Potassium nutrition of ectomycorrhizal Pinus pinaster: overexpression of the Hebeloma cylindrosporum HcTrk1 transporter affects the translocation of both K\(^+\) and phosphorus in the host plant

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Summary

- Mycorrhizal associations are known to improve the hydro-mineral nutrition of their host plants. However, the importance of mycorrhizal symbiosis for plant potassium nutrition has so far been poorly studied. We therefore investigated the impact of the ectomycorrhizal fungus *Hebeloma cylindrosporum* on the potassium nutrition of *Pinus pinaster* and examined the involvement of the fungal potassium transporter HcTrk1.
- *HcTrk1* transcripts and proteins were localized in ectomycorrhizas using *in situ* hybridization and EGFP translational fusion constructs. Importantly, an overexpression strategy was performed on a *H. cylindrosporum* endogenous gene in order to dissect the role of this transporter.
- The potassium nutrition of mycorrhizal pine plants was significantly improved under potassium-limiting conditions. Fungal strains overexpressing *HcTrk1* reduced the translocation of potassium and phosphorus from the roots to the shoots of inoculated plants in mycorrhizal experiments. Furthermore, expression of *HcTrk1* and the phosphate transporter *HcPT1.1* were reciprocally linked to the external inorganic phosphate and potassium availability.
- The development of these approaches provides a deeper insight into the role of ectomycorrhizal symbiosis on host plant *K*\(^+\) nutrition and in particular, the *K*\(^+\) transporter HcTrk1. The work augments our knowledge of the link between potassium and phosphorus nutrition via the mycorrhizal pathway.

Introduction

Ectomycorrhizal (ECM) symbiosis is a mutual association between fungal symbionts and woody plant roots. Although only 3–5% of higher plants are able to form ECM associations, this symbiosis is strongly represented in northern forests and plays a crucial role in the equilibrium of forest ecosystems (Smith & Read, 2008). The fungal symbiont provides nutrients and water in exchange for photosynthetically-derived carbohydrates from the plant (Nehls et al., 2010). These underground ‘trades’ are situated in a specialized plant–fungus organ called the ectomycorrhiza, located at the interface between the root cortical cells and the fungal hyphae that form the Hartig net.

The external fungal hyphae produce two main structures, the fungal sheath and the extra-radical mycelium. The fungal sheath surrounding the root forms a buffer compartment between the inner root and external environment and is thought to act as a storage compartment for nutrients (Bücking et al., 2007). This tissue is described in *Pisolithus tinctorius* ECM as a low permeability zone for certain ions such as potassium (Bücking et al., 2002). Extra-radical hyphae explore a large volume of soil, taking up the nutrients and water that are otherwise inaccessible to the host plant, translocating them to the inner root hyphae.

Studies investigating the nutrient exchanges in ECM symbiosis have focused primarily on nitrogen (Wallenda & Read, 1999; Müller et al., 2007) and inorganic phosphate (Pi) (Plassard & Dell, 2010). However, other nutrients are also traded, including potassium, sulfate and microelements (Casieri et al., 2013). In higher plants, potassium (K\(^+\)) is the most abundant cation in the cytoplasm where it is vital for metabolic processes (Clarkson & Hanson, 1980; Leigh & Wynn Jones, 1984) and plays major roles in numerous other physiological functions such as neutralization of negative charges, osmoregulation, growth, or hydric and salinity stress tolerance (Broadley & White, 2005). Within the plant, K\(^+\) transport has been well studied at both the physiological and molecular levels (Lebadea et al., 2007; Hedrich, 2012; Wang &
Potassium uptake by the mycorrhizal pathway has been demonstrated for both ECM and arbuscular mycorrhizal (AM) symbiosis (Marschner & Dell, 1994). For ECM fungi, the beneficial effects on net plant K+ uptake have been shown (Ryigiewicz & Bledsoe, 1984; Jongbloed et al., 1991). Jentschke et al. (2001) reported that the ectomycorrhizal pathway provides only a small fraction (5–6%) of the total K+ in Norway spruce seedlings. However, this contribution could be important under the limiting conditions that frequently occur in natural forest habitats. Recent studies have highlighted the impact of ECM and AM fungi on plant K+ nutrition in relation to increased salt tolerance in poplar (Li et al., 2012) and Olea europaea (Porras-Soriano et al., 2009), and better adaptation to drought stress in Zea mays (El-Mesbah et al., 2012). Nonetheless, any substantial molecular data regarding the fungal K+ transport systems that could be involved in the K+ nutrition of host plants are lacking.

