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A Phosphate Transporter from *Medicago truncatula* Involved in the Acquisition of Phosphate Released by Arbuscular Mycorrhizal Fungi

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Many plants have the capacity to obtain phosphate via a symbiotic association with arbuscular mycorrhizal (AM) fungi. In AM associations, the fungi release phosphate from differentiated hyphae called arbuscules, that develop within the cortical cells, and the plant transports the phosphate across a symbiotic membrane, called the periarbuscular membrane, into the cortical cell. In *Medicago truncatula*, a model legume used widely for studies of root symbioses, it is apparent that the phosphate transporters known to operate at the root–soil interface do not participate in symbiotic phosphate transport. EST database searches with short sequence motifs shared by known phosphate transporters enabled the identification of a novel phosphate transporter from *M. truncatula*, MtPT4. MtPT4 is significantly different from the plant root phosphate transporters cloned to date. Complementation of yeast phosphate transport mutants indicated that MtPT4 functions as a phosphate transporter, and estimates of the K_m suggest a relatively low affinity for phosphate. *MtPT4* is expressed only in mycorrhizal roots, and the *MtPT4* promoter directs expression exclusively in cells containing arbuscules. MtPT4 is located in the membrane fraction of mycorrhizal roots, and immunolocalization revealed that MtPT4 colocalizes with the arbuscules, consistent with a location on the periarbuscular membrane. The transport properties and spatial expression patterns of MtPT4 are consistent with a role in the acquisition of phosphate released by the fungus in the AM symbiosis.

INTRODUCTION

In natural ecosystems, >80% of vascular flowering plants live in symbiotic associations with arbuscular mycorrhizal (AM) fungi (Smith and Read, 1997). These sophisticated, mutualistic interactions have evolved over many hundreds of million years, and the fossil record indicates that the rudimentary root systems of the earliest land plants were associated with ancestral AM fungi (Remy et al., 1994; Redeker et al., 2000). Today, AM symbioses can be found in ecosystems throughout the world, where they affect plant biodiversity and ecosystem functioning (Newsham et al., 1995; van der Heijden et al., 1998). The underlying mechanisms are not fully understood, but the contribution of AM fungi to plant phosphorus nutrition and the resulting impact on plant health and fitness are widespread (Smith and Read, 1997; van der Heijden et al., 1998). These attributes also make the AM symbiosis an essential component of sustainable agriculture (Jeffries, 1987).

AM fungi are obligate biotrophs that colonize plant roots to obtain carbon from the plant. In addition to growth within the root cortex, they develop an extensive extraradical mycelium in the surrounding soil. The intraradical and extraradical growth phases are a single continuum, and it is via this continuum that the fungus is able to translocate phosphate from the soil to the interior of the root system, where it is released to the plant (Jakobsen, 1995; Harrison, 1997; Smith et al., 2001). Phosphorous is one of the mineral nutrients essential for plant growth and development. It plays diverse regulatory, structural, and energy transfer roles and consequently is required in significant quantities, constituting up to 0.2% of the dry weight of the plant cell (Bieleski and Ferguson, 1983; Schachtman et al., 1998). Although the total phosphorous content of soils may be high, phosphorus exists largely as sparingly soluble complexes that are not directly accessible to plants, and it is one of the mineral nutrients that limits crop production throughout the world (Bieleski, 1973). In symbiotic association with AM fungi, plants profit from phosphate acquired by the extensive network of fine extraradical hyphae that extend beyond root depletion zones to mine new regions of the soil (Jakobsen, 1995).

The process of the development of AM symbiosis varies with the plant and fungal species involved (Smith and Smith, 1997). In most crop species, the fungus grows initially in the intercellular spaces of the root and then differentiates within the inner cortical cells, forming dichotomously branched

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hyphae, called arbuscules (Bonfante-Fasolo, 1984; Gianinazzi-Pearson, 1996; Harrison, 1999). Although located physically within the cortical cell, the arbuscule remains separated from the plant cytoplasm by an extension of the plasma membrane, called the periarbuscular membrane (Figure 1). This envelopment of the arbuscule also results in the formation of a new apoplastic space between the periarbuscular membrane and the arbuscule, called the periarbuscular space (Figure 1) (Bonfante and Perotto, 1995; Harrison, 1997). Thus, the coordinated differentiation of both symbionts is required to achieve arbuscule formation, and analyses of the cytoskeleton of the invaded cells indicate extensive, dynamic rearrangements of the cortical microtubules, which presumably enables the trafficking of membrane and cell wall precursors to the extending membrane and new apoplastic compartment (Genre and Bonfante, 1997, 1998, 1999; Blancaflor et al., 2001). The plant plasma membrane extends ~10-fold to surround the arbuscule (Alexander et al., 1989), and this extensive interface, coupled with the high phosphate content of these cells (Cox and Tinker, 1976; Schoknecht and Hattingh, 1976; Cox et al., 1980), supports the suggestion that phosphate transport between the fungus and the plant occurs at this location.

Although radiotracer studies have demonstrated that phosphate moves from the soil via the fungal hyphae to the plant (Sanders and Tinker, 1971; Smith and Gianinazzi-Pearson, 1988; Pearson and Jakobsen, 1993), relatively little is known about the phosphate transport proteins involved. High-affinity phosphate transporters that are expressed exclusively in the extraradical mycelium have been cloned from two AM fungi, *Glomus versiforme* and *Glomus intraradices* (Harrison and van Buuren, 1995; Maldonado-Mendoza et al., 2001). Expression of these transporters is regulated according to the phosphate conditions surrounding the hyphae, and it is likely that they are responsible for phosphate uptake by the extraradical hyphae before transport to the plant. The mechanisms underlying the subsequent translocation of phosphate to the intraradical hyphae and efflux from the arbuscule are unknown at present. It is speculated that phosphate is translocated as polyphosphates, which then are degraded to phosphate before efflux from the arbuscule to the periarbuscular space (Cox et al., 1980; Solaiman et al., 1999). Phosphate efflux is a process ongoing in most cells. However, calculations of phosphate efflux from the arbuscule indicate rates ~1000-fold higher than those measured in fungal cells growing in culture (Cairney and Smith, 1993). Consequently, it has been proposed that a specialized efflux system exists in the arbuscule membrane (Smith and Smith, 1990; Smith et al., 2001). The final phase of phosphate transport to the plant requires that the plant be able to transport phosphate across the periarbuscular membrane into the cortical cell.

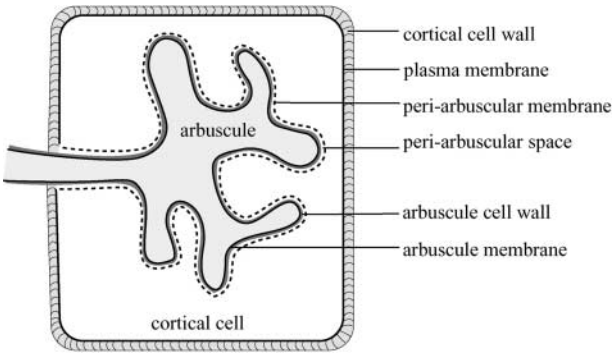

Figure 1. Features of the Arbuscule–Cortical Cell Interface.

The plant periarbuscular membrane (dotted line) and the plasma membrane of the cortical cell are contiguous. The apoplastic compartment resulting from the envelopment of the arbuscule is called the periarbuscular space.

In recent years, two distinct groups of phosphate transporters have been cloned from plants. A low-affinity phosphate transporter that shares sequence similarity with the sodium-coupled symporters of yeast and *Neurospora crassa* was identified in Arabidopsis (Daram et al., 1999). This transporter operates in the chloroplast envelope (Versaw and Harrison, 2002). Members of a second group of transporters share similarity with the proton-coupled symporters of yeast and *N. crassa* and have been cloned from the roots of a range of plant species, including potato (StPT1 and StPT2) (Leggewie et al., 1997), Arabidopsis (AtPT1 and AtPT2) (Muchhal et al., 1996; Smith et al., 1997), *Medicago truncatula* (MtPT1 and MtPT2) (Liu et al., 1998b; Chiou et al., 2001), and tomato (LePT1 and LePT2) (Daram et al., 1998; Liu et al., 1998a). These transporters show higher affinities for phosphate, with K_m values in the low to mid micromolar range, and they are expressed during growth in low-phosphate conditions. LePT1 and MtPT1 have been characterized in the most detail, and both proteins are located in the plasma membrane of root hair and epidermal cells, where they probably play a role in phosphate transport at the root–soil interface (Daram et al., 1998; Liu et al., 1998a; Chiou et al., 2001).

