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A. Benedetto · F. Magurno · P. Bonfante · L. Lanfranco

Expression profiles of a phosphate transporter gene (*GmosPT*) from the endomycorrhizal fungus *Glomus mosseae*

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Abstract Arbuscular mycorrhizal (AM) fungi have long been shown to successfully contribute to phosphate uptake by plant roots. The first step of the fungus-mediated uptake is carried out by fungal membrane Pi transporters (PT) that transfer Pi from the soil into the extraradical hyphae. In the present work we report the identification and characterisation of a PT gene from *Glomus mosseae*, an AM fungus important for natural and agricultural ecosystems. Degenerate primers and rapid amplification of cDNA ends–polymerase chain reaction (PCR) allowed us to obtain a sequence (*GmosPT*) showing a highly significant similarity with *GiPT* and *GvPT*, the only two other PT genes already isolated from AM fungi. Reverse transcriptase–PCR experiments were carried out to study *GmosPT* expression profiles in structures corresponding to different fungal life stages (quiescent and germinated sporocarps, intraradical and extraradical hyphae) and in extra- and intraradical hyphae exposed to high and low Pi concentrations. *GmosPT* showed an expression pattern similar to *GiPT*, the *Glomus intraradices* PT gene, since its transcript was more abundant in the extraradical mycelium treated with micromolar Pi levels. In addition, the intraradical mycelium also showed a significant *GmosPT* expression level that was independent from external Pi concentrations. This finding opens new questions about the role and functioning of high-affinity PT in AM fungi.

Keywords AM fungi · Phosphate transporters · *Glomus mosseae* · Symbiosis

A. Benedetto · F. Magurno · P. Bonfante · L. Lanfranco
Dipartimento di Biologia Vegetale,
Università degli Studi di Torino,
Turin, Italy

P. Bonfante
Istituto per la Protezione delle Piante,
Sezione di Torino,
CNR,
Turin, Italy

L. Lanfranco (✉)
Dipartimento di Biologia Vegetale,
Viale Mattioli 25,
Turin, 10125, Italy
e-mail: luisa.lanfranco@unito.it
Tel.: +39-011-6705982
Fax: +39-011-6705962

Introduction

Phosphorus (P) is an essential nutrient for plant growth and development. It is involved in a number of fundamental processes such as energy generation and photosynthesis (Vance et al. 2003). Hence, P availability in the soil is a parameter that broadly affects plant life, with important consequences both on plant distribution in the ecosystems and on crop culture yield. Total P concentration in the soil is high, but orthophosphate (Pi), the inorganic form preferred by plants, is available only in limited amounts due to its low solubility and slow diffusion rates (Bieleski 1973; Holford 1997); as a consequence, a depletion zone is formed very rapidly around actively absorbing roots. It has been calculated that mean free Pi in the soil does not exceed the concentration of 10 μM (Bieleski 1973).

Plants have developed different strategies to ensure and enhance Pi acquisition: modifying root architecture and extension to explore larger portions of soil and secreting organic acids or phosphatases that allow the release of bound Pi (Marschner 1995; Neumann and Martinoia 2002; Rausch and Bucher 2002). As an alternative, they can establish symbiotic association with soil micro-organisms, in particular, with arbuscular mycorrhizal (AM) fungi (Karandashov and Bucher 2005). Thanks to their wide network of extraradical hyphae, AM fungi are able to overcome the nutrient depletion zone, and this results in a very efficient exploitation of soil resources (Zhu et al. 2001). For this reason these organisms are considered powerful tools for low-input agricultural practices (Jeffries et al. 2003). Mycorrhizal plants therefore can choose to acquire Pi directly from the soil through plant-specific phosphate transporters (PT) or through uptake and trans-

port systems of the fungal symbiont. It has been demonstrated that both systems can work simultaneously, but there is a preferential uptake via fungal hyphae. This seems to occur independently from nutrient availability or growth effect (Smith et al. 2004), thus indicating that Pi transport in the AM symbiosis plays a role that is less obvious than previously expected. In addition, there is evidence of functional diversity in AMs; that is, plants can respond differently to different AM fungi, not only at the level of colonization, Pi uptake and growth, but also at the level of the expression of genes involved in P nutrition (Burleigh et al. 2002).

