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Plant potassium nutrition in ectomycorrhizal symbiosis: properties and roles of the three fungal TOK potassium channels in *Hebeloma cylindrosporum*

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Running title: TOK potassium channels of an ectomycorrhizal fungus

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Originality - Significance Statement

Nutrient transfer in ectomycorrhizal symbiosis requires specialized and bidirectional transport, ensuring uptake of mineral nutrients from the soil by fungal hyphae and secretion towards the plant root. Potassium nutrition has been shown to be improved in ectomycorrhizal plants but the molecular players involved are not yet completely uncovered. Tandem-pore outward-rectifying potassium (TOK) channels that are described in yeast to mediate K^+ efflux have been found specifically in fungi. Here, TOK channels are reported for the first time in an ectomycorrhizal fungus, and represent good candidates to be involved in symbiotic potassium transfer. *Hebeloma cylindrosporum* harbors three TOK channels belonging to two subfamilies with distinct properties and roles.

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Summary

Ectomycorrhizal fungi play an essential role in the ecology of boreal and temperate forests through the improvement of tree mineral nutrition. Potassium (K^+) is an essential nutrient for plants and is needed in high amounts. We recently demonstrated that the ectomycorrhizal fungus *Hebeloma cylindrosporum* improves the K^+ nutrition of *Pinus pinaster* under shortage conditions. Part of the transport systems involved in K^+ uptake by the fungus has been deciphered, while the molecular players responsible for the transfer of this cation towards the plant remain totally unknown. Analysis of the genome of *H. cylindrosporum* revealed the presence of three putative tandem-pore outward-rectifying K^+ (TOK) channels that could contribute to this transfer. Here we report the functional characterization of these three channels through two-electrode voltage-clamp experiments in oocytes and yeast complementation assays. The expression pattern and physiological role of these channels were analyzed in symbiotic interaction with *P. pinaster*. Pine seedlings colonized by fungal transformants overexpressing two of them displayed a larger accumulation of K^+ in shoots. This study revealed that TOK channels have distinctive properties and functions in axenic and symbiotic conditions, and suggested that *Hc*TOK2.2 is implicated in the symbiotic transfer of K^+ from the fungus towards the plant.

Introduction

Potassium (K^+) is the most abundant inorganic cation in plant cells, and participates in many processes, including regulation of osmotic pressure, energization of the plasma membrane and resistance to abiotic and biotic stresses (Wang *et al*., 2013; Benito *et al.*, 2014). Although it is one of the most important elements in the Earth crust, its availability for plant roots is limited due to its strong adsorption to soil particles (Schroeder, 1978). Plants overcome the difficulty to acquire K^+ through various strategies, such as expression of high-affinity transport systems

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and efficient storage capabilities (Kochian and Lucas, 1989; Anschütz *et al*., 2014) as well as interaction with soil microorganisms (Garcia and Zimmermann, 2014).

The mycorrhizal symbiosis is a mutualistic interaction occurring between the roots of most land plants and hyphae of soil-borne fungi. This intimate relationship results in the formation of new structures in plant roots, the mycorrhizae, responsible for nutrient exchanges between both partners (Smith and Read, 2008). Among the different types of mycorrhizal associations, the ectomycorrhizal symbiosis is a dominant component of temperate and boreal forests (Fransson *et al*., 2000). Ectomycorrhizal fungi represent up to 34% of all fungal biodiversity in the soil, where they play major roles in the functioning of these ecosystems (Courty *et al*., 2010; Tedersoo *et al*., 2010). Ectomycorrhizal fungi improve plant water and mineral nutrition, whereas the host provides the fungus with photosynthates. Upon colonization, fungal hyphae penetrate the roots and form a specific structure between plant cortical cells, called the Hartig net. The Hartig net is responsible for nutrient and water exchanges between both partners through the specific expression of a wide range of transport proteins (Casieri *et al.*, 2013; Garcia *et al.*, 2016). Characterization of fungal transport systems active at uptake or release sites in soil-exploring hyphae or at the interface between plant and fungus is a crucial prerequisite for understanding of these biological fluxes. Although phosphorus and nitrogen are the main macronutrients shuttled by ectomycorrhizal fungi from the soil toward plant roots in many ecosystems (Smith and Read, 2008), recent studies have also highlighted a significant improvement of K^+ acquisition in colonized plants (Jentschke *et al.*, 2001; Garcia *et al*., 2014; Garcia and Zimmermann, 2014).

Maritime pine (*Pinus pinaster* Ait.) is a forest-forming tree in Southern Europe typically found in coastal regions where it can tolerate unfavourable environments. Furthermore, *P. pinaster* has a high economic value due to its exploitation as a softwood and resin source (Maaf-IGN, 2016). The ectomycorrhizal fungus *Hebeloma cylindrosporum*

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(Basidiomycota) interacts with a broad range of woody plant species, including its natural host *P. pinaster* (Marmeisse *et al.*, 2004). Both the plant and the fungus are well adapted to acidic sandy soils, like those present along the European Atlantic coast, an environment often suffering from salinity and nutrient deprivation (Lousteau *et al.*, 1995; Gryta *et al.*, 1997; Barbéro *et al.*, 1998). This tolerance is probably due to efficient nutrient absorption, transfer and storage abilities provided by the mutualistic association.

Molecular insights into the ectomycorrhizal fungus *H. cylindrosporum* became facilitated by the generation of an expressed sequence tag library (Lambilliotte *et al.*, 2004), and more recently the sequencing of its whole genome (Kohler *et al.*, 2015) and transcriptomic analyses (Doré *et al.*, 2015, 2017). This collection of data enabled the identification of putative channels and transporters that might be involved in K^+ transfer from the soil to the plant through ectomycorrhizal structures (Garcia and Zimmermann, 2014). We recently described one of these K^+ transporters, *Hc*Trk1, which contributes to the uptake of K^+ from the soil (Corratgé *et al.*, 2007; Garcia *et al.*, 2014). In this study, we aim at functionally characterizing three tandem-pore outward-rectifying K^+ (TOK) channels from *H. cylindrosporum*, and investigating their role in ectomycorrhizal symbiosis.

The TOK channel family is specific to fungi. The first TOK channel was originally identified in yeast, *Sc*TOK1, and functionally characterized by heterologous expression in *Xenopus laevis* oocytes, which revealed outward-rectifying K^+-selective transport properties (Ketchum *et al.*, 1995; Lesage *et al.*, 1996; Reid *et al.*, 1996). Previous studies in yeast had reported a depolarization-dependent outward current that was strongly selective for K^+ (Gustin *et al.*, 1986; Bertl *et al.*, 1993). Later on, TOK channels were found in filamentous fungi, including *Neurospora crassa* (Roberts, 2003), and in several other yeasts (Ramos *et al.*, 2011). TOK polypeptides typically harbor eight transmembrane domains and two pore regions. The functional channel is expected to display a dimeric structure, where the four pore

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regions assemble in the centre of the protein to give rise to the channel pore representing the ion permeation pathway (Baev *et al.*, 2003; Roberts, 2003; Prole and Taylor, 2012). This structure suggests that TOK channels probably evolved from the fusion of two K^+ channels, a Shaker-like voltage-dependent subunit harboring six transmembrane segments and a subunit with two transmembrane segments like the inward rectifiers (Lesage *et al*., 1996; Bertl *et al*., 1998; Saldaña *et al.*, 2002) or the bacterial KcsA channel (Doyle *et al*., 1998). However, in contrast to Shaker-like channels, no voltage-sensor could be identified in TOK channels (Ketchum *et al.*, 1995; Lesage *et al*., 1996).

Two subfamilies of TOK channels have been found in the genome of many ectomycorrhizal fungi, like in *H. cylindrosporum*, but not in the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (Garcia and Zimmermann, 2014). Given the fact that their homologues in yeast and *N. crassa* behave as outward-rectifying K^+ channels allowing K^+ efflux (Ketchum *et al.*, 1995; Lesage *et al*., 1996; Roberts, 2003; Ariño *et al*., 2010), it was tempting to assume that members from these subfamilies in ectomycorrhizal fungi could contribute to K^+ secretion from the fungal Hartig net into the plant apoplasm. Here, we describe three TOK channels from the ectomycorrhizal fungus *H. cylindrosporum*. Their sequences have been analyzed *in silico* and they have been functionally characterized by heterologous expression and voltage-clamp experiments in oocytes, and complementation of a yeast mutant strain defective in K^+ transport systems. We also analyzed their expression patterns in ectomycorrhizae by *in situ* hybridization. Their role in axenic and symbiotic conditions was studied by the phenotyping of transgenic fungal lines overexpressing these proteins.