In *M. truncatula* and potato, it is clear that MtPT1/MtPT2 and StPT1/StPT2 are not involved in phosphate transport in the AM symbiosis, because these phosphate transporter genes are downregulated rapidly as the symbiosis develops (Liu et al., 1998b; Chiou et al., 2001; Rausch et al., 2001). By contrast, *LePT1* continues to be expressed as the fungus invades the roots, and transcripts were located in the cells with arbuscules, suggesting that it also may mediate phosphate transport in mycorrhizal roots (Rosewarne et al., 1999). Recently, a high-affinity phosphate transporter, StPT3, was identified from potato. StPT3 shares 83% amino acid identity with StPT1 and StPT2 but is expressed only in mycorrhizal roots, specifically in cells containing arbuscules; thus, it may mediate phosphate transport at the arbuscular interface (Rausch et al., 2001).

After the cloning of phosphate transporters from AM fungi and *M. truncatula*, we initiated a search for *M. truncatula* phosphate transporters expressed in mycorrhizal roots. In contrast to tomato and potato, hybridization-based approaches using probes corresponding to *MtPT1* and *MtPT2* suggested that *M. truncatula* does not express a phosphate transporter of this type at the mycorrhizal interfaces. Here, we describe the identification of a novel phosphate transporter from *M. truncatula* (MtPT4) and provide evidence for a role in phosphate transport across the periarbuscular membrane.

RESULTS

Identification of a Novel Phosphate Transporter, MtPT4

The motif-building program MEME (Bailey and Elkan, 1994) was used to identify sequence motifs shared by proton- and sodium-coupled phosphate transporters from plants, fungi, bacteria, and mammals. The training set consisted of 15 phosphate transporters, including members of the proton-coupled phosphate transporter family (PHS family) (Pao et al., 1998) and the sodium-coupled transporters of the inorganic phosphate transporter family (PiT family; Saier, 1994, 1999). MEME generated seven motifs of between 10 and 47 amino acids (Table 1). Motifs 1 and 4 were shared by the sodium-coupled transporters of mammalian and fungal origin and also were present in the Arabidopsis PHT2;1 transporter (Daram et al., 1999; Versaw and Harrison, 2002). Motifs 2, 3, 5, 6, and 7 were present in the proton-coupled transporters of plant and fungal origin, and motifs 3, 5, and 6 also were present in the Arabidopsis PHT2;1 sequence, although they did not match the PHT2;1 sequence exactly.

M. truncatula has been adopted as a model species for the analysis of root symbioses and legume biology (Barker et al., 1990; Cook, 1999), and during the past few years, *M. truncatula* genome projects have generated large numbers of EST sequences from a wide range of libraries. Included in this collection are sequences from mycorrhizal cDNA libraries of *M. truncatula* roots colonized with two species of AM fungi, *G. versiforme* and *G. intraradices*. To identify putative phosphate transporters expressed in mycorrhizal roots, the motifs generated by MEME (Table 1) were used in TBLASTN searches of the GenBank Other EST database and the TIGR *M. truncatula* Gene Index. The searches used either single motifs or combinations of motifs, and although this strategy generated a significant number of hits, it was possible to use information from the *M. truncatula* Gene Index (TIGR) to restrict further analysis to those sequences expressed in mycorrhizal roots. As expected, this approach resulted in the identification of ESTs corresponding to the known phosphate transporters MtPT1 and MtPT2. In addition, two novel *M. truncatula* ESTs that shared 73% identity with motif 5 were identified. These ESTs belonged to TC12920 (TIGR tentative consensus), whose members all were from the *M. truncatula*/*G. intraradices* cDNA library.

Primers were designed to the consensus sequence, and a partial cDNA was amplified from an *M. truncatula*/*G. versiforme* cDNA library by PCR. The partial sequence then was used as a probe to enable the identification of a full-length cDNA from the same cDNA library. The cDNA, designated *MtPT4*, is 1850 bp long and is predicted to encode a protein of 59 kD that is suggested to possess the 12 transmembrane domains typical of proteins of the PHS transporter family (Saier et al., 1999). MtPT4 contains four of the five motifs found in the other proton-coupled phosphate transporters, although only one of these shows a match of >95% (Table 2). ClustalW analysis indicates that MtPT4 is significantly different from the other plant phosphate transporters (Figure 2), and it shares only 61% identity at the amino acid level with MtPT1 and MtPT2 (Liu et al., 1998b) and 60% identity with StPT3, the mycorrhiza-specific phosphate transporter from potato (Rausch et al., 2001). The closest match from Arabidopsis, a nonmycorrhizal species, is with PHT6 (Okumura et al., 1998). DNA gel blot analysis indicates that MtPT4 is unique and that the *M. truncatula* genome does not contain other closely related sequences (Figure 3).

Table 1. Phosphate Transporter Motifs Generated by MEME

Motif No.	Width	Sequence
1	18	GLPVSTTHCIVGAVIGVG
2	47	TLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFG
3	22	CGTIPGYWFTAWLVDKLGRKKI
4	10	GANDVANALG
5	38	YSLTFFFANFGPNATTFIVPGEIFPARYRSTCHGISAA
6	20	HGLHLLGTASSWFLLDIAFY
7	13	YWRMKMPETPRYT

Table 2. Motifs Present in the MtPT4 Sequence

Motif No.	Percent Identity	Alignment
1	0	Absent
2	95	^{126}TLCFFRFWLGFGIGGDYPLSATIMSEYANKRTRGAFIAAVFAMQGVG^{172} (MtPT4) TLCFFRFWLGFGIGGDYPLSATIMSEYANK1TRGAFIAAVFAMQG G TLCFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFG (motif)
3	66	^{350}GTFPGYWFTVFFIEKLGRFKI^{370} (MtPT4) GT PGYWFT + ++KLGR KI GTIPGYWFTAWLVDKLGRKKI (motif)
4	0	Absent
5	73	^{404}YGLTFFFANFGPNSTTFVLPAELFPTRVRSTCHAFSAA^{441} (MtPT4) Y LTFFFANFGPN+TTF++P E+FP R RSTCH SAA YSLTFFFANFGPNATTFIVPGEIFPARYRSTCHGISAA (motif)
6	80	^{289}HGRHLIGTMSCWFLLDIAFY^{308} (MtPT4) HG HL+GT S WFLLDIAFY HGLHLLGTASSWFLLDIAFY (motif)
7	0	Absent

The identity and alignment are based on the BLAST algorithm. The positions of the motifs in the MtPT4 open reading frame are indicated at the beginning and end of the sequences.

MtPT4 Encodes a Low-Affinity Phosphate Transporter

The transport activities of plant phosphate transporters have been assayed in two yeast phosphate transport mutants, NS219 (Bun-ya et al., 1991) and PAM2 (Martinez and Persson, 1998). NS219 lacks PHO84, the high-affinity, proton-coupled phosphate transporter, and PAM2 lacks PHO84 and a high-affinity, sodium-coupled transporter, PHO89. To determine whether *MtPT4* encodes a functional phosphate transporter and to analyze the phosphate transport activity of this protein, the *MtPT4* cDNA was cloned into a yeast expression vector, pWV3, under the control of the yeast alcohol dehydrogenase promoter. The resulting plasmid, pWV3-MtPT4, was introduced into NS219 and PAM2. Both NS219 and PAM2 transformants carrying pWV3-MtPT4 displayed an increase in phosphate uptake relative to the yeast strains carrying pWV3 (Figures 4A and 4B). In both strains, phosphate transport followed Michaelis-Menten kinetics. The NS219 strain expressing pWV3-MtPT4 showed an apparent K_m of 1058 $\pm$ 79 μM, whereas the PAM2 strain expressing pWV3-MtPT4 showed an apparent K_m of 1326 $\pm$ 62 μM. NS219 and PAM2 strains carrying pWV3 showed apparent K_m values of 978 and 993 μM, respectively, which correlate well with previous measurements of the low-affinity transport systems for yeast (Figure 4C). Subtraction of the phosphate transport activities derived in the yeast strains carrying pWV3 from those derived in strains expressing *MtPT4* enabled an estimation of the activity resulting from MtPT4. In NS219 and PAM2, the K_m values calculated for MtPT4 were 493 $\pm$ 14 μM and 668 $\pm$ 89 μM, respectively. The highest transport rates were obtained at pH 3; however, in both NS219 and PAM2, the transport activity attributable to MtPT4 was highest at pH 4.0 (Figure 4D and data not shown).

MtPT4 Is Expressed Exclusively in Mycorrhizal Roots

RNA gel blots of RNA isolated from *M. truncatula* roots at 1 to 5 weeks after inoculation with *G. versiforme* were hybridized with the *MtPT4* cDNA. *MtPT4* transcripts were detected exclusively in RNA from mycorrhizal roots at 2 to 5 weeks after inoculation, and the levels increased with increasing colonization of the root system. *MtPT4* transcripts were not present in noncolonized roots or in any other tissues of the plant, including seedlings, primary roots, cotyledons, leaves, stems, flowers, or pods (Figure 5 and data not shown). In addition, BLASTN analysis of the TIGR *M. truncatula* Gene Index, which contains 175,000 ESTs from 32 libraries, indicated that ESTs corresponding to *MtPT4* exist only in mycorrhizal root libraries.