In the fungus-mediated uptake, Pi is absorbed into extraradical mycelium by means of active PT (Harrison and van Buuren 1995; Maldonado-Mendoza et al. 2001) and accumulated in the vacuoles of extraradical hyphae in the form of polyphosphate (polyP) (Callow et al. 1978; Ezawa et al. 2003; Solaiman et al. 1999). PolyP chains are supposed to be transferred by means of a motile tubular vacuolar network (Bago et al. 2002; Cox et al. 1980; Timonen et al. 2001; Uetake et al. 2002) in the intraradical compartment, where Pi ions resulting from polyP hydrolysis are assumed to be released by membrane-passive carriers into the periarbuscular space (Ezawa et al. 2002). Mycorrhiza-specific PT, possibly responsible for plant Pi uptake in arbuscule-containing cells, have recently been characterised in potato, rice and *Medicago truncatula* (Harrison et al. 2002; Karandashov et al. 2004; Paszkowski et al. 2002; Rausch et al. 2001).

Phosphate transporter genes already isolated from the AM fungi *Glomus versiforme* and *G. intraradices*, *GvPT* and *GiPT*, respectively (Harrison and van Buuren 1995; Maldonado-Mendoza et al. 2001), encode for high-affinity proton-coupled transporters. They both share structural and sequential similarity with other plant and fungal high-affinity PT. The apparent K_m of GvPT, evaluated in a heterologous system, is in the micromolar range, a value comparable to free Pi concentration generally found in soil solution. *GvPT* and *GiPT* transcripts are predominantly detected in extraradical mycelium, thus indicating a role in Pi acquisition from the soil. It has been demonstrated that *GiPT* expression responds to external Pi concentrations and also to overall mycorrhiza Pi content (Maldonado-Mendoza et al. 2001).

Here, we report the identification and characterisation of a PT gene from *G. mosseae*, an AM fungus important for natural and agricultural ecosystems. The sequence, named *GmosPT,* shows the highest similarity with *GiPT*, and its expression follows a similar regulation, as the transcript level in extraradical mycelium is higher at micromolar Pi concentrations. These data suggest for *GmosPT* a role in Pi uptake from the soil. Differently from *GiPT*, a significant expression signal has been detected in the intraradical fungal structures present in mycorrhizal roots. This finding opens new questions about the role and functioning of high-affinity PT in AM fungi.

Materials and methods

Sporocarps of *G. mosseae* (BEG 12) were obtained from Biorize (Dijon, France) and surface-sterilized with 3% chloramine T. To induce germination, sterilized *G. mosseae* sporocarps were incubated on water at 25°C for 2 weeks. For gene expression analyses, quiescent and germinated sporocarps were treated with 35 μM Na_2HPO_4 for 48 h.

Plants of *Cucumis sativus* L. cv Marketmore inoculated with *G. mosseae* (BEG 12) were obtained as described in Balestrini et al. (2005). Roots were sampled after 2 months. After inspection with the stereomicroscope, 100 1-cm-long root segments were sampled, stained with the cotton blue and used to evaluate the intensity of root colonization according to Trouvelot et al. (1986).

Mycorrhizal roots were submerged for 24 h in a Long–Ashton solution (Hewitt 1966) without phosphate. Then, a 24-h treatment in the same solution without phosphate or added with 35 μM or 3.5 mM Na_2HPO_4 was performed. This experiment was carried out on two independent series of plants. Extraradical hyphae and mycorrhizal roots, free of extraradical mycelium, were collected and immediately frozen in liquid nitrogen.

Genomic DNA was extracted from about 100 *G. mosseae* sporocarps as described by Lanfranco et al. (1999). PCR reactions, with degenerate primers PT1 (5′-ATGGGT RTYGGIATHGGIGGIGAYTAYCC-3′) and PT2 (5′-GTC GTRTTIGGICCRAARTTYGRAARAAA-3′), were carried out in a final volume of 50 μl, containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.1 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM of each deoxyribonucleoside-triphosphate (dNTP), 3 μM of each primer, 2 units of RED *Taq* DNA polymerase (Sigma, St. Louis, MO) and 10–100 ng of genomic DNA. The PCR programme was as follows: 94°C for 3 min (1 cycle), 92°C for 45 s, 49°C for 45 s, 72°C for 1 min and 15 s (28 cycles) and 72°C for 5 min (1 cycle).