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Results

In silico analysis of TOK channels from the ectomycorrhizal fungus *H. cylindrosporum*

Putative *TOK* channel genes belonging to two subfamilies have been identified in the genome of ectomycorrhizal fungi (Garcia and Zimmermann, 2014). In *H. cylindrosporum*, the TOK family comprises three members, named *Hc*TOK1, *Hc*TOK2.1 and *Hc*TOK2.2. Their *in silico* predicted secondary structure highlighted a major difference between *Hc*TOK1 and the two *Hc*TOK2-type channels in the intracellular domain that connects the two parts of the protein harboring each one pore domain, the Shaker-like moiety (six transmembrane segments) and the inward rectifier-like moiety (two transmembrane segments) (Fig. 1A). This cytosolic loop is about 150 amino acids longer in *Hc*TOK2.1 and *Hc*TOK2.2 compared to *Hc*TOK1. In contrast, *Hc*TOK2.1 and *Hc*TOK2.2 harbored a shorter C-terminal domain than *Hc*TOK1 resulting in a similar total length for all three proteins (around 780 amino acids). Comparison of protein sequences between *H. cylindrosporum*, *S. cerevisiae* and *N. crassa* revealed that *Hc*TOK2.1 and *Hc*TOK2.2 share about 30 % of similarity with *Sc*TOK1 and *Nc*TOKA (Fig. 1B). *Hc*TOK1 shares the same level of similarity, ca. 30%, with *Sc*TOK1, as well as with *Hc*TOK2.1 and *Hc*TOK2.2, but a higher level of similarity, ca. 42%, with *Nc*TOKA. Also, the two pore sequences display differences between species, particularly in the hallmark motif of K^+-selective channels, TxGYGD, which is not strictly conserved (Fig. 1C). The pore P1 is the most variable because the position corresponding to the core tyrosine residue is occupied by a leucine in *Hc*TOK1, like in *Sc*TOK1, or by a phenylalanine in *Hc*TOK2.1 and *Hc*TOK2.2, like in *Nc*TOKA. Concerning the second pore, the P2 sequence of *Hc*TOK1 is more similar to that of *Sc*TOK1or *Nc*TOKA than to that of *Hc*TOK2.1 or *Hc*TOK2.2.

Putative TOK channels were found in basal fungi belonging to the Neocallimastigomycota, Blastocladiomycota, Chytridiomycota, Zoopagomycota and Mucoromycota phyla, as well as in Basidiomycota and Ascomycota, but not in the

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Glomeromycotina *Rhizophagus irregularis* (Fig. 2; Garcia and Zimmermann, 2014). The phylogenetic analysis of the TOK sequences divides them into three main clades: an ancestral clade containing basal fungi, a TOK1 subfamily present only in Ascomycota and Basidiomycota species, and a Basidiomycota-specific clade to which the newly-described TOK2 subfamily belongs (Fig. 2). In summary, this analysis enlarges our previous phylogenetic studies (Garcia and Zimmermann, 2014) and indicates that *Hc*TOK2.1 and *Hc*TOK2.2 are members of a subfamily of TOK proteins that had not been described in fungi thus far.

_Hc_TOK1 and _Hc_TOK2.1 exhibit an outward-rectifying channel activity in _Xenopus laevis_ oocytes

In order to figure out whether the *Hc*TOK1, -2.1 and -2.2 channels are able to transport K^+, their activity was investigated by two-electrode voltage-clamp experiments in *X. laevis* oocytes. Different membrane potentials were imposed to the oocytes (Fig. 3A) and the corresponding currents were recorded. First, oocytes were tested in 1 mM external K^+ at pH 6.5. Ion channel activity could be detected in oocytes expressing *Hc*TOK1 (Fig. 3C) or *Hc*TOK2.1 (Fig. 3D), where it gave rise to outwardly directed currents upon membrane depolarization, but not in oocytes injected with *Hc*TOK2.2 cRNA (Fig. 3E), which displayed no difference in comparison with the control oocytes (Fig. 3B). Both channels displayed an instantaneous and a time-dependent activation as observed for the yeast channel (Lesage *et al*., 1996). However, the time dependent component of activation differed remarkably, the current activation being slower for *Hc*TOK2.1 than for *Hc*TOK1. Steady-state current-voltage curves (I-V) indicated much larger current amplitudes in *Hc*TOK1- than in *Hc*TOK2.1-expressing oocytes in these conditions (Fig. 3F, Fig. S1).

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To further characterize the activity of *Hc*TOK1 and *Hc*TOK2.1, bath solutions containing either 1, 10 or 100 mM K^+ at pH 6.5, or three pH conditions (pH 5.5, 6.5 or 7.5) in 1 mM K^+ were used. These analyses were also performed with *Hc*TOK2.2, but currents different from control oocytes were never recorded (Fig. S1). To summarize, *Hc*TOK1 and *Hc*TOK2.1 behaved as outward-rectifying channels whose currents were enhanced by low external K^+ concentrations shifting activation to more negative potentials (Fig. 3G and H). A slight inward current was observed for *Hc*TOK1 at hyperpolarizing potentials (Fig. 3G) as previously observed for *Nc*TOKA and *Sc*TOK1 (Roberts, 2003; Roller *et al.*, 2008). The reversal potential shifted with the decrease of external K^+ concentrations towards more negative potentials as detectable in I-V curves for *Hc*TOK1 (Fig. 3G) indicating selectivity for K^+. However, tail current analyses revealed that this shift did not follow completely the K^+ Nernst potential, especially at 1 mM K^+ (Fig. S2 A and B), suggesting either permeability to other ions such as Na^+ which replaced external K^+ in this experiment or a reduced sensitivity of K^+ dependent gating at low K^+ as observed for *Nc*TOK (Roberts, 2003). Nevertheless, replacing 100 mM KCl in the bath solution by 100 mM NaCl allowed the observation of large K^+ outward currents as predicted for a reduced external K^+ concentration (Fig. S2 C and D). Concerning *Hc*TOK1, no effect of external pH was observed (Fig. 3I), similarly to *Sc*TOK1 (Lesage *et al*., 1996). In contrast, *Hc*TOK2.1 was regulated by the external pH, the outward currents being increased by acidification of the external solution from 7.5 to 6.5 and 5.5 (Fig. 3J, Fig. S1). Altogether, these results revealed that *Hc*TOK1 displays functional properties reminiscent of the yeast outward-rectifying K^+ channel *Sc*TOK1, and that *Hc*TOK2 behaves also as an outward-rectifying channel but with distinct functional features, at least in terms of pH sensitivity, kinetics and rectification capacity.

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Growth complementation of yeast mutants lacking K^+ transport systems

The *S. cerevisiae* strain PLY246 defective for K^+ transport was transformed with the empty pYES2 plasmid as control, or with pYES2 constructs containing either the *HcTOK1*, *HcTOK2.1*, *HcTOK2.2* or *ScTOK1* coding sequences (the corresponding yeast transformants being named PLY246-pYES2, PLY246-*Hc*TOK1, PLY246-*Hc*TOK2.1, PLY246-*Hc*TOK2.2 or PLY246-*Sc*TOK1). These yeast strains were tested for functional growth complementation by serial drop assays (Fig. 4). The PLY246 strain transformed with the control vector (PLY246-pYES2) was able to grow at high (50 mM) but not at low (10 mM) K^+, confirming its defective phenotype due to the lack of K^+ transport systems (Bertl *et al*., 2003). Cells transformed with the positive control vector containing *ScTOK1* (PLY246-*Sc*TOK1) were able to grow on both media. Yeast cells transformed with the *Hc*TOK1 channel (PLY246-*Hc*TOK1) were also able to grow at 10 mM K^+, although at a rate lower than that of the PLY246-*Sc*TOK1 cells. Such a capacity of *Hc*TOK1 to partially complement the defect in K^+ uptake of the PLY246 strain, indicating actually K^+ influx into the cells, is consistent with the fact that this transporter can mediate small inward currents observed at hyperpolarizing voltages in oocytes (Fig. 3G). On the contrary, *Hc*TOK2.1 did not complement the PLY246 mutant strain, indicating a lack of absorption activity. This result was also consistent with the voltage-clamp recordings highlighting a strict outward rectification capacity of *Hc*TOK2.1 (Fig. 3H). Interestingly, yeast cells transformed with the vector containing *Hc*TOK2.2 were able to grow at 10 mM K^+, and displayed a growth rate on this medium similar to that of the positive control PLY246-*Sc*TOK1 (Fig. 4). This result indicates that *Hc*TOK2.2 encodes a functional polypeptide endowed with K^+ transport activity allowing K^+ influx across the yeast cell membrane.

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The three *TOK* genes from *H. cylindrosporum* have different expression patterns in pine ectomycorrhizae

Recently, transcriptional studies in axenic and symbiotic conditions revealed changes in the expression of *H. cylindrosporum* genes (Doré *et al*., 2015, 2017). Based on these results, the expression levels of the three *HcTOK* channel genes were compared between both conditions (Fig. S3). *HcTOK1* and *HcTOK2.1* expression levels did not undergo major changes. Strikingly, *HcTOK2.2* was more than 30 times induced upon establishment of the symbiotic interaction, suggesting a specific role in ectomycorrhizae.

The spatial expression patterns of the three *HcTOK* genes were investigated in ectomycorrhizae. *In situ* hybridization experiments were performed on colonized *P. pinaster* roots using either *sense* or *antisense* ribosomal probes as negative and positive controls, respectively, or probes for the three TOK channels (Fig. 5). As expected, concerning the control probes, signals (blue coloration) were observed in samples treated with the *antisense* ribosomal probe, but not with the *sense* one (Fig. 5A and B). Similarly, concerning the three TOK channels, hybridization with the *antisense* probes gave rise to signals (Fig. 5C, E and G), while using the *sense* probes did not (Fig. 5D, F and H). The signals indicated that *HcTOK1* was highly expressed in extra-radical hyphae, the sheath and the Hartig net (Fig. 5C). In contrast, *HcTOK2.1* was barely expressed and only inside the root (Fig. 5E). Finally, the signals resulting from hybridization with the *HcTOK2.2 antisense* probe were most apparent in the Hartig net, faint in the fungal sheath surrounding the root, and not detectable in extra-radical hyphae (Fig. 5G).