MtPT4 Is Present in the Membrane Fraction of Mycorrhizal Roots

To enable the detection of MtPT4, antibodies were raised to a peptide corresponding to the 15 amino acids at the C terminus of the protein. Affinity-purified antibodies cross-reacted with the peptide, whereas the preimmune serum showed no cross-reaction (data not shown). Protein gel blot analyses demonstrated the presence of MtPT4 in microsomal protein fractions from *M. truncatula* roots colonized with *G. versiforme* but not from noncolonized roots (Figure 6A). MtPT4 levels increased with increasing colonization of the root system. As a control, the expression of the MtPT1 phosphate transporter also was evaluated in these protein samples. Previous analyses have indicated that MtPT1 is present in noncolonized roots and that the levels decrease as the roots become colonized with AM fungi

(Chiou et al., 2001). As expected, MtPT1 was detected in noncolonized roots, and the levels decreased with increasing colonization of the root system by *G. versiforme* (Figure 6A). Unlike many plant–pathogen interactions, the AM symbiosis is a relatively nonspecific association. MtPT4 also was expressed in roots colonized with *Gigaspora gigantea*, an AM fungus from a different family than *G. versiforme* (Morton and Benny, 1990) (Figure 6B), and MtPT1 also was downregulated in roots colonized with this fungus (Figure 6B).

The *MtPT4* Promoter Directs Expression in Cells Containing Arbuscules

A genomic fragment containing the *MtPT4* gene and 865 bp of upstream sequence was isolated from an *M. truncatula* genomic library. Sequence analysis revealed the presence of a 195-bp intron, 393 nucleotides downstream of the first ATG codon, and both CAAT and TATA boxes, typical of a promoter, within 180 nucleotides 5′ of the ATG. A sequence sharing limited identity (54%) with one of the mycorrhiza and

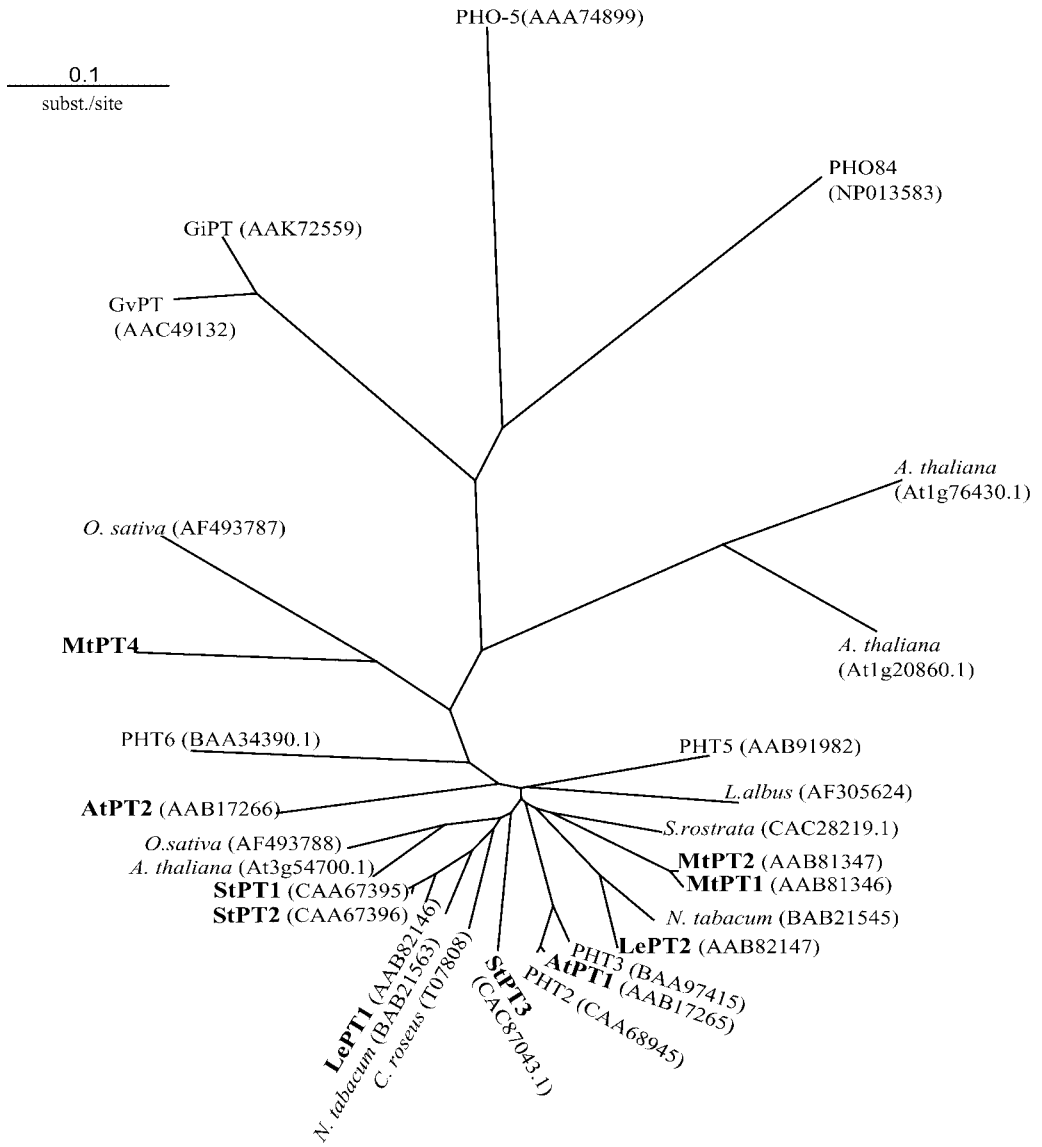

Figure 2. Unrooted Tree Based on a ClustalW Alignment Showing MtPT4 and Other Phosphate Transporters of the PHS Family.

Plant phosphate transporters that have been characterized are shown in boldface.

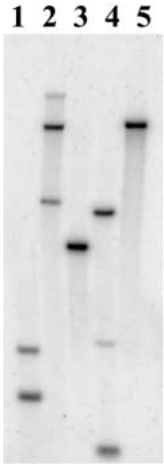

Figure 3. DNA Gel Blot of *M. truncatula* DNA Hybridized with a *MtPT4* Probe Corresponding to the Coding Sequence of the *MtPT4* Gene.

The DNA in lanes 1 to 5 was digested with enzymes DraI, BamHI, EcoRV, HindIII, and EcoRI, respectively. The *MtPT4* gene does not contain EcoRV and EcoRI sites.

resistance–related elements (MRR1) reported by Rausch et al. (2001) also is present in the upstream region.

To examine the expression patterns of *MtPT4*, 865 bp of upstream sequence was fused to the *UidA* gene, and *M. truncatula* plants containing transgenic roots carrying this construct were created according to the procedure of Boisson-Dernier et al. (2001). The plants were inoculated with *G. versiforme*, and after development of the symbiosis, the roots were stained for β-glucuronidase (GUS) activity. Blue staining indicative of GUS activity was present exclusively in mycorrhizal roots, specifically in the cortical cells containing arbuscules (Figures 7A and 7B). The root shown in Figure 7B was counterstained with acid fuschin to enable the visualization of *G. versiforme*, and the arbuscules and intercellular hyphae were visible by epifluorescence microscopy (Figure 7C). It should be noted that acid fuschin also stains the root vasculature. A comparison of the light and epifluorescence micrographs (Figures 7B and 7C) confirmed that GUS staining was present only in cells containing arbuscules and not in adjacent cells or in cells adjacent to intercellular hyphae. This staining pattern was observed in at least four independent transgenic root systems.

MtPT4 Is Present in the *M. truncatula* Periarbuscular Membrane

The arbuscule forms within a cortical cell but remains separated from the cytoplasm of the plant cell by an extension of the plant plasma membrane, called the periarbuscular membrane (Figure 1). This membrane has long been assumed to house a phosphate transporter(s) involved in the uptake of phosphate released by the AM fungus. To determine whether MtPT4 is located on the *M. truncatula* periarbuscular membrane, mycorrhizal roots were hand sectioned, fixed, and stained with the MtPT4 antibody. The MtPT4 antibody was detected with a secondary antibody conjugated to AlexaFluor 488, and the sections were counterstained with a wheat germ agglutinin (WGA)–Texas red conjugate to expose the fungus. Using a low-power fluorescence microscope, the arbuscules showed a strong red fluorescence arising from the WGA–Texas red stain, and a green fluorescent signal, indicating the presence of MtPT4, colocalized to the same cells (data not shown). Confocal laser scanning microscopy was used to examine the arbuscules in greater detail. Figure 8 shows the images of two arbuscules. The individual branches of the arbuscules were clearly visible (Figures 8A and 8D), and the MtPT4 signal colocalized with the arbuscules surrounding the individual branches (Figures 8B, 8C, 8E, and 8F). The signal appeared strongest around the fine branches of the arbuscule, and there was little or no signal associated with the thicker hyphae at the center of the arbuscule (Figure 8). A single optical cross-section through an arbuscule further revealed the colocalization of the MtPT4 signal around the branches of an arbuscule (Figures 9A to 9C).

