Molecular and Functional Characterization of a Na+-K+ Transporter from the Trk Family in the Ectomycorrhizal Fungus Hebeloma cylindrosporum.

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Molecular and Functional Characterization of a Na^+-K^+ Transporter from the Trk Family in the Ectomycorrhizal Fungus *Hebeloma cylindrosporum*[*]

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Ectomycorrhizal symbiosis between fungi and woody plants strongly improves plant mineral nutrition and constitutes a major biological process in natural ecosystems. Molecular identification and functional characterization of fungal transport systems involved in nutrient uptake are crucial steps toward understanding the improvement of plant nutrition and the symbiotic relationship itself. In the present report a transporter belonging to the Trk family is identified in the model ectomycorrhizal fungus *Hebeloma cylindrosporum* and named HcTrk1. The Trk family is still poorly characterized, although it plays crucial roles in K^+ transport in yeasts and filamentous fungi. In *Saccharomyces cerevisiae* K^+ uptake is mainly dependent on the activity of Trk transporters thought to mediate H^+:K^+ symport. The ectomycorrhizal HcTrk1 transporter was functional when expressed in *Xenopus* oocytes, enabling the first electrophysiological characterization of a transporter from the Trk family. HcTrk1 mediates instantaneously activating inwardly rectifying currents, is permeable to both K^+ and Na^+, and displays channel-like functional properties. The whole set of data and particularly a phenomenon reminiscent of the anomalous mole fraction effect suggest that the transport does not occur according to the classical alternating access model. Permeation appears to occur through a single-file pore, where interactions between Na^+ and K^+ might result in Na^+:K^+ co-transport activity. *HcTrk1* is expressed in external hyphae that explore the soil when the fungus grows in symbiotic condition. Thus, it could play a major role in both the K^+ and Na^+ nutrition of the fungus (and of the plant) in nutrient-poor soils.

The symbiotic interaction named mycorrhization, which associates a fungus with the root system of a higher plant, is considered as a major determinant of terrestrial ecosystem productivity. It appeared very early during evolution and was instrumental in the colonization of land by plants (1, 2). The major benefit for the plant is an improvement of its mineral nutrition (nitrogen, phosphorus, potassium, and micronutrients) (3, 4). The ectomycorrhizal symbiosis typically associates woody plants from boreal and temperate forests to fungi belonging to Basidiomycota or Ascomycota (5) through the differentiation of a new organ, named ectomycorrhiza. Therein, highly branched fungal hyphae developing between the root cortical cells form the so-called Hartig net, which constitutes the actual interface between the two partners. Exchanges of solutes and photosynthates from the plant to the fungus and of mineral nutrients from the fungus to the plant, take place at this interface. Developing from the fungal sheath that surrounds the root, external hyphae explore the soil and efficiently take up mineral nutrients (3, 6, 7). Identification at the molecular level and functional characterization of fungal transport systems responsible for the uptake of mineral nutrients from the soil is, thus, crucial to understand the improvement of plant nutrition and, ultimately, the symbiotic relationship. However, very few genes encoding fungal membrane transporters have been identified so far, and the present knowledge principally concerns nitrogen nutrition (8–11). Here we characterize a K^+ transporter from the Trk family in the model fungus *Hebeloma cylindrosporum*, which is so far the only ectomycorrhizal species that can be grown *in vitro* from spore to spore (12), allowing genetic approaches.

An expressed sequence tag (EST)[3] resource prepared from this fungus and analyzed in a previous report enabled the identification of a large set of putative membrane transport systems (13), including a K^+ transporter belonging to the Trk family. Trk-type transporters have probably evolved from minimal bacterial K^+ channel proteins (14–17). The first member of this family was initially identified in *Saccharomyces cerevisiae* (18–20). In this model organism, ScTrk1 is essential to K^+ uptake in the low concentration range, probably by a mechanism involving a co-transport with H^+ (21).

HcTrk1 could be expressed in a functional state in *Xenopus* oocytes, thus providing for the first time detailed electrophysi-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AM396516 and AM396517.

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[3] The abbreviations used are: EST, expressed sequence tag; RT, reverse transcriptase; NMDG, *N*-methyl-D-glucamine; MES, 4-morpholineethanesulfonic acid.

ological data on a K^+ transporter of the Trk family. HcTrk1 displays channel-like properties and is permeable to both K^+ and Na^+. Localization of *HcTrk1* transcripts in the fungus living in symbiotic conditions revealed that the transporter is expressed in external hyphae that spread out from mycorrhizal roots and explore the soil, taking up nutrient ions.