Overall, the expression studies indicate that *HcTOK2.2* was strongly induced and displayed the highest expression level, amongst the three *HcTOK* channel genes, when the fungus engaged in ectomycorrhizal symbiosis. The corresponding *HcTOK2.2* transcripts were

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specifically present in hyphae internally colonizing the root, within the Hartig net, suggesting a function at this interface between fungus and plant.

Analysis of symbiotic *in vitro* co-cultures of *Pinus pinaster* and *HcTOK* overexpression lines

Aiming at analyzing the physiological role of the three TOK channels from *H. cylindrosporum*, we generated transgenic overexpressing fungal lines. Among the lines produced, the two displaying the highest overexpression (OE) levels were selected for each gene after real-time PCR screenings (Fig. S4). These transgenic fungal lines were tested in symbiosis with *P. pinaster* (Fig. 6 A) along with the corresponding wild-type line (h7) and a transgenic line transformed with the empty pPZP133 vector (CT, control). Prior to the harvest, the remaining liquid medium from each tube was collected. The K^+ concentration in the culture medium was decreased in six days from 1 mM right after the last watering to approximately 100 to 150 µM in six days (Fig. 6 B).

The total amounts of K^+ assayed in the plants (roots and shoots) were higher in the plants inoculated with the TOK1-OE2, the line in which the transforming construct resulted in a very high increase in expression level, and in the plants inoculated with the TOK2.1-OE3 and TOK2.2-OE2 and -4 lines, than in the control plants (Fig. 6 C). Expressed on a dry weight basis, the major differences in K^+ contents were displayed by the shoots rather than by the roots in seedlings colonized by TOK2.1-OE3 and TOK2.2-OE2 and -4 lines (Fig. 6D). Finally, when grown in axenic fungal cultures, only the TOK1-OE2 line contained significantly more K^+ than the control lines h7 and pPZP133 (Fig. S5). These results suggest that seedlings inoculated with TOK1-OE2 accumulated more K^+ because of a higher K^+ content in the fungus, whereas the effect observed in the seedlings colonized by TOK2.1-OE3 and TOK2.2-OE2 and -4 lines was due to an enhanced efflux of K^+ towards the plant.

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Discussion

The TOK channel family represented in the ectomycorrhizal fungus *H. cylindrosporum* by three distinct members

Members of the fungal-specific TOK channel family are present in most of the Basidiomycota and Ascomycota species that have been sequenced so far, and also in more basal taxa, including Zygomycota, Neocallimastigomycota and Chytridiomycota (Garcia and Zimmermann, 2014; Fig. 2). Three *TOK* channel genes were found in the genome of the ectomycorrhizal fungus *H. cylindrosporum*. The two recently identified TOK-subfamilies, TOK1 and TOK2, are represented in this fungus (Garcia and Zimmermann, 2014; this study), the first one by a single member, *Hc*TOK1, and the second one by two members, *Hc*TOK2.1 and *Hc*TOK2.2. The present *in silico* analyses reveal different structural features between these two subfamilies. The different position of the largest intracellular loop (Fig. 1), placed at the extreme C-terminus downstream of the P2 in *Hc*TOK1, and between P1 and P2 in *Hc*TOK2 proteins, might modify the functional properties or regulation of the channels.

One particularity of TOK channels is the variation of their K^+-filter signature in the pore domains compared to the canonical TxGYGD sequence. More specifically, the central tyrosine of the P1 pore, a residue that has been shown to play a major role in the structure and selectivity for K^+ in K^+-selective Shaker and KcsA-type channels (Doyle *et al.*, 1998) is replaced by a leucine or a phenylalanine. In the three members of the two subfamilies in *H. cylindrosporum*, the leucine is found in *Hc*TOK1 and the phenylalanine in both *Hc*TOK2 channels. Roller *et al*. (2008) analyzed the significance of such changes by mutating either the leucine of P1 into a tyrosine or the tyrosine of P2 into a leucine, or by introducing these two mutations at the same time. The ion transport activity of the channels was barely altered by any of these mutations, but changes in several parameters of the transport process were

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recorded, including opening speed and amplitude of inward currents. Unexpectedly, the effect of the mutation of the pore P2 yielded very slight changes in the properties of the channel.

The present results indicate that two from the three TOK channels from *H. cylindrosporum*, *Hc*TOK1 and *Hc*TOK2.1, can be expressed heterologously in a functional state in *X. laevis* oocytes, allowing electrophysiological analyses. The reasons why the activity of the third one, *Hc*TOK2.2, is undetectable in this expression system might be linked to *e.g.* inappropriate cytosolic conditions in oocytes or missing regulatory proteins. For instance, several plant K^+ channels from the Shaker family remain electrically silent when expressed in oocytes except when they are co-expressed with CBL-CIPK phosphorylating partners (Xu *et al*., 2006; Cuéllar *et al*., 2013). Nonetheless, expression in mutant yeast cells defective for K^+ transport provides evidence that *Hc*TOK2.2 is permeable to K^+ (Fig. 5).

No member from the TOK2-type subfamily had been characterized so far. The analysis of *Hc*TOK2.1 in oocytes provides the first information on such channels. Overall, the functional properties of this channel, as outward rectification and selectivity for K^+, are reminiscent of those of its *H. cylindrosporum Hc*TOK1 relative, as well as of those of *Sc*TOK1 and *Nc*TOKA from *S. cerevisiae* and *N. crassa* (Ketchum *et al*., 1995; Bertl *et al*., 2003; Roberts, 2003). Even that all three *HcTOK* channels did not display pH-dependent expression levels (Fig. S6), the unique sensitivity of *Hc*TOK2.1 to external pH at the functional level could allow the fungal cells to adapt to pH changes in the soil, the fungal sheath and/or the Hartig net. Indeed, the currents induced by *Hc*TOK2.1 were stronger at pH 5.5 than 6.5 or 7.5, meaning that acidification of the external medium in a physiological range of pH values can enhance the activity of this channel. Moderately acid pH can be expected in forest soils and has been reported for the Landes forest (Jolivet *et al*., 2007). Interestingly, acidification and pH signaling has been described for ectomycorrhizal roots in *Eucalytus globulus-Pisolithus microcarpus* interaction (Ramos *et al*., 2009). Regulation of the TOK

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channel activity by pH as found here for *Hc*TOK2.1 might contribute to the adaptability of the fungus to specific environmental conditions.

Outward rectification was analyzed and controversially discussed for the *S. cerevisiae* channel TOK1. Two models for the possible gating mechanism have been proposed suggesting either an external (presence of other cations) or a protein-intrinsic voltage-dependent block (Ketchum *et al*., 1995; Lesage *et al*., 1996). Further analyses of this ion channel rectification would be interesting for the new fungal channels from *H. cylindrosporum*. Furthermore, distinct behavior found here for *Hc*TOK1 and *Hc*TOK2.1 with respect to the channel activation kinetics, pH dependence and outward rectification could allow addressing questions concerning the corresponding molecular bases and physiological meaning of these differences.

Towards the physiological role of TOK channels in ectomycorrhizal symbiosis

Different types of K^+ and/or Na^+ transport systems have been identified in yeasts, amongst which the TOK channels are considered as the sole systems dedicated to K^+-selective secretion (Ariño *et al*., 2010; Ramos *et al*., 2011). In yeasts, TOK channels have been shown to be involved in control of the cell membrane potentials and in apoptosis mechanisms by allowing K^+ efflux (Ramos *et al*., 2011). Although no more precise function has been described thus far, the conservation of the TOK family across the fungal kingdom provides evidence of its importance in fungal physiology (Bertl *et al*., 2003; Maresova *et al.*, 2006; Roller *et al*., 2008; Zahrádka and Sychrová, 2012).

Overexpression of *HcTOK* channel genes in *H. cylindrosporum* was poorly informative on the roles of the corresponding proteins in the free-living fungal mycelia grown in axenic cultures, except for the TOK1–OE2 line, regarding fungal K^+ content (Fig. S5). Intriguingly, the very strong overexpression of *HcTOK1* in the TOK1-OE2 cells resulted in an

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increase of fungal internal K^+ contents. Such an effect is consistent with the fact that the rectification provided by *Hc*TOK1 is not absolute, allowing this channel to mediate K^+ influx in oocytes (Fig. 3) as well as in yeast (Fig. 4). Another hypothesis would be that the strong overexpression of *HcTOK1* affects the cell membrane potential, shifting the potential towards more negative values and thereby increasing the driving force for K^+ uptake. It is worth to note that, in *S. cerevisiae*, *tok1* KO mutant yeast cells do not display significant changes in K^+ contents, K^+(Rb^+) uptake and K^+ efflux (Bertl *et al.*, 2003).

Here we have investigated the roles of *Hc*TOK channels in ectomycorrhizal symbiosis. The colonization of *P. pinaster* roots by the fungal lines overexpressing the *HcTOK* channel genes resulted in higher K^+ amounts in the plant, in one line out of two in the cases of *Hc*TOK1 and *Hc*TOK2.1, and in both lines in the case of *Hc*TOK2.2. Such an effect is consistent with the fact that TOK channels can mediate K^+ efflux, a property that can enable them, when ectopically overexpressed, to contribute to K^+ transfer towards the host plant. It is worth to note that the increase in the plant K^+ amounts appeared to be essentially due to increased K^+ contents in shoots (Fig. 6C & D). In other words, the K^+ translocation towards the shoots was stimulated in the plants colonized by the fungal strains overexpressing the *TOK* channel genes, suggesting an enhanced K^+ efflux from the fungus towards the plant.

