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SHORT COMMUNICATION



HcPT1.2 participates in Pi acquisition in *Hebeloma cylindrosporum* external hyphae of ectomycorrhizas under high and low phosphate conditions

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ABSTRACT

Ectomycorrhizal fungi improve tree phosphorus nutrition through transporters specifically localized at soil-hyphae and symbiotic interfaces. In the model symbiosis between the fungus *Hebeloma cylindrosporum* and the maritime pine (*Pinus pinaster*), several transporters possibly involved in phosphate fluxes were identified, including three H⁺:Pi transporters. Among these three, we recently unraveled the function of one of them, named HcPT2, in both pure culture and symbiotic interaction with *P. pinaster*. Here we investigated the transporter named HcPT1.2, by analyzing inorganic phosphate transport ability in a yeast complementation assay, assessing its expression in the fungus associated or not with the plant, and immunolocalizing the proteins in ectomycorrhizas. We also evaluated the effect of external Pi concentration on expression and localization of HcPT1.2. Our results revealed that HcPT1.2 is involved in Pi acquisition by *H. cylindrosporum* mycelium, irrespective of the external Pi concentrations.

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TEXT

Phosphorus nutrition of trees from boreal and temperate forests is mostly ensured by the ectomycorrhizal (ECM) symbiosis.¹ ECM fungi are able to cover a large portion of soil, and to solubilize and assimilate inorganic orthophosphate (Pi) through extra-radical and mantle hyphae. Pi is then transported towards a network of hyphae colonizing the host roots called the Hartig net, where it is released into a symbiotic interface and taken up by plant cortical cells. Molecular mechanisms facilitating Pi fluxes in ectomycorrhizas is still barely known.^{2,3} In the model ECM fungus *Hebeloma cylindrosporum*, two H⁺:Pi transporters were functionally characterized in heterologous systems so far.⁴ Among them, HcPT1.1 seems mainly involved in Pi acquisition from the soil under limiting conditions.⁵ However, HcPT1.1 proteins were also localized in Hartig net hyphae, suggesting a possible role at the symbiotic interface too. More recently, we revealed that the second identified H⁺:Pi transporter of *H. cylindrosporum*, HcPT2, seems having a dual function in both Pi acquisition from the soil and Pi release towards the host cortical cells.⁶ With the recent genome and transcriptome sequencing of *H. cylindrosporum*,^{7–9} we also identified a third transporter that we named HcPT1.2 because of a higher level of similarity shared with HcPT1.1 (65.7 %) than with HcPT2 (48 %). Recently, an updated phylogenetic analysis of selected phosphorus transporter classes using the predicted proteins (<https://jgi.doe.gov/JGI>) of 35 fungal species, including 14 ECM ones, showed that both HcPT1.1 and HcPT1.2 proteins belong to the same subgroup (Ia) of H⁺:Pi transporter.³ Here, we report (i) the functional characterization of HcPT1.2 in yeast using non-labelled Pi, (ii) the expression of *HcPT1.2* in both axenic and symbiotic

conditions under high and low Pi supply, and (iii) the protein immunolocalization in *Pinus pinaster* ectomycorrhizas.

