plants and fungi. PHO1 shows highest homology with the Rcm1 mammalian receptor for xenotropic murine leukemia retroviruses and with the *Saccharomyces cerevisiae* Syg1 protein involved in the mating pheromone signal transduction pathway. *PHO1* is expressed predominantly in the roots and is upregulated weakly under Pi stress. Studies with *PHO1* promoter–β-glucuronidase constructs reveal predominant expression of the *PHO1* promoter in the stelar cells of the root and the lower part of the hypocotyl. There also is β-glucuronidase staining of endodermal cells that are adjacent to the protoxylem vessels. The Arabidopsis genome contains 10 additional genes showing homology with *PHO1*. Thus, PHO1 defines a novel class of proteins involved in ion transport in plants.

INTRODUCTION

The radial movement of ions from root epidermal and cortical cells to the xylem can be mediated by two major pathways. In the apoplastic pathway, ions move radially toward the stele through the extracellular space, whereas in the symplastic pathway, ions move intracellularly from cell to cell via plasmodesmata (Bowling, 1981; Clarkson, 1993). Although a third pathway is possible, namely, one in which ions move from cell to cell through a successive uptake and release of ions from and into the extracellular space, the high energy requirement of this pathway makes it unlikely to play a major role in ion transport to the xylem.

The movement of ions and water through the apoplast of the root is blocked at the level of the endoderm by the Casparian strip, a zone in which the cell wall is impregnated with hydrophobic compounds such as suberin and lignin. Thus, passage of ions beyond the Casparian strip and toward the stele must proceed via the symplasm. Once in the cells of the stele, the release of ions into the xylem requires their efflux out of the stelar cells. Thus, radial transport of ions from the external solution to the xylem requires a minimum of two passages across the plasma membrane, once for the uptake of ions into the epidermal, cortical, or outer surface of the endodermal cells, and then again for the efflux of ions out of the stelar cells before entering the xylem vessel (Bowling, 1981; Clarkson, 1993).

The uptake of anions such as Pi into a cell is an energy-requiring process. The negatively charged phosphate ion (HPO_4^{-2} or $H_2PO_4^-$) must move against an electrical gradient, the interior of the cell being negatively charged (~ -100 mV), as well as against a concentration gradient, the intracellular concentration of Pi being 1000 to 10,000 times higher than the extracellular concentration (the concentration of Pi in soil solution typically is 1 to 10 μM, whereas the cytoplasmic Pi concentration is ~10 mM). The energy required to move Pi into cells is mediated by H^+-ATPase, which pumps protons outside the cell and thus creates a proton concentration gradient across the plasma membrane. The movement of protons back into the cells is linked to Pi influx via a H^+-Pi cotransporter. In contrast to influx, the efflux of Pi out of cells can be passive because Pi movement is in the same direction as the electrical and Pi concentration gradients across the membrane. Thus, Pi efflux could be mediated by uniporters or ion selective channels.

Our knowledge of the proteins involved in Pi transport and homeostasis in higher plants has been extended in the past 5 years by the cloning of several genes encoding the H^+-Pi

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cotransporters involved in Pi uptake into cells. The *AtPT1* gene from Arabidopsis was the first high-affinity H^+-Pi cotransporter identified in higher plants (Muchhal et al., 1996). At least nine genes showing similarity to *AtPT1* have been identified in Arabidopsis from expressed sequence tags and genome sequence analysis, although the expression of only *AtPT1* and *AtPT2* are readily detectable by RNA gel blot analysis (Muchhal et al., 1996; Lu et al., 1997; Mitsukawa et al., 1997; Smith et al., 1997; Okumura et al., 1998; Raghothama, 2000). Homologs of *AtPT1* also have been identified in *Catharanthus roseus* (*CrPT1*; Kai et al., 1997), potato (*StPT1* and *StPT2*; Leggewie et al., 1997), tomato (*LePT1* and *LePT2*; Daram et al., 1998; Liu et al., 1998a), *Medicago trunculata* (*MtPT1* and *MtPT2*; Liu et al., 1998b), tobacco (*NtPT1*), and wheat (*TaPT1*) (Raghothama, 2000). All members of this family of high-affinity phosphate transporters share extensive amino acid homology with each other and show significant homology with the yeast Pho84 H^+-Pi cotransporter. Each protein consists of 12 membrane-spanning regions that are separated into two groups of 6 membrane-spanning domains by a larger hydrophilic region, a characteristic topology shared by numerous solute transporters belonging to the major facilitator superfamily (Pao et al., 1998). The majority of H^+-Pi cotransporters cloned in plants are expressed preferentially in the roots, with transcript abundance increasing after Pi stress (Muchhal et al., 1996; Leggewie et al., 1997; Smith et al., 1997; Liu et al., 1998a, 1998b; Muchhal and Raghothama, 1999).

A gene homologous with the animal Na-Pi cotransporter gene has been isolated in Arabidopsis that encodes a low-affinity H^+-Pi cotransporter expressed preferentially in leaves (*Pht2;1*; Daram et al., 1999). Although Pht2;1 also is a member of the family of transporters having 12 membrane-spanning regions, it shows no homology with the plant high-affinity H^+-Pi cotransporter family. The presumed role of Pht2;1 is in the transport of Pi into the cells of the shoot (Daram et al., 1999).

Mutants defective in the acquisition of Pi have been isolated in Arabidopsis in an effort to identify novel genes involved in Pi homeostasis. The *psr1* mutant was identified as deficient in the induction of ribonucleases and acid phosphatases involved in the scavenging of Pi from organic sources under Pi-limiting conditions (Chen et al., 2000). The *pho1* and *pho2* mutants were identified in a screen for plants having altered levels of Pi in leaves of plants growing in soil, and the *pho3* mutant was isolated by screening roots for acid phosphatase activity (Poirier et al., 1991; Delhaize and Randall, 1995; Zakhleniuk et al., 2001). The *pho2* mutant shows high levels of Pi accumulation in leaves despite normal Pi uptake in isolated roots, indicating a deficiency either in Pi loading to the phloem vessels of leaves or in a regulatory protein involved in the control of leaf Pi level (Delhaize and Randall, 1995; Dong et al., 1998). The *pho3* mutant shows reduced accumulation of Pi in both leaves and roots (Zakhleniuk et al., 2001). Neither *PHO2* nor *PHO3* has yet been isolated.

The *pho1* mutant is characterized by a severe deficiency in shoot Pi level but normal root Pi content. Studies of Pi transport into the mutant revealed normal uptake of Pi into roots but deficiency in transferring Pi to the root xylem vessels for its subsequent transport to the leaves. The amount of other inorganic ions was normal in shoots of *pho1*, and the transport of sulfate from the roots to the shoot was unaffected (Poirier et al., 1991). These data indicate that *PHO1* represents a gene involved specifically in the loading of Pi to the xylem in roots. Here, we report the isolation and characterization of the *PHO1* gene.