_Hc_TOK2.2 is a promising candidate for K^+ movements in ectomycorrhizae

With respect to the fungal capacity to transfer K^+ to the host plant, the whole set of results supports the hypothesis that a single TOK channel is principally dedicated to this function, *Hc*TOK2.2. Indeed, *Hc*TOK2.2 was strongly and preferentially expressed in the Hartig net, and its expression was remarkably induced, by more than 30 times, in symbiotic condition compared to fungal pure culture. Furthermore, the colonization of pine roots by the fungal strains ectopically overexpressing *HcTOK2.2* led to a high accumulation of K^+ in the host

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plant, particularly in the aerial parts, suggesting an increased K^+ translocation from the mycorrhizal roots to the shoot. Bücking and Heyser (2000) reported that, in some conditions, K^+ can be retained by the hyphae of the Hartig net, without a transfer to the host cortical cells. Therefore, the effect we observed here could be due to a decrease of this K^+ retention in the mycelium due to the overexpression of *HcTOK2.2*.

In contrast, *HcTOK1* expression was detected in all hyphae forming the ectomycorrhizae, without any specific localization, and *HcTOK2.1* transcripts were barely labeled in our *in situ* hybridization experiments probably due to a reduced expression level within ectomycorrhizae (Fig. S3). Furthermore, the transcript levels of these two channels were not enhanced upon mycorrhizal association. Altogether, these results indicate that *Hc*TOK1 and *Hc*TOK2.1 are not essentially dedicated to K^+ secretion in the Hartig net towards the cortical cells of the host roots, but are involved in fungal K^+ homeostasis through the control of K^+ efflux and partly of K^+ influx, as already proposed for *Sc*TOK1 (Maresova *et al.*, 2006).

Conclusion

In summary, our results indicate that the three TOK channels from *H. cylindrosporum* display specific rather than redundant functions. *Hc*TOK1 is closer to the previously characterized *Sc*TOK1 and *Nc*TOKA than the other two channels and could play a role in the basal K^+ homeostasis of the fungus. Moreover, we described and characterized for the first time another subfamily of TOK channels. Both TOK2 members of *H. cylindrosporum* display original properties compared to *Hc*TOK1, which could help in investigating the structure-function relationships of these channels. The first member from the *H. cylindrosporum* TOK2 subfamily, *Hc*TOK2.1, is not likely to play a major role in K^+ secretion towards the host plant, but could provide a pH sensing mechanism in the regulation of K^+ homeostasis. In

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contrast, the strong induction of the second one, *Hc*TOK2.2, upon mycorrhizal symbiosis, its preferential localization in the Hartig net, and the fact that its overexpression results in higher accumulation of K^+ in colonized plants provide evidence for a role of this channel in K^+ secretion towards the host root in mycorrhizal symbiosis.

Experimental Procedures

Fungal materials and cultures

The *H. cylindrosporum* (Romagnesi) monokaryotic strain h7 used in our studies was isolated from the germination of a basidiospore by Debaud and Gay (1987). Fungal cultures were maintained on solid YMG medium (Rao and Niederpruem, 1969) at 26ºC in the dark.

The mycelia were grown on N6 medium (Louche *et al.*, 2010; 6 mM KNO_3, 3 mM NaH_2PO_4, 1 mM $MgSO_4$, 4 mM KCl, 0.5 mM $CaCl_2$, 0.04 mM iron (III) citrate, 0.3 µM thiamine HCl, 0.2 ml·l^{-1} Morizet & Mingeau (1976) microelements solution, 5 g·l^{-1} glucose, pH 5.5; Morizet & Mingeau (1976) microelements solution: 3.08 g·l^{-1} $MnSO_4·1H_2O$, 4.41 g·l^{-1} $ZnSO_4·7H_2O$, 2.82 g·l^{-1} H_3BO_3, 0.98 g·l^{-1} $CuSO_4·5H_2O$, 0.29 g·l^{-1} $Na_2MoO_4·2H_2O$). For pH-assays, culture medium pH was adjusted additionally to pH 4.5, 6.5, and 7.5 by HCl or $Ca(OH)_2$. Fungal transgenic lines were obtained by Agrotransformation using *Agrobacterium tumefaciens* LBA1126 as described by Ngari *et al.* (2009) and Garcia *et al.* (2013).

Ectomycorrhization assays

Plant seeds (*P. pinaster* Soland in Ait. From Medoc, Landes-Sore-VG source, France) were sterilized in 30% H_2O_2 for 15 min, rinsed with sterile water, hydrated at 4ºC for 48-72 h and germinated on solid medium (2 g l^{-1} glucose, 10 g l^{-1} agar) for two weeks. The device used for co-cultures was described by Plassard *et al.* (1994). Briefly, four implants of

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H. cylindrosporum solid culture and a pine seedling were placed in a glass tube between a Whatman paper (cat. No. 1542-240) and the tube wall. Six biological replicates were prepared per condition. Each tube was filled with 20 ml of N1 medium (0.2 mM $Ca(NO_3)_2$, 0.6 mM KNO_3, 0.2 mM KH_2PO_4, 1 mM $MgSO_4$, 0.2 mM KCl, 0.02 mM iron (III) citrate, 0.3 µM thiamine-HCl, complemented with 0.2 $ml \cdot l^{-1}$ Morizet and Mingeau (1976) microelements solution; pH 5.5). Plants were watered every week with a fresh solution, and harvested after 2 months. The remaining nutritive solution was measured after the harvest.

Cloning and *in silico* analyses

Total RNAs were extracted from 2-week old *H. cylindrosporum* axenic cultures grown in N6 medium by tissue grinding in liquid nitrogen, followed by a TRIzol® Reagent purification (Invitrogen, Carlsbad, CA, USA). RNAs were treated with DNAse I (Invitrogen), checked for integrity by electrophoresis in 1 % agarose gel, and retrotranscribed to cDNA using the Superscript III enzyme (Invitrogen). Primers with flanking SpeI restriction sites were designed to amplify the sequences coding for *Hc*TOK1 (Protein ID 31571), *Hc*TOK2.1 (Protein ID 129509) and *Hc*TOK2.2 (Protein ID 127201) (*H. cylindrosporum* h7 genome database v2.0; http://genome.jgi-psf.org/Hebcy2/Hebcy2.home.html; Table S1). A poly-A tail was added to the product of the previous reaction and the construct was inserted into a TOPO-TA cloning vector (Invitrogen). The *Hc*TOK coding sequences were amplified by PCR with primers with flanking HindIII (for *Hc*TOK1) or EcoRI (for *Hc*TOK2.1 and *Hc*TOK2.2) restriction sites and cloned in the Zero Blunt® vector (Invitrogen) (Table S1).

For the generation of *Hc*TOK1, *Hc*TOK2.1 and *Hc*TOK2.2 overexpressing (OE) fungal lines, three plasmids named pPZP-OE-TOK1 or -2.1 or -2.2 were constructed by digestion of the TOPO-TA-TOK1, -2.1 or -2.2 vectors using SpeI and ligation into the pPZP-Pgpd vector (Ngari *et al*., 2009; Garcia *et al*., 2013).

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In silico analysis of the secondary structure was performed with the online TMHMM software (Krogh *et al.*, 2001) and the predicted structures were represented with the Protter website tool (Omasits *et al.*, 2014). The phylogenetic analysis was done by choosing TOK protein sequences from representative members of the publicly available fungal tree (https://genome.jgi.doe.gov/programs/fungi/index.jsf; Grigoriev *et al.*, 2011 and 2014), aligning them using MUSCLE and Gblocks, building the tree with SeaView 4 (Gouy *et al.*, 2010), and calculating bootstrap values using the PhyML method (Guindon and Gascuel, 2003). The phylogenetic tree was created using iTOL tool.

In situ hybridization

Two PCR reactions on cDNA were performed to obtain 350 bp *sense* and *antisense* probes for the *in situ* detection of *HcTOK1*, *-2.1* and *-2.2* transcripts, as described by Garcia *et al.* (2014), using specific sets of primers (Table S2). PCR products were purified by ethanol precipitation and *in vitro* transcription was made using the MAXIscript® T7 Transcription Kit (Invitrogen) to synthetize digoxigenin-UTP-labelled RNA probes. The hybridization and detection of the probes were performed on 8 µm-thick sections of mycorrhizal roots fixed in paraffin as described by Garcia *et al.* (2014). Images were taken on a wide-field microscope Leica DM6000 (Leica Microsystems, Wetzler GmbH, Germany) and analyzed with the Volocity Acquisition 5.1.0 software (Perkin Elmer, Boston, MA, USA) and FiJi (Schindelin *et al.*, 2012).

Electrophysiological analysis

In order to investigate the functional properties of the *Hc*TOK1, -2.1 and -2.2 channels, the two-electrode voltage-clamp (TEVC) experiments were performed using *X. laevis* oocytes. The TOPO-TA plasmids containing the *Hc*TOK1, -2.1 or -2.2 coding sequences were

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digested with SpeI and the cDNA inserted in the pGEM*Xho* vector (derived from pGEMDG; D. Becker, Wu□rzburg), which was then linearized and transcribed *in vitro* with the mMessage mMachine® T7 Ultra kit (Invitrogen). Oocytes were injected with water for negative control or with either 10, 30 or 50 ng of the *in vitro* described cRNA of *HcTOK1*, -*2.1* or -*2.2* respectively, and incubated in ND96 medium (mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 2.5 sodium pyruvate; supplemented with 50 µg ml^{-1} gentamycin; pH 7,4) at 18ºC for 3 days before TEVC recordings.