Switching mechanism at the 5′ end of the RNA transcript (SMART) cDNA, obtained from *G. mosseae* external mycelium mRNA according to the manufacturer's instructions (SMART cDNA synthesis kit, Clontech, Palo Alto, CA), was used in RACE experiments carried out coupling SMART universal PCR primers and specific primers designed on the available partial sequence. Sequence data for a 150-bp fragment at the 3′ end of the gene, kindly received from Dr. Natalia Requena (Karlsruhe University), were also used to design RACE primers. The reactions were performed as already described for PCR amplifications; the programme was as follows: 94°C for 3 min (1 cycle), 92°C for 30 s, annealing temperature variable (depending on the primers used and the reaction stringency chosen) for 45 s, 72°C for 1 min (30–35 cycles) and 72° for 5 min (1 cycle).

PCR products, amplified from genomic DNA and cDNA, were extracted and purified from agarose gel using the QIAEX II gel extraction kit (Qiagen, Valencia, CA) and directly cloned into the pGem-T vector (Promega, Madi-

son, WI). XL-2 Blue ultracompetent cells were transformed and plated onto selective medium following the manufacturer's instructions. Plasmid DNAs were prepared with the Qiaprep Spin miniprep kit (Qiagen). DNA sequences were determined by MWG Biotech (Ebersberg, Germany) using T7 and SP6 primers. The *GmosPT* sequence has been submitted to GenBank under the accession number DQ074452. DNA sequences analyses were performed with the BLASTX software (http://www.ncbi.nlm.nih.gov/), Expasy molecular biology server (http://www.expasy.org/) and ClustalW (http://www.ebi.ac.uk/clustalw/). Phylogenetic analysis was carried out with the ClustalX programme (http://www.ebi.ac.uk/clustalw/), and the secondary structure was determined using a TopRed 2 programme (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html, Claros and von Heijne 1994).