Relationships between K+ and phosphorus (P) in mycorrhizal symbiosis are also poorly studied, even though K+ is considered as one of the major counter-ions of the soluble short-chain polyphosphates (Bücking & Heyser, 1999), which forms a phosphate reserve for the host plant in AM symbiosis (Takanishi et al., 2009). Related to the co-localization of K+ and P in the vacuoles of ECM basidiomycota such as Pisolithus tinctorius (Ashford et al., 1999), K+ could be a key element in forming and stabilizing polyphosphates in vacuoles.

In this study, we examined the contribution of the ECM symbiosis to K+ nutrition in the host plant Pinus pinaster and investigated the involvement of a fungal K+ transport system, HcTrk1. HcTrk1 belongs to the Trk/Ktr/HKT transporter family (Corratgé-Faillie et al., 2010; Vieira-Pires et al., 2013), and was earlier identified in an EST library of the model fungus Hebeloma cylindrosorum (Lambilliotte et al., 2004). This transporter is known to complement yeast Δtrak1-Δtrak2 mutants defective in K+ uptake. Its electrophysiological characterization in Xenopus laevis oocytes has shown it to be a Na+–K+ transporter (Corratgé et al., 2007). To dissect the role of this transporter within the fungus and the ectomycorrhizal symbiosis, we investigated the expression of HcTrk1, the localization of the encoded protein in ectomycorrhiza and its involvement in K+ nutrition of the host plant Pinus pinaster using an overexpression strategy. Finally, we examined the consequence of impaired K+ translocation due to HcTrk1 overexpression for plant P nutrition.

Materials and Methods

Wild-type and transgenic fungal strains

The homokaryotic strain h7 of the ECM basidiomycota Hebeloma cylindrosorum Romagnesi (Deboud & Gay, 1987) was grown in the dark at 26°C in standard medium YMG (Yeast extract, Malt extract, Glucose; Rao & Niederpruem, 1969) either on agar-solidified Petri dishes or in liquid cultures without shaking.

For Agrotransformations of the strain h7 (Combier et al., 2003), thalli growing in liquid medium were ground in 80 ml of YMG using an ultra-turrax (IKA, http://www.ika.com/), and transferred to Petri dishes (90 mm diameter). For 1 month, successive grindings were made until a new thallus was formed in 1 d that was suitable for transformation. The Agrotransformation method, mitotic stability test and the production of the control fungal strain pPZP-133 (transformed by the empty vector) have been detailed in previous work (García et al., 2013). Using the same protocol, translational fusion and overexpressing fungal lines, which are carboxin resistant, were obtained for HcTrk1 studies.

Potassium, sodium and phosphorus shortage in fungal pure cultures

Wild-type and transgenic fungal strains were cultivated for 14 d in control N6 liquid medium (Louche et al., 2010) containing 10 mM K+ (6 mM KNO3, 4 mM KCl) with fresh N6 supplied at days 7 and 12. Thalli were washed five times with N6-K, N6-Na or N6-P media (N6 medium without added K+, Na+ or P, respectively), cultivated in these media and periodically sampled for quantification of their intracellular ion content and for qRT-PCR analyses. In some experiments, following the deprivation treatment, they were re-supplied with complete N6 medium before sampling for qRT-PCR experiments.

Ectomycorrhiza production

Maritime pine seeds (Pinus pinaster Soland in Ait. from Medoc, Landes-Sore-VG source, France) were sterilized with 37% H2O2 (Ali et al., 2009) and sown on Petri dishes containing agarose (Eurobio Molecular Biology Grade) and 0.2% glucose. Germinated seeds were co-cultivated with plugs of fungal strains in a glass tube between filter paper and the wall of the tube (Supporting Information Fig. S1). Standard K+ (SK, 1 mM K+) liquid medium (0.2 mM Ca(NO3)2, 0.6 mM KNO3, 0.2 mM KH2PO4, 1 mM MgSO4, 0.2 mM KCl, 0.5 ml l−1 ferric citrate 1%, 0.2 ml l−1 Morizet & Mingeau (1976) micronutrient solution, 1 ml l−1 thiamine (100 lg l−1)) was used in these plant–fungus cultures for in situ hybridization and protein localization in ectomycorrhizas. For functional analyses with HcTrk1 overexpressing fungal strains, SK and low K+ (LK, 0.05 mM K+) (0.5 mM Ca(NO3)2, 0.2 mM NaH2PO4, 1 mM MgSO4, 0.05 mM KCl, 0.5 ml l−1 ferric citrate 1%, 0.2 ml l−1 Morizet & Mingeau (1976) micronutrient solution, 1 ml l−1 thiamine (100 lg l−1)) liquid media were used for K+-sufficient and K+-deficient culture conditions, respectively. For each experiment, four to five 2-month-old plants were collected for quantification of K+, Na+ and P in roots and shoots.