RESULTS

Genetic Mapping of PHO1

The *pho1-1* mutant was isolated from an ethyl methanesulfonate–mutagenized population of Arabidopsis (Poirier et al., 1991). Mapping of the *PHO1* gene to a chromosome was accomplished initially by the segregation analysis of F2 plants from a cross between the *pho1-1* mutant with Arabidopsis line W-100 containing the *an-1*, *ap-1*, *bp-1*, *cer2-2*, *er-1*, *gl1-1*, *hy2-1*, *ms1-1*, *py-201*, and *tt3-1* mutant alleles. Linkage of *PHO1* with the *GL1* gene on chromosome 3 was detected, with an estimated distance of 12 centimorgan (cM; data not shown). Mapping using polymorphic markers was accomplished subsequently with a mapping population derived from a cross between *pho1-1* and the Landsberg *erecta* accession. Analysis of an initial population of 204 F2 plants revealed 20 and 11 recombination events between *PHO1* and markers m105 and g4711, respectively, whereas no recombination events were detected with marker m255.

Several yeast artificial chromosomes were isolated that hybridized to marker m255. One of the largest yeast artificial chromosome clones was yUP4G10, with a length of $\sim$250 kb. One end of clone yUP4G10 (probe 4G10) gave one recombination event among 204 F2 plants, whereas probe pr4, derived from the other end of yUP4G10, showed no recombination events in the same population. Analysis of an extended F2 population of 764 plants with markers 4G10, m255, pr4, and g4711 revealed 6, 0, 0, and 105 recombination events relative to *PHO1* (Figure 1). Analysis of the same population of plants with the cleaved amplified polymorphic sequence (CAPS) marker 32C9 derived from one end of the bacterial artificial chromosome (BAC) clone T8K21 (TAMU32C9) also showed no recombination events among 764 plants. Although the physical distance between m255 and 32C9 was estimated to be 100 kb, the genetic distance between these two markers in the F2 population analyzed was $<$0.065 cM.

These data indicated an unusually low recombination frequency in the area of *PHO1* between Columbia and Landsberg *erecta*, compared with the average of 185 kb/cM calculated for chromosome 4 (Schmidt et al., 1995). Thus, a

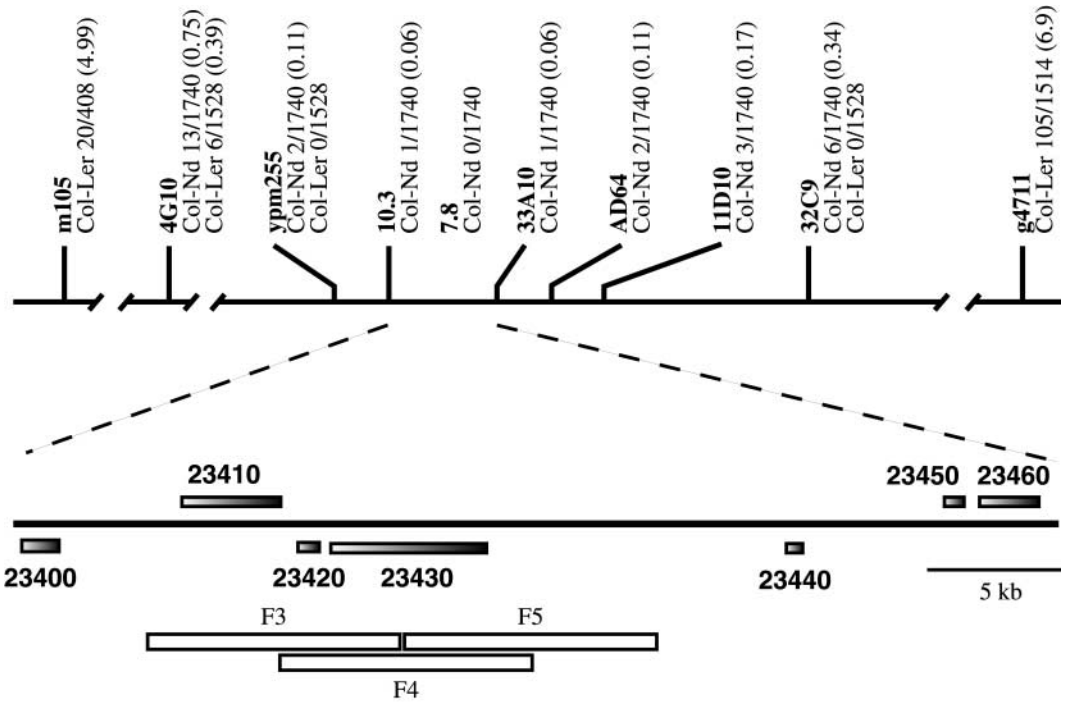

Figure 1. Mapping of *PHO1* in Arabidopsis.

The positions of polymorphic markers surrounding *PHO1* on chromosome 3 are shown at top. The distance (cM) between *PHO1* and each marker is indicated in parentheses beside the ratio of the number of recombination events detected to the total number of chromosomes analyzed for two distinct mapping populations, namely, plants derived from a cross between the Columbia and Landsberg *erecta* accessions (Col-Ler) and plants derived from a cross between the Columbia and Niederzenz accessions (Col-Nd). Below the genetic map is a physical map derived from a portion of the P1 clone mLM24. The locations of potential genes identified from the analysis of genomic DNA are indicated by shaded boxes labeled with the corresponding GenBank accession numbers (all accession numbers start with the prefix At3 g). The three overlapping genomic fragments (F3, F4, and F5) used in the complementation of the *pho1* mutant are indicated by open boxes.

second F2 mapping population of 870 plants was derived from a cross between *pho1-1* and wild-type Niederzenz accession. In this population, 13, 2, and 6 recombination events were detected between *PHO1* and markers 4G10, m255, and 32C9, respectively. CAPS marker 11D10, derived from one end of the BAC clone T3L10 (TAMU11D10), showed three recombination events, whereas CAPS marker AD64, derived from a λ clone, showed two recombination events. Three more CAPS markers were derived from the T3L10 clone and the overlapping BAC T9A10 (TAMU33A10). These markers, designated 10.3, 7.8, and 33A10, showed one, zero, and one recombination event in the population of 870 plants. Restriction enzyme analysis of BACs T9A10 and T3L10 indicated a distance of ~30 kb between markers 10.3 and 33A10.

The P1 clone mLM24 was found to encompass the region defined by markers m255, 10.3, and 7.8 but ended a few kilobases upstream of marker 33A10. Analysis of the sequence of mLM24 between marker 10.3 and the end of the clone toward marker 33A10 revealed the presence of seven potential open reading frames (ORFs) (Figure 1). Of these, the hypothetical protein At3 g23430 showed the presence of several potential transmembrane-spanning domains, a feature typical of solute transporters. Thus, complementation analysis was focused on the area encompassing At3 g23430. Three overlapping genomic fragments (F3 to F5; Figure 1) of 10 kb were amplified by polymerase chain reaction (PCR) and cloned in the binary vector pPZP112.