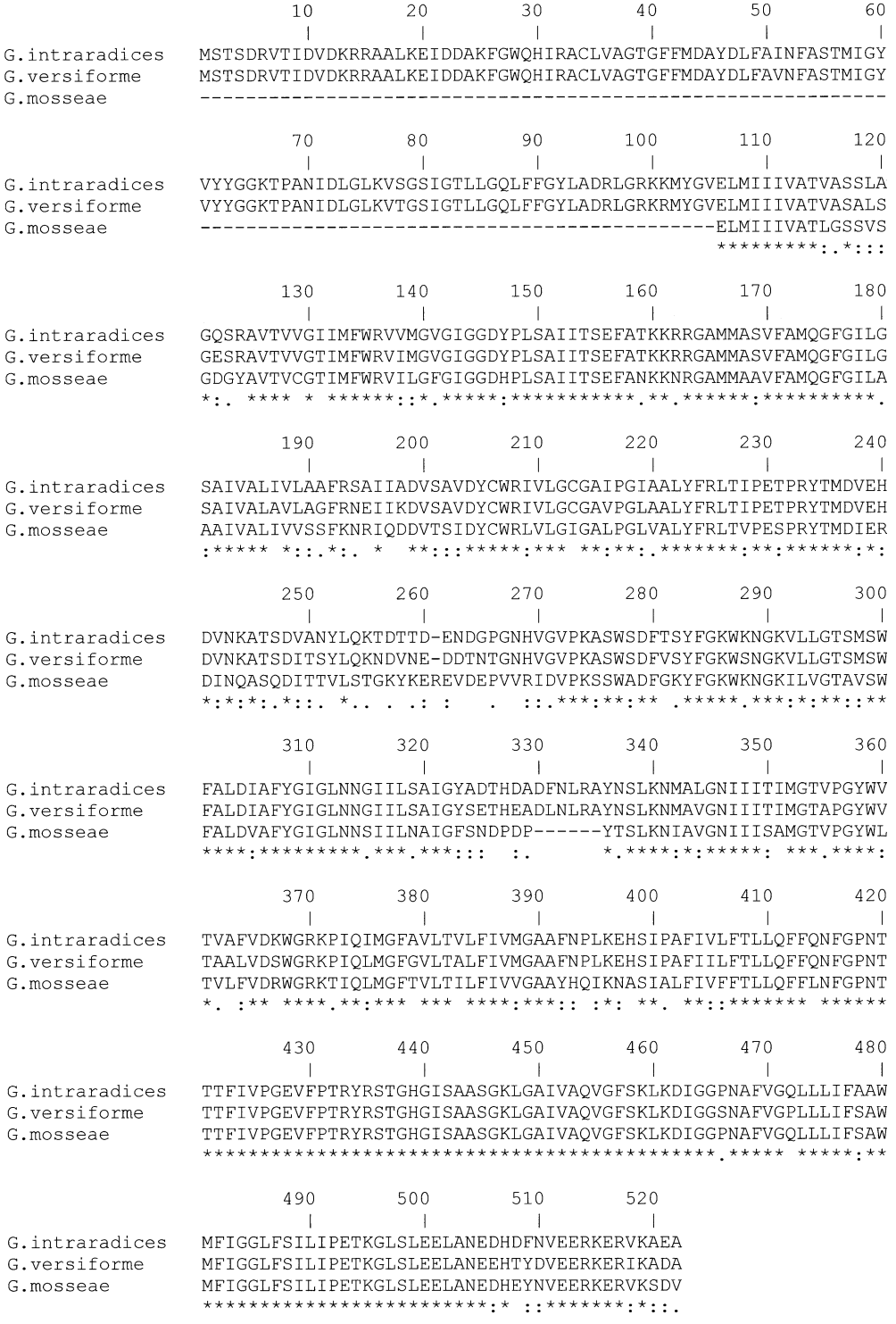

Fig. 1 Clustal W alignment of the protein sequence deduced from *GmosPT* gene with P transporters from *G. versiforme* (Harrison and van Buuren 1995) and *G. intraradices* (Maldonado-Mendoza et al. 2001). Identity (*), strongly similar (:), and weakly similar (.).

Ribonucleic acid was extracted from 50 sporocarps, 50 germinated sporocarps, 10–50 mg of mycorrhizal roots and 5–10 mg of extraradical hyphae using the SV Total RNA Isolation System kit (Promega). Total RNA was precipitated in 6 M LiCl and re-suspended in 20 µl of DEPC water. To exclude DNA contaminations, all the RNA samples were checked by RT-PCR analyses, conducted with the 18S rRNA universal primers NS1–NS2 (White et al. 1990), in the presence or absence of reverse transcriptase. RT-PCR reactions were performed using the One-step RT-PCR kit (Qiagen) in a final volume of 25 µl containing 5 µl of 5× buffer, 400 µl dNTPs, 0.6 µl of each primer, 1 µl of One-step RT-PCR enzyme mix and 0.1–2 µl of total RNA. Samples were incubated for 30 min at 50°C followed by a 15-min incubation at 95°C. Amplification reactions (92°C for 30 s, 60°C for 30 s and 72°C for 45 s) were run for a maximum of 32 cycles. PCR reactions were allowed to proceed for a different number of cycles to determine the exponential phase of amplification.

