The expression of GintPT, the phosphate transporter of Rhizophagus irregularis, depends on the symbiotic status and phosphate availability

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Valentina Fiorilli, Luisa Lanfranco and Paola Bonfante

Abstract
The development of mutualistic interactions with arbuscular mycorrhizal (AM) fungi is one of the most important adaptation of terrestrial plants to face mineral nutrition requirements. As an essential plant nutrient, phosphorus uptake is acknowledged as a major benefit of the AM symbiosis, but the molecular mechanisms of its transport as inorganic phosphate (Pi) from the soil to root cells via AM fungi remain poorly known. Here we monitored the expression profile of the high-affinity phosphate transporter (PT) gene (GintPT) of Rhizophagus irregularis (DAOM 197198) in fungal structures (spores, extraradical mycelium and arbuscules), under different Pi availability, and in respect to plant connection. GintPT resulted constitutively expressed along the major steps of the fungal life cycle and the connection with the host plant was crucial to warrant GintPT high expression levels in the extraradical mycelium. The influence of Pi availability on gene expression of the fungal GintPT and the Medicago truncatula symbiosis-specific Pi transporter (MtPT4) was examined by qRT-PCR assay on microdissected arbusculated cells. The expression profiles of both genes revealed that these transporters are sensitive to changing Pi conditions: we observed that MtPT4 mRNA abundance is higher at 320 than at 32 µM suggesting that the flow towards the plant requires high concentrations. Taken on the whole, the findings highlight novel traits for the functioning of the GintPT gene and offer a molecular scenario to the models describing nutrient transfers as a cooperation between the mycorrhizal partners.

Abbreviations
AM
Arbuscular mycorrhizal
ARB
Arbuscule-containing cells
Ct
Threshold cycle
ERM
Extraradical mycelium
IRM
Intraradical mycelium
LMD
Laser microdissection
P
Phosphorus
Pi
Inorganic orthophosphate
PT

Phosphate transporter
ROCs
Root organ cultures

Introduction

As an essential mineral nutrient involved in plant growth and development processes, phosphorus (P) is an element whose availability has significant consequences on both plant ecology and crop yield. P is a nonrenewable resource that is present in the soil in the form of inorganic orthophosphate (Pi) which is readily sequestered by cations (e.g., Ca^{2+}, Al^{3+} and Fe^{3+}), mostly under acid conditions (Tinker and Nye *2000*): as a consequence, the mean free Pi in the soil does not usually exceed a 10 µM concentration (Bieleski *1973*). In addition, due to the scarce mobility of Pi, plant uptake, via root epidermal cells and root hairs, leads to a rapid exhaustion of Pi availability near the root system, and creates a depleted zone (Smith et al. *2011*).
In order to increase Pi uptake, plants have evolved different strategies, such as the enhanced growth of lateral roots and root hairs, and/or the solubilization of soil Pi by means of organic acid and phosphatase secretion: all these processes are probably orchestrated by a systemic signalling that is triggered during Pi starvation (Doener *2008*; Lambers et al. *2011*; Péret et al. *2011*) and involves specific gene expression regulators, as has been demonstrated in Arabidopsis (Pérez Torres et al. *2009*). Another widespread and evolutionary ancient strategy is the establishment of arbuscular mycorrhizal (AM) symbiosis which involves the majority of land plants and fungi belonging to the Glomeromycota phylum (Parniske *2008*; Bonfante and Genre *2010*). AM fungi ensure that their host plants have an efficient supply of mineral nutrients, particularly P and nitrogen (Allen and Shachar-Hill *2009*; Tian et al. *2010*; Smith and Smith *2011*). Thanks to the hyphal network they develop in the soil (up to 100 fold more extensive than root hairs), AM fungi acquire nutrients not only for their own needs, but also for delivering them to the root cortical cells via a fast but not fully understood mechanism (Smith et al. *2011*; Fellbaum et al. *2012*). In return, up to 20 % of the photosynthesis products of the host can be allocated to the fungus, which is unable to complete its life cycle without the host (Smith and Read *2008*).
Mycorrhizal plants preferentially take up Pi via fungal hyphae (Smith et al. *2003*, *2004*): unlike the so-called “direct pathway”, this indirect pathway first requires Pi uptake from the soil through high-affinity fungal Pi transporters (PT) in the extraradical mycelium (ERM). Current data suggest that Pi is stored in vacuoles as polyphosphate (poly-Pi) and translocated to the intraradical hyphae (Callow et al. *1978*; Solaiman et al. *1999*; Ezawa et al. *2003*). After hydrolysis, Pi is exported from the AM fungus to the periarbuscular space, where it is taken up by plant cortical cells (Javot et al. *2007*). Poly-Pi is the largest P storage and a mediator of long distance P translocation in AM fungi (Hijikata et al. *2010*). It remains uncertain whether P is translocated as poly-Pi without turning over, or whether it is translocated as Pi through a dynamic regulation of poly-Pi synthesis/hydrolysis (Ezawa et al. *2001*). Regardless of this, it is known that up to 100 % of the plant P can come from the mycorrhizal pathway, even in the absence of a plant growth effect (Pearson and Jakobsen *1993*; Ravnskov and Jakobsen *1995*).
Recent data have demonstrated that the establishment of AM symbiosis should not be considered as a strategy to increase Pi uptake exclusively during Pi starvation: AM activity in fact is not always suppressed in high Pi fertilized soils (Smith et al. *2011*). For instance, field experiments have shown that some AM fungi, such as Rhizophagus irregularis (Syn. Glomus intraradices; Krüger et al. *2012*), are deemed more tolerant to a high Pi concentration, while other species are absent under this nutrient condition (Verbruggen and Kiers *2010*). High Pi availability enhances root growth and

reduces the colonized versus non-colonized root length ratio (Smith et al. *1992*; Marschner *1995*; Smith et al. *2011*). In addition, a very high Pi concentration can markedly modify root colonization, and reduce Pi uptake via the fungal pathway, arbuscule development, and the AM fungal biomass in both the roots and soil (Nagy et al. *2009*; Breuillin et al. *2010*; Smith et al. *2011*). High Pi conditions have also been shown to have an impact at the very early stages of the interaction with a dramatic decrease in hyphopodia formation (Balzergue et al. *2011*): this effect is probably due to a negative regulation of stimulatory compounds, such as strigolactones (Laparre et al. *2011*). These data raise questions on the dependency of the fungal phosphate transporter (PT) expression on the Pi concentration.

