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SHORT COMMUNICATION

*Hc*TOK1 participates in the maintenance of K^+ homeostasis in the ectomycorrhizal fungus *Hebeloma cylindrosporum*, which is essential for the symbiotic K^+ nutrition of *Pinus pinaster*

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ABSTRACT

Most land plants rely on root symbioses to complement or improve their mineral nutrition. Recent researches have put forward that mycorrhizal fungi efficiently absorb and transfer potassium (K^+) from the soil to host plant roots, but the molecular mechanisms involved are not completely elucidated yet. We have recently revealed that K^+ is likely released from the fungal Hartig net to the plant by TOK channels in the ectomycorrhizal model *Hebeloma cylindrosporum – Pinus pinaster*. *H. cylindrosporum* harbours three TOK members. Herein, we report that one of them, *Hc*TOK1, has similar features than the yeast *Sc*TOK1. Moreover, we propose a role for this channel in the transport of K^+ from the medium to ectomycorrhizal roots under K^+ starvation.

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Text

Potassium (K^+) is one of the main macronutrients for plants, but its low availability often hampers the absorption by roots.[1] Plants can face this constraint by establishing mycorrhizal symbioses, in which the fungus overcomes the lack of K^+ with efficient soil exploration and specialized transport systems at both uptake and release sites of symbiotic structures.[2–4] In ectomycorrhizal symbiosis, the fungus differentiates three pseudo-tissues called extra-radical hyphae involved in nutrient acquisition, mantle isolating the roots from the soil, and Hartig net where trophic exchanges take place with plant epidermal and cortical cells.[5] In the symbiotic model between the ectomycorrhizal fungus *Hebeloma cylindrosporum* and the host plant *Pinus pinaster*, the Na^+-K^+ symporter *Hc*Trk1 acts as an uptake mechanism in extra-radical and mantle hyphae,[6,7] whereas secretion of K^+ to colonized roots is still barely known. Excitingly, we have recently described three tandem-pore outward-rectifying K^+ (TOK) channels of *H. cylindrosporum*,[8] of which at least one, *Hc*TOK2.2, is likely to transfer K^+ from Hartig net hyphae to the symbiotic interface. Another channel, named *Hc*TOK1, is phylogenetically close to the only member from *Saccharomyces cerevisiae, Sc*TOK1, and shows similarities in its transport properties.[8] In the present study, we describe the subcellular localization of *Hc*TOK1 and provide additional information about its possible role in symbiotic transport of K^+ within ectomycorrhizas under K^+-limiting conditions.

The *S. cerevisiae* strain ply232 was transformed with the pFL61 plasmid[9] containing a *Hc*TOK1-eGFP fusion, which led to a strong fluorescence signal at the plasma membrane (Figure 1). To study the K^+ homeostasis of *H. cylindrosporum* when this outward-rectifying channel was overexpressed, two transgenic lines transformed with *Hc*TOK1-OE constructs already described in Guerrero-Galán et al.[8] along with two control lines (wild type h7 and empty pPZP-133 vector CT) were cultured in a K^+-rich medium (N6, described in Louche et al., 2010).[10] After two weeks, fungal mycelia were transferred to the same medium but without K^+ (N6-K, described in Garcia et al., 2014)[7] and sampled 0, 12, 24 and 48 hours later. Tissues were dried, weighted and mineralized in 0.1 N HCl for 24 h before measuring the K^+ and Na^+ contents by atomic absorption spectrophotometry (SpectrAA 220 FS, Varian). The *Hc*TOK1-OE2 line, which showed the strongest overexpression of *HcTOK1*,[8] displayed a higher K^+ accumulation than the two control lines (Figure 2), but no change in Na^+ contents (data not shown). The same lines were tested in symbiosis with *Pinus pinaster*, in comparison to non-inoculated plants as an additional control (Figure 3). Seedlings were cultured *in vitro* for two months before harvest in low K^+ medium and analyzed as described in Garcia et al.[7] Inoculation increased the K^+ nutrition of all the seedlings, compared to non-mycorrhizal controls. Moreover, plants inoculated with the lines overexpressing *HcTOK1* accumulated more K^+ in shoots but not in roots, indicating that translocation of K^+ was increased in these plants (Figure 3). On the contrary, the Na^+ contents of seedlings did not change (data not shown). The overexpression of *HcTOK1* did not affect the biomass of fungal cultures, nor that of the inoculated seedlings, compared to the corresponding controls (data not shown).