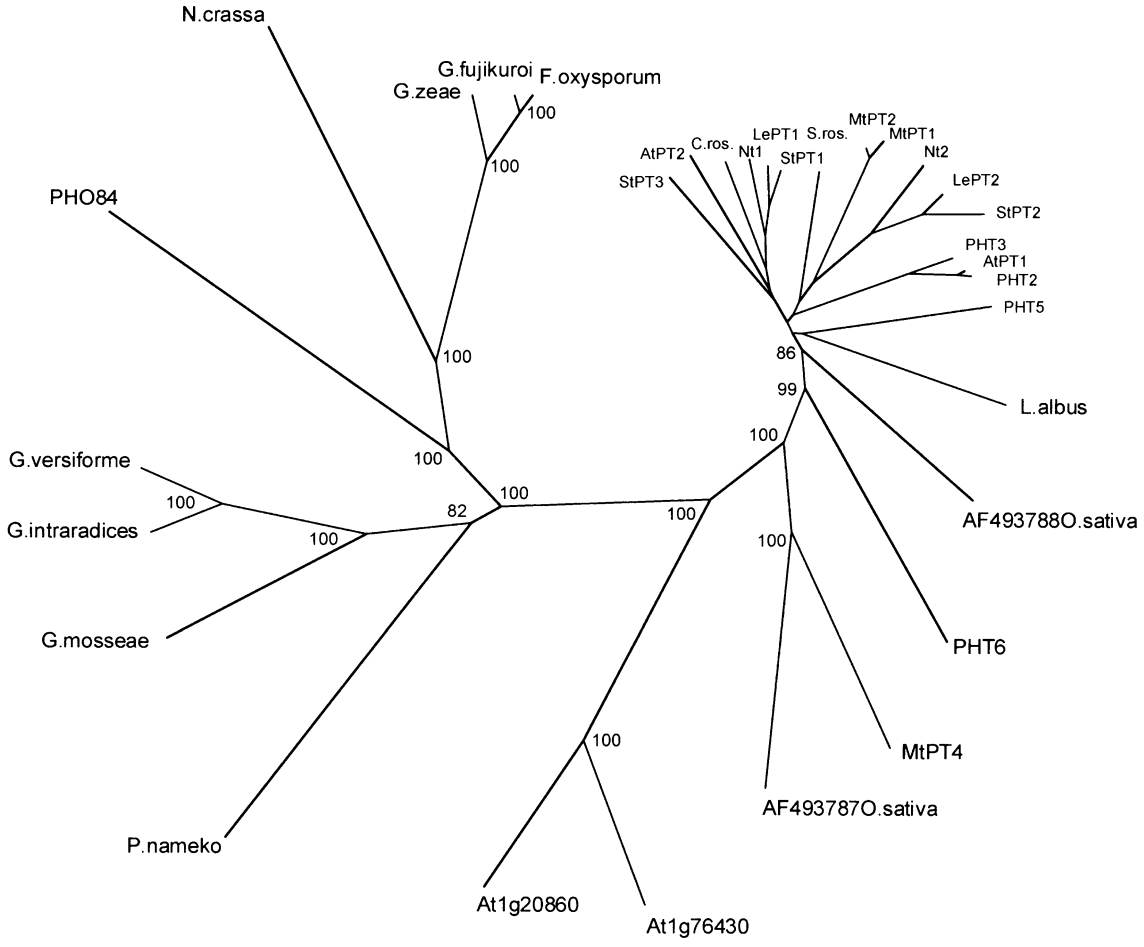

Fig. 2 Neighbor-joining analysis of protein sequences coding for fungi and plants P transporters. *Arabidopsis thaliana*: *AtPT1*, AAB17265; *AtPT2*, NP_181428; NP_173510; and AAP37683; *PHT2*, NP_199151; *PHT3*, NP_199150; *PHT5*, NP_180842; and *PHT6*, BAA34390; *Catharanthus roseus*, BAA20522; *Fusarium oxysporum*, BAC65212; *Gibberella fujikuroi*, BAC65214; *G. intraradices*: *GiPT*, AAL37552; *G. versiforme*, *GvPT*, S67491; *Gibberella zeae*, XP_388070; *Lupinus albus*, AAK38196; *Lycopersicon esculentum*: *LePT1*, AAB82146 and *LePT2*, AAB82147; *M. truncatula*: *MtPT1*, AAB81346; *MtPT2*, AAB81347; and *MtPT4*, AAM76744; *Neurospora crassa*, AAA74899; *Nicotiana tabacum*: *Nt1*, BAB21545 and *Nt2*, BAB21563; *Oryza sativa*: *AF493787*; AAM14592; *Pht1*, *AF493788* and *Pht2*, AAM14593; *Pholiota nameko*, BAB43910; *Saccharomyces cerevisiae*: *PHO84*, P25297; *Sesbania rostrata*, CAC28219; and *Solanum tuberosum*: *StPT1*, CAA67395; *StPT2*, CAA67396; and *StPT3*, CAC87043

Reverse transcriptase–PCR products were quantified on agarose gel by comparison with the Low DNA Mass Ladder (Invitrogen, Carlsbad, CA). RT-PCR experiments were conducted in duplicate on two independent samples. Ribosomal primers 5.21 (5′ CCTTTTGAGCTCGGTCTC GTG 3′) and NDL22 (5′ TGGTCCGTGTTTCAAGACG 3′) (van Tuinen et al. 1998) were used to calibrate RNA amounts in each sample, and the specific oligonucleotides PT2A (5′ GAATCGTATTCAAGATGATGTTA 3′) and PT2B (5′ CGAATGATCCCGATCCTTATACT 3′) were employed for the evaluation of *GmosPT* mRNA levels. PT2A–PT2B primer pair was tested on DNA/RNA samples from *G. mosseae* and *C. sativus* using standard PCR/RT-PCR conditions with an annealing temperature of 60°C.