To date, high-affinity PT encoding genes that share homology with the yeast high-affinity transporter PHO84 (Bun-Ya et al. *1991*) have been identified from three AM fungi: Glomus versiforme (GvPT), Rhizophagus irregularis (GintPT), and Glomus mosseae (GmPT) (Harrison and van Buuren *1995*; Maldonado-Mendoza et al. *2001*; Benedetto et al. *2005*). These genes are expressed in the ERM, indicating a role in Pi acquisition from the soil, as well as in the intraradical mycelium, IRM (Benedetto et al. *2005*; Balestrini et al. *2007*; Tisserant et al. *2012*). Moreover, when ERM is considered, GintPT and GmPT expression responds to external Pi concentrations (Maldonado-Mendoza et al. *2001*; Benedetto et al. *2005*) while in the IRM, GmPT does not seem to be influenced by the external Pi availability (Benedetto et al. *2005*).

In spite of the huge amount of data that deal with the transfer of the fungal Pi towards the plant, a molecular analysis which examines the expression dynamics of the fungal PT along the diverse stages of the symbiosis and in the context of the nutritional exchanges with the plant is not available. To reach this goal, we used root organ cultures (ROCs) and bicompartimented Petri dishes (Hammer et al. *2011*; Kiers et al. *2011*), since this system allows both plant and fungal compartments to be manipulated. We therefore examined the expression profile of the high-affinity PT gene (GintPT) of the AM fungus R. irregularis in the fungal structures (spores, ERM and arbuscules) and under different Pi availability as well as the impact of the plant connection on GintPT expression.

As a further step, since the exchange of nutrients is considered the driving force for the evolutionary stability of AM symbiosis (Parniske *2008*; Humphreys et al. *2010*; Kiers et al. *2011*), we wondered whether the dynamics of the fungal GintPT expression could be related to that of other plant and fungal genes involved in nutrients exchanges. We therefore investigated the MtPT4 gene, encoding a phosphate transporter of Medicago truncatula, which is expressed in arbuscule-containing cells and is responsible for the uptake of Pi released by the fungus in the periarbuscular space (Harrison et al. *2002*; Javot et al. *2007*). We also investigated the MST2 gene, the fungal hexose transporter which is possibly involved in C uptake (Helber et al. *2011*).

Our results conclusively demonstrate that, under controlled conditions, the AM fungus constitutively expresses GintPT, and that a connection with the host plant is required to support high expression levels in the extraradical hyphae. Lastly, thanks to the setting up of a quantitative RT-PCR assay applied to laser-microdissected cells, we have shown that the fungal and plant PTs in arbusculated cells are sensitive to changes in Pi concentrations. These data reveal how PTs are crucial markers for detecting the nutritional interactions created by the mycorrhizal partners under changing Pi availability.

Materials and methods

Biological material

Rhizophagus irregularis (DAOM 197198), recently reassigned from Glomus intraradices Schenck and Smith (Krüger et al. *2012*), was produced in monoaxenic cultures maintained on Agrobacterium rhizogenes-transformed chicory roots (Bécard and Fortin *1988*) in two-compartment Petri plates, as described in Pérez-Tienda et al. (*2011*). Plates were incubated in the dark at 24 °C until the fungal compartment, which contained a solid M medium without sucrose (M–C medium), was profusely colonized by the fungus (approximately 6 weeks). In order to collect the spores, the fungal compartment of the solid monoaxenic cultures was blended and sieved. About 6,000 spores were recovered with a pipette under a binocular microscope, frozen in liquid N_2 and stored at −80 °C.

To obtain the ERM, the fungal compartment content was removed and replaced by a liquid M–C medium. The newly formed ERM was collected from the liquid medium after about 2 weeks. In all the experiments, the mycelia were collected with forceps, rinsed with sterilized water, dried with sterilized filter paper, immediately frozen in liquid N and stored at −80 °C.

Seeds of Medicago truncatula Gaertn cv Jemalong (originally provided by Massimo Delledonne, University of Verona, Italy) were scarified using P180-200 sandpaper, sterilized with 5 % commercial bleach for 3 min and rinsed three times for 10 min with sterile distilled water. Germination was induced under sterile conditions in 0.6 % agar/water, and the seeds were incubated for 5 days in the dark and then exposed to light for 4 days.

Mycorrhizal roots were obtained by means of the sandwich method, inoculating M. truncatula seedlings with R. irregularis ERM in between two sterile nitrocellulose membranes, as described in Guether et al. (*2009*). Plants were grown in a growth chamber under a 14 h light (24 °C)/10 h dark (20 °C) regime for 2 months.

