

Fluorescent in situ RT-PCR to visualise the expression of a phosphate transporter gene from an ectomycorrhizal fungus

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Abstract Expression of a mycorrhizal fungal-specific phosphate (P) transporter gene (*HcPT1*) was studied in mycelium of the ectomycorrhizal fungus *Hebeloma cylindrosporium*, by in situ reverse transcriptase polymerase chain reaction using amplification of complementary DNA sequences. The expression of *HcPT1* was visualised under two different P treatments. Mycelium was transferred to liquid medium with or without P and incubated for 5 days. Under P starvation, mycelium growth and vitality was reduced and the expression of *HcPT1* up regulated. Enzyme-labelled fluorescent substrate was used to detect gene expression in situ with epi-fluorescence microscopy and to visualise it at the level of the individual hyphae both in starved and non-starved hyphae. Up-regulation of *HcPT1* was observed as a more intense fluorescent signal and from the larger proportion of hyphae that showed expression.

Keywords Fluorescence microscopy · Gene expression · *Hebeloma cylindrosporium* · In situ RT-PCR · Phosphate transporter

Introduction

Identification of the mechanisms involved in the acquisition of nutrients by mycorrhizal symbionts will lead to a better understanding of the ecological functioning of mycorrhizal symbioses and of nutrient transfer processes to mycorrhizal plants (Martin et al. 2001; Chalot et al. 2002). Total phosphorus (P) contents of soils can be high, but due to low mobility caused by adsorption, precipitation or conversion to organic forms, its availability for uptake by plants or microorganisms is reduced. Mycorrhizal fungi have developed several mechanisms to increase P uptake, including enzyme activities (mainly phosphatases), excretion of organic acids (see review by Bolan 1991) and up-regulation of phosphate transporters (Harrison and van Buuren 1995; Maldonado-Mendoza et al. 2001). P transporter genes, both of plant and fungal origin, showing enhanced expression during mycorrhizal symbiosis, have been studied in detail in the arbuscular mycorrhiza. Some plant P transporters have been shown to be expressed specifically in arbuscule-containing cells, thus indicating a role in the acquisition of P released by the fungus (Rosewarne et al. 1999; Rausch et al. 2001; Harrison et al. 2002; Karandashov et al. 2004). P transporters from the external mycelium of arbuscular mycorrhizal fungi have also been studied (Harrison and van Buuren 1995; Maldonado-Mendoza et al. 2001; Benedetto et al. 2005). P transporters of the external mycelium have been found to be up regulated at low P concentrations (Maldonado-Mendoza et al. 2001; Benedetto et al. 2005), and Harrison and van Buuren (1995) suggested that the P transporter of *Glomus versiforme* (GvPT) plays a role in the P acquisition from soil.

Several sequences corresponding to genes that could play a role in nutrient uptake or secretion have been identified in an expressed sequence tags database of the

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ectomycorrhizal fungus *Hebeloma cylindrosporum*, including two P transporters (Chalot et al. 2002; Lambilliotte et al. 2004) that have recently been characterised (Tatry 2003). One P transporter gene, *HcPT1*—a putative member of the fungal H⁺/Pi transporter family—was found to be expressed when the fungus grows in pure culture. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) showed that its expression increases with P starvation and that expression patterns are consistent with a role in P acquisition from the soil solution by ectomycorrhizal *Pinus pinaster* roots (Tatry 2003). However, no studies have yet been made to visualise the gene expression in situ in ectomycorrhizal structures.

In situ RT-PCR is a technique that allows in situ visualisation of gene expression at much lower levels than by using in situ hybridisation (Morel et al. 1998). The aim of this study was to develop an in situ RT-PCR protocol to visualise the expression pattern of *HcPT1* in mycelium of *H. cylindrosporum* in response to P availability. The fungus was grown in liquid culture with either sufficient P (plus P treatment) or without P (no P treatment), and in situ expression patterns of *HcPT1* were compared with *EF1-α*, a constitutively expressed housekeeping gene.