EXPERIMENTAL PROCEDURES

Fungus, Plant, and Ectomycorrhizae—A dikaryotic strain (D2) of the ectomycorrhizal basidiomycete *H. cylindrosporum* Romagnesi, resulting from a cross between the homokaryotic strains h1 and h7 (12), was used. Mycelia were grown in the dark, at 24 °C, in standard medium (modified Pachlevski-Oddoux medium (22)) either in liquid cultures without shaking (23) or on agar-solidified medium (13). For Northern blot analyses, mycelia were grown in standard liquid medium containing ~5 mM K^+ at pH 6.0 for 15 days and then transferred for 3 further days either in fresh standard medium (control treatment), in K^+-free medium (potassium salts being replaced by ammonium salts in the standard medium), in Na^+ supplemented medium (standard medium supplemented with 3 mM NaCl), or in standard medium adjusted to pH 4.5 or 8.0 with HCl or KOH, respectively.

Ectomycorrhizae were obtained in sterile conditions with the fungus cultivated together with maritime pine seedlings grown from seeds (*Pinus pinaster* Soland in Ait from Medoc, Landes-Sore-VG source). Actively growing mycelia were placed close to lateral pine roots in test tubes for 2 months (24). The pine seedlings were then transferred either in rhizoboxes filled with a thin layer of forest soil (25) or on vertical agar plates (cellophane-over-agar method 26) on MMN/2 medium (27) for two additional months. Ectomycorrhizal roots and external hyphae exploring the soil were then separately harvested, immediately frozen in liquid nitrogen, and subsequently stored at −80 °C before RNA isolation and RT-PCR analyses.

Isolation of HcTrk1 cDNA and Gene—The *HcTrk1* cDNA was identified in a collection of about 4000 *H. cylindrosporum* ESTs, obtained by sequencing a cDNA library prepared from strains D2, h1, and h7 and constructed in the yeast expression vector pFL61 (13). RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) experiments were performed using the FirstChoice RLM-RACE kit following manufacturer's recommendations (Ambion, Austin, TX) revealed that the EST clone corresponded to a full-length cDNA. The complete *HcTrk1* cDNA sequence has been deposited in the EMBL data base with accession number AM396516. The genomic DNA was purified from *H. cylindrosporum* mycelium according to Van Kan (28) to determine the number of gene copies by Southern blot analysis. A genomic library constructed in λ-GEM2 from the *H. cylindrosporum* strain h1 (29) was screened (about 5000 phages corresponding to ~5 times the size of the *Hebeloma* genome) using full-length *HcTrk1* cDNA as a probe and following standard protocols (30). Hybridization of digested (BamHI, EcoRV, SacI, HindIII, XbaI, EcoRI, XhoI, and AccI) DNA from three positive phages with two probes corresponding to the full-length clone and to the 5′ end, respectively, was carried out to clone fragments of the *HcTrk1* gene. Fragments of two isolated phages containing the *HcTrk1* gene were subcloned in pBluescript and sequenced (Genome Express, Grenoble, France). Intron locations in the *HcTrk1* gene were deduced from sequence alignments and search for splice junctions. The obtained genomic DNA sequence has been submitted to the EMBL data base with accession number AM396517.

Sequence Analyses—Accession numbers of protein sequences used for comparison are the following: *Neurospora crassa* NcTrk1, CAA08813; *S. cerevisiae* ScTrk1, CAA89424; *S. cerevisiae* ScTrk2, CAA82128; *Schizosaccharomyces pombe* SpTrk1, P47946; *Arabidopsis thaliana* AtHKT1, Q84TI7; *Triticum aestivum* TaHKT1, AAA52749. Similarity search was done with the BLAST program at www.ncbi.nlm.nih.gov/BLAST (31). Transmembrane regions were predicted by using the TMpred program. Multiple sequence alignment was performed using Multalin (32).