Results

Several PT amino acid sequences from plants and fungi were aligned with ClustalW software to design degenerate primers PT1 and PT2. These primers were employed in PCR amplifications on *G. mosseae* genomic DNA. An 800-bp fragment was obtained showing the highest similarity with *GvPT*, the gene coding for a high-affinity PT in *G. versiforme* (accession number S67491; Harrison and van Buuren 1995). The isolated fragment, named *GmosPT*, matched to the central portion of *GvPT* gene, lacking 3′ and 5′ ends. Therefore, 3′ and 5′ RACE experiments on SMART cDNA from *G. mosseae* external mycelium were performed to obtain the full-length sequence. A 1,237-bp cDNA, complete at the 3′ end, was identified, corresponding to about 80% of the expected protein sequence (411 over 521 amino acids) and sharing the highest similarity (*E* value=$4e^{-171}$, 73% identity, 86% similarity) with *G. intraradices GiPT* (accession number AAL37552; Maldonado-Mendoza et al. 2001).

The alignment of *GmosPT*-deduced amino acid sequence with the protein sequences of *GiPT* and *GvPT* revealed a strong similarity (Fig. 1). The phylogenetic analysis, as shown in Fig. 2, showed that GmosPT groups close the other two PTs isolated from AM fungi within the fungal transporters cluster and separated from the plant PT sequences.

The prediction of the secondary structure (data not shown) resulted in a nine-membrane-spanning domains protein, very similar to the typical structure of P transporters from plants and fungi which consists of 12 (6+6) membrane-spanning domains. The other three transmembrane domains at the N terminal probably lie in the 5′-lacking end of *GmosPT* cDNA.

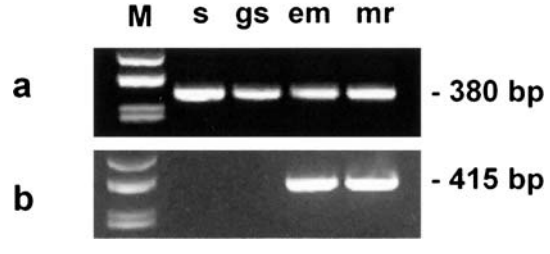

Fig. 3 RT-PCR products obtained with fungal 28S rDNA primers (**a**) or *GmosPT*-specific primers (**b**) from the following samples: *s* indicates quiescent sporocarps; *gs*, germinated sporocarps; *em*, extraradical mycelium; and *mr*, mycorrhizal roots. *M* indicates pUC18 + *Hae*III

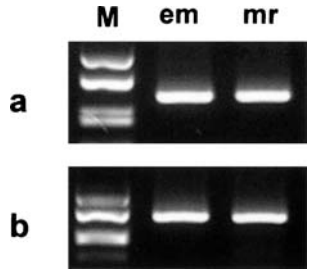

Fig. 4 Semi-quantitative RT-PCR analysis using fungal 28S rDNA primers (**a**) or *GmosPT*-specific primers (**b**) on extraradical mycelium (*em*) and mycorrhizal roots with extraradical hyphae removed (*mr*) treated with 35 μM Na_2HPO_4. *M* indicates pUC18 + *Hae*III

GmosPT expression was analysed by RT-PCR. Sequence-specific primers (PT2A/PT2B) were initially tested on DNA and RNA extracted from the host plant *C. sativus*. The negative results obtained from these reactions (data not shown) allowed us to exclude any cross-hybridisation with the plant genome. Total RNA extracted from quiescent sporocarps, sporocarps germinated in water, extraradical hyphae and mycorrhizal roots was then used to identify the stages of fungal life cycle showing *GmosPT* expression. *C. sativus* roots, 2 months after inoculation, showed a good percentage of colonization which, in different experiments, varied from 50 to 70% of root segments. The presence of fungal RNA in the different samples was verified by using *G. mosseae*-specific 28S rRNA primers (5.21/NDL22, van Tuinen et al. 1998) that did not recognise any sequence in the plant genome (data not shown). These reactions gave a PCR product of 380 bp in all samples (Fig. 3a). Using *GmosPT*-specific primers, an amplified fragment of the expected size (415 bp) was obtained from both external hyphae and mycorrhizal roots but not from quiescent and germinated sporocarps (Fig. 3b). No amplified product was obtained from quiescent or germinating sporocarps treated with 35 μM Pi (data not shown).