Materials and methods

Experimental procedure

Agar plugs with a diameter of 8 mm of mycelium of *H. cylindrosporum* Romagnesi [dikaryotic strain D2 resulting from plasmogamy of two compatible homokaryotic strains h1 and h7 (Debaud and Gay 1987)] were transferred to Petri dishes containing 25 ml liquid medium. This liquid medium consisted of 5 g l⁻¹ glucose, 10 mg l⁻¹ ferric citrate, 6 mM KNO₃, 4 mM KCl, 1 mM NaH₂PO₄, 0.5 mM CaCl₂, 1 mM MgSO₄·7H₂O, 0.3 μM thiamine-HCl, supplemented with 0.2 ml l⁻¹ of micronutrient solution, and pH was adjusted to 6.0 (Scheromm et al. 1990). The fungus was left to grow in the dark at 26°C, and the medium was replaced weekly. P treatment was performed by transferring the mycelia into Petri dishes containing 25 ml of the liquid medium either without P (no P treatment) or normal medium with P (plus P treatment) for 2 or 5 days. Five days of treatment was chosen because at that time the mycelium was expected to be starved for P in the no P treatment (Tatry 2003).

Primer design

The fungal phosphate transporter gene *HcPT1* was amplified using a specific set of primers based on 3' parts of complementary DNA (cDNA) and genomic DNA sequences

(accession number AJ970310 and AJ970313 in the EMBL bank, respectively; M.V. Tatry, E. El Kassis, R. Lambilliotte, C. Corratgé, I.M. van Aarle, S. Zimmerman, H. Sentenac and C. Plassard, unpublished results). The gene-specific primer pair was *HcPT1*Sforward (5'-CTTCATCATCATGGGTTT CGGATACG-3') and *HcPT1*Sreverse (5'-CTCGCACAC CTCTGACGAAACCGTC-3'). PCR on the cDNA library from *H. cylindrosporum* (Lambilliotte et al. 2004) resulted in an expected single product of 400 bp (see also Fig. 5). The primers were specifically designed to cover introns and did not produce amplification with *H. cylindrosporum* genomic DNA.

Amplification of *EF1-α* (accession number AJ970312) encoding elongation factor-1 alpha, an abundant protein in eukaryotic cells, was used as a positive control of the vitality of fungal mycelium. The gene-specific primer pair used was *EF1*forward (5'-CCTCCTCGATGCCATCGATGCCATT GAAC-3') and *EF1*reverse (5'-CTTGAAGTGGAAAGGC GACGTTTCCACGAC-3'), which generated a single PCR product of 310 bp when the cDNA library was used as a template (see also Fig. 5). An amplification product was observed also with genomic DNA (approximately 400 bp), as the primers did not cover introns.

Sample preparation and fixation

Mycelium was washed at harvest, and the youngest, most distal part of the fungal colony was collected. This mycelium was rapidly cut into small pieces of less than 2-mm length, immediately fixed in Eppendorf tubes containing freshly prepared PAA [2% (v/v) paraformaldehyde, 63% (v/v) ethanol and 5% (v/v) acetic acid] and stored overnight at 4°C (Koltai and Bird 2000; Cellier et al. 2004). Paraformaldehyde is a fixative commonly used in in situ RT-PCR experiments because it limits the degradation of RNA, inhibits the formation of secondary structures and thus favours cDNA synthesis (see the review of Morel et al. 1998). On the other hand, paraformaldehyde is known to interfere with the poly-A chain of mRNA, thus lowering cDNA synthesis efficiency. However, the use of hexamer primers or gene-specific primers minimises the effect of this interference, as the RT reaction will not depend on the availability of the poly-A chain. Fixed pieces of mycelium were washed extensively to remove fixative with four changes of diethyl pyrocarbonate (DEPC)-treated water over 30 min under regular agitation.