The bath solutions that were percolated contained 2 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM HEPES-Tris, pH 6.5. They were supplemented with K^+ and Na^+ provided as gluconate salts, the concentration of K^+ being 1, 10 or 100 mM, and the sum of the concentrations of the two cations K^+ and Na^+ being 100 mM. The effect of the external pH on TOK channel activity was investigated with bath solutions at pH 5.5 (the HEPES buffer present in the other bath solutions being then substituted by MES buffer), 6.5 and 7.5. The TEVC protocol started at a holding potential of -60 mV and then imposed voltage pulses to the oocyte membrane from hyperpolarizing potentials (-100 mV or -60 mV) to depolarizing potentials (+80 mV) in steps of +10 mV. Voltage-clamp protocols, measurements and data analyses were performed using the pClamp10 software (Axon Instruments, Foster City, CA, USA).

Functional complementation in yeast

Saccharomyces cerevisiae strains PLY232 (WT) and PLY246 (*trk1*Δ*, trk2*Δ*, tok1*Δ) were used for yeast complementation assays (Bertl *et al*., 2003) using the pYES2 plasmid. Plasmids allowing expression of either *HcTOK1*, *HcTOK2.1* or *HcTok2.2* were obtained by the digesting the Zero Blunt®-TOK1, -2.1 or -2.2 vectors and ligation into pYES2. A pYES2 plasmid harboring *ScTOK1* cDNA from *S. cerevisiae* was used as positive control, and the empty pYES2 vector as negative control. Each of these constructs was transferred into

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PLY246 cells using the transformation method as described by Gietz and Schiestl (2007). PLY232 (WT) strain was transformed with the empty pYES2 for use as control. Functional complementation for K^+ uptake was tested by serial drop assays. Overnight cultures of yeast were rinsed with sterile water and diluted to an OD_{600} of 1. Three tenfold serial dilutions were prepared and 10 µl-drops were plated onto selective agar medium containing either 10 or 50 mM KCl, added in the arginine-phosphate medium described by Rodríguez-Navarro and Ramos (1984).

Real-time quantitative PCR analyses

Total RNAs were extracted from 2-week old cultures of wild-type and transgenic lines of *H. cylindrosporum* and transcribed to cDNA. Real-time PCRs were performed in the LightCycler® 480 Real-Time system (Roche, Mannheim, Germany) using a reaction mix containing 10 ng cDNA, 5 µl SYBR® Premix Ex Taq ™ II Tli RNaseH Plus (TaKaRa Bio Inc., Dalian, China) and 0.5 µM of each forward and reverse primer (Table S3). The amplification protocol had the following steps: 95ºC for 30 s, 45 cycles of [95ºC for 10 s, 60ºC for 10 s, 72ºC for 15 s] and cooling to 40ºC for 40 s. Two housekeeping genes were used for data normalization, α-tubulin and elongation factor 1α (Protein IDs 24108 and 439881; http://genome.jgi-psf.org/Hebcy2/Hebcy2.home.html) (Table S3). Data analyses were performed as described by Cuéllar *et al.* (2010).

Phenotyping of transgenic fungal lines in axenic and symbiotic conditions

Fungi were cultured in N6 liquid medium in axenic conditions for two weeks. Mycelia were then sampled, washed twice in water for 1 min, weighed, dried at 60ºC for 7 days and weighed again. K^+ ions were extracted from the mycelia by 0.1 N HCl (5 ml per sample) for 24 h. For the tests in symbiotic conditions, roots and shoots of 2-month old mycorrhizal pine

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seedlings were weighed and dried at 60ºC for 14 days. Acid extraction was performed with 5 ml of HCl 6 N at 110ºC for 16 h. K^+ contents in fungal and plant tissues were determined by atomic absorption spectrometry (SpectrAA 220 FS, Varian).

Statistical analyses

The statistical treatment of the data was performed with R Studio with default parameters (RStudio Team, 2015). Data were analyzed with the Kruskal-Wallis and Wilcoxon-Mann-Whitney tests.

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Table and Figure legends

Figure 1. Protein sequence analysis of *H. cylindrosporum* TOK channels. A. Secondary structure prediction of three TOK channels from *Hebeloma cylindrosporum*. Each channel harbors 8 transmembrane segments and 2 pore domains named P1 and P2. The canonical K^+ selectivity filter sequences between the transmembrane segments 5 - 6 and 7 - 8 (circled) are represented as inserted in the membrane. *Hc*TOK1 has a C-terminal cytosolic tail of 259 amino acids, while *Hc*TOK2.1 and *Hc*TOK2.2 contain a cytosolic region of 251 and 242 amino acids between the two pores. B. Protein similarity analysis. *Sc*TOK1 is equally distant to all three TOK from *H. cylindrosporum*, while *Nc*TOKA is closer to *Hc*TOK1 than *Hc*TOK2.1 and *Hc*TOK2.2. C. Comparison of the pore sequences. The sequence of the canonical selectivity filter of K^+-selective channels (TxGYGD) is highly conserved in the second (P_2) pore domain, whereas the tyrosine residue is substituted by a leucine or phenylalanine in the first pore domain (P_1) in all the *Hc*TOK channels.

Figure 2. Maximum Likelihood phylogenetic tree of fungal TOK channels. The branches with bootstrap support of over 500 (out of 1000 replicates) are indicated with grey dots. The radial tree was generated using iTOL v4.0.3 (http://itol.embl.de/) (Letunic and Bork, 2016). Basal fungi have an ancestral version of the TOK channels. Channels belonging to the TOK1 subfamily are highlighted with branches in green and channels belonging to the TOK2 subfamily with branches in magenta. Bold characters indicate the Basidiomycota branches. The TOK1 subfamily is present in Ascomycota and Basidiomycota fungi, whereas the TOK2 subfamily is specific of the latter clade. The list of species used is shown in Table S4. The three TOK channels of *H. cylindrosporum* are marked in red and other channels described previously are indicated in blue, namely *Sc*TOK1 (Ketchum *et al*., 1995), *Nc*TOKA (Roberts,

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2003), *Ca*TOK1 (Baev *et al*., 2003), as well as *Hw*TOK1A and *Hw*TOK2A (Plemenitaš *et al*., 2014). Units of branch length ("Tree Scale") are in amino acid substitutions per site.

Figure 3. Analysis of *Hc*TOK1, *Hc*TOK2.1 and *Hc*TOK2.2 K^+ transport activity by two-electrode voltage-clamp in *Xenopus laevis* oocytes. A. Protocol of voltage pulses imposed to the oocytes. B – E. Examples of currents obtained in 1 mM K^+ at pH 6.5 recorded in response to the voltage pulses in oocytes injected with water (B), *HcTOK1* (C), *HcTOK2.1* (D) or *HcTOK2.2* cRNA (E). F. Current-voltage (I-V) responses to the imposed membrane potentials in 1 mM K^+ at pH 6.5. Significant currents were observed in oocytes injected with *HcTOK1* or *HcTOK2.1* cRNA (n = 11 and n = 18, respectively). The currents observed in *HcTOK2.2*-injected oocytes (n = 9) were negligible and very similar to the control currents recorded in oocytes injected with water (n = 9). G and H. Dependence of the normalized *Hc*TOK1 (n = 14) and *Hc*TOK2.1 (n = 9) currents to the external K^+ concentration at pH 6.5. The normalization was performed using the current values recorded at +60 mV in the 1 mM K^+ bath solution. I and J. Dependence of the normalized *Hc*TOK1 (n = 10) and *Hc*TOK2.1 (n = 10) currents on the external pH in 1 mM K^+. Normalization was performed using the current values recorded at +60 mV in the pH 6.5 bath solution. The insert in J shows a representative example of *Hc*TOK2.1 current increase at pH 5.5.

Figure 4. Functional complementation of the potassium transport-defective yeast strain PLY246 (*trk1*Δ *trk2*Δ *tok1*Δ) in a serial-drop test on selective arginine-phosphate medium containing either 50 or 10 mM K^+. *Hc*TOK2.2 strongly complemented at 10 mM K^+ for the absence of K^+ absorption systems at the same level as the *Sc*TOK1 channel. The *Hc*TOK1-transformed yeast grew to a lesser extent. *Hc*TOK2.1 displayed no difference compared to the

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control strain PLY246-pYES2. PLY232-pYES2 was used as positive control for yeast growth.

Figure 5. *In situ* hybridization of *HcTOK1/2.1/2.2* transcripts in *P. pinaster* - *H. cylindrosporum* ectomycorrhizae. The left column (A, C, E, G) shows the hybridizations with *antisense* probes, and the right column (B, D, F, H) the controls with *sense* probes. Sections hybridized with the sense probe (negative control) did not show any significant signal. Cross-sections were hybridized with 18s ribosome probes (A and B), *HcTOK1* (C and D), *HcTOK2.1* (E and F), and *HcTOK2.2* (G and H) probes. The *HcTOK1* transcripts were detected in all hyphae of ectomycorrhizae (C). Transcripts from *HcTOK2.*1 were weakly detected in the Hartig net (E). *HcTOK2.2* transcripts were mostly present in the Hartig net, but a faint staining was also detected in the extra-radical hyphae (G). Arrows indicate the regions with detected signals for the three *TOK* channels. Magnification X20. exh, extra-radical hyphae; Hn, Hartig net; sh, sheath; cc, cortical cells.