Transgenic plants obtained by transformation of these genomic fragments into *pho1-4* plants were analyzed for growth in soil and Pi content in leaves of 33-day-old plants (Figure 2). Although plants transformed with the genomic fragments F3 and F5 showed a phenotype similar to that of the *pho1-4* mutant in size, appearance, and leaf Pi content, transgenic plants transformed with fragment F4 were wild type for all phenotypes analyzed (Figure 2). Although fragment F4 contains two complete ORFs, the hypothetical protein At3 g23420, which shows no significant homology with a known protein in GenBank, could not encode PHO1 because this ORF also is found completely within fragment F3,

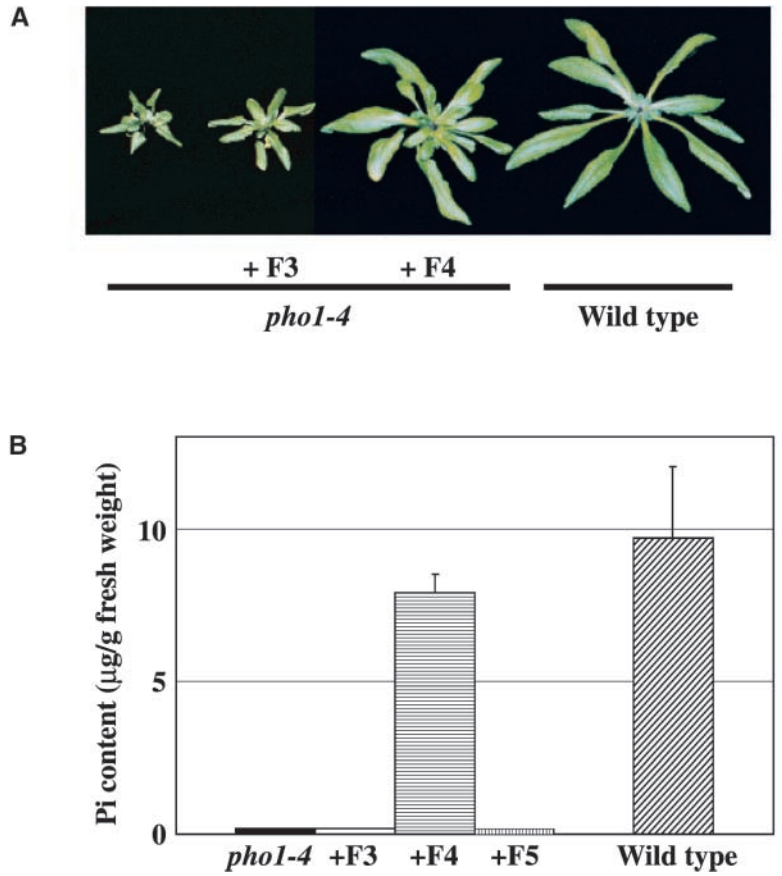

Figure 2. Complementation of *pho1*.

The *pho1-4* mutant was transformed independently with genomic fragments F3, F4, and F5 shown in Figure 1.
(A) Phenotypes of *pho1-4* mutant plants, transgenic *pho1-4* plants transformed with the F3 and F4 fragments, and wild-type plants grown in soil. Transgenic *pho1-4* plants transformed with the F5 fragment had the same appearance as untransformed *pho1-4* plants and transgenic *pho1-4* plants transformed with the F3 fragment (data not shown).
(B) Analysis of the Pi content of leaves from *pho1-4* mutant plants, transgenic *pho1-4* plants transformed with the F3, F4, and F5 fragments, and wild-type plants.

which does not complement the *pho1* phenotype. Thus, ORF At3 g23430 was identified tentatively as the *PHO1* gene.

Sequence Analysis of PHO1 and Mutant Alleles

A 2.7-kb cDNA encoding the full-length PHO1 protein was isolated using a combination of library screening and reverse transcriptase–mediated PCR. PHO1 encodes a protein of 782 amino acids. Analysis of the hydropathy profile of the protein revealed that the first half of the protein is hydrophilic, whereas the second half is mostly hydrophobic and contains six potential transmembrane-spanning domains (Figure 3). The *PHO1* gene spans an area of 5.8 kb and contains 14 introns. The sequence of *PHO1* is different from the predicted coding sequence of gene At3 g23430 only in the last exon because of the choice of a splicing acceptor site. Analysis of PHO1 with the BLAST program revealed homology with human and mouse cell surface receptors for the xenotropic and polytropic murine leukemia viruses (Battini et al., 1999; Tailor et al., 1999; Yang et al., 1999). Sequence alignment of PHO1 with the human retrovirus receptor Rmc1 showed 29% identity and 47% homology in the last 512 amino acids of PHO1 (Figure 4). PHO1 also showed homology with the *SYG1* gene of *Saccharomyces cerevisiae*. Truncated forms of Syg1 were shown to interact with the β-subunit of the heterotrimeric G protein involved in the signal transduction pathway of the response of yeast to the mating pheromones (Spain et al., 1995). PHO1 showed 23% amino acid identity and 39% homology with Syg1 (Figure 4). The hydropathy profiles of both Rmc1 and Syg1 are similar to that of PHO1, that is, with a long hydrophilic portion at the

N-terminal end followed by a hydrophobic portion containing several putative membrane-spanning domains (Figure 3).

Sequence analysis of the four *pho1* alleles was performed using reverse transcriptase–mediated PCR products. The *pho1-1* and *pho1-2* alleles have point mutations introducing stop codons at positions 749 and 340 of the protein sequences, respectively (Figure 5). The alleles *pho1-3* and *pho1-4* have point mutations at different intron-exon junctions, leading to abnormal splicing of the transcript and resulting in frame shifts and the synthesis of truncated proteins (Figure 5). Thus, all four *pho1* alleles showed point mutations leading to the synthesis of a truncated PHO1 protein. These data, combined with the complementation of the mutant phenotype with genomic fragment F4 encompassing the *PHO1* coding region, unambiguously identified the *PHO1* gene.

PHO1 Is Expressed in Cells of the Vascular System of Roots

The expression profile of *PHO1* was examined first by RNA gel blot analysis. *PHO1* was found to be expressed predominantly in roots, but weak expression also was detected in rosette leaves, stems, cauline leaves, and flowers with developing siliques (Figure 6A). The expression of *PHO1* was regulated weakly by Pi availability, with roots of plants grown in medium with a low Pi concentration showing less then a twofold increase in steady state mRNA compared with plants grown in medium with abundant Pi (Figure 6B).

To more precisely determine the cells that express *PHO1* in the various tissues, transgenic plants expressing the β-glucuronidase (GUS) gene under the control of fragments encompassing 0.55, 1.1, 1.6, and 2.1 kb of the promoter region of *PHO1* were analyzed. The expression profiles for all constructs were similar. The results shown in Figure 7 are for the 1.6- and 2.1-kb promoter fragments. Analysis of 10-day-old seedlings revealed a predominant accumulation of GUS in the vascular system of the lower section of the hypocotyl (Figures 7C and 7F) and in the mature zones of the roots (Figures 7A, 7D, and 7E). No GUS staining was detected at the root tip or in the elongating zone of the root (Figure 7B). The absence of GUS staining also was evident in the emerging lateral roots (Figures 7A, 7C, and 7E). Transversal sections of mature roots and hypocotyls revealed the presence of GUS in cells of the stele, including the pericycle and xylem parenchyma cells (Figures 7D to 7F). No GUS staining was observed in epidermal or cortical cells of the root and hypocotyl. Although the majority of cells forming the endoderm did not show GUS staining, we regularly observed one or two cells of the endoderm staining for GUS. These cells were found typically within the axis formed by the large xylem vessels (Figures 7D to 7F).

Although GUS expression in roots and hypocotyls was detected readily after 15 to 30 min of reaction, no GUS expression could be detected reliably in cotyledons, mature leaves, flowers, or siliques of plants grown in soil, even after a staining period of 12 to 24 hr.