ERM detachment from mycorrhizal roots and NBT staining

To evaluate the expression of the fungal PT when detached from the root, 6-week-old bi-compartmental plates with fully colonized fungal compartments (solid medium) were selected. The root compartment was eliminated to break the connection between the ERM and the roots. After 2 days from the detachment, ERM was harvested and the solid medium was dissolved with a 10 mM pH 6.0 citrate buffer (0.018 ml mix of 0.1 M citric acid and 0.082 ml of 0.1 M sodium citrate to reach a final volume of 50 ml with sterile distilled water). An aliquot of a liquid medium containing 35 µM Pi was added to a subset of the detached fungal compartments, immediately after the root detachment. For each treatment three biological replicates were considered.

The vitality of the detached ERM was assessed by means of succinate dehydrogenase activity staining. Freshly harvested detached and undetached ERM were placed in a Petri dish containing 5 ml of an ice cold 10 % sorbitol solution and 5 ml of the nitro blue tetrazolium (NBT) chloride working solution (1 mg/ml of NBT, 0.5 mmol Tris/HCl pH 7 and 4.5 µmol $MgCl_2$, 2.5 mmol sodium succinate). The reaction was then incubated for 1 h at 37 °C. The mycelium was rinsed with distilled water and mounted onto a slide; the formazan precipitates were assessed under a light microscope (Primo Star Zeiss with a Leica DFC425 digital camera attached).

ERM treatment with different Pi concentrations

To evaluate the expression of the fungal PT when the ERM was treated with diverse Pi concentrations, the liquid medium, in which the ERM had developed for about 2 weeks, was removed and the hyphae were rinsed three times with sterile water. A fresh liquid M medium (18 ml), containing low (35 µM KH_2PO_4), intermediate (320 µM KH_2PO_4) or high (3.2 mM KH_2PO_4) Pi concentrations, was then added. The ERM from the three conditions was harvested 24 h later to

perform RNA extractions, while the Pi content was measured before the addition of the new medium and at the time of ERM harvesting in the fungal compartment containing 35 µM or 320 µM Pi using a Malachite Green Phosphate Detection Kit (R&D Systems, Minneapolis, MN, USA). A seven-point standard curve was prepared using twofold serial dilutions of the 100 µM phosphate stock in an Assay Buffer (Tris-HCl 100 mM pH 8.0). The optical density of each well was determined using a microplate reader spectrophotometer (TECAN Infinite M200, Grödig, Austria) set at 620 nm. Three biological replicates were considered for both gene expression analysis and Pi content measurement.

Mycorrhizal root treatment with different Pi concentrations

A set of M. truncatula mycorrhizal plants, obtained using the sandwich method, was watered with a Long-Ashton solution containing a low Pi concentration (32 µM $Na_2HPO_4 \cdot 12H_2O$) (Hewitt *1966*) and another set containing a higher Pi concentration (320 µM $Na_2HPO_4 \cdot 12H_2O$). The whole root system was collected 2 months post-inoculation; a portion was immediately frozen in liquid nitrogen, stored at −80 °C and used for RNA extractions, another portion was destined for microdissection analysis and a third one was used to assess the mycorrhization level according to Trouvelot et al. (*1986*).

Nucleic acid extraction and cDNA synthesis

Total genomic DNA was extracted from R. irregularis ERM and M. truncatula shoot using the DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. Each primer pair was first tested on plant or fungal genomic DNA as a positive control and to exclude cross-hybridization. Total RNA was extracted from spores, ERM, and mycorrhizal M. truncatula roots using the Plant RNeasy Kit (Qiagen), according to the manufacturer's instructions. Samples were treated with TURBO™ DNase (Ambion) according to the manufacturer's instructions. The RNA samples were routinely checked for DNA contamination by means of RT-PCR (One-RT-PCR, Qiagen) analysis, using GintEF1α and MtTef primers (Table 1). For single-strand cDNA synthesis about 700 ng of total RNA was denatured at 65 °C for 5 min and then reverse-transcribed at 25 °C for 10 min, 42 °C for 50 min and 70° for 15 min in a final volume of 20 µl containing 10 µM random primers, 0.5 mM dNTPs, 4 µl 5× buffer, 2 µl 0.1 M DTT and 1 µl Super-ScriptII (Invitrogen).

Table 1
List of primers used in this study

Primer ID	Primer sequences [5'–3']
GintEFαf	GCTATTTTGATCATTGCCGCC
GintEFαr	TCATTAAAACGTTCTTCCGACC
GintPTf	AACACGATGTCAACAAAGCAAC
GintPTr	AAGACCGATTCCATAAAAAGCA
MST2f	GGCAGGATATTTGTCTGATAG
MST2r	GCAATAACTCTTCCCGTATAC
MtTEFf	AAGCTAGGAGGTATTGACAAG
MtTEFr	ACTGTGCAGTAGTACTTGGTG
MtPT4f	TCGCGCGCCATGTTTGTTGT

Primer ID	Primer sequences [5'–3']
MtPT4r	CGCAAGAAGAATGTTAGCCC

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was performed using an iCycler apparatus (Bio-Rad). Each PCR reaction was carried out in a total volume of 20 µl containing 1 µl diluted cDNA (1:2), 10 µl 2× SYBR Green Reaction Mix and 3 µl of each primer (3 µM). The following PCR programme was used: 95 °C for 90 s, 50 cycles of 95 °C for 15 s, 60 °C for 30 s. A melting curve (80 steps with a heating rate of 0.5 °C per 10 s and a continuous fluorescence measurement) was recorded at the end of each run to exclude that the primers had generated non-specific PCR products (Ririe et al. *1997*). All reactions were performed on at least three biological and three technical replicates. Baseline range and threshold cycle (Ct) values were automatically calculated using iCycler software. Transcript levels were normalized to the Ct value of GintEF1α (Gonzàlez-Guerrero et al. *2010*) for the fungal gene and MtTef (Hohnjec et al. *2005*) for plant gene. Only Ct values leading to a Ct mean with a standard deviation below 0.5 were considered. Statistical tests were carried out through one-way analysis of variance (one-way ANOVA) and Tukey's post hoc test, using a probability level of $P < 0.05$.