Presence of RNA and DNA in fresh and fixed samples of mycelium was confirmed and compared using acridine orange (Merck, Whitehouse Station, NJ, USA) staining. Briefly, samples were washed in 1% acetic acid (v/v) for 30 s, rinsed with H₂O and incubated successively for 2 min in 0.1 M phosphate buffer (pH 6) and 3 min in 0.01% acridine orange in phosphate buffer (v/v). Samples were

then rinsed once in 11% CaCl₂ (w/v) and twice in phosphate buffer. They were mounted in phosphate buffer and observed using a Zeiss LSM510 AX70 confocal laser scanning microscope equipped with Zeiss LSM software. The excitation wavelength was 488 nm, emission wavelengths were between 505 and 530 nm for the green image (DNA-bound acridine orange) and higher than 585 nm for the red image (RNA-bound acridine orange). Intact fresh or fixed samples mounted in DEPC water were observed to determine the autofluorescence of the mycelium.

In situ reverse transcription and PCR amplification

The protocol used for in situ reverse transcription (RT) was modified from Koltai and Bird (2000) and Cellier et al. (2004). For reverse transcription, pieces of fixed mycelium were incubated in PCR tubes in 40 µl RT mix [1× RT buffer (Promega, Madison, WI, USA), 0.31 mM of each deoxyribonucleotide triphosphate (dNTP) and 0.75 µM HCPT11Srev or EF1rev primer]. The samples with RT mix were heated to 65°C for 5 min and then put on ice for 2 min. Moloney murine leukemia virus reverse transcriptase H(-) (Promega) was added to each sample to a final concentration of 5 U µl⁻¹, and samples were incubated at 42°C for 1 h. Mycelium was not subjected to RNase A or DNase treatment for negative control of samples, as Koltai and Bird (2000) reported that a relative high concentration of these enzymes over a long incubation period does not appreciably reduce staining.

After reverse transcription, the RT mix was removed, and the samples were washed three times. For each washing, 100 µl of DEPC-treated water was added to the samples in the PCR tubes, the samples were inverted during 1 min, spun shortly, and the liquid was carefully removed. Forty microlitres of PCR mix [1× PCR buffer (Invitrogen, Carlsbad, CA, USA), 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.25 µM each of the gene-specific primers, 0.25 nM digoxigenin-11-2'-deoxyuridine 5'-triphosphate (dUTP; Roche Diagnostics, Mannheim, Germany) and 1 U Taq DNA Polymerase (Invitrogen)] was added to each sample. Thermocycling was performed at 72°C for 2 min and 20 cycles (92°C for 30 s; 60°C for 30 s; 72°C for 45 s) for both genes. The PCR mix was removed after amplification, and the samples were post-fixed in 100% ethanol.

Negative controls (no-RT) were prepared for each gene by omitting the reverse transcription. For this, the samples were put in 40 µl of DEPC-treated water during the RT step and were in the following steps treated as the other samples. Mock in situ RT-PCR was performed by leaving fixed material during the whole in situ RT-PCR amplification in DEPC-treated water. These mock in situ RT-PCR samples were used to monitor the effect of the thermocycling on the morphology and the activity of endogenous phosphatase.

Experiments were repeated at least three times and representative results are shown.

In situ detection of amplified cDNA

All solutions were filtered through a non-pyrogenic filter (0.2 µm, Schleicher & Schuell, BioScience, Dassel, Germany) before use. Post-fixed samples in PCR tubes were washed three times for 10 min in phosphate-buffered saline (PBS; 5 mM Na₂HPO₄, 300 mM NaCl, pH 7.5) under gentle agitation, and then blocked with 0.1% BSA in PBS for 30 min. Samples were subsequently incubated under agitation at room temperature for 1 h with alkaline phosphatase-conjugated anti-digoxigenin-Fab fragment (Roche Diagnostics) diluted 1:1,000 in blocking solution. The samples were washed three times for 3 min in PBS and two times for 10 min in a washing buffer (30 mM Tris, 1.5 M NaCl, 0.05% Triton® X-100, pH 8.0) to remove excess antibody and stored in washing buffer at 4°C overnight.