Northern Blot and Reverse Transcription PCR—Total RNA was extracted from *in vitro* grown mycelia using Trizol following the manufacturer's recommendations (Invitrogen) or from ectomycorrhizal hyphae growing on soil according to Kiefer *et al.* (33) and used for Northern blot and RT-PCR analyses, respectively. For Northern blot experiments, 30 μg of each RNA sample were loaded in a 1.2% agarose-formaldehyde gel and blotted onto a nylon membrane (Biotrans(+) nylon membranes, ICN, Irvine, CA). Hybridization was performed as described (34) with a full-length ^{32}P-labeled *HcTrk1* cDNA as probe. After autoradiography, the membrane was stripped and reprobed with a ^{32}P-labeled *Hebeloma EFIα* probe. For RT-PCR analysis, first-strand cDNA was synthesized from 0.5 μg of total RNA from each sample using Moloney murine leukemia virus reverse transcriptase H minus (Promega, Madison, WI) with poly(T) as primer, according to the manufacturer's instructions. One μl of the RT product was then used for PCR amplification (40 cycles) with Taq polymerase (Invitrogen) using the following primers: 5′-GGATTGCTTCAAGGATTCTTCCGGA-3′ and 5′-GGGTGCAAGGCTCGCAACTGGAAC-3′ for *HcTrk1* and 5′-ATCTCTGCAGAGAAAGCTCACCACG-3′ and 5′-CATACCCTCACCGACGTACCAGTGC-3′ for *HcTUB* (*H. cylindrosporum* α-tubulin.

Complementation Assays Using S. cerevisiae—*S. cerevisiae* WΔ6 (Mata *ade2 ura3 trp1 trk1*Δ::*LEU2 trk2*Δ::*HIS3* (35)) defective for K^+ uptake was transformed with *HcTrk1* cDNA cloned in the yeast expression plasmid pDR196 or with the empty plasmid. A K^+ channel from *A. thaliana,* AKT1, previously shown to complement the yeast K^+ uptake deficiency (36), was used as a positive control. Cells were transformed according to Dohmen *et al.* (37) and plated for 3 days on YNB medium (yeast nitrogen base, 1.7 g/liter; $(NH_4)_2SO_4$, 5 g/liter; D-glucose, 20 g/liter; agar, 20 g/liter) supplemented with 50 mM K^+ and the appropriate amino acids for transformant selection. For drop tests, cells from an overnight culture in YNB supplemented with 50 mM KCl were harvested by centrifugation, washed 3 times, and resuspended in distilled water to an optical density of 1 at 600 nm. Serial 10-fold dilutions of this suspension were made and dropped (10 μl) onto 2% agar plates of arginine phosphate medium (38) containing 0.1 mM K^+ (nominal concentration) or supplemented with 1 or 3 mM KCl. The plates were incubated for 3 days at 30 °C and then photographed.