The channel *Hc*TOK1 is unambiguously localized at the plasma membrane, as previously suggested by results obtained by yeast complementation and two-electrode voltage-clamp in Guerrero-Galán et al.[8] The same distribution pattern has been

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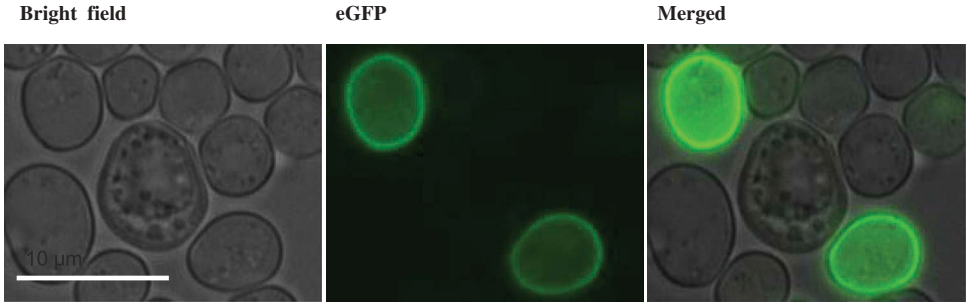

Figure 1. Subcellular localization of *Hc*TOK1 channels in *Saccharomyces cerevisiae*.
A fluorescence signal, obtained with *HcTOK1*-cDNA-eGFP fusions expressed in *Saccharomyces cerevisiae*, is observed at the plasma membrane.

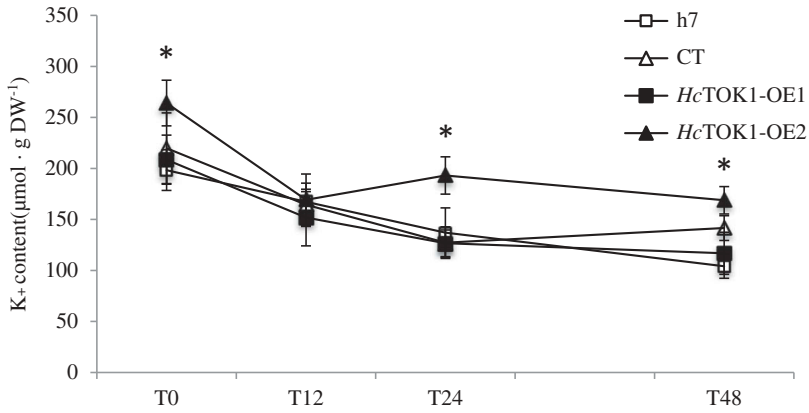

Figure 2. Potassium accumulation in *HcTOK1*-overexpressing lines of *Hebeloma cylindrosporum*.
Potassium contents of *Hebeloma cylindrosporum* h7 and empty pPZP133 (CT) controls and two lines overexpressing *HcTOK1* cDNA (*Hc*TOK1-OE1 and -OE2) in K^+ starvation kinetics. Mycelia were cultured 2 weeks in complete N6 medium and then transferred to an N6 medium without K^+. Samples were harvested at 0, 12, 24 and 48 hours (T0, T12, T24 and T48). Overexpression of *Hc*TOK1 induced an accumulation of K^+ in the fungus. *Significant difference compared to controls (Wilcoxon-Mann-Whitney test; $p < 0.05$). n = 6–9.