In situ hybridization

Sense and antisense probes (300 bp) were generated by two successive PCR amplifications of HcTrk1 cDNA contained in the pFL61 vector. The first amplification was prepared with a primer containing the T7 promoter fused to the cDNA of HcTrk1.
ISHTrk–T7–F, and the specific ISHTrk-R primer (Table S1) to specifically select the \( HcTrk1 \) cDNA. For the second amplification, ISHT7-Prom and specific Trk-R primers (Table S1) were used with a 1 : 100 dilution of the first PCR. Preparation of hybridization probes and ectomycorrhizal samples from test tube cultures and in situ hybridization followed the protocol previously described by García et al. (2013).

**Construction of plasmids for localization and functional analyses of \( HcTrk1 \)**

For localization of \( HcTrk1 \) proteins, a translational fusion vector was constructed, allowing expression of \( HcTrk \) fused to EGFP under control of the \( HcTrk1 \) promoter region. Sequences of \( HcTrk1 \) without its STOP codon and of its promoter region (\( PTrk1 \)) were amplified from cDNA and from a gDNA library of \( h1 \) strain using PromTrk1-Trk1-F/FTTrk1-Spel-R and Prom-Trk1-Spel-F/PromTrk1-Trk1-R primers, respectively (Table S1). The two amplified fragments were fused using the PCR fusion strategy and replaced the \( Pgpd \) promoter of pPZP-Pgpd-E plasmid (García et al., 2013), to form the pPZP-PTrk1-Trk1::E vector.

In order to overexpress \( HcTrk1 \) in transformed fungi, a vector pPZP-OETrk1 was constructed. The cDNA sequence of \( HcTrk1 \) (2376 bp) was amplified using Trk1-SpeI-F and OETrk1-SpeI-R primers (Table S1) and inserted in the pPZP-Pgpd vector.

**Protein localization using confocal microscopy**

Localization of \( HcTrk1 \) proteins was analyzed in ectomycorrhizas produced using fungal strains transformed with the pPZP-PTrk1-Trk1::E vector. EGFP fluorescence pictures were acquired using a confocal microscope (Axiovert 200M Zeiss LSM 510 Meta, with a C-Apochromat 40×/1.2 objective, Montpellier RIO Imaging Platform). Mycorrhizas were embedded in 4% agarose and sliced (50 µm) with a vibratome (Microm HM650V; Thermo Scientific, Waltham, MA, USA). To specifically mark the chitin, cut tissues were incubated in a Petri dish containing 1× PBS buffer and Wheat Germ Agglutinin (WGA), conjugated with 5 µg ml\(^{-1}\) Alexa Fluor® 555 (Life Technologies, Carlsbad, CA, USA) for one night under constant conditions of shaking, then washed three times with 1× PBS buffer. Detection of EGFP and Alexa Fluor® 555 was performed using an argon laser with 488 and 555 nm excitation wavelengths, respectively. The fluorescence emission was observed using an adapted spectral filter (band-pass 505–530 and 530–600 nm, respectively). Image analyses were carried out with Zeiss LSM Image Browser v4.2,0,121 software.

**Quantification of potassium, sodium and phosphorus contents**

Mycelia and plant tissue samples were weighed, dried (65°C for 1 wk) and weighed again (dry weight, DW). Dried samples were mineralized in 6 N HCl at 110°C for 16 h, centrifuged and supernatants collected. Appropriate dilutions were made to determine the K\(^+\) and Na\(^+\) contents using flame atomic absorption spectrophotometry (SpectrAA 220; Agilent Technologies, Santa Clara, CA, USA). Total phosphorus contents were determined using the Malachite green method (Ohno & Zibilske, 1991). Samples were incubated in 1.25 M sodium acetate (1:6 v/v) before dilution in water for colorimetric assay. Quantifications were made using an ELx 808 microplate photometer (DIALAB, http://dialab.at) at 630 nm and the software Gen5 (BioTek, www.biotek.fr).