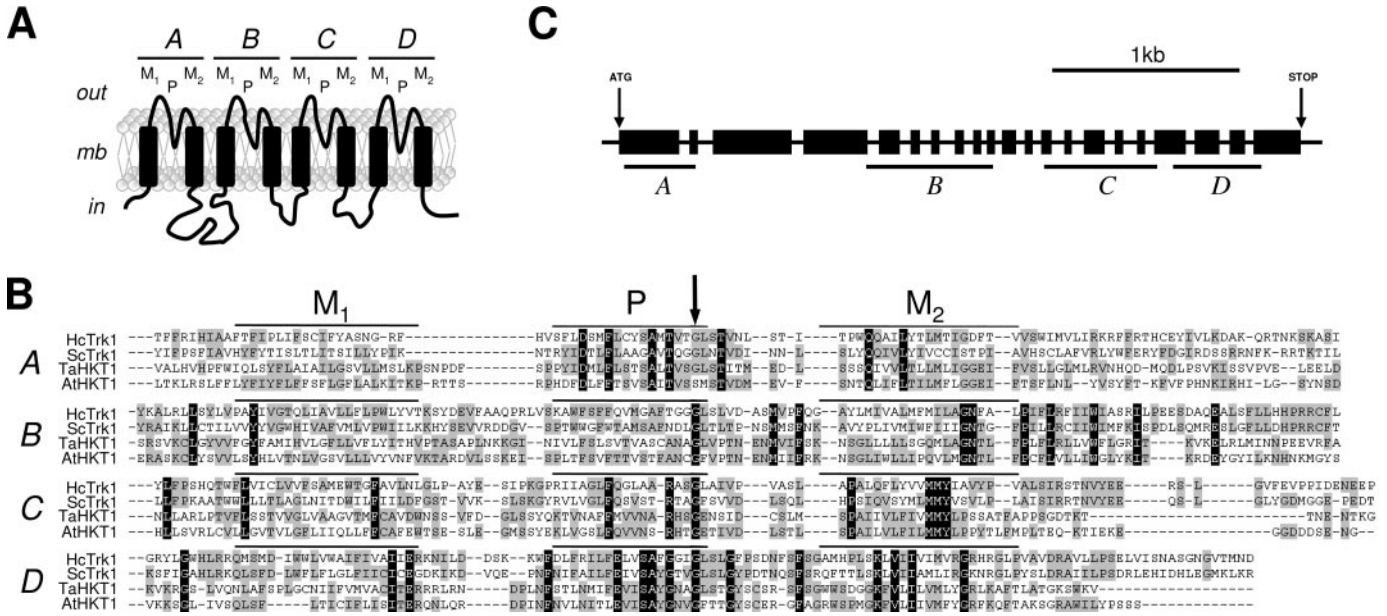

FIGURE 1. **Identification of HcTrk1, a Trk transporter in the ectomycorrhizal basidiomycete *H. cylindrosporum*.** *A*, schematic structural model of Trk transporters. Trk transporters display four tandemly repeated MPM domains (named *A–D*), each comprising two transmembrane (*mb*) segments (M_1 and M_2) surrounding a pore region (*P*). *B*, sequence alignments in the regions corresponding to the four MPM motifs of *H. cylindrosporum* HcTrk1 and of Trk transporters from other fungal or plant species: *S. cerevisiae* ScTrk1, *T. aestivum* TaHKT1, and *A. thaliana* AtHKT1. Identical amino acids are on a *black* (conserved in all four proteins) or *gray background*. The *arrow* indicates a highly conserved residue shown to be essential for transport selectivity (60). *C*, gene structure of *HcTrk1*. Exons are designated by *black boxes*. The mean intron size is 60 bp. *kb*, kilobase(s).

HcTrk1 Expression and Electrophysiological Analysis in Xenopus laevis Oocytes—The coding region of *HcTrk1* cDNA, fitted with BamHI and XbaI restriction sites introduced by PCR at the 5′ and 3′ ends, respectively, was subcloned into the BamHI and XbaI sites of a modified pGEMHE vector (39) (gift from D. Becker, Department of Molecular Plant Physiology and Biophysics, Würzburg, Germany) under the control of the T7 promoter. *HcTrk1* cRNA was transcribed from linearized (NheI) plasmid using mMESSAGE mMACHINE T7 Ultra kit (Ambion). Oocyte preparation, injection, and measurements were performed as described (40). Oocytes were injected with 50 ng of *HcTrk1* cRNA in 50 nl of diethyl pyrocarbonate (DEPC)-treated water, control oocytes being injected with 50 nl of DEPC-treated water only. The oocytes were kept at 18 °C in a medium containing 96 mM NaCl, 2 mM KCl, 1.8 mM $MgCl_2$, 1 mM $CaCl_2$, 2.5 mM sodium pyruvate, 5 mM HEPES-NaOH, pH 7.4, supplemented with 50 mg/ml gentamycin. Whole-cell currents were recorded using the two-electrode voltage-clamp technique, 3–5 days after injection. The ionic strength of the bath solutions was kept constant by adding *N*-methyl-D-glucamine chloride (NMDG-Cl) to compensate for the changes in concentrations of the tested monovalent cations (K^+ and Na^+ in most experiments, introduced as chloride salts) in a background medium containing 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM MES, 5 mM Tris, pH 6.5. Voltage-pulse protocol application, data acquisition, and data analyses were performed using pClamp9 (Axon Instruments, Foster City, CA) and Sigmaplot (Jandel Scientific, Erkrath, Germany). The voltage pulses ranged between +20 mV and −130 mV in −15-mV steps from a holding potential of −40 mV. To extract the HcTrk1-mediated currents, mean endogenous current values obtained from three control oocytes (water-injected) from the same oocyte batch in parallel experiments were subtracted from the currents recorded on *HcTrk1*-expressing oocytes.