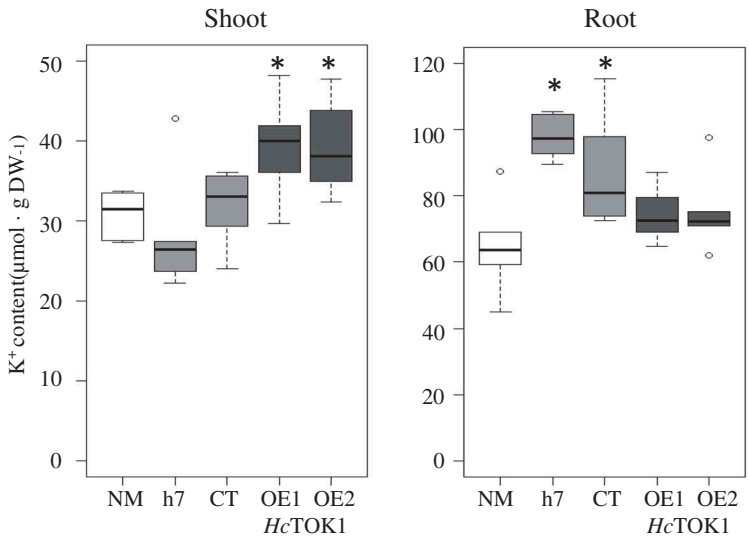

Figure 3. Distribution of potassium in *Pinus pinaster* shoots and roots colonized with *HcTOK1*-overexpressing fungal lines.
Distribution of K^+ contents in *Pinus pinaster* seedlings non-inoculated (NM) or inoculated with *Hebeloma cylindrosporum*, after 2 months of *in vitro* culture in K^+ starvation conditions (0.05 mM). Mycorrhizal plants were inoculated with h7 and CT controls and the two *Hc*TOK1-OE lines. In low K^+ conditions, plants inoculated with *H. cylindrosporum* displayed a greater K^+ content, particularly in roots. Overexpression of *Hc*TOK1 channel enhanced the transfer of K^+ to the shoot. *Significant difference compared to NM controls (Wilcoxon-Mann-Whitney test; $p < 0.05$). n = 5–6.

found for *Sc*TOK1.[11] *HcTOK1* is expressed at very low levels in *H. cylindrosporum*, compared to the other two channels of the family, *HcTOK2.1* and *HcTOK2.2*.[8] The two overexpressing lines chosen for this study, *Hc*TOK1-OE1 and -OE2, show different levels of induction (40X and 100X, respectively), and the one with the strongest activity induced

an accumulation of K^+ in the fungus. Even though this result may seem contradictory with the already described role of *Hc*TOK1 as an outward-rectifying channel,[8] it actually fits well in the description of the yeast *Sc*TOK1 channel. Indeed, Maresova et al.[11] demonstrated that overexpressing *Sc*TOK1 hyperpolarized the plasma membrane. In this situation, even when the K^+ concentration gradient is strong, the activity of the channel would be blocked, as its activation relies on depolarization. Moreover, under hyperpolarizing membrane potentials, *Hc*TOK1, as *Sc*TOK1,[12,13] shows a slight inward current, that would be responsible for higher K^+ contents in *Hc*TOK1-OE2 mycelium compared to control strains.[8] This behavior was conserved along the K^+-starvation kinetic assay, in which the overexpression of *HcTOK1* did not imply a faster loss of K^+. The fact that Na^+ contents did not vary in the fungus support the idea that *Hc*TOK1 is selective for K^+.

In vitro inoculation of *P. pinaster* with *Hc*TOK1-OE lines resulted in a higher K^+ content in shoots, compared to inoculated and non-inoculated controls. Two hypotheses could explain this result. First, K^+ accumulation in the *Hc*TOK1-OE2 line (and presumably, also in *Hc*TOK1-OE1) would imply that plants are directly interacting with a greater "source" of K^+ than plants mycorrhized with control lines when cultured under K^+ starvation. In this case, the root would act itself as a "sink", maintaining a stronger medium-fungus-plant flux. Second, *Hc*TOK1 is overexpressed in all fungal hyphae types which could lead to a dual role: K^+ accumulates in extra-radical hyphae and its transfer towards plant roots through the Hartig net increases.

In conclusion, we revealed that *Hc*TOK1 is localized at the plasma membrane, shows similar physiological properties than *Sc*TOK1, and is probably a key regulator of K^+ homeostasis in the fungus at the cellular level.[14] Furthermore, its role as uptake channel, albeit not strong, might be complementary to that of putative high affinity K^+ transporters (unpublished results). For now, the study of TOK channels has led to a detailed description of the TOK1 subfamily, well conserved in all fungi. It will be interesting to focus future researches in analyzing members of the TOK2 subfamily, described in our recent publication, which presents new functional properties and could have unexpected roles not only in the physiology of the fungus, but also in ectomycorrhizal symbiosis.[8]

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed

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