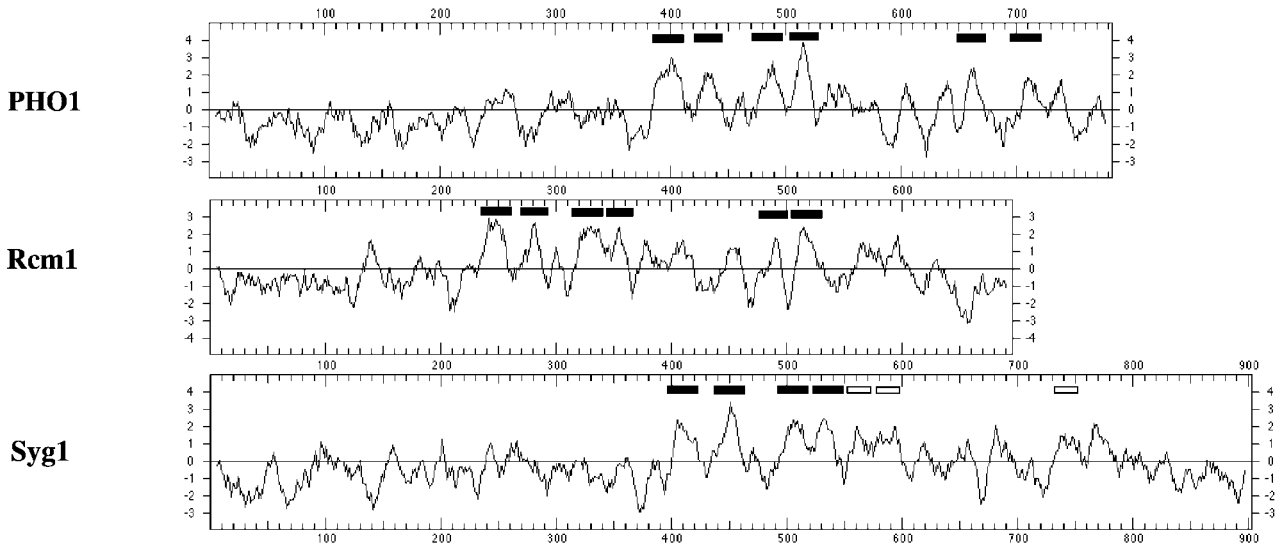

Figure 3. Hydropathy Profiles of PHO1, Rcm1, and Syg1.

Evaluation of the potential transmembrane-spanning domains was performed using the program TMHMM2.0 (Krogh et al., 2001; http://www.cbs.dtu.dk/services/TMHMM/) and was transposed on a Kyte-Doolittle plot using a window of 11 amino acids. Transmembrane segments having a probability score of at least 1.0 are indicated by closed rectangles, whereas those having a probability score between 0.5 and 1.0 are indicated by open rectangles.

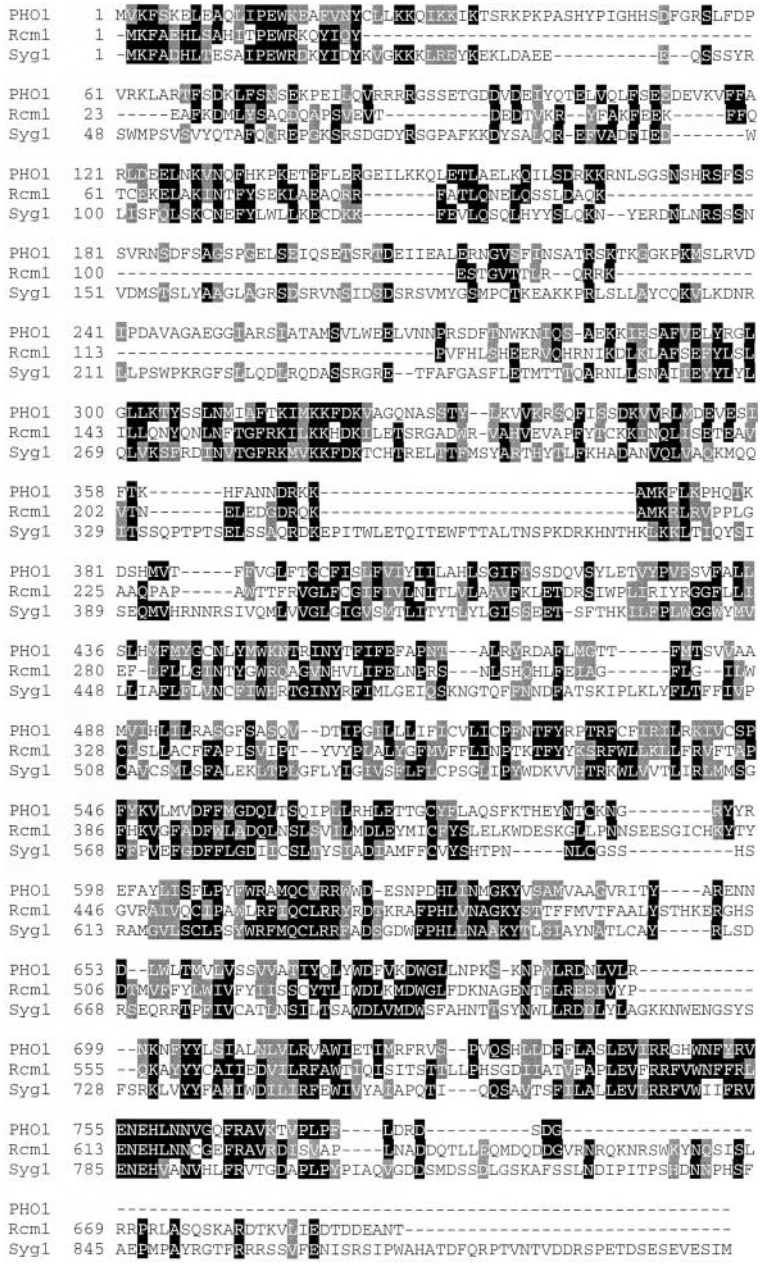

Figure 4. PHO1 Is Homologous with the Mammalian Rcm1 Receptor for Murine Leukemia Retroviruses and the *S. cerevisiae* Syg1 Protein.

The protein sequence of PHO1 was aligned with the sequences for the human cell surface receptor for the xenotropic and polytropic murine leukemia viruses (Rcm1) and the *S. cerevisiae* Syg1 protein using the ClustalW program (http://www.ebi.ac.uk/clustalw/). Gray and black boxes represent homologous and identical amino acids, respectively.

PHO1 Is One Member of a Family of Genes

Analysis of the Arabidopsis genome sequence revealed the presence of 10 additional putative genes showing homology with *PHO1* (http://cbs.umn.edu/arabidopsis/atprotdb/fam98.htm). The hydropathy profiles of these PHO1 homologs are comparable to that of PHO1, that is, the N-terminal half is mostly hydrophilic, whereas the C-terminal half is mostly hydrophobic, with a number of potential transmembrane-spanning domains. Although the amino acid sequences of

these homologs are deduced at present from a prediction of exons identified from genomic sequences, pairwise analysis of the members of this gene family revealed a level of amino acid similarity between 45 and 92%. None of the *PHO1* homologs mapped to the same chromosome area as *PHO2*, the gene mutated in the Pi overaccumulator described by Delhaize and Randall (1995). The genes At2 g03240, At2 g03250, and At2 g03260 all mapped to the top of chromosome 2, near marker mi320, whereas *PHO2* mapped to the bottom of chromosome 2, near marker m429 (Delhaize and Randall, 1995).