Laser microdissection (LMD)

Medicago truncatula mycorrhizal roots, obtained using the sandwich method, were dissected, fixed and embedded in paraffin according to the method described in Pérez-Tienda et al. (*2011*). A Leica AS LMD system (Leica Microsystem) was used to collect arbuscule-colonized cortical cells from paraffin root sections, as described by Balestrini et al. (*2007*). 5,000 arbuscule-containing cells (ARB) from M. truncatula roots (2,500 cells for each of the two biological replicates) were microdissected using LMD. RNA was extracted following the Pico Pure kit (Arcturus Engineering, Mountain View, CA, USA) protocol. A DNAse treatment was performed using an RNA-free DNase Set (Qiagen) in a Pico Pure column, according to the manufacturer's instructions. RNA was then quantified using a NanoDrop 1000 spectrophotometer. DNA contamination in RNA samples was evaluated using GintEF1α and MtTef primers (Table 1) by means of RT-PCR assays carried out using One-Step RT-PCR kit (Qiagen).

Quantitative RT-PCR reactions were performed with iCycler apparatus (Bio-Rad) in a total volume of 25 µl, containing 2 µl RNA, 12.5 µl 2× SYBR Green RT-PCR Reaction Mix, 0.5 µl of each primer (10 µM) and 0.5 µl of iScript Reverse Transcriptase of the One-Step RT-PCR kit (Bio-Rad). The following PCR programme was used: 50 °C for 10 min, 95 °C for 5 min, 50 cycles of 95 °C for 10 s, 60 °C for 30 s. Amplification reactions were performed using specific primers for the GintEF1α and MtTef housekeeping gene, and for the genes of interest, GintPT (accession no. AY037894), MtPT4 (accession no. AY116211) (Zocco et al. *2011*), and MST2 (accession no. HM143864) (Helber et al. *2011*). A melting curve was obtained as described above. All the reactions were performed on two biological and two technical replicates. Baseline range and Ct values were automatically calculated using the iCycler software. Transcript levels were normalized to the Ct value of GintEF1α for the fungal genes and MtTef for plant gene.

The setting up of a real-time PCR-based quantitative analysis on arbusculated cells RNA allowed us to calculate the percentage of the fungal and plant transcripts in the arbuscule-containing cells. Assuming that the expression levels of the fungal and plant Tef genes were similar, we obtained the Ct values for both the fungal and plant Tef in each of the two biological replicates of

microdissected cells. We calculated the difference ($Ct_f - Ct_p$) between the fungal (Ct_f) and plant (Ct_p) housekeeping Ct, and the relative amount of plant transcripts was assumed to be $2 \wedge Ct_f - Ct_p$ We then converted this value into a percentage considering the total amount of transcripts present in the cell to be 100.

Phosphate quantification in mycorrhizal roots

Phosphate (Pi) was extracted from tissue samples powdered under liquid nitrogen in a mortar. Aliquots of about 3 mg of dry material were suspended in 1 ml of twice-distilled water containing 20 mg of insoluble PVPP to remove phenolic compounds. After shaking for 1 h at 4 °C, samples were boiled for 15 min, and centrifuged for 5 min and then for 10 min, at 16,000g and 4 °C (centrifuge 5402, Eppendorf, Engelsdorf, Germany). The clear supernatant was used for Pi analysis by anion-exchange chromatography. In all samples anions were separated on a IonPac column (AS9-SC, 250 × 4 mm; CDM Dionex) eluted with a mixture of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ at a flow rate of 1.1 ml/min. Pi was detected by a conductivity detector module (CDM, Dionex). Six biological replicates were considered for each biological condition. Statistical tests were carried out through one-way analysis of variance (one-way ANOVA) and Tukey's post hoc test, using a probability level of $P < 0.05$.

Results

GintPT expression over main fungal developmental stages

The expression profile of the high-affinity Pi transporter (GintPT) of R. irregularis was investigated in semi-quantitative RT-PCR assays on spores and ERM obtained in the in vitro monoaxenic cultures and on arbusculated cells collected through the laser microdissection technology from M. truncatula mycorrhizal roots. The amount of mRNAs obtained from the different samples was first calibrated with fungus specific Tef primers (Fig. 1a). Normalized RNA samples were then amplified with GintPT specific primers. A signal was obtained in all the three analysed samples (Fig. 1b), indicating that GintPT is constitutively expressed over the main fungal life cycle steps, including arbuscules.

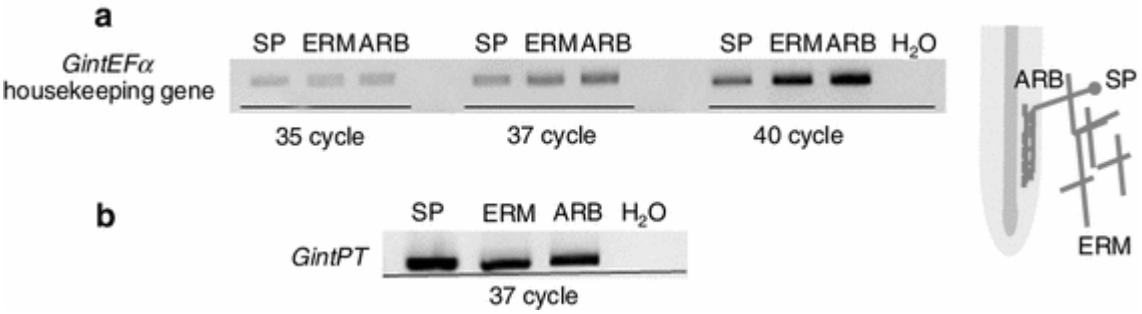

Fig. 1
Gel electrophoresis of RT-PCR products obtained from different R. irregularis fungal structures using GintEFα (a) and GintPT (b) primers. SP spores; ERM extraradical mycelium; ARB arbusculated cortical cells collected by laser microdissection; H_2O no DNA