Two more washes in washing buffer were performed the next day, before detection of alkaline phosphatase was carried out using the ELF-97 endogenous phosphatase detection kit (Molecular Probes, Leiden, The Netherlands). The protocol used was modified from Van Aarle et al. (2001) and Olsson et al. (2002). Briefly, enzyme-labelled fluorescent (ELF) substrate was diluted 1:40 in the alkaline detection buffer, vigorously shaken and filtered through a 0.22-µm filter (Millex®-GV, Millipore, Bedford, USA). Samples were incubated in 20 µl ELF substrate-buffer solution for 20 min in the dark and transferred to washing buffer. Three washings of 1 min were performed, before the samples were mounted. Microscopical observations and image acquisitions were done between 25 and 30 min from the start of the incubation. This protocol gave a high level of specific staining for the mycelium samples with low background staining. The optimal incubation time in ELF substrate-buffer solution was determined by microscope observations of samples and their negative controls using an Olympus BX61® microscope equipped with an epifluorescence condenser and a Hoechst/DAPI filter set. The objectives used were Olympus UPlanFI 20× (numerical aperture 0.5) and Olympus UPlanFI 40× (numerical aperture 0.75), and images were taken with a Color View II® camera using Analysis® software (Soft Imaging System, Munster, Germany). Micrographs were imported in Adobe Photoshop (version 8). Contrast and light levels were adjusted for every micrograph, so as to get the best resolution of hyphae compared to the background. Then, coloured images were transferred to grey scale and inverted.

For detection of alkaline phosphatase-conjugated antibody with the commonly used nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP), a ready-to-use stock solution (Roche Diagnostics) was used.

Samples were washed three times 3 min in PBS and two times 10 min in a 10× washing buffer (100 mM Tris, 150 mM NaCl, pH 9.5) as described before. The NBT/BCIP was diluted 1:100 in washing buffer (1×), and samples were incubated in 50 µl of this solution. Incubation was performed in the dark for several min, until colour development of the negative control samples could be observed by eye. The reaction was stopped by transferring the samples into sterile ultra pure water, and the samples were washed several times. Samples were mounted and observed immediately, and images were taken as described above. Micrographs were used without any further treatment with Photoshop.

In vitro reverse transcription and PCR amplification

After collection of samples for in situ RT-PCR, the rest of the washed mycelium was used to extract total RNA according to the method described by Ausubel et al. (1996). In vitro reverse transcription reaction was carried out on 0.5 µg of total RNA, using specific reverse primers. The same procedure was used as described for in situ RT-PCR. RT product (0.5 µl) was then used to carry out PCR amplification with Taq polymerase (Invitrogen) using the manufacturer's instructions and the same conditions of thermocycling as described for in situ RT-PCR.

Results

Controls for specificity of in situ RT-PCR

The morphology and autofluorescence of mycelium subjected to mock in situ RT-PCR was compared to that of fresh material. No significant changes were observed in mycelium structure or in the intensity of blue autofluorescence. Endogenous alkaline phosphatase activity, which was generally present in fresh samples, had disappeared completely in mock in situ RT-PCR samples. Intact fresh samples observed using laser confocal microscopy exhibited a low level of autofluorescence that was set to