DISCUSSION

The *PHO1* Locus Lies in an Area of Low Recombination Frequency between Columbia and Landsberg *erecta*

The *PHO1* gene was identified by a map-based cloning strategy. Initial mapping of *PHO1* in an F2 population derived from a cross between Columbia (*pho1-1*) and Landsberg *erecta* indicated close linkage of *PHO1* with the polymorphic marker m255 on chromosome 3. Further mapping revealed an abnormally low recombination frequency of 1 cM $\cong$ 1500 kb in an area of $\sim$100 kb surrounding *PHO1* defined by polymorphic markers m255 and 32C9 (Figure 1). In contrast, the recombination frequency in the same area in a mapping population between Columbia (*pho1-1*) and Niederzenz was $\sim$1 cM $\cong$ 220 kb, a value close to the average recombination frequency calculated for chromosome 4 of 1 cM $\cong$ 185 kb (Schmidt et al., 1995). Although we have no data on the structure of the chromosome around the *PHO1* locus in Landsberg *erecta*, it is likely that the low recombination frequency detected near *PHO1* between Columbia and Landsberg *erecta* results from changes at the chromosomal level that prevent appropriate local chromosome alignment and crossing over during meiosis, such as rearrangements or inversions. In this context, it is interesting that Landsberg *erecta* is a laboratory mutant developed after radiation of the wild-type Landsberg accession (Rédei, 1962) and that it differs from the wild-type parent, as well as from Columbia, in the position of the 5S rRNA locus on chromosome 3 (Fransz et al., 1998).

PHO1 Is Homologous with the Mammalian Rcm1 Murine Retrovirus Receptor and the Yeast Syg1

Transformation of the *pho1-4* mutant with overlapping fragments of 10 kb derived from BAC clones enabled the identification of the gene complementing the mutant phenotype. Cloning of the corresponding cDNA and sequence analysis of the gene in four independent *pho1* mutants confirmed that the *PHO1* gene has been identified. The hydropathy profile of the PHO1 protein reveals the presence of two major domains, the N-terminal half of PHO1, which is mainly hydrophilic, and the C-terminal half, which shows the presence of six potential membrane-spanning domains (Figure 3). Although the presence of membrane-spanning domains is a feature expected of integral membrane proteins, such as ion transporters, the structure of PHO1 appears quite distinct from the structures of Pi transporters cloned in various organisms that are members of the major facilitator superfamily. These transporters, which include the *S. cerevisiae* Pho84 H^+-Pi cotransporter as well as all of the plant H^+-Pi cotransporters identified to date, are mostly hydrophobic proteins having 12 membrane-spanning domains grouped in two clusters of 6 domains separated by a larger hydrophilic region (Pao et al., 1998). It is striking that PHO1 shows no significant homology (BLAST E value $\geq 10^{-5}$) with any characterized solute transporter. Instead, PHO1 shows highest homology with the mouse and human Rcm1 receptor for the xenotropic and polytropic murine leukemia retroviruses (Battini et al., 1999; Tailor et al., 1999; Yang et al., 1999) and with Syg1 of *S. cerevisiae*, a protein involved in the signal transduction pathway of the response of yeast to the mating pheromones (Spain et al., 1995).

The Pattern of Expression of PHO1 Is Consistent with Its Role in Pi Loading to the Xylem

The *pho1* mutant is deficient specifically in loading Pi to the xylem. The uptake of Pi into root epidermal and cortical cells is normal in the mutant, as is the movement of Pi from the xylem of the hypocotyl to the shoot (Poirier et al., 1991). Together, these results indicate that PHO1 is likely to be involved in the efflux of Pi out of the stelar cells for its transport into the xylem. The expression pattern of *PHO1* is consistent with this interpretation. RNA gel blot analysis showed that *PHO1* is expressed mainly in the root. The expression pattern of the GUS reporter gene under the control of the *PHO1* promoter confirmed the predominant expression of *PHO1* in the root. The *PHO1* promoter also was active in the lower part of the hypocotyl, which represents a zone of transition between the vascular elements of the root and the shoot. Cross-sections through the root and the lower hypocotyl revealed GUS expression in the stelar cells, including the pericycle and xylem parenchyma cells. No GUS staining was detected in the epidermal or cortical cells of the root or the hypocotyl.

Although the majority of endodermal cells did not show GUS expression, we regularly observed one or two endodermal cells staining for GUS. These GUS-positive endodermal cells always were found adjacent to the protoxylem. Careful observation of GUS staining revealed a gradient of GUS expression within the pericycle, with cells adjacent to the protoxylem showing stronger staining than cells farther away (Figures 7D to 7F). The significance of this pattern and its relevance to Pi transport are unclear at present. Pericycle and endodermal cells adjacent to the protoxylem poles have

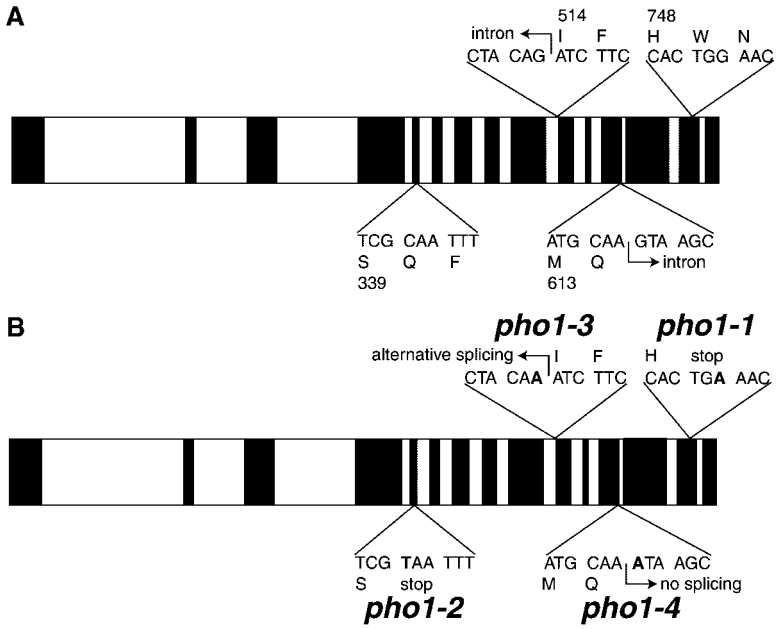

Figure 5. Structures of Wild-Type and Mutant Alleles of *PHO1*.

The structure of the *PHO1* gene is shown with exons indicated by black boxes and introns indicated by white boxes. The sequences of selected regions are highlighted, with the amino acids indicated by a single letter above the corresponding codons. The 5′ and 3′ junctions of selected introns are indicated by right and left crooked arrows, respectively.

(A) Structure of the wild-type *PHO1* gene.