As a further step, we analysed GintPT mRNA abundance in the ERM grown in the hyphal compartment of monoaxenic cultures in association with roots or after a physical detachment from the host. After the root compartment was removed, the fungal compartment was left for 2 days to allow the reprogramming of gene expression and to reduce the influence of the previous symbiotic condition. In order to verify the presence of metabolically active hyphae, succinate-NBT

vital staining, which reveals the activity of the mitochondrial succinate dehydrogenase enzyme (Vierheilig et al. *2005*; Oger et al. *2009* and references therein), was performed on the fungal material detached or undetached from the root system. No qualitative difference was observed between the detached ERM and the undetached ERM (Suppl. Fig. S1). By contrast, GintPT expression was dramatically reduced (11.25-fold) when the ERM was detached from the host roots (Fig. 2). The supply of fresh medium containing 35 µM Pi to the detached ERM, although inducing a slight increase in the GintPT transcript (2.5-fold with respect to the detached ERM), did not restore the expression levels of the ERM connected to the roots (Fig. 2). These results suggest that the connection with the host root influences GinPT expression in the ERM.

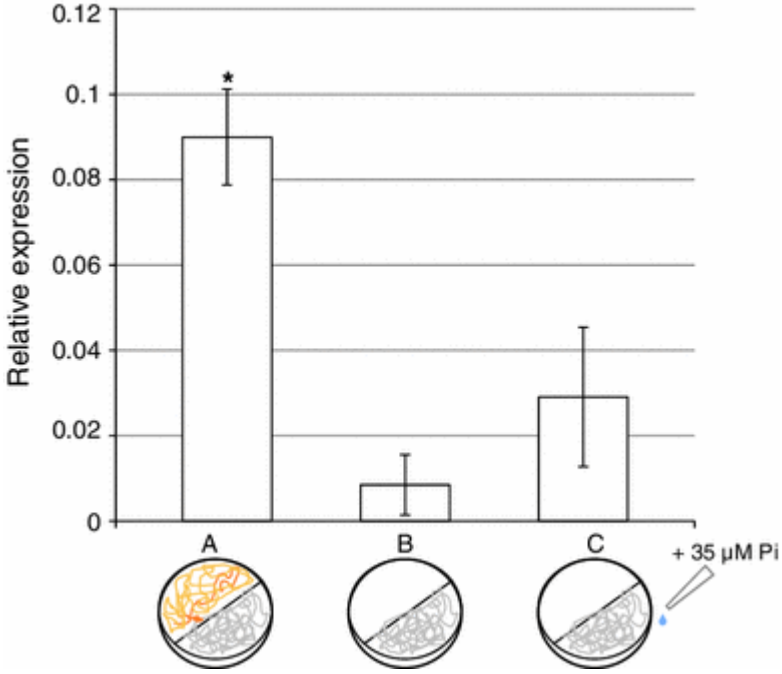

Fig. 2
Quantitative RT-PCR expression analysis of GintPT in the extraradical mycelium (ERM) of R. irregularis. Petri dish A, ERM grown in fungal compartment in association with roots developed in the root compartment; dish B, ERM detached from mycorrhizal roots; dish C, ERM detached from roots and added with fresh medium containing 35 µM Pi. Error bars represent SD of three replicates; *P < 0.05

GintPT response to different Pi treatments in the ERM

The expression of PT genes in the ERM is known to be responsive to different Pi concentrations (Maldonado-Mendoza et al. *2001*; Benedetto et al. *2005*). However, only two highly different Pi concentrations (35 µM vs. 3.5 mM) were compared in the previous studies. We wanted to test an additional intermediate concentration. In order to address this issue, the ERM grown in a liquid medium in the hyphal compartment of monoaxenic cultures was exposed for 24 h to 35 µM, 320 µM or 3.2 mM Pi and GintPT transcript levels were investigated by means of qRT-PCR. In agreement with literature data, the ERM exposure to the millimolar Pi concentration almost completely abolished GintPT expression. Interestingly, an up-regulation (3.2-fold) of GintPT was observed in the ERM exposed to 320 µM Pi in comparison to the ERM treated with very low Pi (35 µM) (Fig. 3). This result suggests that a moderately high Pi availability increases the ability of the ERM to acquire Pi from the surrounding medium. To verify this, we measured the final Pi concentration in the medium in which the ERM was kept for 24 h in 35 or 320 µM Pi. We obtained 4.9 and 206.4 µM Pi for the 35 µM and the 320 µM Pi conditions, respectively. Thus, upon exposure to 320 µM, the ERM was able to acquire 3.7-fold more Pi than upon 35 µM Pi exposure.

This result supports a positive correlation between the transcriptional regulation of GintPT and the Pi transport capability of the ERM.