Arbuscules are transient structures that develop and then degenerate over a period of 6 to 10 days, depending on the species (Toth and Miller, 1984; Alexander et al., 1988, 1989). Figures 9D to 9F show images of a root segment containing arbuscules at early, partially developed, and mature stages of development. The mature arbuscules showed strong MtPT4 staining, and a faint MtPT4 signal was visible on the arbuscule that was partially developed. Staining was not visible on the very young arbuscules that were composed of only the first few dichotomous branches. As the arbuscule branches began to degenerate, the MtPT4 staining appeared punctate, and arbuscules in which the branches had collapsed entirely did not show MtPT4 staining (Figures 9G to 9I). The punctate staining of the degenerating arbuscules and the lack of staining of the collapsed arbuscule were not the result of poor penetration of the antibody, because mature arbuscules adjacent to these arbuscules showed strong staining. These data suggest that MtPT4 expression is coordinated with the life of the arbuscule.

Although the periarbuscular membrane and the plasma membrane of the cell represent a single continuum, MtPT4 staining was strongest around the fine branches of the arbuscule, weaker or absent around the trunk of the arbuscule, and absent from the plasma membrane of the cell. From these data and the biochemical data indicating that MtPT4 was present in the membrane fraction, we conclude that MtPT4 is located in the periarbuscular membrane.

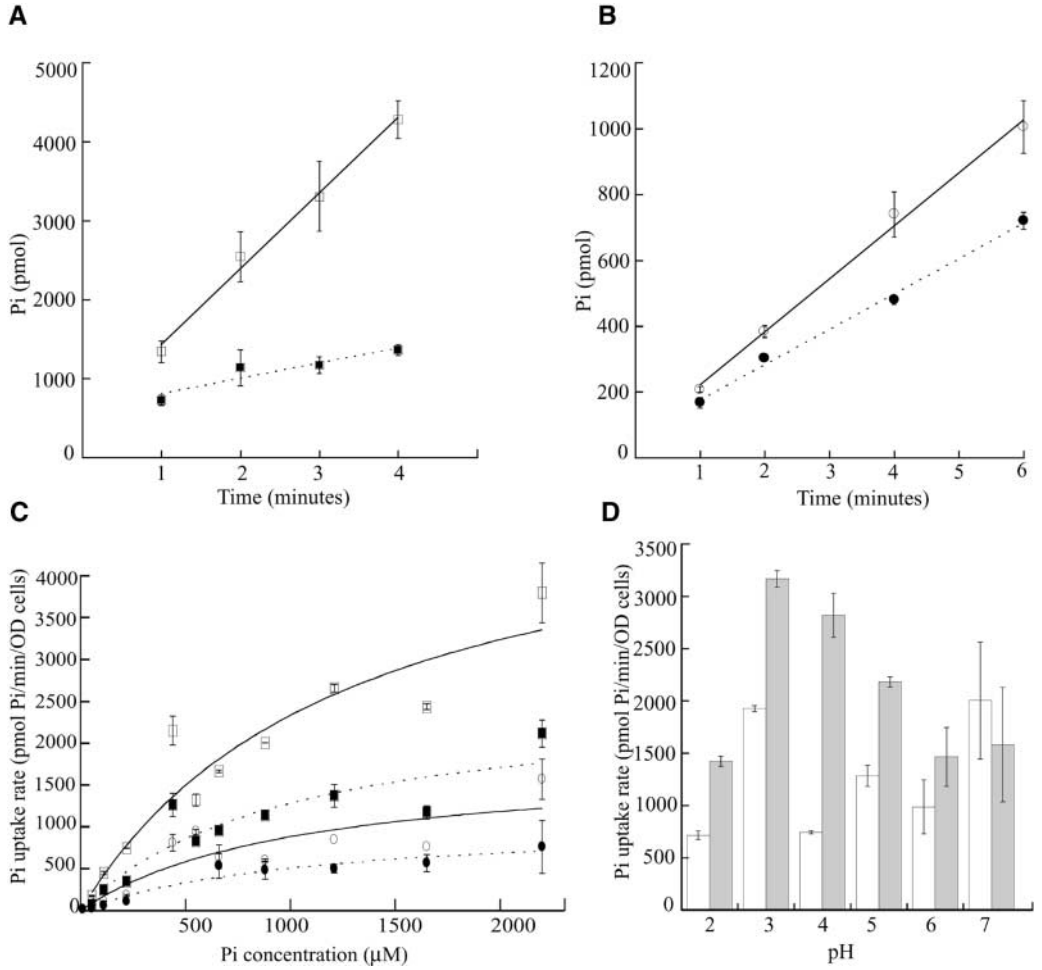

Figure 4. Phosphate Transport Properties of NS219 and PAM2 Expressing *MtPT4*.

(A) Phosphate uptake in PAM2 expressing pWV3-MtPT4 (open squares) and pWV3 (closed squares). The external Pi concentration was 880 μM, and the pH was 4.7. Values are means of duplicate samples ± SD.

(B) Phosphate uptake in NS219 expressing pWV3-MtPT4 (open circles) and pWV3 (closed circles). The external Pi concentration was 110 μM, and the pH was 4.7. Values are means of triplicate samples ± SD.

(C) Phosphate uptake rates plotted as a function of phosphate concentration in PAM2 expressing pWV3-MtPT4 (open squares) and pWV3 (closed squares) and NS219 expressing pWV3-MtPT4 (open circles) and pWV3 (closed circles). The external pH was 4.7. Values are means of duplicate samples ± SD. Curves were fitted by nonlinear regression. K_m values were derived from three replicate experiments for each strain. Results from one representative experiment are shown.

(D) Phosphate uptake in PAM2 expressing pWV3-MtPT4 (gray bars) and pWV3 (white bars) as a function of pH. Values are means of duplicate samples ± SD.

DISCUSSION

The vast resource of sequence information that has become available during the past few years, coupled with the development of bioinformatics tools, has provided new opportunities for the identification of proteins. As a first step to identify novel phosphate transporters from *M. truncatula*, sequence motifs shared by known phosphate transporters of the PiT (Saier et al., 1999; Saier, 2000) and PHS (Pao et al., 1998) families were generated by MEME (Bailey and Elkan, 1994). Interestingly, a chloroplast phosphate transporter, PHT2;1 (Versaw and Harrison, 2002), and homologs from ice plant and Medicago were the only proteins to share motifs from both groups. PHT2;1 shares sequence similarity with the sodium-coupled phosphate transporters of the PiT family, but functional analyses suggest that it probably operates via

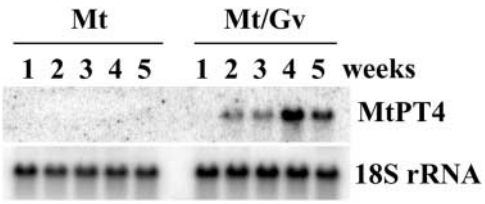

Figure 5. Expression of *MtPT4*.

RNA gel blot of RNA isolated from noncolonized *M. truncatula* roots (Mt) and *M. truncatula* roots colonized with *G. versiforme* (Mt/Gv). Roots were harvested at 1 to 5 weeks after inoculation. The blot was hybridized with an *MtPT4* probe (top) and then stripped and rehybridized with an 18S rRNA probe (bottom).

proton coupling (Daram et al., 1999; Versaw and Harrison, 2002). Because PHT2;1 and its homologs share relatively little sequence identity with PHS family transporters, these motifs may be informative in determining regions of the protein important for proton cotransport.

Most of the plant PHS family phosphate transporters cloned to date share significant sequence similarity. For example, the *M. truncatula* phosphate transporter MtPT1 shares 98% identity with both MtPT2 (Liu et al., 1998b) and a third transporter, MtPT3 (J. Liu, W.K. Versaw, and M.J. Harrison, unpublished data). These three transporters share 75 to 80% identity with the phosphate transporters StPT1, StPT2, and StPT3 from potato (Leggewie et al., 1997; Rausch et al., 2001), LePT1 and LePT2 from tomato (Daram et al., 1998; Liu et al., 1998a), and AtPT1 and AtPT2 from Arabidopsis (Muchhal et al., 1996; Smith et al., 1997). In contrast, MtPT4, the cDNA obtained as a result of the motif searches, encodes a protein that is significantly different at the sequence level; it shares only 61% identity with MtPT1 and MtPT2 and between 59 and 60% identity with the transporters from potato, tomato, and Arabidopsis, including LePT1 and StPT3, the two transporters expressed in mycorrhizal roots. Surprisingly, the protein that shares the most similarity with MtPT4 is a putative phosphate transporter from rice, Pht1-1, that shares 69% identity with MtPT4. The expression patterns of the rice transporter gene are unknown.