Figure 6. Determination of the K^+ content in mycorrhizal pine seedlings. Plants were inoculated with wild-type (h7) and transgenic (pPZP133, represented as CT) control fungal lines and two isolates overexpressing *HcTOK1* (TOK1-OE1/2), *HcTOK2.*1 (TOK2.1-OE2/3) and *HcTOK2.2* (TOK2.2-OE2/4) and cultured for 2 months before harvesting. A. Example of the *in vitro* set-up used for symbiotic cultures of *Pinus pinaster* and *Hebeloma cylindrosporum*. B. K^+ concentration in the remaining nutritive solution of the seedlings 6 days after watering. C. Total amount of K^+ in the whole plants. The TOK1-OE2, TOK2.1-OE3 and both TOK2.2-OE transgenic lines contain more K^+ than the h7 and CT controls. D. Distribution of K^+ contents in the shoots and roots of the seedlings. Those mycorrhized with TOK2.1-OE3, TOK2.2-OE2 and TOK2.2-OE4 contain significantly more K^+ in the

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shoots, whereas the K^+ contents in the root remain constant. * Significant difference compared to the mycorrhized controls h7 and CT ($p < 0.05$). n = 5-6.

Table S1. Primers used for amplification and cloning of the three TOK channels from *Hebeloma cylindrosporum.*

Table S2. Primers used for *in situ* hybridization experiments on *Hebeloma cylindrosporum* – *Pinus pinaster* ectomycorrhizae.

Table S3. Primers used for Real-time PCR experiments.

Table S4. Sequences used in the phylogenetic tree and abbreviations of their names and species.

Figure S1. Mean currents of *Xenopus* oocytes expressing *Hc*TOK1, *Hc*TOK2.1 or *Hc*TOK2.2 or injected with water determined by voltage-clamp in dependence on external K^+ (A) or pH (B).The mean currents are shown for +60 mV, n=5-13.

Figure S2. Determination of the reversal potential of the *Hc*TOK channels by the analysis of tail currents measured in *Xenopus* oocytes by voltage-clamp. A. From top to bottom, example of tail-current protocols to determine the reversal potential of the *Hc*TOK1 and *Hc*TOK2.1 channels, and the corresponding currents measured. B. Values of the reversal potential (E_{rev}) for *Hc*TOK1 (n = 10) and *Hc*TOK2.1 (n = 7) in dependence to the external K^+ concentration. The line represents the theoretical E_{rev} calculated with the Nernst equation (at 10-fold decrease in the K^+ concentration, the E_{rev} shifts -58 mV). At lower K^+ the reversal potential

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does not coincide with the E_{rev}. C and D. Current-voltage curves for oocytes expressing *Hc*TOK1 (n = 8) and *Hc*TOK2.1 (n = 5) in 100 mM KCl or 100 mM NaCl.

Figure S3. Expression of *HcTOK1*, *HcTOK2.1* and *HcTOK2.2* in pure culture and ectomycorrhizae, determined by RNA-seq experiments from Doré *et al*. (2015). The expression of *HcTOK1* and *HcTOK2.1* did not change significantly between the culture conditions (threshold = 5-fold change). *HcTOK2.2* was 31-fold overexpressed in ectomycorrhizae. FLM, free-living mycelium; ECM, ectomycorrhizae; RPKM, Reads Per Kilobase of exon model per Million mapped reads.

Figure S4. Determination of transcript levels of *HcTOK1*, *HcTOK2.1*. and *HcTOK2.2* in *Hebeloma cylindrosporum* pure cultures. Transcript levels were determined in the control (h7 and pPZP133, represented as CT) and overexpressing lines (TOK1-OE1 and 2, TOK2.1-OE2 and 3 and TOK2.2-OE2 and 4) and expressed as relative expression representing the fold-change in comparison to the basal expression in h7. Absolute expression values are given as numbers between parentheses indicating the number of copies per ng total RNA. Note that the expression level of *HcTOK1* was very low in the control lines (approximately 10 copies per ng of total RNA) compared to *HcTOK2.1* and *HcTOK2.2*. n=3 pools of 3 fungal cultures.

Figure S5. Phenotypical analysis of the transgenic lines overexpressing *HcTOK1*, *HcTOK2.1*. and *HcTOK2.2*. K^+ contents in the mycelia. The only line showing any difference in comparison with the two control lines (h7 and pPZP133, represented as CT), TOK1-OE2, accumulates more K^+ in its tissues. * Significant difference compared to both controls ($p<0.05$). n=8-9.

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Guerrero, M. D. C., Delteil, A., GARCIA, K., Houdinet, G., Conejero, G., Gaillard, I., Sentenac, H., Zimmermann, S. (Auteur de correspondance) (2018). Plant potassium nutrition in ectomycorrhizal symbiosis: properties and roles of the three fungal Kir potassium channels in Hebeloma cylindrosporum . Environmental Microbiology, 20 (5), 1873-1887. , DOI : 10.1111/1462-2920.14122

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Figure S6. Analysis of the effect of external pH on the transcript levels of *HcTOK1*, *HcTOK2.1* and *HcTOK2.2* in *Hebeloma cylindrosporum* pure cultures. Transcript levels were determined in *H. cylindrosporum* (h7) mycelia grown for two weeks in liquid cultures at pH 4.5, 5.5, 6.5 or 7.5. Note that the expression level of *HcTOK1* was very low in all conditions (8-28 copies per ng of total RNA; *cf.* also Fig. S4) explaining differences in the relative expression. For each *TOK* gene, the lowest expression level that was observed was used for normalization and given the value of 1. n=3 pools of 2 fungal cultures.

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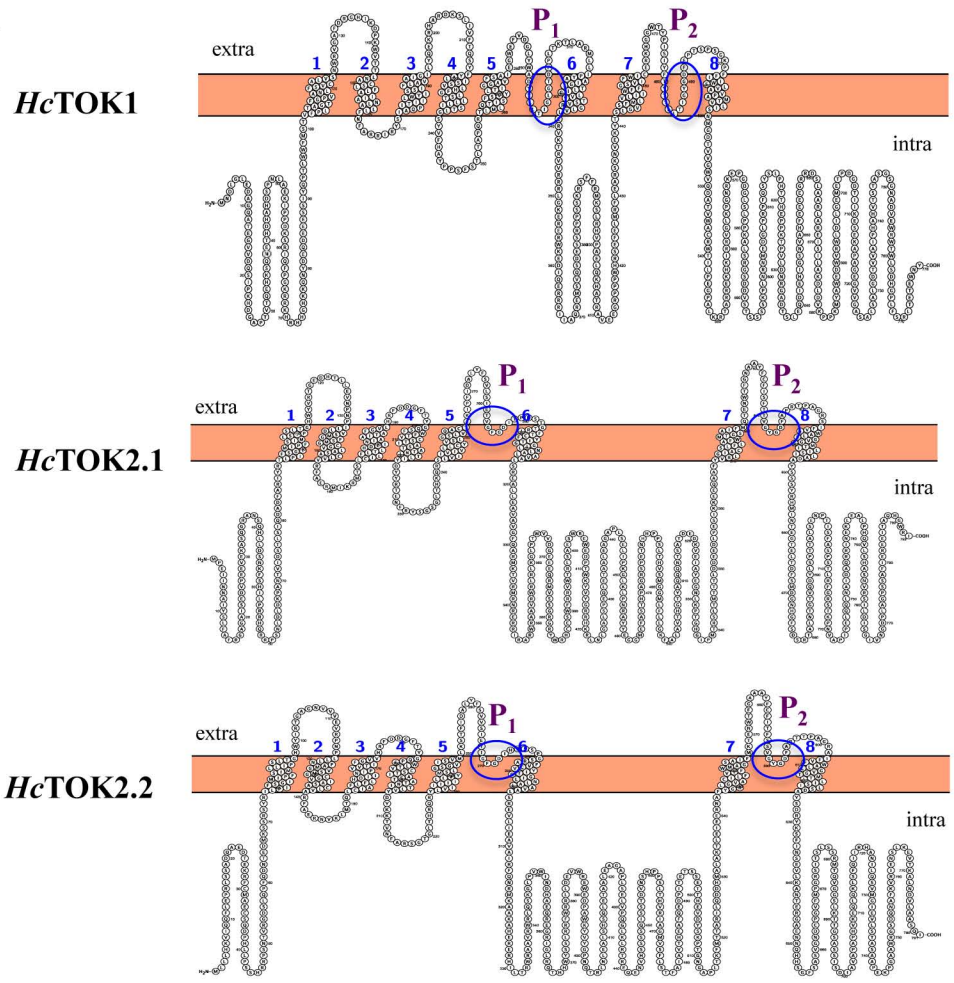
A



B

	*Sc*TOK1	*Nc*TOKA	*Hc*TOK 1	*Hc*TOK2.1	*Hc*TOK2.2
***Sc*TOK1**		39.1 %	31,7 %	32,2 %	30 %
***Nc*TOKA**			41,9 %	29,4 %	27 %
***Hc*TOK1**				29 %	29 %
***Hc*TOK2.1**					60,8 %
***Hc*TOK2.2**					