The PHO84 deficient yeast strain MB192 (*MATa pho3-1 pho84::HIS3 ade2 leu2-3, 112 his3-532, trp1-289 ura3-1,2 can1*)¹⁰ was transformed with an empty vector (pFL61, carrying the *ura* gene) or with the pFL61 vector containing *HcPT1.2* construct (Figure 1). To build this latter vector, the coding sequence of *HcPT1.2* was amplified in two steps from *H. cylindrosporum* cDNA using two sets of primers (HcPT1.2-F1 – CCTTGCGGCCGCACTAGTATGCCGAGCATCATCGAGAG, HcPT1.2-R2 – ACTATCTCCCGCAGCACATC, HcPT1.2-F2 – GTCAGGCCGTTCTATCTGGA, and HcPT1.2-R2 – TCAAAAACCCCTGCGACGG). Both fragments were reassembled using *Bam*HI restriction enzyme, and cloned into pFL61 using *Not*I restriction enzyme.¹¹ Yeast were transformed and selected on YNB medium (1.7 g l⁻¹; (NH₄)₂SO₄, 5 g l⁻¹; glucose, 20 g l⁻¹; agar, 20 g l⁻¹) supplemented with the appropriate amino acids (Adenine, Leucine, Tryptophan) for transformant selection as previously described.¹² Presence of *HcPT1.2* coding sequence was checked by PCR using HcPT1.2-F and HcPT1.2-R. Two independent strains that incorporated *HcPT1.2* were selected randomly from a collection of 10 transgenic cells for the complementation, *HcPT1.2-a* and *HcPT1.2-b* (Figure 1). *HcPT1.2-a*, *HcPT1.2-b*, wild-type MB192 (WT), and pFL61 strains were incubated at O.D. = 0.1 in liquid synthetic medium (SM) containing 50 μM of Pi supplied as KH₂PO₄, and the decrease in Pi concentration in the medium was measured at 24, 30, and 42 hours. One liter of SM medium contained 20 g glycerol, 5 g glucose, 0.8 g KCl, 0.5 g MgSO₄·7H₂O, 0.5 g CaCl₂, 1.08 g (NH₄)₂SO₄, 4 ml of microelement solution (containing per liter: 125 mg H₃BO₃, 67.5 mg

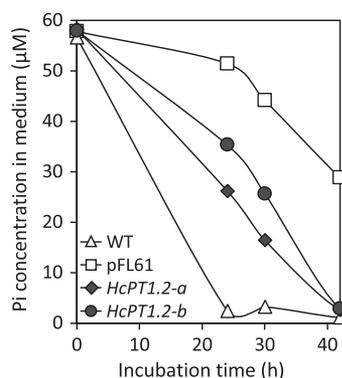


Figure 1. Functional complementation of *PHO84* deficient yeast strain MB192 by HcPT1.2 Pi transporter of *Hebeloma cylindrosporium*. Phosphate absorption by wild-type (WT) or *PHO84* deficient strains transformed with an empty vector (*pFL61*) or *PHO84* deficient strains transformed with an empty vector (*pFL61*) or *HcPT1.2* (*HcPT1.2-a* and *HcPT1.2-b* are two independent transformed strains) was determined by measuring Pi concentration directly in culture media at 24, 30 and 42 hours after inoculation. (n = 3).

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 25 mg KI, 500 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 50 mg $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 650 mg $\text{Fe}_2(\text{SO}_4)_3$, 100 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 30 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 25 mg $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$, 1 ml of vitamin solution (containing per liter: 5 g panthotenate, 20 g inositol, 2 g nicotinic acid, 250 mg pyridoxal hydrochloride, 250 mg thiamine hydrochloride, 10 mg of biotine) and auxotrophy factors. Just after the inoculation, we also measured the actual Pi concentration in the medium which was a bit higher than 50 μM due to the inoculum, but comparable between all strains (Figure 1). While WT strain consumed all available Pi in 24 hours, less than 20 % was taken up by the pFL61 strain, and only 50 % of initial Pi was remaining after 42 hours. *HcPT1.2-a* and *HcPT1.2-b* strains consumed around 50 % of Pi after 24 and 30 hours, respectively, and almost 100 % after 42 hours. We also checked the expression of *HcPT1.2* in pure culture and in ectomycorrhizas under high and low P conditions by RT-qPCR with the internal marker *HcTub*, as recently described (Fig. 2).⁶ *HcPT1.2*-qPCR-F and *HcPT1.2*-qPCR-R primers (CATTATG GCCGAGCGTAC and CTCCGAGAGCGATTGGATC, respectively) were used for the amplification. In pure culture, expression of *HcPT1.2* was determined in two-week-old mycelia grown in liquid medium supplemented with 1 mM of Pi, in mycelia transferred in a Pi-free medium for 24 hours (Figure 2a) or 5 days (Figure 2b), and in mycelia re-enriched with 1 mM of Pi for 24 hours. Ectomycorrhizas were produced in rhizoboxes on thin soil layers enriched or not in P as described previously (Fig. 3).⁶ Similar expression levels were observed in all tested conditions, indicating that external Pi availability did not affect the expression of *HcPT1.2* in both axenic and symbiotic culture. Finally, we immunolocalized *HcPT1.2* proteins in ectomycorrhizas produced on high and low P soil conditions using a recently detailed protocol.⁶ Briefly, images were obtained by confocal microscopy on 50 μm cross-sections labelled with *HcPT1.2* primary antibodies and Dylight 405-conjugated secondary antibodies (Figure 3). *HcPT1.2* proteins were detected in extra-radical and mantle hyphae, but not in the Hartig net (Figure 3(a,c)). Consistently with the expression level data described above, no difference in signal intensity was detected under high or low P supply.