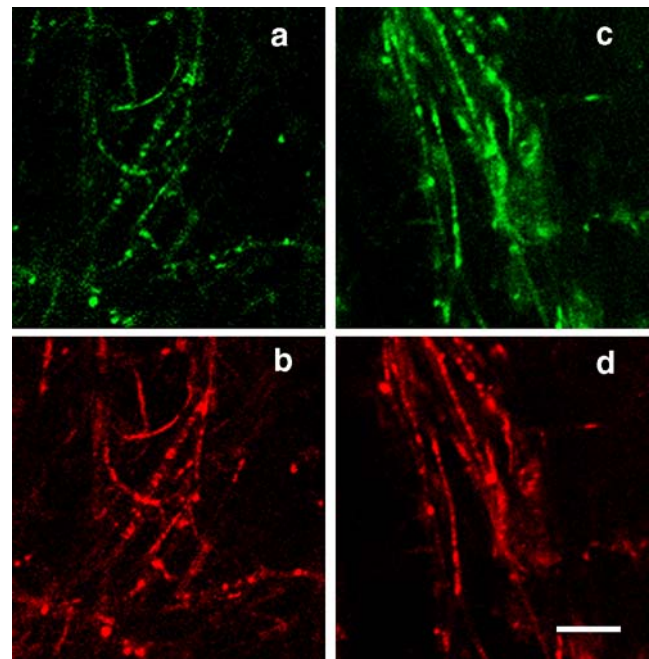


Fig. 1 DNA and RNA in fixed mycelium of *H. cylindrosporum* visualised with acridine orange staining. Presence of DNA (**a, c**) and RNA (**b, d**) was visualised in *H. cylindrosporum* mycelium either fixed (**a, b**) or fresh (**c, d**). Autofluorescence of hyphae was set to zero first in intact fresh samples mounted in DEPC water. All images were then taken using these settings, as autofluorescence level observed in intact fixed samples was extremely low. Scale bar is 10 µm. Micrographs were taken at ×400 magnification

zero before observe acridine orange stained samples. Autofluorescence of fixed samples was also extremely low, confirming that fixation did not change hyphae chemical properties. Acridine orange stained samples displayed similar red and green images (Fig. 1), confirming the presence of RNA (in red) as well as DNA (in green) in fresh and fixed material. Taken as a whole, these results indicate that fixation and subsequent thermocycling of samples had no significant effect on the detection of RNA or DNA.

Occasionally, a low background staining resulting from non-specific precipitation of the ELF substrate was observed due to relatively long incubation times (Table 1). In negative controls or RT samples, ELF precipitates could

Table 1 Staining intensity of the alkaline phosphatase-conjugated antibody with NBT/BCIP or ELF from in situ RT-PCR amplified samples

Treatment	Days of treatment	Staining intensity		
		<i>EF1-α</i>		HcPT1
		NBT/BCIP	ELF	ELF
No phosphorus	2	Not determined	Not determined	Low
	5	Low–intermediate	Intermediate	Intense–very intense
Plus phosphorus	5	Low–intermediate	Intense	Low–intermediate
Control (no RT)	5	Not specific	Very low or not specific	No or not specific

sometimes be observed in the mounting medium. In this case, background staining was not specifically associated with the mycelium, and the ELF precipitates were located in another focal plane, making it easy to differentiate between gene-specific staining and background. Overall gene expression patterns, as observed after specific ELF staining, were consistent within the treatments and controls, and a high repeatability was obtained.

Comparison of detection methods

Both the detection methods (NBT/BCIP and ELF) were performed on RT and negative control samples for the housekeeping gene *EF1- α* . Samples were divided into two sub-samples after incubation with the antibody but before staining. When stained with NBT/BCIP, the negative control samples did not show any specific staining (Table 1, Fig. 2a). These samples were on average light grey. In general, staining of the RT samples with NBT/BCIP resulted in a low-to-intermediate purple-brownish staining of the mycelium with a continuous and homogenous distribution (Table 1; Fig. 2b,c). This homogenous staining suggested diffusion of stain around the place of gene expression. No individual crystals, resulting from the formazan reduction product of the tetrazolium salt, could be observed under bright field, dark field or differential interference settings. In samples with only a light staining, it was difficult to distinguish mycelium with gene expression from mycelium without gene expression, especially