C

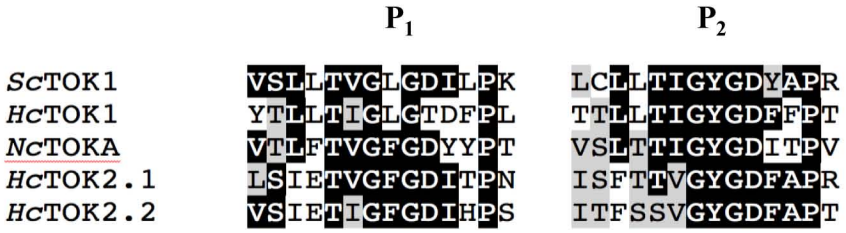


Figure 1

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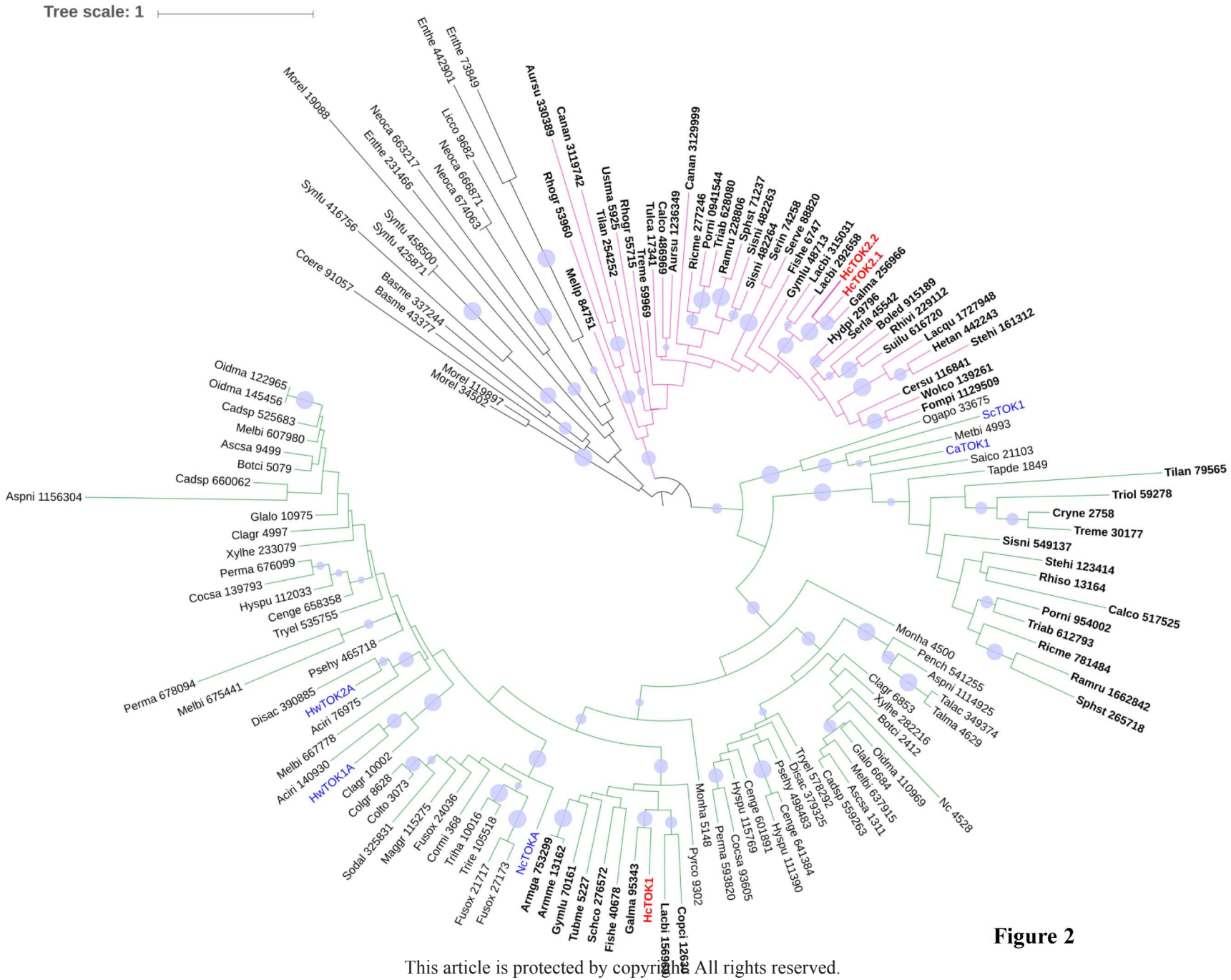

Figure 2

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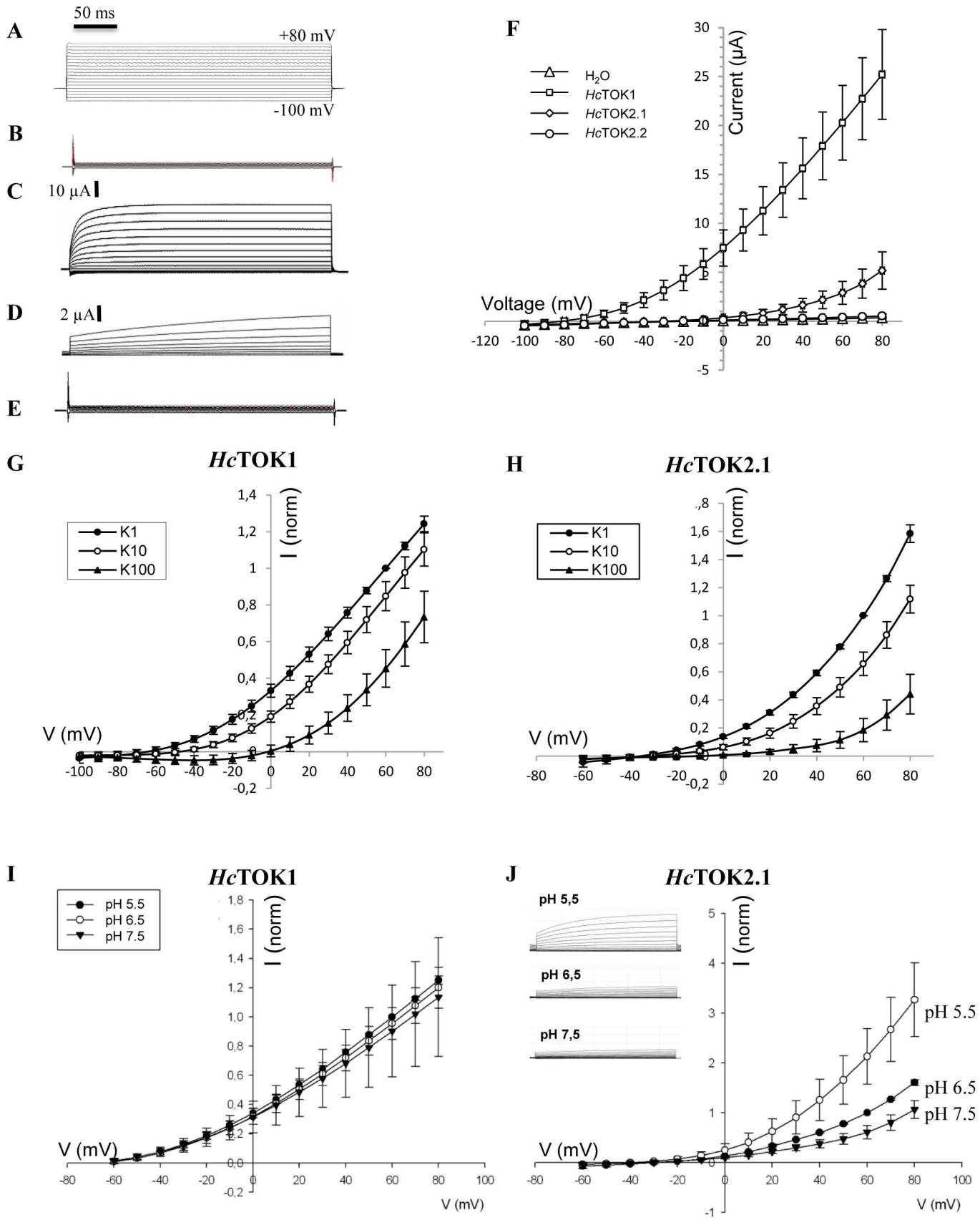