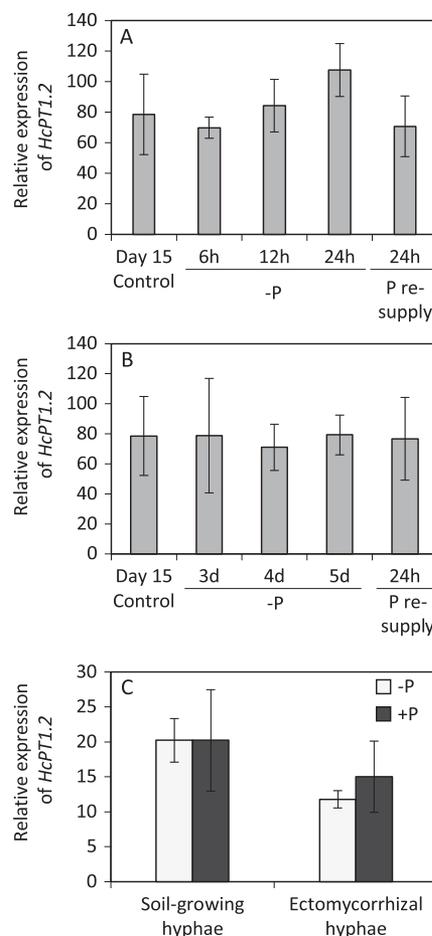


Figure 2. Expression of *HcPT1.2* in pure culture and ectomycorrhizal symbiosis with *Pinus pinaster* under P-sufficient and -limiting conditions. (a,b) Expression of *HcPT1.2* in pure culture was determined by RT-qPCR on two-week-old mycelia grown in liquid medium supplemented with 1 mM of Pi (Day 15 Control), and then in mycelia transferred in a Pi-free medium during 24h (a) or 5 days (b). Mycelia were finally transferred in a Pi-rich medium for 24h (1 mM, P resupply). (c) Expression of *HcPT1.2* in symbiotic condition was determined in extra-radical (Soil-growing hyphae) or root-associated hyphae (Ectomycorrhizal hyphae), obtained in rhizoboxes on thin soil layers enriched in P (+ P) or not (-P). No difference was recorded in any conditions. Values were normalized with housekeeping gene (*HcTub*). Data are means \pm standard deviation (n = 4).

Recently, we showed that transcript levels of *HcPT1.2*, the third H^+ :Pi transporter from *H. cylindrosporium*, were much lower in free-living mycelia and ectomycorrhizas than those of *HcPT1.1* and *HcPT2*, but we did not describe further this candidate.^{6,8} Here, we revealed its function, expression, and localization in the fungus in axenic and symbiotic conditions. Although we were not able to fully characterize *HcPT1.2* because we did not use radio-labeled Pi, we have clearly demonstrated that *HcPT1.2* proteins are able to partially complement the *PHO84* deficient yeast strain MB192. This indicates that *HcPT1.2* can participate in Pi acquisition in the ECM fungus *H. cylindrosporium*. We also did not observe a Pi-dependent regulation of *HcPT1.2* expression and protein localization. This feature set *HcPT1.2* apart from other H^+ :Pi fungal transporters characterized so far. Indeed, *HcPT1.1* and *HcPT2* transcripts are up-regulated by Pi limiting or sufficient conditions, respectively,^{4,5} and *BePT*, *RIPT*, and