Phosphate transport assays revealed that MtPT4 also showed different transport activities compared with the high-affinity phosphate transporters cloned previously, particularly with StPT3 and LePT1. The apparent K_m for phosphate of StPT3 was estimated at 64 μM (Rausch et al., 2001), whereas that of LePT1 was estimated at 30 μM (Daram et al., 1998). By contrast, in both of the yeast strains tested, MtPT4 showed an apparent K_m that was considerably higher, ~1 mM when expressed in NS219 and 1.3 mM when expressed in PAM2. Both NS219 and PAM2 have intact low-affinity phosphate transport systems (Bun-ya et al., 1991; Martinez and Persson, 1998). If it is assumed that the endogenous yeast phosphate transport activity and MtPT4 activity are simply additive, then the K_m of MtPT4 derived in these two strains is 493 ± 14 μM and 668 ± 89 μM, respectively. These data suggest that MtPT4 has a relatively low-affinity phosphate transport activity, with a pH optimum consistent with the proton cotransport mechanism shared by the PHS family of phosphate transporters.

MtPT4 transcripts were detected exclusively in mycorrhizal roots, and analysis of transgenic roots carrying a *MtPT4* promoter–*UidA* fusion revealed that the *MtPT4* promoter was active only in cells that contained arbuscules. This expression pattern is the same as that reported for *StPT3* and is similar to that reported for *LePT1* (Rosewarne et al., 1999; Rausch et al., 2001), although *LePT1* also shows nonsymbiotic expression in the root cap, stele, epidermis, and root hairs of noncolonized roots as well as in leaves (Daram et al., 1998; Liu et al., 1998a). In tobacco, two H^+-ATPase genes also are expressed in cells containing arbuscules, and such proteins may be responsible for generating the proton gradients required for the cotransport of phosphate (Gianinazzi-Pearson et al., 2000). It is interesting that these two H^+-ATPase genes also show non-

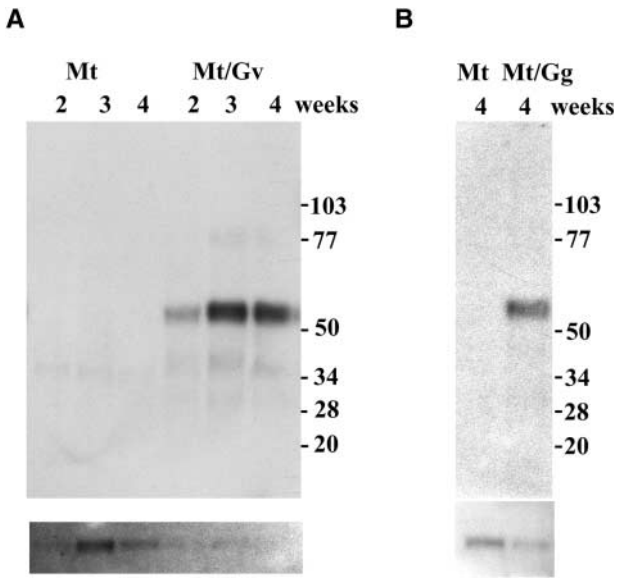

Figure 6. Expression of MtPT4.

(A) Protein gel blot of microsomal proteins isolated from *M. truncatula* roots (Mt) and *M. truncatula* roots colonized with *G. versiforme* (Mt/Gv) harvested at 2, 3, and 4 weeks after inoculation.
(B) *M. truncatula* roots (Mt) and *M. truncatula* roots colonized with *G. gigantea* (Mt/Gg) harvested at 5 weeks after inoculation. The blots were probed with affinity-purified MtPT4 (top) and MtPT1 (bottom) antibodies.
Size markers (kD) are shown (right).

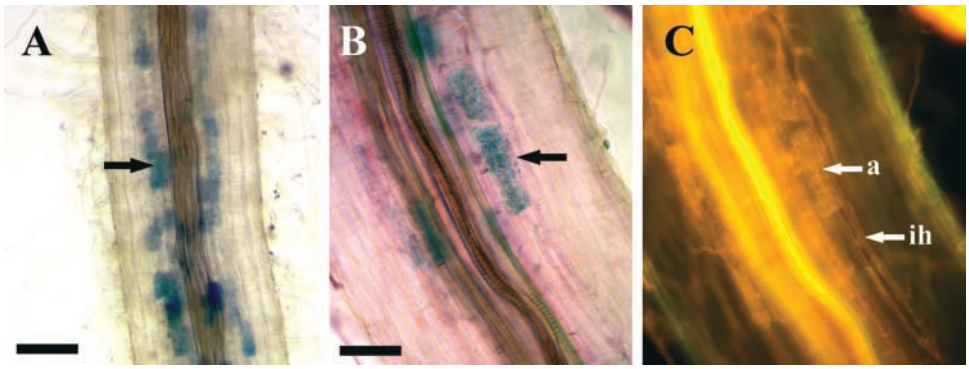

Figure 7. Cell Type–Specific Expression of the *MtPT4* Promoter.

(A) and **(B)** Histochemical staining for GUS activity in *M. truncatula* roots carrying an *MtPT4* promoter–*UidA* fusion. Roots were colonized with *G. versiforme*. Arrows indicate positive GUS staining in cells with arbuscules. The root shown in **(B)** was counterstained with acid fuschin. Bars = 200 μm **(A)** and 50 μm **(B)**.

(C) Epifluorescence micrograph of the root shown in **(B)** revealing arbuscules (a) and intraradical hyphae (ih). Acid fuschin also stained the vascular tissue.

symbiotic expression patterns similar to that of *LePT1*. To date, relatively little is known about the mechanisms that regulate gene expression at the arbuscular interface, but based on these data, it seems that the establishment of the appropriate transport activities involves both the redeployment of genes that have nonsymbiotic functions and the expression of new genes that function exclusively in the symbiosis.

In the mycorrhizal symbiosis, it is predicted that phosphate released by the fungus to the periarbuscular space is transported across the periarbuscular membrane into the cortical cell (Smith and Smith, 1989). To determine whether MtPT4 is located in the periarbuscular membrane and might mediate this process, antibodies were raised to a peptide corresponding to the C terminus of the transporter protein. Consistent with the analyses of *MtPT4* transcripts, the MtPT4 protein was detected in microsomal fractions from roots colonized with *G. versiforme* or *G. gigantea* but not from noncolonized roots. Immunolocalization analysis revealed MtPT4 surrounding the branches of the arbuscule. MtPT4 is predicted to be an integral membrane protein, and from the biochemical evidence of location in the membrane fraction and the cell biology data indicating the location surrounding the arbuscules, we conclude that MtPT4 is present in the periarbuscular membrane, where it likely plays a role in phosphate transport into the cell. MtPT4 is the only protein shown to date to be located exclusively on the periarbuscular membrane, and MtPT4 antibodies will aid in the development of biochemical fractionation and purification schemes for the periarbuscular membrane.

Arbuscules develop via continuous dichotomous branching that results in a structure containing a hierarchy of branches, beginning with a thick trunk and ending with extremely fine branches (Bonfante-Fasolo, 1984). MtPT4 staining was particularly intense on the membrane surrounding the fine branches of mature arbuscules and was not visible surrounding the thicker trunk hyphae. It has been shown previously that ATPase staining, consistent with proton pump activity, also was strongest on the fine branches of the arbuscule (Marx et al., 1982; Gianinazzi-Pearson et al., 1991). Arbuscules are transient structures that develop and then collapse and decay, leaving the cortical cell intact and capable of hosting another arbuscule (Cox and Sanders, 1974; Bonfante-Fasolo, 1984; Toth and Miller, 1984; Alexander et al., 1988). A series of images from roots containing arbuscules at different stages of development revealed that MtPT4 was not detectable in very young arbuscules, which had only one or two dichotomous branches, but was detected as the number of branches increased. Staining was strongest in mature arbuscules but absent from collapsed arbuscules, suggesting that expression is coordinated with arbuscule development and decay. This expression pattern also correlates with a previous report in which H^+-ATPase activity staining was noted to disappear from the periarbuscular membrane as the arbuscule aged (Gianinazzi-Pearson et al., 1991). Together, these data support the hypothesis that phosphate absorption occurs maximally in mature arbuscules.