RESULTS

Identification of a Trk-type Transporter in the Ectomycorrhizal Fungus H. cylindrosporum—A sequence related to the Trk family of K^+ transporters, initially described in *S. cerevisiae*, was identified in a *H. cylindrosporum* EST library (13). The corresponding cDNA clone (2455 bp) was sequenced, revealing an open reading frame of 2373 bp encoding a putative 791-amino acid protein with an expected mass of 87.9 kDa, designated as HcTrk1 (*H. cylindrosporum* Transporter of K^+). RNA ligase-mediated rapid amplification of cDNA ends experiments confirmed that the cDNA clone comprised the full-length coding sequence. The deduced HcTrk1 amino acid sequence displays 12 hydrophobic domains, like other members of the Trk family initially thought to possess 12 transmembrane segments (19, 41–43). Latterly, based on multiple sequence alignments and residue conservation, Durell *et al.* (14) have proposed that members of the Trk superfamily, which comprises Trk and KtrAB from prokaryotes, Trk from yeast, and HKT from plants, have evolved from minimal bacterial K^+ channel proteins displaying two transmembrane segments, M1 and M2, surrounding a hydrophobic pore P, forming the so-called MPM structure (44). Trk transporters present four sequential MPM motifs, named *A* to *D* (Fig. 1*A*). The amino acid sequence of HcTrk1 shares the highest levels of similarity (~40%) with the potassium transporters SpTrk1, ScTrk2, and NcTrk1 of *S. pombe, S. cerevisiae*, and *N. crassa.* The sequence alignments in the hydrophobic core corresponding to the four MPM motifs (Fig. 1*B*) reveal much higher levels of similarity (62% between HcTrk1 and ScTrk1).

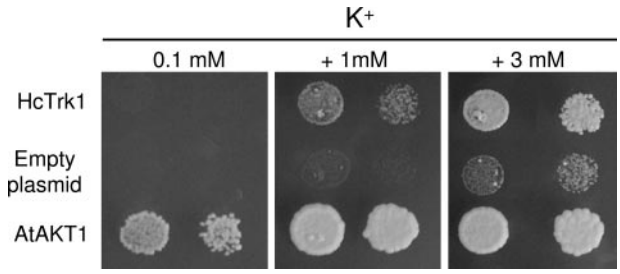

FIGURE 2. **HcTrk1 complements *S. cerevisiae* mutant cells defective for K^+ uptake.** Cells from the yeast mutant strain WΔ6 (*trk1*-Δ::LEU2 *trk2*-Δ::HIS3) defective for K^+ uptake were transformed with either *HcTrk1* cDNA cloned in the yeast expression plasmid pDR196 or the empty plasmid or with the *A. thaliana* K^+ channel *AKT1* cDNA as a positive control. Drop tests were performed on arginine phosphate medium containing about 0.1 mM K^+ (nominal concentration) or on AP medium supplemented with 1 or 3 mM KCl. The plates were incubated for 3 days at 30 °C.

A clone containing the full-length *HcTrk1* gene was obtained by screening a *H. cylindrosporum* genomic library using the full-length cDNA fragment as a probe. Alignment of the nucleotide sequence of *HcTrk1* cDNA with the genomic sequence revealed that the CDS comprises 20 introns, all displaying a similar size, close to 60 bp (Fig. 1*C*). Southern blot analyses carried out using the full-length cDNA fragment as a probe indicated that the *HcTrk1* gene is probably present in a single copy (data not shown).

HcTrk1 Complements a Yeast Mutant Defective for K^+ Uptake—*S. cerevisiae* mutant strains defective for K^+ uptake can be complemented by expression of heterologous Trk transporters from *e.g. S. pombe* (45) or *N. crassa* (43). Expression of *HcTrk1* in *S. cerevisiae* WΔ6, a mutant defective for K^+ uptake, was found to restore yeast growth on media containing K^+ in the mM concentration range (Fig. 2). No complementation was observed when the concentration of K^+ was decreased to 0.1 mM. Thus, in this type of growth test, HcTrk1 appears less efficient than the *Arabidopsis* AKT1 channel (used as a positive control (36)). Adding 3 mM Na^+ in the growth medium did not increase the efficiency of HcTrk1 to restore the yeast mutant growth on low K^+ medium (data not shown).

HcTrk1 Mediates Na^+ and K^+ Transport in Xenopus Oocytes—Electrophysiological analyses were carried out to further investigate the functional properties of HcTrk1 using the two-electrode voltage-clamp technique after heterologous expression in *Xenopus* oocytes. Currents were recorded in bath solutions containing either 100 mM K^+ or 100 mM Na^+. Typical recordings obtained in control or *HcTrk1*-expressing oocytes are shown in Fig. 3*A*. *HcTrk1* expression gave rise to instantaneously activating inwardly rectifying currents, of greater magnitude in the Na^+ bath than in the K^+ bath. The bath concentration of K^+ or Na^+ was then varied in the 10–100 mM range, and the magnitude of HcTrk1 current was plotted *versus* the concentration of the monovalent cation. In the case of K^+, the resulting isotherm was quasi-linear (Fig. 3*B*, *left panel*). In the case of Na^+, a hyperbolic relationship appeared (Fig. 3*B*, *right panel*), indicative of a saturation process that could be described using Michaelis-Menten formalism with a K_m parameter of 26 mM.