(B) Structures of the four *pho1* alleles. Point mutations are indicated by boldface letters. Mutations in *pho1-1* and *pho1-2* create stop codons, whereas mutations in *pho1-3* and *pho1-4* are in the last and first base of an intron, respectively, resulting in abnormal splicing of the mRNA.

features that distinguish them from neighboring cells. For example, lateral roots initiate from the pericycle cells immediately adjacent to the two protoxylem poles (Laskowski et al., 1995). Furthermore, in numerous plants, including Arabidopsis, the initial formation of the Casparian band in the anticlinal walls of the endodermal cells is followed by the deposition of suberin lamellae into the entire walls (reviewed by Peterson and Enstone, 1996). These changes in the endodermis begin opposite the phloem strands and spread toward the protoxylem, resulting in the presence of thin-walled endodermal cells, called passage cells, opposite the protoxylem poles. Passage cells are thought to offer a lower resistance pathway for water flow into the stele (Peterson and Enstone, 1996). Although experiments with barley roots showed that the movement of phosphate through the endodermal cells was not inhibited by the development of suberin lamellae, the expression of *PHO1* in passage cells may indicate a differential flux of Pi through these cells (Clarkson et al., 1971).

No GUS staining was detected in any other aboveground tissues, including stems, leaves, and flowers, despite the low *PHO1* expression detected in these tissues by RNA gel blot analysis. The apparent discrepancy between the absence of GUS expression in the aerial parts of the plant and the weak positive signal detected by RNA gel blot analysis may be attributable to differences in the sensitivity of these methods to detect *PHO1* gene transcription. It also is possible that elements other than the 2.1-kb region upstream of the *PHO1* gene are required for the weak expression in aboveground tissues. Nevertheless, RNA gel blot and GUS expression analyses indicate that *PHO1* is expressed primarily in the stelar cells of the root and of the lower section of the hypocotyl.

Plants have been shown to respond to Pi stress through the upregulation of a number of genes involved in the acquisition of Pi from the extracellular environment, its transport into the cells, and its metabolism. Genes that have been shown to be upregulated at the transcriptional level by Pi stress include RNases, phosphatases, H^+-Pi cotransporters, and several genes encoding riboregulators or signal peptides (reviewed by Raghothama, 1999). Pi stress led to a significant increase in *PHO1* transcript abundance in roots (Figure 6B). However, this increase was relatively low compared with the upregulation of genes such as the H^+-Pi cotransporter *AtPT2* (Muchhal et al., 1996). Although the significance of the increase of *PHO1* transcript under Pi stress needs further investigation, it is known that ion efflux into the xylem can be regulated independently of ion influx

into the root and that, under nutrient stress, both ion influx into the root and transfer of the ion to the xylem are enhanced (Clarkson, 1985; Engels and Marschner, 1992). Thus, the regulation of *PHO1* transcription could be involved in the modulation of Pi transport to the root xylem under Pi stress.

What Is the Role of PHO1 in Pi Loading to the Xylem?

Study of the physiology of the *pho1* mutant indicates that PHO1 is involved in Pi loading to the xylem and may encode a transporter that mediates Pi efflux out of root stelar cells. Such a transporter could be a uniporter or a channel allowing the passage of Pi along its electrochemical gradient. Thus, PHO1 could be analogous to the SKOR protein, a K^+ outwardly rectifying channel expressed in the stelar cells of the root that is involved in the release of K^+ into the xylem sap (Gaymard et al., 1998).

Although PHO1 showed no significant homology with any known solute transporter, the homology with the Rmc1 receptor for xenotropic and polytropic murine leukemia viruses is interesting because several retrovirus receptors have been shown to encode solute transporters. These include transporters for neutral and basic amino acids (Wang et al., 1991; Rasko et al., 1999) and two distinct Na^+-Pi cotransporters (Kavanaugh and Kabat, 1996). No transporter activity has yet been assigned to Rmc1, and expression of the protein in *Xenopus* oocytes failed to elicit a distinct phosphate-inducible current (M. Kavanaugh, personal communication).

PHO1 has been expressed in both *Xenopus* oocytes and yeast, as revealed by protein gel blot analysis of microsomes using an anti-peptide antibody (D. Hamburger, C. Roth, and Y. Poirier, unpublished data). No changes in either influx or efflux of Pi was detected in yeast or *Xenopus* oocytes expressing PHO1 compared with controls using ^{33}Pi as a tracer (D. Hamburger, C. Roth, and Y. Poirier, unpublished data). Furthermore, no novel Pi-inducible currents were detected in *Xenopus* oocytes expressing PHO1 (I.C. Forster and D. Hamburger, unpublished data). Thus, we have no direct evidence that PHO1 is itself a Pi transporter. It is possible that the yeast and *Xenopus* oocyte systems used to express PHO1 do not allow a functional reconstitution of transporter activity or that the protein is not targeted to the plasma membrane. Alternatively, it is possible that PHO1 represents only one subunit of a multisubunit transporter and that the presence of other unidentified components is essential for Pi transport activity.

Finally, it is possible that PHO1 may not be involved directly in Pi transport but that it influences the activity of a plasma membrane Pi exporter indirectly. This possibility is exemplified by the Pho86 protein of yeast. Screening for yeast mutants deficient in Pi stress response led to the identification of four genes, named *Pho86*, *Pho87*, *Pho88*, and *Gtr1* (reviewed by Persson et al., 1995). Several of the corresponding proteins, including Pho86, have potential membrane-spanning domains, indicating that they are associated with membranes. Disruption of these genes resulted in impaired uptake of Pi, and many models have depicted the corresponding proteins interacting with the Pho84 H^+-Pi cotransporter at the plasma membrane. However, the Pho86 protein was shown recently to be an endoplasmic reticulum resident protein required for the targeting of Pho84 to the plasma membrane (Lau et al., 2000).

PHO1 showed good homology with the Syg1 protein of *S. cerevisiae*. In yeast, the mating pheromone–initiated signal is transduced by a heterotrimeric G protein composed of α, β, and γ subunits (reviewed by Kurjan, 1992). Deletion of the Gα subunit (*gpa1* mutant) resulted in cell death caused by unchecked signaling from the G$\beta\gamma$ heterodimer. The *Syg1* gene was identified in a screen aimed at suppressing *gpa1* lethality (Spain et al., 1995). Whereas the full-length Syg1 protein was a weak suppressor of *gpa1*, the deletion mutant

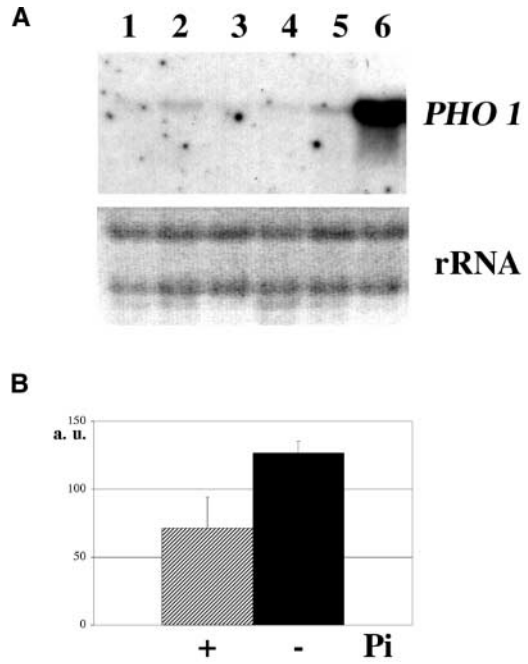

Figure 6. Expression of *PHO1* in Various Tissues.

RNA gel blot analysis of *PHO1* was performed with 10 μg of total RNA isolated from various tissues.