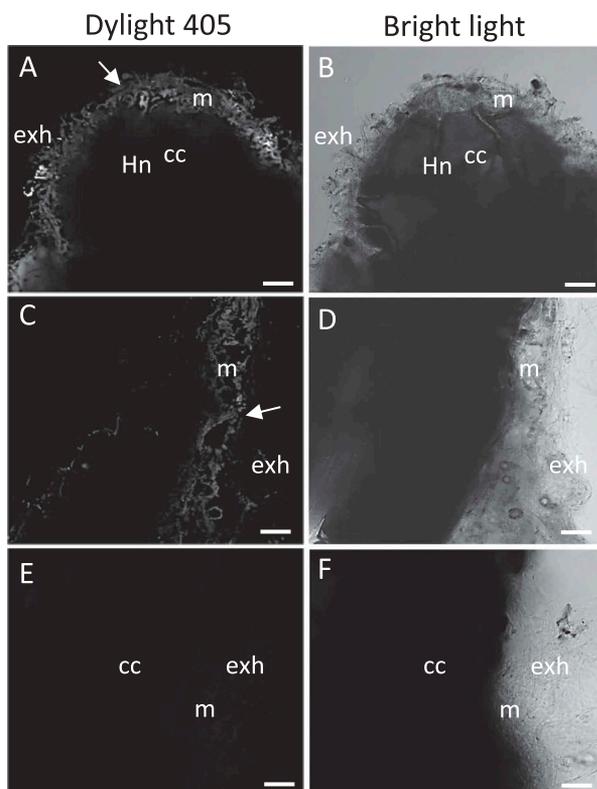


Figure 3. Immunolocalization of HcPT1.2 in *Hebeloma cylindrosporum* hyphae forming ectomycorrhizas with *Pinus pinaster* under P-sufficient and -limiting conditions. Ectomycorrhizas were obtained in rhizoboxes on thin soil layers enriched in P (a,b) or not (c,d). Cross-sections labelled with secondary antibodies only show no signal (E). Images were obtained by confocal microscopy on 50 μm cross-sections labelled with HcPT1.2 primary antibodies and Dylight 405-conjugated secondary antibodies. m, hyphal mantle; exh, extra-radical hyphae; Hn, Hartig net; cc, cortical cell.

LbPT transcripts of *Boletus edulis*, *Rhizopogon luteolus*, and *Leucocortinarius bulbiger*, respectively, are up-regulated at low Pi.^{13,14} The absence of Pi-dependent regulation supposes that *HcPT1.2* is more versatile than *HcPT1.1* and *HcPT2*, and even if its expression is low it may play a critical role for a basal and stable Pi acquisition, particularly under fluctuating environmental conditions. However, we cannot exclude that P availability may impact the activity of HcPT1.2 proteins through post-translational modifications as described in plants and yeasts,^{15,16} and further experiments are still necessary to investigate this question. Localization of HcPT1.2 exclusively at the uptake sites of ectomycorrhizas (i.e. extra-radical hyphae and mantle) is consistent with its possible role in Pi acquisition from the soil. Once again, the absence of protein detection in the Hartig net sets HcPT1.2 apart from HcPT1.1 and HcPT2 since both of them localize in all hyphae of ectomycorrhizas.^{5,6} The generation of transgenic *H. cylindrosporum* strains up- or down-regulating the expression of candidate genes is a powerful approach to unravel their function in axenic and symbiotic conditions,^{6,17–20} and will help us to validate the Pi import role of HcPT1.2 in ectomycorrhizal symbiosis.

To conclude, although we have now started to investigate all H⁺:Pi transporters identified in *H. cylindrosporum*, important paths still remain unexplored. For example, efforts are now

needed to unravel their shared and specific regulations by external P or unknown molecules originated from the host,^{6,21} the biosynthesis, movement, and degradation of polyphosphates,²² and the other putative candidates that might be involved in Pi and organic P transport we recently identified, e.g. HcPHO1 and HcPho90.⁶ Understanding the molecular mechanisms evolved by ECM fungi for P acquisition, transport, and delivery to the host is crucial to harness this symbiosis for a better and more sustainable production of crop trees.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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