**qRT-PCR analyses**

qRT-PCR experiments were performed as previously described (Garcia et al., 2013). Expression levels of the transporter and channel genes \( HcTrk1 \) (445173), \( HcTrk2 \) (176376), \( HcHAK \) (435192), \( HcPT1.1 \) (446637), \( HcSKC \) (79961), \( HcTOK1 \) (31571), \( HcTOK2.1 \) (129509) and \( HcTOK2.2 \) (127201) were determined relative to the internal control \( \alpha\)-tubulin (24108) on mycelium samples (see Table S1 for primers). Numbers represent protein IDs from the \( H. \) clynderosporum \( h7 \) genome database v2.0 (http://genome.jgi-psf.org/Hebcy2/Hebcy2.home.html).

**Results**

**Ectomycorrhizal association with Hebeloma clynderosporum improves the potassium nutrition of Pinus pinaster under K\(^+\)-limiting conditions**

In order to evaluate the influence of K\(^+\) supply on the growth of \( P. \) pinaster and to investigate the impact of ECM symbiosis on K\(^+\) uptake by the host plant, nonmycorrhized and mycorrhized plants were cultivated in two different culture conditions. Growth and the K\(^+\) content of the plants reflecting their K\(^+\) uptake were compared after 2 months in either K\(^+\) sufficient conditions (SK) or K\(^+\) deficiency conditions (LK). Pine plants were co-cultivated with \( H. \) clynderosporum \( h7 \) wild-type strain or with the strain transformed with an empty vector.

The biomass of both the nonmycorrhized and mycorrhized plants subjected to LK conditions was reduced compared to plants cultivated in the higher K\(^+\) medium (Fig. 1a), a visual observation that was confirmed by the whole plant DW measurements (Fig. 1b). Between nonmycorrhized and mycorrhized plants, no significant difference was observed in either culture conditions after 2 months.

When grown in SK medium, no significant difference in K\(^+\) contents between mycorrhized and noninoculated plants was observed (Fig. 1c). By contrast, in LK medium, the mycorrhized plants contained significantly higher K\(^+\) contents in both the roots (37%) and shoots (32%) than the noninoculated plants (Fig. 1d). Thus, ECM colonization strongly improved the plant K\(^+\) nutrition in conditions of low external K\(^+\).

**Localization of the \( HcTrk1 \) transporter transcripts and proteins in ectomycorrhizas**

In order to determine the localization of the potassium transporter \( HcTrk1 \) (Corratgé et al., 2007) within ectomycorrhizas,
**in situ** hybridization and translational fusion approaches were used to detect, respectively, the relevant transcripts and proteins. Ectomycorrhizas were produced by co-culturing *H. cylindrosporum* and *P. pinaster* for 2 months in SK medium (the expression of *HcTrk1* did not depend on external K⁺ or Na⁺ concentrations (Fig. S2)). For **in situ** hybridization, cross-sections of ectomycorrhizas were treated and marked with antisense and sense rRNA 18S and *HcTrk1*-specific probes (Fig. 2). Control sense probes displayed no signal (Fig. 2a,c). Blue signals were detected in extraradical hyphae, the fungal mantle and the Hartig net when the *HcTrk1* antisense probe was used (Fig. 2b). The control rRNA 18S antisense probes marked all cell types within the ectomycorrhizas (Fig. 2d).

A translational fusion strategy using EGFP as a reporter was applied to localize *HcTrk1* proteins within the ectomycorrhizas. Representative pictures of two independent lines (isolates 3 and 8) are shown (Fig. 3). Mycorrhizal association was first verified by fungal chitin staining (Fig. 3, second panels). Strong WGA-Alexa-555 signals were observed in the cross-sections of all mycorrhizas, formed by either the empty vector fungal transformant (Fig. 3a) or by each of the two strains carrying the translational fusion construct (Fig. 3b,c). EGFP fluorescence was observed in the fungal sheath and, to a lesser extent, in the extraradical hyphae of mycorrhized plant roots with fungi transformed with *HcTrk1*-EGFP constructs (Fig. 3b,c, third and fourth panels). No fluorescence signal was detected within the Hartig net or in the mycorrhizal tissues produced with the empty vector fungal transformant (Fig. 3a, third panel).