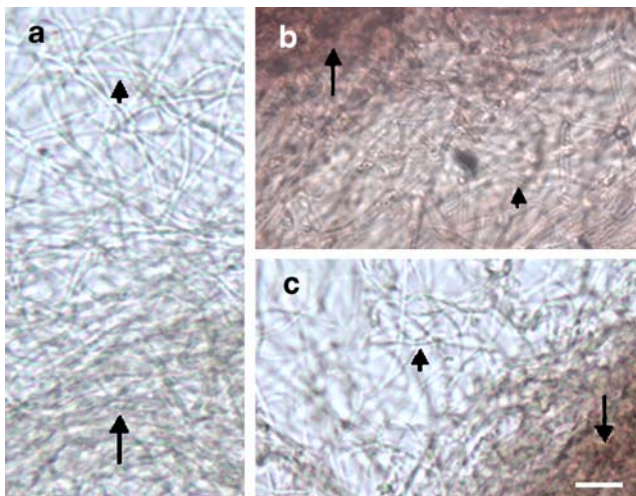


Fig. 2 In situ RT-PCR amplification in *H. cylindrosporum* detected with NBT/BCIP. In situ RT-PCR of *EF1- α* , a housekeeping gene, in mycelium of *H. cylindrosporum* that was grown in plus P (a, b) or no P (c) containing medium. In (a), the negative control (sample without RT) is shown, and in (b, c), the RT samples are shown. Detection of the alkaline phosphatase-conjugated antibody was carried out with NBT/BCIP and could be detected as a purple-brownish staining. Depending on the thickness of the mycelium, some areas were less intensely stained (small arrows) than others (large arrows). Scale bar is 10 μ m. Micrographs were taken at $\times 200$ (a, c) and $\times 400$ (b) magnification

when hyphal density was low. In such cases, the negative control samples were needed for comparison to confirm a change in colour of the RT samples (compare small and large arrows, Fig. 2).

When ELF staining was performed on negative control samples, the mycelium exhibited a blue autofluorescence that can be observed in the micrograph as light grey structures (see the small arrow, Fig. 3a). Contrary to NBT/BCIP, a bright green crystalline precipitation due to the formation of ELF crystals was observed at the place of gene expression. Due to image processing, these crystals are shown as dark precipitates in the hyphae. Few ELF crystals were observed in the negative control (large arrow, Fig. 3a, Table 1), that could be easily distinguished from background staining (asterisk, Fig. 3a). The crystals should correspond to the staining of the genomic DNA in the nuclei of *H. cylindrosporum*, as the image obtained is comparable to the image obtained after specific staining of the nuclei (E. El Kassis, personal communication). These observations are thus in agreement with the PCR reaction occurring only at the level of genomic DNA in negative controls. In contrast, in RT samples, numerous crystals were distributed over the whole hyphae (Fig. 3b,c; Table 1). Although it is not possible to distinguish the staining in the nuclei from the mRNA expression, the fungal cells displayed a staining pattern clearly different in the RT

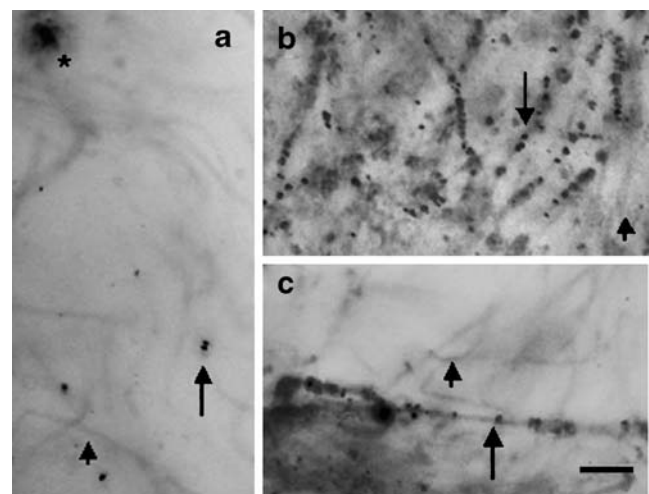


Fig. 3 In situ RT-PCR amplification in *H. cylindrosporum* detected with ELF. In situ RT-PCR of *EF1- α* , a housekeeping gene, in mycelium of *H. cylindrosporum* that was grown in plus P (a, b) or no P (c) containing medium. In (a), the negative control (sample without RT) is shown with a low amount of background staining (asterisk), and in (b, c), the RT samples are shown. Detection of the alkaline phosphatase-conjugated antibody was carried out with ELF-97 substrate. When no gene expression was detected, the mycelium fluoresced blue and can, in the micrographs, be observed as light grey structures (small arrows). Upon gene expression, a green fluorescent precipitate was observed which, in the micrographs, is visualised as the dark precipitate (large arrows). Scale bar is 10 μ m. Micrographs were taken at $\times 400$ magnification

samples and in the negative control samples. With the ELF method, it was possible to visualise gene expression at the level of the individual hyphae (large arrows, Fig. 3b,c) and to locate areas where gene expression was absent (small arrows, Fig. 3b,c). Furthermore, under the severe P starvation of 5 days, a smaller proportion of the mycelium showed gene expression for *EF1- α* , compared to the plus P treatment indicating that the mycelium lost its vitality (Table 1). These observations are in agreement with in vitro RT-PCR (Fig. 5).