(A) RNA from rosette leaves of wild-type plants (lane 1), rosette leaves of *pho1-4* mutant plants (lane 2), and stems (lane 3), cauline leaves (lane 4), flowers (lane 5), and roots (lane 6) of wild-type plants. An RNA gel blot was hybridized to the *PHO1* cDNA (top). The intensity of the rRNA bands after staining an agarose gel with ethidium bromide is shown at bottom.

(B) RNA from roots of plants grown in medium with (+) or without (−) Pi. The *y* axis represents arbitrary units (a.u.).

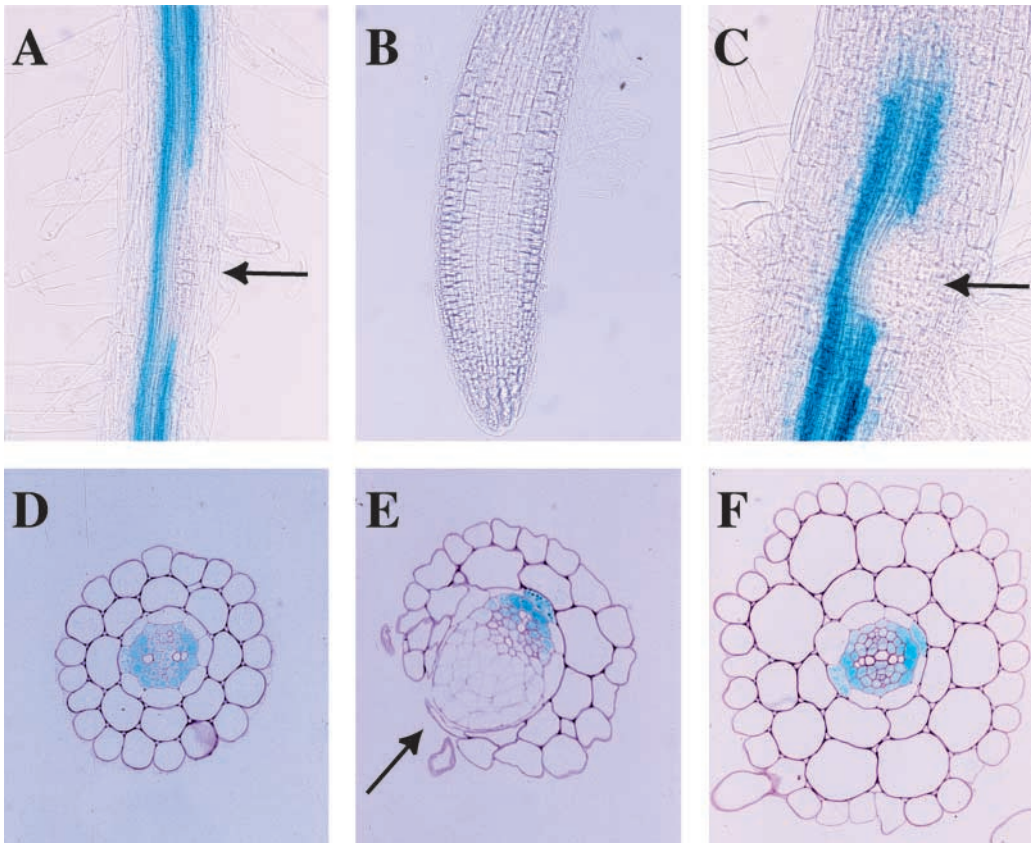

Figure 7. Expression Pattern of the *PHO1* Promoter.

The GUS reporter gene was expressed under the control of a 2.1-kb (**[A]**, **[B]**, **[D]**, and **[E]**) or a 1.6-kb (**[C]** and **[F]**) fragment of the *PHO1* promoter region. Longitudinal and transverse views of the mature zone of roots (**[A]**, **[D]**, and **[E]**), root tip and elongation zone **(B)**, hypocotyl-root junction **(C)**, and hypocotyl **(F)** are shown. Arrows indicate regions where lateral roots are emerging.

expressing only the hydrophilic portion of the protein was a strong suppressor. Interestingly, the truncated Syg1 was shown to interact with the Gβ subunit in a two-hybrid assay (Spain et al., 1995). Although Syg1 may not be involved normally in the pheromone response pathway, these data suggest that Syg1 may respond to or transduce a signal in yeast via Gβ or Gβγ. The Gβγ heterodimer has been shown to be involved in numerous cellular processes mediated by direct interactions with a variety of downstream effectors. In particular, Gβγ has been shown to activate the muscarinic K^+ channel in heart cells (Logothetis et al., 1987).

Although the normal function of Syg1 in yeast remains unclear at present, the interaction between Syg1 and subunits of the heterotrimeric G protein complex raises the question of whether PHO1 controls Pi loading to the xylem via a signaling pathway involving G proteins. Regulation of the activity of K^+ channels in guard cells by G proteins has been demonstrated (Assmann, 1996). Furthermore, it is interesting that the yeast Gtr1 shown to be necessary for Pho84-mediated Pi uptake harbors sequence similarity with a number of GTP binding proteins of the ras family and contains the tripartite consensus element for binding GTP. Such observations have led to the proposal that Gtr1 might exert a regulatory function on the Pho84 Pi transporter through GTP binding (Persson et al., 1995). In view of these findings, it will be important to identify any proteins with which PHO1 might interact, because this may give essential information on the mode of action of PHO1 in Pi loading to the xylem.

PHO1 Is One Member of a Gene Family in Arabidopsis

A search of the Arabidopsis database with PHO1 revealed the presence of 10 additional putative proteins having high homology with PHO1. Pairwise comparison of all homologs by the BLASTP program revealed a level of amino acid simi-

larities ranging from 45% between At1 g68740 and At2 g03250 to 92% between At1 g26730 and At1 g35350. The hydropathy profiles of all homologs are comparable to those of *PHO1*.

The presence of a large family of PHO1-related proteins raises the question of their roles in ion homeostasis. Preliminary experiments showing the expression of the GUS reporter gene under the control of the promoter of some of these homologs reveal an expression pattern distinct from that of PHO1, such as expression in the pollen or in leaf vascular tissue (C. Ribot, E. Rezzonico, D. Hamburger, and Y. Poirier, unpublished data). Although some homologs of PHO1 could be involved in Pi transport in other tissues, it is possible that some may be involved in the transport of other ions.

METHODS

Pho1 Mutants

All *pho1* mutants have been derived from ethyl methanesulfonate–mutagenized populations of *Arabidopsis thaliana* accession Columbia. The *pho1-1* and *pho1-2* mutants were described by Poirier et al. (1991) and Delhaize and Randall (1995), respectively. The *pho1-3* and *pho1-4* mutants were isolated by C. Benning (Michigan State University, East Lansing) and Y.P., respectively. The Arabidopsis line W100 was obtained from the ABRC (Columbus, OH).