**Production of HcTrk1 overexpression lines of Hebeloma cylindrosporum**

In order to dissect the role of the K⁺ transporter *HcTrk1* within the symbiotic association, *H. cylindrosporum* transgenic lines displaying increased levels of *HcTrk1* transcripts were created (Fig. 4). From the OE-Trk1 fungal lines obtained, two lines with the highest *HcTrk1* expression — OE-Trk1-7 and OE-Trk1-9 — were selected for further analyses (Fig. 4a) and compared to the empty vector control line, which behaved like the wild-type h7 strain (data not shown). T-DNA integration of the two selected lines was verified using PCR on gDNA (Fig. 4b).

**Phenotype analyses of HcTrk1 overexpressing lines of Hebeloma cylindrosporum**

The consequences of *HcTrk1* overexpression on fungal cell growth (biomass) and mycelium ion contents were investigated using lines OE-Trk1-7 and OE-Trk1-9. In addition, qRT-PCR analyses performed on these lines revealed slight changes in the expression levels of other putative K⁺ transporters (*HcTrk2* and...
**Fig. 2** Expression of HcTrk1 transcripts in ectomycorrhiza. *In situ* hybridization on mycorrhizas from 2-month-old co-cultures of *Pinus pinaster–Hebeloma cylindrosporum* with HcTrk1-specific (a, b) and rRNA 18S probes (c, d). Control sense probes did not show any signals (a, c). HcTrk1 transcripts were detected in extraradical hyphae, in the fungal sheath and in the Hartig net (b). Blue signals were observed in all cells using control antisense rRNA 18S probes (d). Bars, 100 µm. exh, extraradical hyphae; Hn, Hartig net; sh, sheath.

**Fig. 3** Localization of HcTrk1 proteins in external hyphae of the ectomycorrhiza formed by Hebeloma cylindrosporum and Pinus pinaster. *P. pinaster* and Hebeloma cylindrosporum transgenic lines were co-cultivated for 2 months. (a) Empty vector fungal strain. (b, c) Two independent transgenic strains expressing HcTrk1-EGFP translational fusion proteins (isolate 3 (b) and 8 (c)). First lane, bright field microscopy. Second lane, wheat germ agglutinin (WGA) conjugated with Alexa Fluor® 555 fluorophore, used to identify the structures of the fungal partner. Third lane, EGFP signals observed in fungal transformants with the HcTrk1-EGFP fusion proteins (b, c) in sheath and weakly in soil exploratory hyphae (arrowheads). Fourth lane, WGA-Alexa 555–EGFP merged pictures. Magnification: × 40 (a); ×20 (b, c). exh, extraradical hyphae; Hn, Hartig net; sh, sheath.

HcHAK) and channels (HcTOK1, HcTOK2.1, HcTOK2.2 and HcSKG; Fig. S3).

Biomass production was measured on fungal pure cultures grown for 2 wk in liquid medium (K⁺ 10 mM). Strains overexpressing HcTrk1 displayed a lower DW production than the control lines (Fig. 5a). Ion contents were monitored in mycelia transferred into medium without added K⁺ (Fig. 5b,c). Before the transfer, the OE-Trk1-7 and OE-Trk1-9 strains...
displayed K⁺ contents similar to those of the control lines. Culturing in the absence of K⁺ resulted in decreased K⁺ contents in both types of strains. After 48 h of K⁺ deprivation, K⁺ contents were, however, significantly higher in the OE-Trk1-7 and OE-Trk1-9 strains (Fig. 5b), indicating that HcTrk1 overexpression resulted in improved K⁺ 'retention' probably due to re-uptake from the medium. By contrast, no difference in Na⁺ accumulation was detected between the fungal lines despite a general Na⁺ increase in response to K⁺ deprivation (Fig. 5c).

Potassium and sodium status of plants mycorrhized with HcTrk1 overexpressing fungal lines

In order to study the impact of HcTrk1 on the functioning of the symbiotic association, the control and HcTrk1 overexpressing fungal lines were co-cultivated with maritime pine seedlings for 2 months under standard (SK) or low K⁺ (LK) conditions. Under SK conditions, no significant differences in K⁺ concentration were observed in roots or shoots between nonmycorrhized and mycorrhizized plants (Fig. 6a). In LK conditions, all mycorrhized plants accumulated a greater amount of K⁺ in the roots than the nonmycorrhized plants (Fig. 6b). Higher K⁺ contents were also observed in shoots of plants mycorrhized with the two control lines. Interestingly, plants mycorrhized with the two HcTrk1 overexpressing transformants accumulated less K⁺ in shoots than other mycorrhized plants, despite their higher K⁺ content in roots.