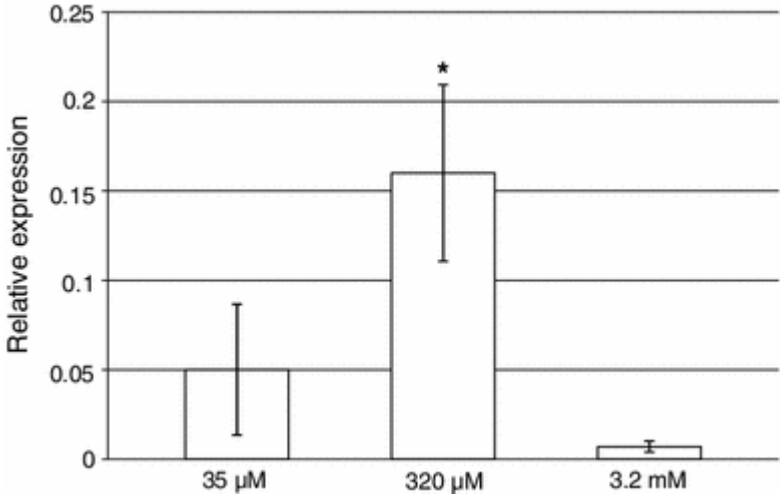

Fig. 3
Quantitative RT-PCR expression analysis of GintPT in ERM grown in liquid medium containing different phosphate concentrations (35, 320 or 3.2 µM). Error bars represent SD of three replicates; *P < 0.05

Response of mycorrhizal roots to different Pi concentrations

Given that GintPT is also expressed in the intraradical phase, we investigated whether different levels of Pi in the medium in contact with the ERM could also modulate the GintPT expression inside root tissues. At the same time, we also considered the plant phosphate transporter, MtPT4, which is known to be expressed in arbusculated cells and responsible for the Pi uptake from the periarbuscular space (Javot et al. *2007*). M. truncatula seedlings were inoculated using the sandwich method and watered with a nutrient solution containing 32 or 320 µM Pi. Two months later, M. truncatula roots showed a well-developed colonization and both sets of plants had similar values of mycorrhization parameters (Table 2), suggesting that the higher Pi availability did not influence the colonization level. qRT-PCR conducted on the whole mycorrhizal roots, from which the external hyphae had been removed, revealed an enhanced expression level of MtPT4 (1.98-fold) in the roots exposed to the higher Pi concentration, while GintPT expression did not seem to be affected by the external Pi level (Fig. 4a).

Table 2
Mycorrhization level in M. truncatula roots exposed to different Pi concentrations (32 µM; 320 µM KH_2PO_4) at 60 dpi

	F %	**M %**	**a %**	**A %**
32 µM	93.1	31.6	81.4	25.2
320 µM	91.7	35.9	84.1	27.0

F % frequency of mycorrhiza in the root system, M % intensity of the mycorrhizal colonisation in the root system, a % arbuscule abundance in mycorrhizal parts of root fragments, A % arbuscule abundance in the root system

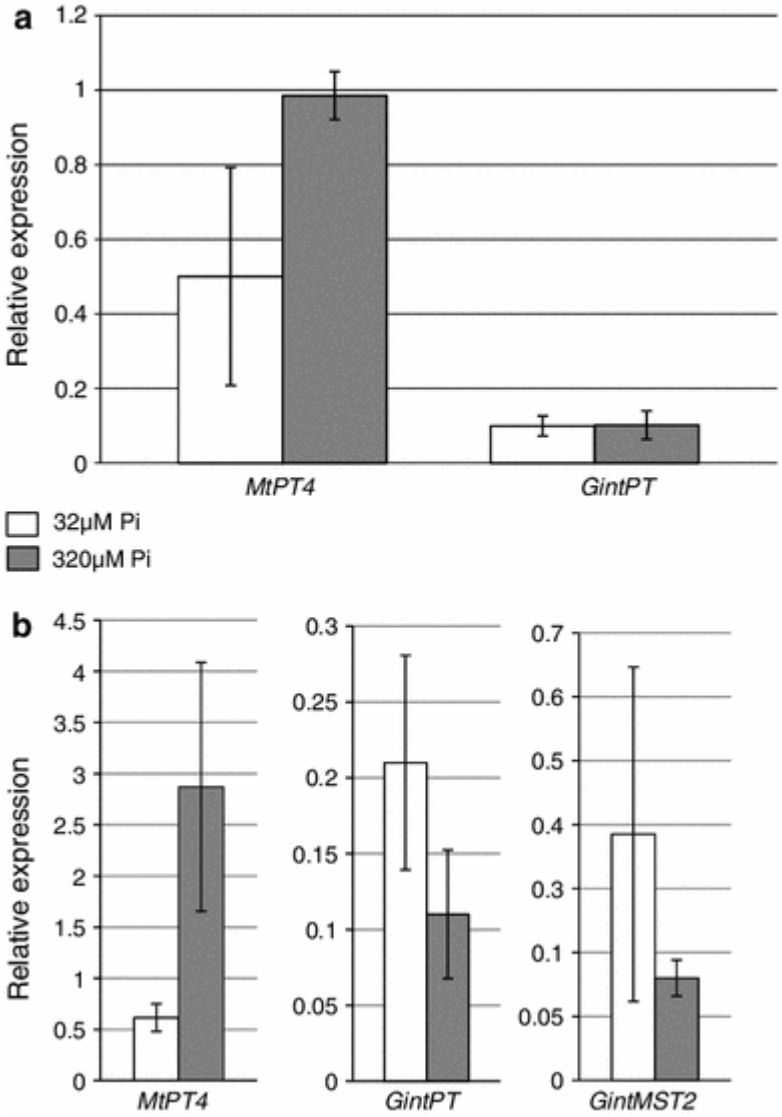

Fig. 4

Quantitative RT-PCR expression analyses of plant and fungal genes in M. truncatula mycorrhizal roots grown at different phosphate concentrations (32 or 320 µM). a Relative expression of MtPT4 and GintPT in whole mycorrhizal roots. b Transcript accumulation of MtPT4, GintPT and GintMST2 in arbuscule-containing cells collected by laser microdissection. Error bars represent SD of two replicates

With the aim of investigating more closely the site of the nutrient exchange between the fungus and the plant, arbusculated cells from the two sets of mycorrhizal plants were collected taking advantage of laser microdissection (LMD) technology, and a real-time RT-PCR-based quantitative assay was performed. This also allowed us to calculate the percentages of fungal and plant transcripts in the arbuscule-containing cells. Considering threshold cycle (Ct) values obtained with specific primers for both the fungal and plant Tef housekeeping genes, we estimated that, in this cell population, the plant transcripts were about 80 % while the fungal transcripts accounted for 20 %. Compared with the gene expression analysis carried out on the whole roots, the LMD approach revealed an even stronger up-regulation of MtPT4 (4.63-fold) and a down-regulation (two fold) of the fungal GintPT expression in the roots exposed to 320 µM Pi (Fig. 4b).