Arbuscules develop deep within the inner cortical cell layer of the root; consequently, analyses of the pH and phosphate concentration within the periarbuscular space are difficult. Measurements obtained with pH-sensitive fluorochrome dyes suggest that the arbuscular apoplast has a pH of ~4.25, and analyses with neutral red also suggest an acidic pH (Smith et al., 2001). In yeast, MtPT4 showed maximum activity at pH 4, which is consistent with conditions

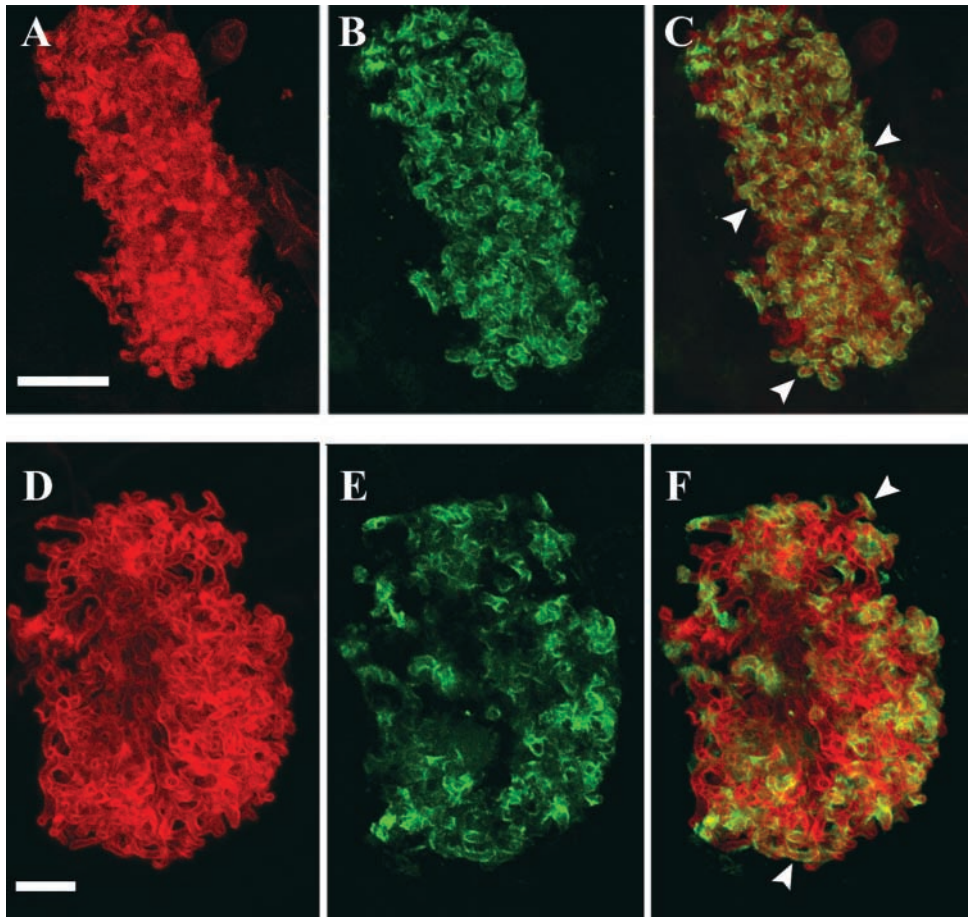

Figure 8. Immunolocalization of MtPT4.

Laser scanning confocal microscopy images of mycorrhizal roots of *M. truncatula*/*G. versiforme* probed with MtPT4 antibodies. The MtPT4 antibodies were visualized with a secondary antibody conjugated with AlexaFluor 488. The roots were counterstained with WGA–Texas red to visualize *G. versiforme*.

(A) and **(D)** WGA–Texas red staining revealing *G. versiforme* arbuscules.

(B) and **(E)** Corresponding images showing green fluorescence from MtPT4 immunostaining.

(C) and **(F)** Merged images showing both red and green fluorescence. The MtPT4 signal is visible surrounding the branches of the arbuscule (arrowheads).

(A) to **(C)** show projections of 39 optical sections taken at 0.16-μm intervals; bar = 30 μm. **(D)** to **(F)** show projections of 43 optical sections taken at 0.3 μm intervals; bar = 10 μm.

predicted in the periarbuscular space. The concentration of phosphate in the periarbuscular space also is unknown, but estimates of the flux across the arbuscule have been made. Phosphate flow into mycorrhizal roots is significantly higher than that into nonmycorrhizal roots, and depending on the phosphate concentration of the external medium, fluxes of between 8 and 100 $\times$ 10^{-13} mol·m^{-1} (root)·s^{-1} have been measured (Cox and Tinker, 1976; Smith et al., 1994; Smith and Read, 1997; Dickson et al., 1999). Various lines of evidence suggest that most of the phosphate moves into mycorrhizal roots via the fungus (Pearson and Jakobsen, 1993; Liu et al., 1998b; Chiou et al., 2001), and estimates of phosphate flow into hyphae correlate well with the root measurements, providing further support for this theory (Jakobsen et al., 1992). Based on the phosphate inflow measurements and estimations of the area of the arbuscules, it is suggested that phosphate fluxes across the arbuscular interface range between 2 and 29 nmol·m^{-2}·s^{-1} and are comparable to transport rates measured in plant and fungal cells in high-phosphate environments (Cox and Tinker, 1976; Smith et al., 1994; Smith and Read, 1997; Dickson et al., 1999). The apparent K_m for MtPT4 is in the range expected for optimal

activity in a relatively high-phosphate environment. By contrast, the K_m values of the high-affinity transporters from potato (Rausch et al., 2001) and tomato (Daram et al., 1998) suggest optimal function in low-phosphate environments. The membrane locations of StPT3 and LePT1 are not known, and it is possible that they are not located in the periarbuscular membrane. Alternatively, it also is possible that each species possesses both high- and low-affinity systems at the periarbuscular membrane.

In addition to forming AM symbioses, legumes are unique

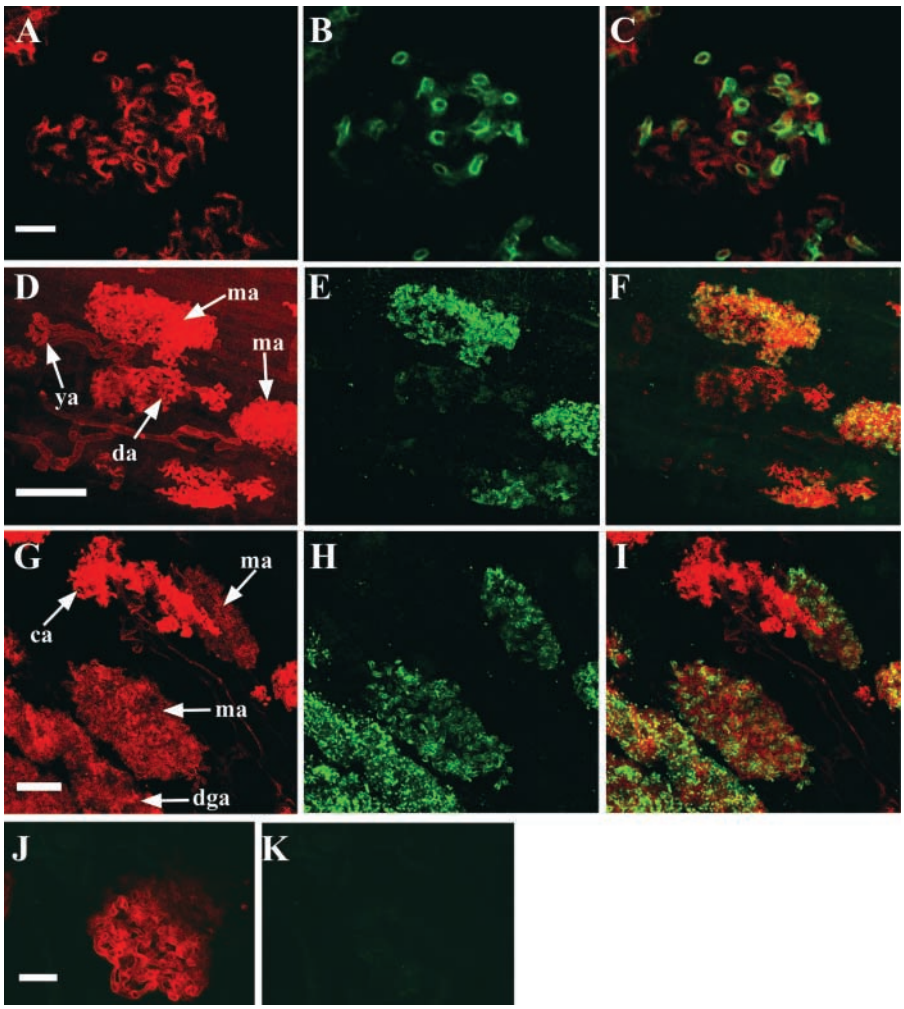

Figure 9. Immunolocalization of MtPT4.