Gene expression of a P transporter

As expected from the primer design, no staining was observed in the negative control samples (Table 1, Fig. 4a), and staining of the hyphae in RT samples corresponded to the expression of *HcPT1* (Table 1, Fig. 4b–d). In the plus P treatment, the individual hyphae displayed a low staining intensity, characterised by rare ELF crystals in the hyphae (small arrow, Fig. 4b). Mycelium that had grown for only 2 days in no P medium had a staining pattern (Fig. 4c) similar to that of the plus P treatment (Fig. 4b). In contrast, although the mycelium incubated for 5 days under no P conditions exhibited a reduced vitality based on *EF1- α* expression, much of the mycelium showed

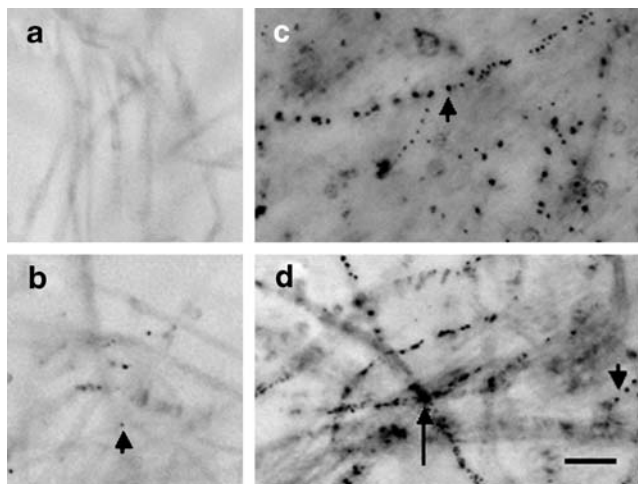


Fig. 4 In situ gene expression of a mycorrhizal fungal-specific phosphate transporter. In situ RT-PCR of *HcPT1*, a mycorrhizal fungal phosphate transporter, on mycelium of *H. cylindrosporum* that was grown in plus P (a, b) or no P (c, d) medium. In (a), the negative control (sample without RT) is shown, and in (b–d), the RT samples are shown. In (c), the mycelium was not yet starved, whereas in (d), the mycelium was starved for P. Detection of the alkaline phosphatase-conjugated antibody was carried out with ELF-97 substrate and could be detected as a green fluorescent precipitate which, in the micrographs, is visualised as the dark precipitate. The small arrow indicates the precipitation of separate ELF precipitates and large arrow indicates an intense precipitation leading to an almost continuous staining. Scale bar is 10 μ m. Micrographs were taken at $\times 400$ magnification

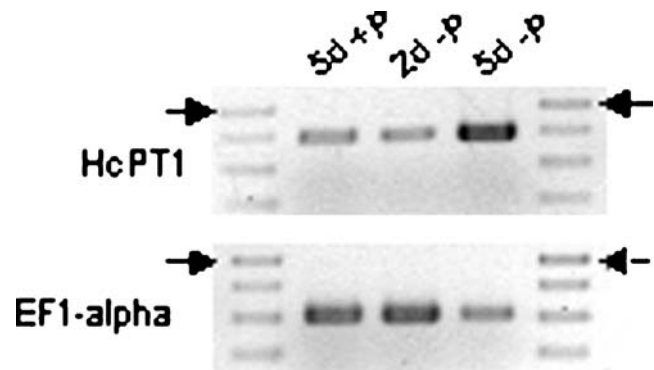


Fig. 5 Semi-quantitative expression of genes extracted from the mycelium of an ectomycorrhizal fungus *H. cylindrosporum*. Expression was done for *HcPT1*, a mycorrhizal fungal phosphate transporter (top), and *EF1- α* , a housekeeping gene (bottom). The mycelium was grown in plus phosphorus (5d +P) medium or the mycelium was grown in medium without phosphorus (-P). At 2 days after transfer to medium without P, the mycelium was not yet starved (2d -P), whereas at 5 days, the mycelium was starved for P (5d -P). RT reaction was carried out on 0.5 μ g of total RNA per sample. The arrows indicate the molecular weight of 0.5 kb

HcPT1 gene expression, and often, an almost continuous staining was observed (large arrow, Fig. 4d). Taken together, these data confirm in vitro RT-PCR (Fig. 5) that the expression of *HcPT1* was up regulated by the 5-day treatment in no P medium.