On the basis of the plant and fungal PT expression levels in the arbusculated cells, and assuming a direct correlation between the mRNA level and Pi transport activity, Pi transfer towards the plant seems more efficient at 320 µM than at 32 µM. In line with this hypothesis the Pi content of

mycorrhizal roots grown at 320 µM resulted to be higher than that of mycorrhizal roots grown at 32 µM (Fig. 5).

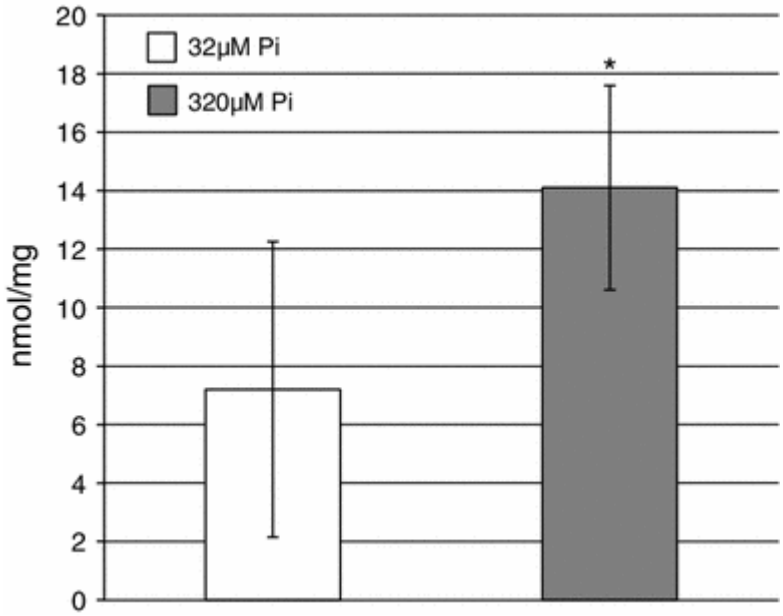

Fig. 5
Phosphate content in M. truncatula mycorrhizal roots grown at 32 or 320 µM Pi. Error bars represent SD of six replicates; *P < 0.05

Since it has been proposed that the exchange of Pi versus carbon compounds in AM symbiosis is interconnected (Kiers et al. *2011*), we also investigated the expression profile of GintMST2, the R. irregularis hexose transporter recently characterized by Helber et al. (*2011*) in arbusculated cells. The gene is in fact possibly involved in the uptake of hexoses released by the plant in the periarbuscular space. GintMST2 showed a slight up-regulation in the arbusculated cells from plants grown at 32 µM Pi compared to 320 µM (Fig. 4b).

Discussion

Starting from the seminal description of the first phosphate transporter (PT) in an AM fungus (Harrison and van Buuren *1995*), one of the main goals of AM research has been to understand Pi uptake by the fungus, its delivery to the plant and, more recently, systemic Pi signalling (Yang and Paszkowski *2011*). Since PT genes exert a primary role in P transfer, the study of fungal and plant PT gene expression represents a starting point to decipher how the P flow is regulated in a mycorrhizal root.

GintPT is constitutively expressed over the main fungal life cycle steps

In order to examine the expression dynamics of the fungal PT over the main steps of the fungal life cycle, we used spores and ERM proliferating in the fungal compartment of bicompartmented Petri dishes and associated with mycorrhizal roots from ROCs, while arbusculated cells were collected from “in sandwich” plants using a laser microdissector. Under these conditions, the fungal GintPT always resulted to be expressed, confirming its constitutive expression in spores, ERM and arbuscules. On one hand, the data validate the microarray experiment performed by Tisserant et al. (*2012*), while on the other hand they point out that Pi uptake is a basic need in itself for fungal viability and its metabolism.

Unlike GmosPT, whose transcripts were not detected in dormant sporocarps of Glomus mosseae collected from pot cultures (Benedetto et al. *2005*), GintPT expression has been observed in spores. Spores are considered the main storage structures of AM fungi. R. irregularis grown in ROCs has been shown to accumulate Pi and other nutrients in spores and hyphae in low C availability conditions (Hammer et al. *2011*). The detection of GintPT transcripts in spores could be related to the growth conditions created by ROCs: A. rhizogenes-transformed roots are grown in a C-rich medium, which becomes depleted during the 2-month period of mycorrhizal colonization. Under these conditions, we can speculate that the nutrient exchange model, according to which the symbionts can reciprocally stimulate a C or P flux by providing nutrients to the other partner (Kiers et al. *2011*), becomes altered. The host is not able to reward the fungus in the best way by providing C, and on the other hand, R. irregularis, which is considered a cooperative fungal species (Kiers et al. *2011*), stores Pi resources in the spores. In agreement with this hypothesis, it has been shown that the uptake of P by the ERM and its translocation to the mycorrhizal roots was stimulated in response to increased carbohydrate availability (Bücking and Shachar-Hill *2005*).