Laser scanning confocal microscopy images of mycorrhizal roots of *M. truncatula/G. versiforme* probed with MtPT4 antibodies.
(A), **(D)**, **(G)**, and **(J)** Red fluorescence from WGA–Texas red staining.
(B), **(E)**, and **(H)** Corresponding images showing green fluorescence from MtPT4 immunostaining.
(C), **(F)**, and **(I)** Merged images showing both red and green fluorescence.
(K) Immunostaining with MtPT4 preimmune serum.
(A) to **(C)** show a single optical section through the branches of an arbuscule; bar = 10 μm. **(D)** to **(F)** show projections of 18 optical sections taken at 0.4-μm intervals. MtPT4 signals are not detected around the very young arbuscules (ya). The MtPT4 signal is just detectable around the developing arbuscule (da). Mature arbuscules (ma) in the same area of the root are associated with strong MtPT4 immunostaining. Bar = 50 μm. **(G)** to **(I)** show projections of 22 optical sections taken at 0.3-μm intervals. The older, collapsed arbuscule (ca) does not show MtPT4 immunostaining. Punctate staining is visible surrounding the degenerating arbuscules (dga). Strong MtPT4 immunostaining is visible surrounding the mature arbuscules. Bar = 30 μm. **(J)** and **(K)** show projections of 15 optical sections taken at 0.5-μm intervals. The MtPT4 preimmune serum did not stain the arbuscules. Bar = 10 μm.

in their ability to form symbioses that assist them with the acquisition of nitrogen (Shultze and Kondorosi, 1998), and nodules, the symbiotic organ resulting from the union of rhizobia and legume roots, have features analogous to those of the AM symbiosis (Perotto et al., 1994; Parniske, 2000). In the nodules, differentiated bacteria, called bacteroids, are encased within a plant-derived membrane, called the peribacteroid membrane. There, they convert atmospheric nitrogen to ammonia, which then is transported to the plant. Like the periarbuscular space, the peribacteroid space also is an acidic, apoplastic compartment, and the concentration of ammonium is estimated to be high, ~12 mM (Streeter, 1988). The transport system involved in the transport of ammonium across the peribacteroid membrane has not been cloned, but a voltage-gated ion channel present in the membrane has the appropriate characteristics and has been implicated in this process (Tyerman et al., 1995).

The periarbuscular membrane is an extension of the plasma membrane of the cell, and cytochemical data indicate that it retains many of the characteristics of the plasma membrane (Gianinazzi-Pearson and Gianinazzi, 1988; Perotto et al., 1994). The first evidence for the differentiation of this region of the membrane came from patterns of H^+-ATPase activity staining (Gianinazzi-Pearson et al., 1991). The finding that the MtPT4 protein is located exclusively on the periarbuscular membrane demonstrates that this region of the membrane contains proteins not found on the peripheral region of the plasma membrane and that it contains activities specialized for its function. These data also suggest that specific targeting mechanisms must operate to ensure location only on the section of the membrane surrounding the fungus. In plants, the location of the auxin efflux carrier is a classic example of an asymmetric location of a transport protein within a membrane. The auxin efflux carrier, which functions in basipetal auxin transport, is located in the membrane at the basal end of the xylem parenchyma cells, and recent data indicate that the actin cytoskeleton is essential for this localization (Gälweiler et al., 1998; Muller et al., 1998; Geldner et al., 2001). During arbuscule development, there is considerable reorganization of both the microtubules and the microfilaments of the cortical cell (Genre and Bonfante, 1997, 1998, 1999; Blancaflor et al., 2001), and it is possible that the targeted localization of MtPT4 is mediated through one or both of these systems. It will be interesting to determine if a particular domain of the protein is necessary for targeting to the periarbuscular membrane.

In summary, the phosphate transport activity of MtPT4 and the distinct cellular and membrane location of this protein strongly support a role in the acquisition of phosphate released by the fungal symbiont in the AM symbiosis. Transgenic approaches will be necessary to determine the full extent of the contribution of this transporter and whether, as has been seen for many important plant transport processes, functional redundancy exists to guard against the loss of this transport activity.

METHODS

Plant Growth Conditions

Medicago truncatula cv Jemalong, line A17, was used throughout this study. Plants were grown in growth rooms under an 18-h-light (25°C)/6-h-dark (22°C) regime. The growth rooms contained F4012/Triten 50 light bulbs (www.lightbulbs4sale.com), and the light intensity at shelf height was 260 $\mu E \cdot m^{-2} \cdot s^{-1}$.

Mycorrhizal fungi (*Glomus versiforme* and *Gigaspora gigantea*) were maintained on leek and Bahia grass, respectively. *G. gigantea* was kindly provided by D. Douds (U.S. Department of Agriculture, Philadelphia, PA). Plants were inoculated with *G. versiforme* as described previously (Harrison and Dixon, 1993). Briefly, seedlings were grown in sterilized Turface (A.H. Hummert Seed Co., St. Louis, MO) until they had one fully opened trifoliate leaf. Then, they were transplanted to either cones (1 seedling per cone) or pots (7 to 10 seedlings per 11-cm pot) containing sterile Turface and inoculated with either 750 sterile spores per cone or 5000 sterile spores per pot. *M. truncatula*/*G. gigantea* mycorrhizas were established in a similar manner except that all experiments were performed in cones and the plants received 30 spores per cone. Spores were surface-sterilized according to Bécard and Fortin (1988). In all experiments, control plants were mock inoculated with the final distilled water wash from the spore sterilization procedure. The plants were fertilized twice weekly with half-strength Hoagland solution (Arnon and Hoagland, 1940) containing 20 μM phosphate. Plants were harvested between 1 and 5 weeks after inoculation, and a random sample of the root system was assessed for colonization via a modified grid-line intersect method (McGonigle et al., 1990). The remaining tissue was frozen in liquid N_2 and stored at −80°C for subsequent RNA and protein isolations. The *M. truncatula*/*G. versiforme* mycorrhizal root samples harvested at 1 to 5 weeks after inoculation and used for RNA gel blot analysis showed colonization levels of 11.8, 17.4, 44, 59, and 69% of root length colonized, respectively. The samples harvested at 2 to 4 weeks for protein analysis showed colonization levels of 39, 53, and 75% of root length colonized, respectively. The *M. truncatula*/*G. gigantea* mycorrhizal root sample harvested at 5 weeks after inoculation showed colonization levels of 42% of root length colonized.

Sequences Used in MEME

Sequences of the following proteins were used to generate the phosphate transporter motifs in the motif-building program MEME (Bailey and Elkan, 1994): *Solanum tuberosum* (StPT1), *Arabidopsis thaliana* (AtPT1, PHT2;1), *Medicago truncatula* (MtPT1, MtPHT2;1), *Triticum aestivum* (partial phosphate transporter cDNA, PT1), *Oryza sativa* (partial phosphate transporter cDNA), *Glomus versiforme* (GvPT), *Saccharomyces cerevisiae* (PHO84, PHO89), *Neurospora crassa* (PHO-5, PHO-4), *Cricetulus griseus* (sodium-dependent Pi transporter), *Homo sapiens* (PiT-2), and *Sinorhizobium meliloti* (PIT).

World Wide Web Sites for Sequence and Analysis Programs

M. truncatula genome programs funded by the Samuel Roberts Noble Foundation (http://www.noble.org/medicago/index.html and http://www.ncgr.org/mgi/index.html) (Bell et al., 2000), the National Science Foundation (http://www.medicago.org/), the Institut National de la Recherche Agronomique–Centre National de la Re-

cherche Scientifique, France (http://medicago.toulouse.inra.fr/Mt/public/ESTMtruncatula.html), and Stanford University (http://bio-srl8. stanford.edu/) were responsible for the production of the majority of the *M. truncatula* ESTs. They are available through GenBank (http://www.ncbi.nlm.nih.gov/), the TIGR *M. truncatula* Gene Index (http://www.tigr.org/tdb/mtgi/) and the Medicago Genome Initiative http://www.ncgr.org/mgi/index.html). MEME is available at http://www.ucsd.edu/MEME.

Identification of MtPT4 cDNA and Genomic Clones

The primers 5′-CAAATGATTACCCTCTTTGG-3′ and 5′-CATAAACCAGCTAAACACATC-3′ designed to the *M. truncatula* TIGR TC12920 were used in PCR to amplify part of the *MtPT4* sequence from an *M. truncatula/G. versiforme* cDNA library. The PCR fragment was labeled with α-^{32}P-dATP and used as a probe to screen an *M. truncatula/G. versiforme* cDNA library (van Buuren et al., 1999). Labeling and hybridization procedures were based on standard protocols (Sambrook et al., 1989). Positive clones were converted to pBluescript− (Stratagene) plasmids according to the manufacturer's instructions, and clones containing full-length inserts were sequenced on both strands. To identify a genomic clone, the *MtPT4* cDNA was labeled with α-^{32}P-dATP and used as a probe to screen an *M. truncatula* genomic library carried in Lambda Fix II (Stratagene). A 2647-bp genomic fragment consisting of the *MtPT4* open reading frame, a 195-bp intron, and 865 bp of upstream sequence was sequenced. The MtPT4 cDNA and gene sequences have been deposited in GenBank.