Much larger Na⁺ contents were found in all plants grown in LK conditions compared to SK conditions (Fig. 6c,d). By contrast, no significant difference in Na⁺ contents was observed between nonmycorrhized and mycorrhizized plants, indicating no impact of the HcTrk1 overexpression on Na⁺ uptake and distribution.

Phosphorus contents of Pinus pinaster seedlings mycorrhized with HcTrk1 overexpressing fungal lines

Total P content assays revealed no significant differences between mycorrhized and nonmycorrhized plants when grown in SK conditions (Fig. 7a). In LK conditions, mycorrhized and nonmycorrhized plants displayed an increased total P content in roots and shoots compared to SK conditions. Remarkably, mycorrhization with the HcTrk1 overexpressing fungal lines OE-Trk1-7 and OE-Trk1-9 led to lower shoot P contents compared to...
control plants (Fig. 7b) despite similar P contents in roots. In addition, qRT-PCR analyses of gene expression in fungal mycelia growing in Pi-free or K\(^+\)-free medium (Fig. S4) revealed that Pi deprivation for 48 h resulted in increased expression of the K\(^+\) transporter gene \(HcTrk1\) and K\(^+\) deprivation resulted in increased expression of the previously identified Pi transporter \(HcPT1.1\) (Tatry et al., 2009; Garcia et al., 2013).

**Discussion**

Hebeloma cylindrosporum improves potassium uptake in *Pinus pinaster* subjected to low potassium availability

Growth of young pine seedlings was significantly reduced after culturing in K\(^+\)-limiting conditions for 2 months (Fig. 1). This K\(^+\) shortage dramatically decreased the K\(^+\) content in all parts of the plants, but was compensated by an increase in Na\(^+\). The maintenance of plant fitness during this period implies that *Pinus pinaster* seedlings are able to tolerate K\(^+\)-limiting conditions, confirming the capacity of this species to adapt to various environmental conditions as already shown for drought (Eilmann & Rigling, 2012) or frost (Korhonen et al., 2013).

The symbiotic association of young pine seedlings with *H. cylindrosporum* improved the K\(^+\) nutrition of mycorrhized plants under K\(^+\)-limiting conditions, although no obvious effect on host plant growth was observed during the early stages of co-culture. A remarkable increase in the K\(^+\) content of roots and shoots of c. 35% was observed under these culture conditions. This demonstrates the importance of the *H. cylindrosporum* fungus in plant adaptation to resource scarcity. This increase in K\(^+\) content by mycorrhization is much higher than the reported 5–6% found in other nutrient conditions and in another symbiotic association (Jentschke et al., 2001). Clearly, the increase of K\(^+\) uptake in mycorrhized plants can be expected to play a more important role in plant nutrition when the nutrient is limited, as in most natural forest ecosystems.

The localization of the \(HcTrk1\) transporter suggests a role in potassium uptake in *Pinus pinaster–Hebeloma cylindrosporum* mycorrhizas

The potassium transporter \(HcTrk1\) has been previously identified and characterized as a first candidate for K\(^+\) (and Na\(^+\)) uptake by the ECM fungus *H. cylindrosporum* (Corratgé et al., 2013).
Overexpression of HcTrk1 affects the translocation of K\(^+\) from roots to shoots in Pinus pinaster

Overexpression of genes in \(H.\ \text{cylindrosporum}\) has previously been used to express constitutively GFP markers (Müller et al., 2006; Rekangalt et al., 2007; Garcia et al., 2013) and to investigate the function of a metallotheinein of \(P.\ \text{involutus}\) (Bellion et al., 2007). To our knowledge, we present the first example of an overexpression strategy aiming at altering the expression of an endogenous gene in an ECM fungus. When grown in pure culture conditions, \(HcTrk1\) overexpressing lines have a superior ability to adapt to \(K^+\)-limiting conditions than the control strains. The use of these overexpressing fungal lines in mycorrhizal assays revealed a defective translocation of \(K^+\) from roots to shoots in a \(K^+\)-deficient medium. Thus, \(K^+\) appears to be retained in root tissues, perhaps due to competition for \(K^+\) uptake at the fungus–plant interface at the expense of mutualism. Overexpression of a \(K^+\) uptake transport system in all types of hyphae, particularly in the Hartig net where \(HcTrk1\) proteins are normally undetectable, could lead to a less ‘cooperative’ fungus that does not sufficiently transfer the \(K^+\) accumulated from the soil to the host plant when \(K^+\) is limited. The fungal \(K^+\) released into the apoplast could be recovered by the fungus, and a pool of \(K^+\) would be retained in roots and not transferred to the shoot. Consequently, artificial ectopic upregulation of the \(HcTrk1\) transporter of \(H.\ \text{cylindrosporum}\) could not improve the global host plant \(K^+\) nutrition.