Semi-quantitative RT-PCR analyses were performed on samples corresponding to external hyphae and mycorrhizal roots from which extraradical hyphae were removed. The amount of mRNA obtained from different samples was first calibrated by PCR amplification with fungus-specific

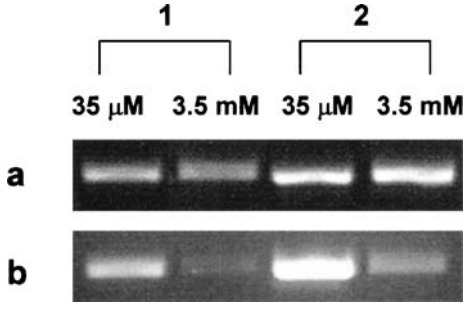

Fig. 5 Semi-quantitative RT-PCR analysis using fungal 28S rDNA primers (**a**) or *GmosPT*-specific primers (**b**) on two independent groups of samples (*1* and *2*) obtained from extraradical mycelium treated with 35 μM or 3.5 mM Na_2HPO_4

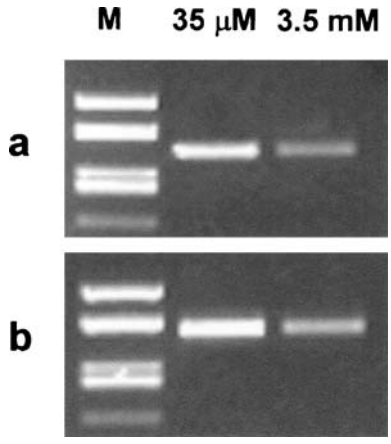

Fig. 6 Semi-quantitative RT-PCR analysis using fungal 28S rDNA primers (**a**) or *GmosPT*-specific primers (**b**) on intraradical mycelium samples obtained from plants treated with 35 μM or 3.5 mM Na_2HPO_4. *M* indicates pUC18 + *Hae*III

28S rRNA primers (Fig. 4a). Calibrated amounts of each mRNA sample were then amplified with *GmosPT*-specific oligonucleotides. A signal of similar intensity was obtained from intraradical and extraradical hyphae (Fig. 4b), suggesting that the gene was expressed at comparable levels.

To verify whether *GmosPT* expression responds to different Pi concentrations in the surrounding medium, *GmosPT* transcript level was investigated in extraradical mycelium, and intraradical fungal structures from plants kept for 24 h in a medium containing micro- or millimolar concentrations of Na_2HPO_4. After calibration on the bases of 28S rRNA transcript (Fig. 5a), RNAs from external hyphae exposed to millimolar concentration of Pi showed a down-regulation of *GmosPT* transcript in comparison to samples from external hyphae treated with micromolar concentration (Fig. 5b). In contrast, we did not observe significant changes in *GmosPT* mRNA abundance in intraradical hyphae from both treatments (Fig. 6).

Discussion

According to recent researches on the contribution of AM fungi to P uptake by plants, the emerging idea is that the regulation of P uptake and transfer in AMs, a crucial aspect of this symbiosis, is pretty more complex than previously expected. Common parameters, such as plant growth responses and P content, does not seem to be directly correlated to the extent of contribution in Pi uptake exerted by the fungus (Burleigh et al. 2002; Smith et al. 2004). The study of PT and their functioning thus represents a key point to fully understand this process.