Markers for Fine Mapping

A number of cleaved amplified polymorphic sequence markers polymorphic between Columbia and Niederzenz were derived from the ends of bacterial artificial chromosomes, from lambda clones, or from genomic sequences. The oligonucleotides and restriction enzymes used to detect these various cleaved amplified polymorphic sequences are as follows: marker 10.3, 5′-CCCGGGAGAAGAAACAGAAGAG-3′ and 5′-GGGCCCATTGTTTATGACCAAT-3′, BsmBI; marker 33A10, 5′-ATTGAAAAGGTAAGGTCTACAGGA-3′ and 5′-GGATCCCAATGTTCTCAACGTTTT-3′, MseI; marker AD64, 5′-TCAACTATCATCACATTTTTTCACG-3′ and 5′-CAAGAACATGTCATCAATCAGACTC-3′, PalI; and marker 11D10, 5′-AAGCTTCATTAGTTTTAGAGGGTAG-3′ and 5′-AAGCTTCATATATACGATCTGAATG-3′, MseI. Marker 7.8 detects a polymorphism between Columbia and Niederzenz after polymerase chain reaction (PCR) using oligonucleotides 5′-TGTAATGGTGATGGCGAGACTA-3′ and 5′-TGTTGAAAATCGGTCGGTATCT-3′. Descriptions of all other markers can be found on the Arabidopsis Information Resources Database (http://www.arabidopsis.org/).

Complementation of the *pho1* Mutant

Fragments of 9.8 kb (F3), 10.2 kb (F4), and 8.9 kb (F5) were amplified by PCR from the bacterial artificial chromosomes T3L10 and T9A10 using oligonucleotides creating unique KpnI sites at each extremity. PCR products from two independent reactions were cloned into the KpnI site of the binary vector pPZP112 (Hajdukiewicz et al., 1994), and the resulting constructs were transferred by electroporation into *Agrobacterium tumefaciens* strain GV3101. The *pho1-4* mutant was transformed with the various constructs by the floral dip method (Clough and Bent, 1998). Transgenic plants were selected by growing surface-sterilized plants on Murashige and Skoog (1962) medium containing 50 μg/mL kanamycin. Transgenic plants were grown subsequently in soil under constant illumination.

The determination of Pi in leaves was performed according to Chapin and Bieleski (1982) with some modifications. Surface-sterilized seed were grown for 10 days on nutrient medium plates before being transferred to soil. The nutrient medium contained 20 mM NH_4NO_3, 5 mM KNO_3, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 100 μM KH_2PO_4, 100 μM Fe-EDTA, 50 μM H_3BO_4, 12 μM $MnSO_4$, 1 μM $ZnCl_2$, 1 μM $CuSO_4$, 3 μM KI, and 0.2 μM $NaMoO_4$. After a growth period of 23 days in soil under constant illumination, leaves from plants were harvested, weighed, and freeze-dried. The material (15 to 25 mg) then was extracted in 1 mL of methanol:chloroform:formic acid:water (12:5:1:2, v/v) at −20°C for 20 min under strong agitation. To the supernatant was added 400 μL of chloroform and 200 μL of water. The mixture was blended thoroughly and centrifuged, and the aqueous phase was transferred to a fresh tube. The tissue residues were extracted further in 20% (v/v) methanol containing 2% (v/v) formic acid, and the supernatant was combined with the aqueous phase before drying under vacuum. The pellet was resuspended in 325 μL of water, to which was added 50 μL of 1 N perchloric acid, 100 μL of 20 mM ammonium molybdate, and 25 μL of 0.1 M triethylamine-HCl, pH 5.0. The precipitate was collected by centrifugation, resuspended in 1 mL of 2 M ammonium hydroxide, and dried under vacuum. The pellet was resuspended in water, and the Pi was measured according to the procedure of Ames (1966).

Isolation of *PHO1* cDNA and Analysis of *pho1* Alleles

A partial cDNA of 1.4 kb was obtained from the PRL2 Arabidopsis cDNA library made from a collection of tissues (Newman et al., 1994). To obtain a full-length cDNA of *PHO1*, reverse transcriptase–mediated (RT)-PCR was performed on total RNA isolated from Arabidopsis roots using primers located at positions 0, −23, −55, −125, and −249 upstream of the putative ATG start codon. The longest RT-PCR product (2.4 kb) that could be amplified reliably was obtained with oligonucleotide 5′-TTTCTTCCAAGTCACTATTAGCAA-3′ located 55 bp upstream of the ATG start codon and oligonucleotide 5′-CCGAAATGCTTCCTCTAAATACT-3′ located 62 bp downstream of the TAA stop codon. The resulting cDNA sequence was compared with the genomic sequence of the P1 clone mLM24 released by the Japanese Arabidopsis sequencing group (http://www.kazusa.or.jp/arabi) to establish the exon-intron borders and define the promoter region. Analysis of the four *pho1* alleles was performed by direct sequencing of cDNAs obtained from several independent RT-PCR procedures using total root RNA isolated from the different mutants.

Analysis of *PHO1* Expression by RNA Gel Blot Analysis

Total RNA was isolated from various Arabidopsis organs using a phenol/SDS extraction protocol (Sambrook et al., 1989). Rosette

leaves, cauline leaves, flowers, and stems were harvested from soil-grown plants, whereas roots were collected from plants growing submerged in a solution of half-strength Murashige and Skoog (1962) medium supplemented with 1% Suc. RNA gel blot analysis was performed using formaldehyde (Sambrook et al., 1989). The fragment used as a probe was a 1.4-kb cDNA fragment encompassing the last two-thirds of the gene, and hybridization was performed under high stringency. For the analysis of *PHO1* in roots under Pi stress, plants were grown in liquid nutrient medium (composition described above) for the first 8 days with 10 mM potassium phosphate followed by 12 days in the same medium without phosphate. Quantification of the signal obtained by RNA gel blot analysis was performed by densitometric analysis of autoradiograms and ethidium bromide–stained gels using the program NIH Image (http://rsb.info.nih.gov/nih-image/).

Analysis of *PHO1* Promoter Activity in Transgenic Plants

Genomic fragments of 0.54, 1.1, 1.6, and 2.1 kb located upstream of the *PHO1* transcribed sequence were isolated by PCR. The oligonucleotides used were 5′-TCGCATATAATTGGATCCGTTTTGATT-3′, introducing a BamHI restriction site 16 bp upstream of the *PHO1* ATG start codon, combined with oligonucleotide 5′-ATATAAAGCTTATTTGTAAAATGGATT-3′ (545-bp fragment), 5′-ACAGTAAGCTTTCAAATCAGTTATTGA-3′ (1.1-kb fragment), 5′-TACCGTTGAAAGCTTTTTTGTTCTTGT-3′ (1.6-kb fragment), or 5′-CTTTCAGTTTAAGCTTCTATTATTCCTA-3′ (2.1-kb fragment), all of which introduced a HindIII restriction site at the other extremity of the PCR products. Each fragment was cloned in front of the β-glucuronidase (GUS) gene in the binary vector pBI121 (Malik and Wahab, 1993). Transgenic Arabidopsis (accession Columbia) plants expressing the GUS gene under the control of the various *PHO1* promoter fragments were obtained by the floral dip method, as described above. Whole 6-day-old seedlings, as well as leaves, stems, flowers, and pollen grains from soil-grown plants, were stained for GUS activity according to the protocol of Lagarde et al. (1996) with the exception that tissues were not vacuum infiltrated to better preserve structures. Stained tissues were kept at 4°C in 70% ethanol before dehydration in a graded series of ethanol and embedding in LR White medium-grade resin (Polysciences, Warrington, PA). Semifine sections were cut at 2 μm using a Reichart ultracut microtome (Leica, Wetzlar, Germany), mounted on glass slides, and counterstained with periodic acid-Schiff.

Accession Numbers

The GenBank accession numbers for the sequences described in this article are AF474076 (PHO1), AF089744 and AF099082 (human retrovirus receptor Rmc1), and U14726 (*S. cerevisiae* Syg1).

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