The overexpression of a transport protein able to mediate \(K^+\) efflux (secretion) towards the plant root cortical cells within the Hartig net would be a worthwhile challenge. In this perspective, dissection of the role of the complete set of fungal \(K^+\) transport systems would provide an integrated view of all the transport systems involved in \(K^+\) uptake and transfer to the host plant. The recent availability of the \(H.\ \text{cylindrosporum}\) genome (http://genome.igi-psf.org/Hebcy2/Hebcy2.home.html) does allow the completion of the initial set of transport systems putatively involved in \(K^+\) homeostasis (\(HcTrk1\) and \(HcSKC\)) by identifying further candidates, namely two \(K^+\) transporters, \(HcTrk2\) and \(HcHA\), and three \(K^+\) channels, \(HcTOK1\), \(HcTOK2.1\) and \(HcTOK2.2\). Specifically, regarding \(K^+\) absorption from the soil, analysis of \(HcHA\) and \(HcTrk2\), in addition to that of \(HcTrk1\), should highlight the individual contribution of each \(K^+\) uptake system under diverse conditions, as performed in \(S.\ \text{occidentalis}\) (Banuelos et al., 2000) or \(N.\ \text{crassa}\) (Rivetta et al., 2013). The overexpression and RNAi strategies, possible thanks to the \(H.\ \text{cylindrosporum}\) Agrotransformation, combined with gene expression and protein localization approaches (this study; Garcia et al., 2013), provide a promising ‘tool-box’ for further investigating the protein actors involved in nutrient exchange in ECM symbiosis.

The alteration of the translocation of potassium affects that of phosphorus in pine

Total \(P\) quantification in mycorrhized pine seedlings led to unexpected results. Plants mycorrhized with the \(HcTrk1\) overexpressing strains contained less \(P\) in the shoots than did the control plants. This defect might result from the retention of \(K^+\) in roots. Previous reports (Ashford et al., 1999) have proposed \(K^+\) as one of the major counter-ions of polyphosphates, the main storage form of \(P\) in fungi. Moreover, Bücking & Heyser (1999) have hypothesized that a relationship exists between \(K^+\) and \(P\) transport along the mycelium of ECM fungi. Our results, revealing that altered \(K^+\) transfer towards the host plant affects fungal \(P\) translocation to the shoots, are consistent with this hypothesis. In addition, in the wild-type strain of \(H.\ \text{cylindrosporum}\), \(Pi\) deficiency led to an increase in \(HcTrk1\) expression and, reciprocally, \(K^+\) deficiency triggered an upregulation of the \(Pi\) transporter gene \(HcPT1.1\) (Tatry et al., 2009; Garcia et al., 2013). Such cross responses of \(HcTrk1\) and \(HcPT1.1\) gene expression to \(Pi\) and \(K^+\) deprivation reinforces the hypothesis of interactions between these two nutrients regarding their membrane transport and transfer to the host plant in ECM symbiosis. Thus, \(K^+\) seems to play a role as a regulator of \(P\) transport. Clearly, the functions of \(K^+\) in mycorrhizal symbiosis and the roles of the transport systems responsible for \(K^+\) uptake and translocation towards the host plants deserve to be further investigated.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 In vitro method of co-culture of Pinus pinaster host plant and Hebeloma cylindrosporum ectomycorrhizal fungus.

Fig. S2 Quantification of the expression level of the potassium transporter HcTrk1 under K⁺ and Na⁺ deprivation in Hebeloma cylindrosporum h7.

Fig. S3 Expression of putative potassium transport systems of Hebeloma cylindrosporum in HcTrk1 overexpressing fungal lines.

Fig. S4 Quantification of the expression level of the potassium transporter HcTrk1 and phosphate transporter HcPT1.1 under Pi and K⁺ deprivation, respectively, in Hebeloma cylindrosporum h7.

Table S1 Primer list

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