In this work we describe the isolation of the partial sequence of a PT gene from the AM fungus *G. mosseae* and its characterisation in terms of transcript profiles. Only two other genes coding for Pi transporters from AM fungi, *GvPT* and *GiPT*, have been previously identified (Harrison and Van Buuren 1995; Maldonado-Mendoza et al. 2001). *GmosPT* shares with them a very high sequence similarity (73% identity and 86% similarity with *GiPT*). The phylogenetic analysis showed that *GmosPT*, *GiPT* and *GvPT* group close together. According to the last classification, based on 18S ribosomal sequences (Schüßler et al. 2001), the genus *Glomus* is non-monophyletic: *G. mosseae* and *G. intraradices* belong to Glomerales, while *G. versiforme* belongs to Diversisporales. Additional PT sequences from other AM fungi would be needed to confirm these phylogenetic relationships.

The predicted secondary structure analogy of GmosPT with plant and fungi PT structures supports the idea that *GmosPT* codes for a protein belonging to the Pi/H^+ co-transporters family (Saier et al. 1999). These proteins exploit the proton gradient generated by membrane H^+–ATPases. Interestingly, in the last few years, two sequences coding for membrane H^+–ATPases were isolated from *G. mosseae* (Ferrol et al. 2000; Requena et al. 2003). These genes might be involved in the generation of the electrochemical gradient required for Pi uptake.

GmosPT expression was observed in extra- and intraradical mycelium but not in germinated and dormant sporocarps. This result suggests therefore that *GmosPT* plays a role in Pi transfer during its interaction with the plant. The expression of *GmosPT* in extraradical mycelium is located in the rhizosphere and is consistent with an activity of Pi uptake from the soil, as described by Harrison and van Buuren (1995) and Maldonado-Mendoza et al. (2001), for *GvPT* and *GiPT*, respectively. Interestingly, we also observed a relatively abundant expression level in intraradical fungal structures inside the root. Although the signal was weak, *GvPT* transcript was also detected by Harrison and van Buuren (1995) in *M. truncatula* roots colonized by *G. versiforme*. The presence of *PT* transcripts in intraradical mycelium can suggest that inside the root, the efflux of phosphate is occurring in competition with its uptake and that the fungus exerts a control over the amount of phosphate delivered to the plant.

It would be interesting to verify if AM PT gene expression and Pi uptake are also regulated by photosynthetic activity partners. Cucumbers, the host plants used in our experiments, are well known as a plant showing rapid growth, and an elevated synthesis of sieve tube exudates proteins (van Bel et al. 2002; Walz et al. 2004). We can hypothesize that these features require a higher P availability. Irrespective of that, AM fungi PT transporters might play a role in balancing and controlling the amounts of Pi released to the plant. This would be in agreement with the emerging idea that Pi fluxes between the two symbionts rely on complex mechanisms. According to these hypotheses it has recently been demonstrated that in axenic cultures of transformed mycorrhizal roots, the uptake of Pi by the fungus and its transfer to the host is stimulated by the transfer of carbon from plant to fungus across the mycorrhizal interface (Bücking and Shachar-Hill 2005).

A typical feature of fungal and plant PT coding genes is represented by their responsiveness to external P concentration; in particular, high-affinity PT (apparent K_m up to 30–40 μM) are induced at a transcriptional level by low

amounts of Pi comparable to micromolar concentration found in the soil. *GiPT* expression, analysed in the hairy roots in vitro system, showed the same regulation pattern (Maldonado-Mendoza et al. 2001). Also, in our experiments performed on external mycelium of *G. mosseae* collected from more natural conditions (mycorrhizal plants grown in pot cultures), *GmosPT* expression was induced by micromolar Pi concentration. These data suggest that *GmosPT* also belongs to the high-affinity PT family. The expression level in intraradical hyphae seemed not to be affected by external Pi concentration. Expression of the PT gene in *G. intraradices* has been shown to depend not only on the surrounding medium but also on plant P content (Maldonado-Mendoza et al. 2001). The differential response observed in *G. mosseae* intraradical mycelium might reflect, as discussed above, a stronger influence from plant P status.

In conclusion, the identification of a *G. mosseae* PT gene, which phylogenetically groups with the two other genes previously identified in AM fungi, suggests that PT are highly conserved in the Glomeromycota. However, the discovery that this gene is consistently expressed inside the root provides a new scenario for the plant–fungus nutrient exchanges, suggesting that, at least when the plant is actively growing, the partners do not simply exchange P vs C, but can compete for the same nutrients.

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