Phosphate Transport Analyses in Yeast

The *MtPT4* coding region was amplified by PCR using a primer set designed to create BamHI and XhoI sites at the 5′ and 3′ ends of the DNA fragment, respectively. The PCR fragment was cloned directionally into BamHI and XhoI sites of pWV3, creating pWV3-MtPT4. This places the *MtPT4* gene under the control of the ADH1 promoter. pWV3-MtPT4 was introduced into the yeast phosphate transport mutants NS219 (Bun-ya et al., 1991) and PAM2 (Martinez and Persson, 1998). pWV3 lacking an insert was introduced into these strains and used as a control. Phosphate transport assays were performed as described previously (Ueda and Oshima, 1975) The phosphate uptake experiments were performed with duplicate or triplicate samples. The apparent K_m values reported for the NS219 and PAM2 strains are averages of three replicate experiments, each of which contained duplicate samples.

DNA and RNA Gel Blot Analyses

DNA and RNA gel blot analyses were performed according to standard molecular biology procedures under high-stringency conditions (Sambrook et al., 1989).

Antibody Production and Affinity Purification

Antibodies to a peptide corresponding to the C-terminal 15 amino acids of the MtPT4 protein (5′-DRPAGIRQDSRTEKM-3′) were prepared by Covance (Denver, CO). An additional Cys residue was added to the N terminus of the peptide to enable coupling to affinity matrices. Rabbits were maintained on an alfalfa-free diet, and preimmune serum was collected before immunization. Serum was collected and analyzed for the presence of antibodies that recognized the peptide by protein gel blot analysis. An affinity resin containing the peptide was prepared using the SulfoLink kit (Pierce, Rockford, IL), and antibodies were purified by affinity chromatography according to the manufacturer's instructions. The affinity-purified antibodies cross-reacted with immunoblots of MtPT4 peptide (data not shown), whereas the preimmune serum showed no cross-reaction. The affinity-purified antibodies were used at a dilution of 1:500 on protein gel blots and at dilutions of 1:100 and 1:500 for immunolocalization analysis.

Preparation of Microsomal Proteins and Protein Gel Blot Analysis

Proteins were isolated from *M. truncatula* roots according to Chiou et al. (2001) and Bush (1989). Roots were ground to a fine powder in liquid N_2 and mixed with extraction buffer (230 mM sorbitol, 50 mM Hepes, 10 mM KCl, 3 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride, pH 7.8) at a ratio of 5:1 (v/w). The slurry was centrifuged at 10,000*g* for 8 min, and the supernatant was removed carefully. The microsomal fraction was pelleted from this supernatant by centrifugation at 100,000*g* for 1 h. The pellet was resuspended in resuspension buffer (330 mM sorbitol, 2 mM Hepes, 10 mM KCl, and 0.1 mM DTT, pH 7.8), and the protein concentration was measured using the Bio-Rad protein reagent. Microsomal samples were separated by SDS-PAGE (Laemmli, 1970) on NuPAGE 10% Bis-Tris gels (Invitrogen, Carlsbad, CA), and the proteins were transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA) using a Bio-Rad semidry blotting apparatus. Immunodetection was performed using the WesternBreeze chemiluminescent system according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). Purified MtPT1 (Chiou et al., 2001) and MtPT4 antibodies were used at a dilution of 1:500. The MtPT4 preimmune serum did not show any cross-reaction with microsomal proteins from either noncolonized or colonized roots.

Preparation of a *MtPT4* Promoter–*UidA* Fusion

An 865-bp fragment corresponding to the *MtPT4* promoter and including the first 18 bp of the MtPT4 coding sequence was amplified using primers 5′-TCCAAGCTTCTGCAGACTCGATCCACAACAAAG-3′ and 5′-TTACCAAAGGCCATGGCAAGGACTTCTAATCCCAT-3′. The 5′ and 3′ primers contain PstI and NcoI sites, respectively, and the PCR fragment was digested and ligated into the PstI and NcoI sites of the pCAMBIA 3301 vector (CAMBIA, Canberra, Australia). The 3′ primer was designed so that ligation to pCAMBIA 3301 would result in a translational fusion of the first six amino acids of the MtPT4 protein to the *UidA* open reading frame. The cloning strategy also resulted in the insertion of one additional amino acid, an Ala residue, before the first Met of the *UidA* open reading frame. The pCAMBIA 3301 vector containing the *MtPT4* promoter region was transformed into *Agrobacterium rhizogenes* strain ARqual using standard methods.

Preparation of *M. truncatula* Plants Containing Transformed Roots

M. truncatula plants with transformed roots were created as described by Boisson-Dernier et al. (2001). Briefly, *M. truncatula* seeds

were surfaced-sterilized and allowed to germinate. The root tip of each seedling was excised, and the remaining root was inoculated with Agrobacterium strain ARqua I containing pCAMBIA 3301 carrying the *MtPT4* promoter–*UidA* fusion or pCAMBIA 3301. The seedlings were grown on modified Fahraeus medium containing 5 mg/L phosphinothricin. After 25 days, significant root development had occurred, and the plants were transplanted to cones and inoculated with *G. versiforme* spores as described above. Roots were harvested at 14, 20, and 30 days after inoculation, and expression of the *UidA* gene was evaluated by β-glucuronidase staining in 0.1 M phosphate buffer, pH 7.0, containing 0.5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 10 mM EDTA, 1 mg/mL 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid, and 1% (v/v) *N*,*N*-dimethylformamide. Roots were counterstained with acid fuschin to enable the localization of *G. versiforme* within the roots.

Immunolocalization of MtPT4

Immunolocalization was performed essentially as described by Blancaflor et al. (2001) with minor modifications. Root pieces were fixed for 2 h in 4% formaldehyde and 5% (v/v) DMSO in PME buffer (50 mM Pipes, 5 mM $MgSO_4$, and 10 mM EGTA), pH 6.9, and hand sectioned using a double-edged razor blade. The root segments were fixed temporarily to a cover slip with a thin layer of agar as described by Brown and Lemmon (1995) and then were digested in 1% cellulase RS, 0.01% pectolyase Y23 (Karlan Research Products, Santa Rosa, CA), and 0.1% BSA in PME buffer for 10 min. After digestion, the segments were washed three times for 5 min each with PME buffer and then incubated in 1% BSA in PBS (135 mM NaCl, 25 mM KCl, and 10 mM Na_2HPO_4), pH 7.5, for 30 min. The BSA was removed, and the segments were incubated overnight with MtPT4 antibody (1:100 and 1:500) in PBS containing 0.5% (w/v) BSA. The segments were washed five times in PBS and incubated in the secondary antibody conjugate, a 1:80 dilution of goat anti-rabbit IgG–AlexaFluor 488 conjugate (Molecular Probes, Eugene, OR) in PBS for 2 h. After five washes in PBS, the segments were incubated in 0.1 mg/mL wheat germ agglutinin–Texas red (Molecular Probes) in PBS for 30 min to stain the fungus (Genre and Bonfante, 1997). After five more washes in PBS, the sections were mounted in 20% (w/v) Mowiol 4-88 (Calbiochem, La Jolla, CA) containing 0.1% (w/v) phenylenediamine in PBS, pH 8.5, and viewed by epifluorescence and confocal microscopy.

Confocal Microscopy

A Bio-Rad 1024 ES confocal laser scanning microscope equipped with ×40 and ×63, numerical aperture 1.2, water-immersion objectives was used for imaging. The excitation and emission wavelengths for the AlexaFluor 488 dye were 488 and 500 to 550 nm, respectively. The excitation and emission wavelengths for the Texas red dye were 568 and 590 nm, respectively. Optical sections were collected at 0.16- to 0.4-μm intervals with Kalman averaging. Images were assembled using Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for noncommercial research purposes.

Accession Numbers

The accession numbers for the sequences mentioned in this article are as follows: *Medicago truncatula* ESTs sharing identity with MEME motif 5, AL384768 and AL384522, respectively, MtPT4 cDNA and gene sequences, AY116210 and AY116211, respectively; *Mesembryanthemum crystallinum* putative phosphate permease cDNA, U84890; *Oryza sativa* partial phosphate transporter cDNA and Pht1-1 cDNA, AF271893 and AF493787; *Solanum tuberosum* StPT1, CAA67395; *Arabidopsis thaliana* AtPT1 and PHT2;1, AAB17265 and AAM53960, respectively; *Medicago truncatula* MtPT1 and MtPHT2;1 cDNA, AAB81346 and AF533081, respectively; *Triticum aestivum* PT1 cDNA, AF110180; *Glomus versiforme* GvPT, AAC49132; *Saccharomyces cerevisiae* PHO84 and PHO89, NP_013583 and NP_009855, respectively; *Neurospora crassa* PHO-5 and PHO-4, AAA74899 and P15710, respectively; *Cricetulus griseus* sodium-dependent Pi transporter, I48084; *Homo sapiens* PiT-2, NP_006740; *Sinorhizobium meliloti* PIT, NP_384292.

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NOTE ADDED IN PROOF

A gene from *Oryza sativa* cv Nipponbare, encoding a protein identical to that of the *O. sativa* Pht1-1 gene mentioned in this manuscript, has been shown to be expressed exclusively in *O. sativa*/*Glomus intraradices* mycorrhizal roots (Paszkowski et al., Proc. Natl. Acad. Sci USA, in press).