Discussion

In situ RT-PCR was developed in the early 1990s (see review by Morel et al. 1998) and is now well established for animal and plant tissues. Using the in situ RT-PCR method, which enables the study of low-expressed genes, we have been able to visualise the expression of an ectomycorrhizal fungal P transporter gene in mycelium and its up-regulation by P starvation. Combining the in situ RT-PCR with a fluorescent substrate enhanced the sensitivity of the method allowing detection of gene expression in individual hyphae.

During the thermocycling of the in situ RT-PCR, the endogenous alkaline phosphatase is denatured. It is thus possible to use alkaline phosphatase-conjugated antibodies, contrary to in situ hybridisation, where endogenous alkaline phosphatase is still present at the time of antibody detection (I.M. van Aarle, personal observation). Overall *EF1- α* and *HcPT1* gene expression have been consistent for the treatments and for the controls, and a high repeatability has been shown in our study. In vitro RT-PCR confirmed the in situ RT-PCR results. The in situ RT-PCR results mainly differed in amount of background staining, and not much variation was seen in relative gene expression,

confirming the robustness of the expression results. It should be noted that primers that amplify the genomic DNA in the nuclei could even be used for *H. cylindrosporium*, as clearly different pictures were obtained for *EF1- α* without RT and with RT reaction. The use of ELF as a substrate for the alkaline phosphatase-conjugated antibody allows detection of gene expression in individual hyphae of *H. cylindrosporium*. Furthermore, preliminary studies indicate the suitability of this substrate to study gene expression in highly coloured tissue sections, such as those of mycorrhizal pine roots (personal observation). This method provides a foundation for in situ detection of gene expression in ectomycorrhizal hyphae. Further development of this methodology as well as, for example, the application of phosphatase inhibitors in the washing buffers to inhibit background staining could improve the in situ analysis to a level where spatial gene expression patterns can be more finely resolved. Such a precise localisation of expression will be valuable for a better understanding of the molecular functioning of the mycorrhizal symbiosis.

The present work confirms in vitro RT-PCR studies on *HcPT1* in *H. cylindrosporium* (Tatry 2003) by showing that under P starvation, the level of gene expression is higher than under conditions of P availability. In addition, in situ RT-PCR indicates that the level of expression of *HcPT1* is not just increased but also that a larger part of the mycelium is involved in P acquisition. The expression of the P transporter is up regulated by P limitation in mycelium showing a loss of vitality. For arbuscular mycorrhizal symbiosis, it was found that the mycorrhizal P transporter of the extraradical mycelium is not only regulated by the P content in the environment but also by the P status of the mycorrhizal fungus (Maldonado-Mendoza et al. 2001) and the age of mycelium (Olsson et al. 2006). This has implications in natural conditions where P is limited, and availability is not homogeneously distributed. However, detailed in vitro studies of the ectomycorrhizal P transporter gene showing the expression pattern under P starvation over time are needed to confirm the presence of such a mechanism.

In conclusion, in situ RT-PCR has been used to visualise the expression of an ectomycorrhizal fungal P transporter gene within mycelium. The use of the ELF substrate to reveal activity of the alkaline phosphatase-conjugated antibody used in the technique enables visualisation of expression in individual hyphae. It should thus be possible to visualise gene expression in the symbiosis and relate it to specific structures. The localisation of fungal gene expression will be of great value to the understanding of the molecular functioning of the mycorrhizal symbiosis. Further development of the methodology to detect spatial gene expression patterns and to determine the cellular location of gene expression within individual fungal hyphae is necessary.

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