Figure 3

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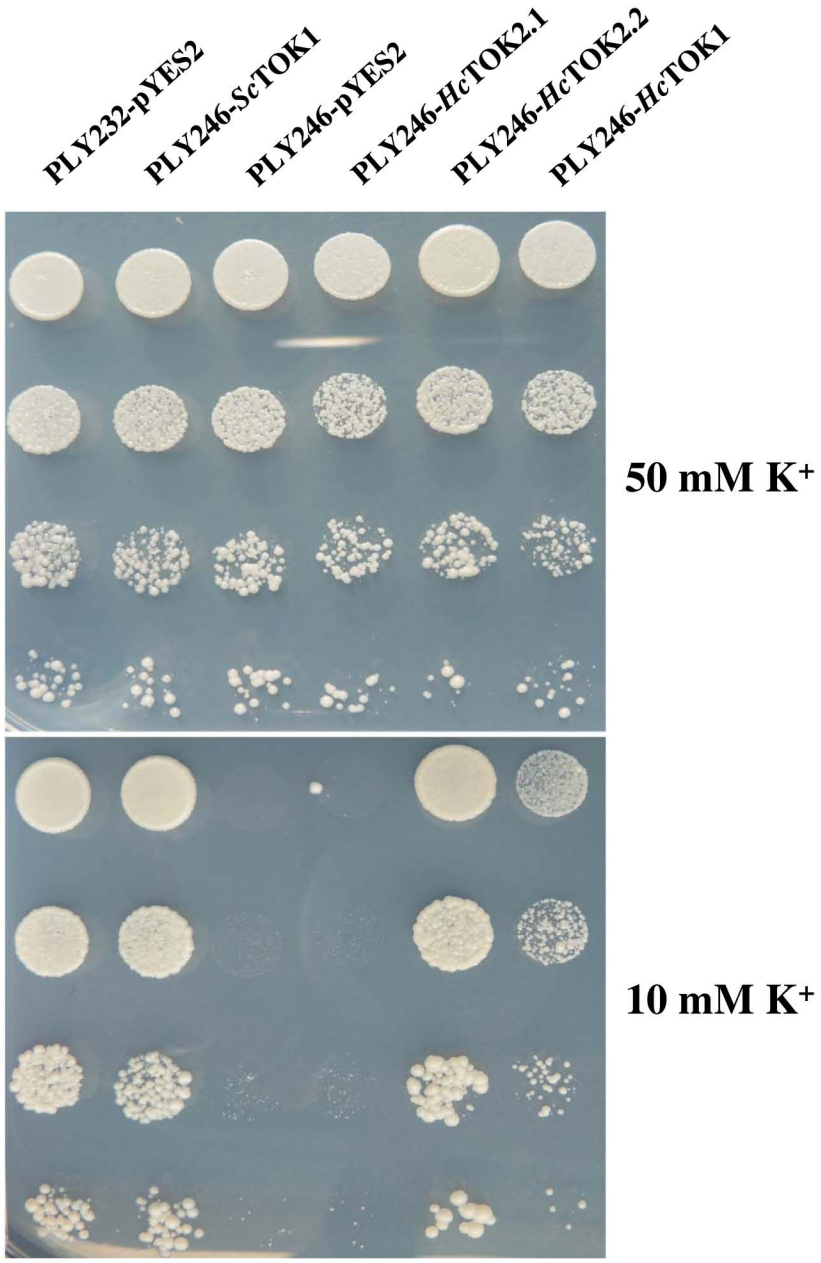

Figure 4

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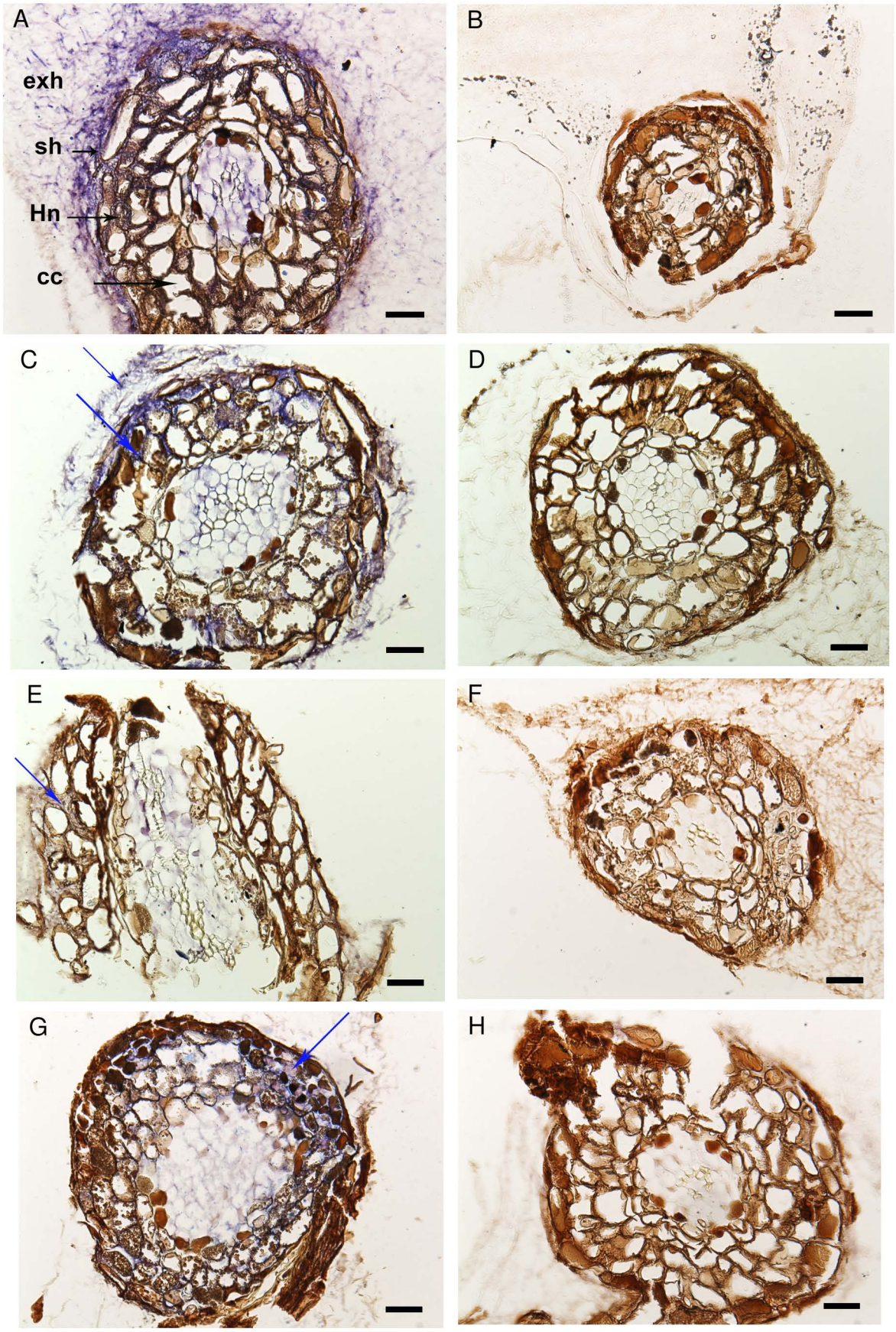

Figure 5

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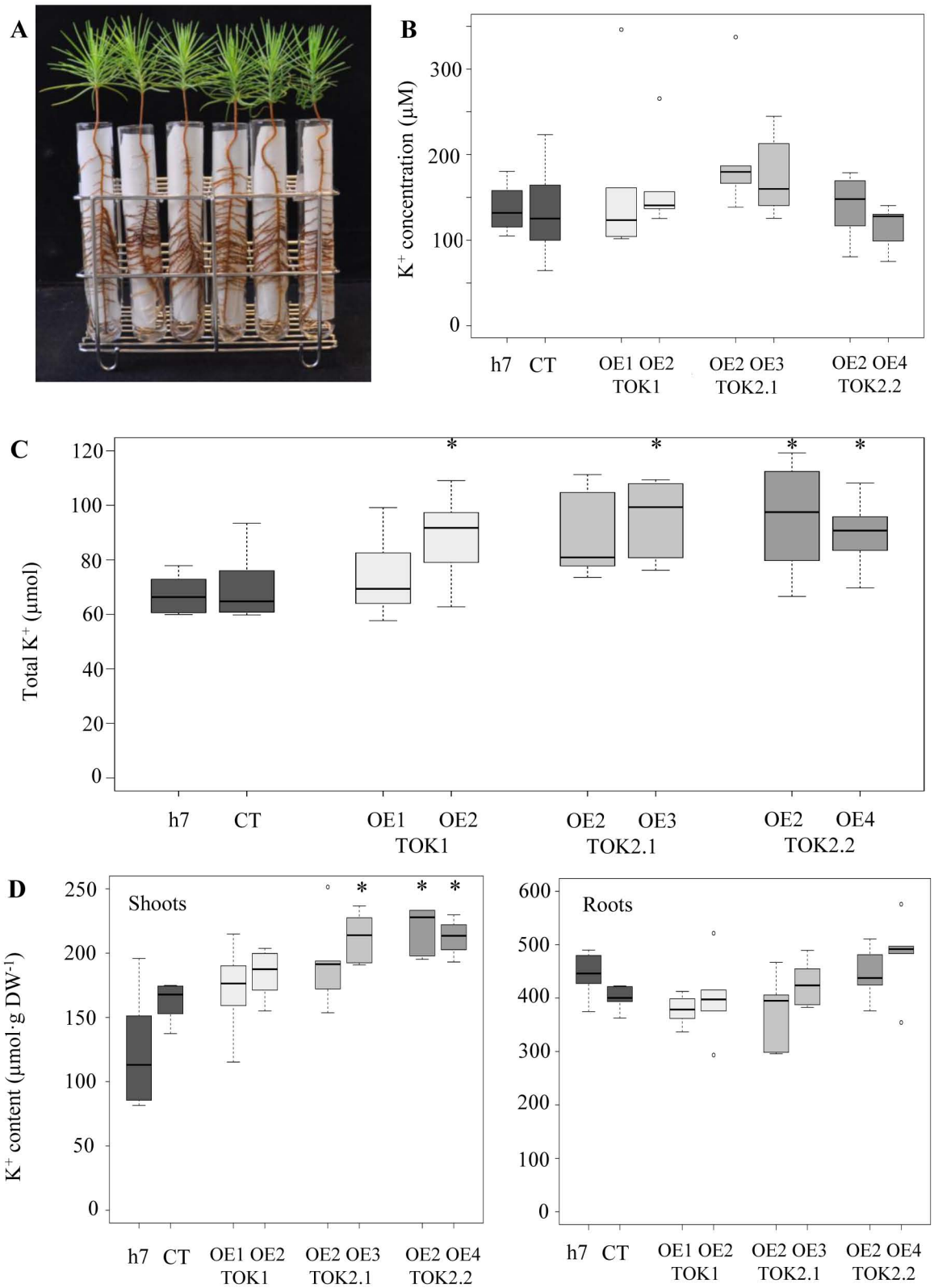

Figure 6

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