GintPT expression in the ERM depends on the symbiosis and on the Pi concentration

As a second step, we wanted to more clearly understand to what extent the ERM expression pattern was modulated by the presence of the host roots and therefore by the symbiotic status. Even though the ERM was detached from the mycorrhizal roots for 2 days, its viability was similar to that of the undetached ERM. Surprisingly, in this condition, GintPT expression showed a strong down-regulation. Not even the exposure of the detached ERM to Pi-containing fresh medium could restore the GintPT expression level of the ERM connected to the host root. These data show that ERM GintPT expression is dependent to a great extent on the symbiotic status: the IRM and the host plant may act as a strong sink for Pi, and, as a consequence, the ERM has to increase its Pi uptake efficiency to warrant Pi delivery to the intraradical hyphae as well as to the host cells. As an alternative, or as an additional point of control, the fungus may turn off GintPT when the carbon flux is missing, which again points to a strong interconnection between P and C exchanges (Bücking and Shachar-Hill *2005*; Kiers et al. *2011*). On the basis of these results, GintPT could be considered a possible functional marker of the symbiotic phase.

In our experiments, different Pi concentrations (low 35 µM, intermediate 320 µM or high 3.2 mM) were used to investigate whether changes in Pi availability in the surrounding medium modulate the Pi uptake of the ERM. Measurements of the Pi content in the media showed that the ERM acquires more Pi when exposed to 320 µM compared to 35 µM Pi. This was also mirrored by the highest level of GintPT transcripts, confirming a good correlation between GintPT expression levels and Pi uptake efficiency of the ERM. In agreement with literature data (Maldonado-Mendoza et al. *2001*), we observed that the ERM treated with 35 µM Pi, nearly completely sequestered the Pi present in the medium in the first 24 h and also that GintPT expression was almost totally abolished when the ERM was exposed to a millimolar Pi concentration.

In conclusion, the roots are crucial to regulate the whole fungal P metabolism, which in addition is also under the control of the Pi levels present in the environment. The fungus seems to be capable to rapidly optimize its Pi uptake capabilities according to the external Pi availability.

GintPT expression in the arbusculated cells opens new scenarios to plant–fungal interactions

The analysis of laser-microdissected arbusculated cells has revealed that GintPT is consistently expressed in arbusculated cells where the Pi flux is commonly expected to be directed towards the

host cells. The presence of fungal PT transcripts (GvPT, GmosPT, GintPT) in the intraradical phase has already been reported (Harrison and Van Buuren *1995*; Benedetto et al. *2005*; Balestrini et al. *2007*; Gomez-Ariza et al. *2009*; Tisserant et al. *2012*), although rarely discussed. Taken together, these findings raise the question of whether the fungus may reabsorb the Pi released in the periarbuscular space and thus exerts a control over the amount of Pi delivered to the host. Current experimental evidences have indicated that a similar fungal re-uptake mechanism may also occur in arbusculated cells for nitrogen resources (Pérez-Tienda et al. *2011*). However, it is worth to note that for these transporters there is no evidence of a direct correlation between the abundance of mRNAs and the activity of the corresponding gene products. In addition, in AM fungi a long distance movement of mRNAs molecules from extraradial hyphae to arbuscules can also be envisaged thanks to the coenocytic nature of hyphae.

We have performed additional experiments on M. truncatula mycorrhizal roots grown under two different Pi regimes (32 or 320 µM Pi): the expression levels of GintPT and the M. truncatula periarbuscular membrane-located phosphate transporter, MtPT4, are in fact affected by an external Pi concentration. Exposure to the higher Pi concentration led to a slight down-regulation of GintPT and a slight up-regulation of MtPT4 which is mirrored by a higher amount of P inside mycorrhizal roots (Fig. 5). These results suggest that when the fungus is exposed to 320 µM Pi, it absorbs more Pi from the surrounding environment (according to previous experiment, Fig. 4b), and, once its needs have been satisfied, it orients most of the Pi flux towards the host.

In conclusion, the plant might take a fungal-mediated advantage by the increased Pi. However, we do not know whether plants take up more Pi for their needs or whether this is an example of the so-called luxury consumption phenomenon, previously described for a wide array of vascular plants (Lawrence *2001*; Tripler et al. *2002*; Boivin et al. *2004*; Sharma and Sahi *2005*; Sharda and Koide *2010*), in which plants acquire Pi resources beyond their requirement levels and then store them. This also suggests that the low Pi level is not only perceived by the host as a starvation condition, but also by the fungus, provoking a sort of competition for Pi uptake between the two symbionts inside the arbusculated cells. This complex mechanism would require fine-tuning between the two partners: quantification of the Pi levels inside arbusculated cells and/or labelling experiments to monitor Pi flux in both directions would be instrumental to confirm this hypothesis.

We wondered whether our data could fit to the emerging model of the reciprocal rewards in which the cooperation between partners in the AM symbiosis is at the basis of the nutrient transfer (Kiers et al. *2011*; Selosse and Rousset *2011*; Fellbaum et al. *2012*). In our experiments the regulation of the only available marker of a C flux from the fungus to the plants, the fungal hexose transporter GintMST2 (Helber et al. *2011*), was not in line with the model since an up-regulation of the GintMST2 in arbusculated cells of roots grown at 320 µM Pi compared to 32 µM was not observed (Fig. 4b). However, it is worth noting that GintMST2 transcripts were detected not only in arbuscules but also in intercellular hyphae (Helber et al. *2011*), suggesting that the periarbuscular space may not be the only site in which the exchange of P for C occurs. In addition, we could envisage that other sugar transporters may be involved in supplying C to the fungus in mycorrhizal roots, or that GintMST2 is not the only one controlling the P versus C exchanges in arbusculated cells.

In conclusion, our research has shown that the molecular mechanism at the basis of the reciprocal rewards appears more complex than expected.

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