HcTrk1 Ionic Selectivity—Selectivity of HcTrk1 for alkali cations was then investigated in *Xenopus* oocytes using bath solutions containing 100 mM concentrations of either Li^+, Na^+, K^+, Rb^+, Cs^+, or NMDG (this large organic cation being used as non-permeant control). Comparison of *HcTrk1*-expressing oocytes and control oocytes revealed that HcTrk1 was significantly permeable to K^+ and Na^+ only (Fig. 4).

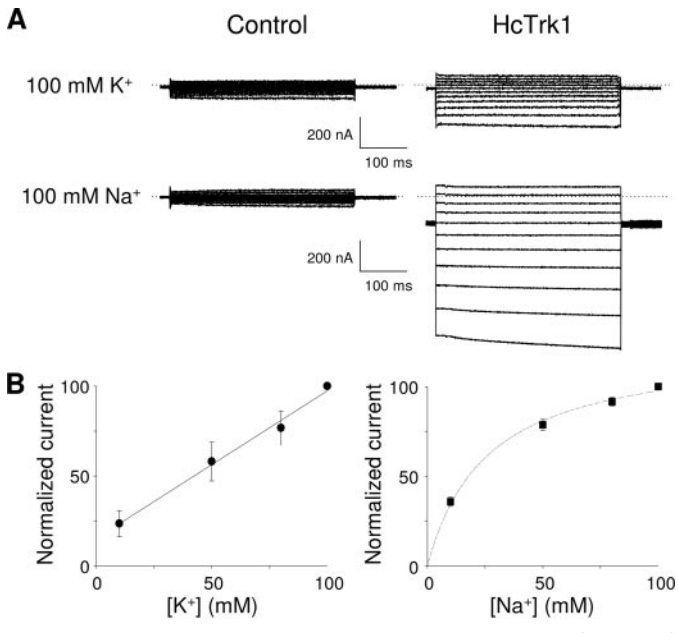

FIGURE 3. **HcTrk1 expressed in *Xenopus* oocytes mediates K^+ and Na^+ transport.** *A*, examples of current traces recorded in control oocytes (injected with water, *left panels*) or in *HcTrk1*-expressing oocytes (*right panels*) recorded in 100 mM KCl (*upper panels*) or in 100 mM NaCl (*lower panels*). The voltage step ranged between +20 mV and −130 mV in −15-mV steps from a holding potential of −40 mV. *B*, effect of K^+ or Na^+ concentration on HcTrk1 current. For each oocyte in each bath solution, the HcTrk1 current was defined as the total current in *HcTrk1*-expressing oocyte minus the control current (mean current obtained for three control oocytes from the same batch in the same bath). The bath solution contained 1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM MES, 5 mM Tris, pH 6.5, as standard background medium, and 10, 50, 80, or 100 mM KCl (*left panel*) or NaCl (*right panel*). The ionic strength of the solutions was kept constant by adding *N*-methyl-D-glucamine to compensate for the change in K^+ or Na^+ concentration (no added NMDG in 100 mM K^+ and in 100 mM Na^+). Normalized HcTrk1 current was HcTrk1 current at −115 mV in the presence of a given concentration of K^+ (Na^+) expressed as percent of the HcTrk1 current recorded at the same potential in 100 mM K^+ (Na^+). Data are the means (± S.D.) obtained from 8–12 *HcTrk1*-expressing oocytes.

Effect of Monovalent Cations on HcTrk1 Current in the Presence of Na^+—Changing the external pH from 5.5 to 8 in 100 mM external Na^+ concentration did not affect HcTrk1 current (data not shown). The effect of external alkali cations on HcTrk1 current was studied by adding 20 mM K^+, Rb^+, Li^+, Cs^+, or NMDG to a bath solution containing 80 mM Na^+. Li^+, which is not permeant through HcTrk1 (Fig. 4), was without any significant effect on the HcTrk1 current when compared with the corresponding NMDG control treatment. The other cations reduced the current, with the non-permeant species Rb^+ and Cs^+ probably acting as blockers and the permeant K^+ as a competitor (Fig. 5).

Interaction between Na^+ and K^+ during HcTrk1 Permeation—The effects of the permeant K^+ and non-permeant Cs^+ on HcTrk1 currents in the presence of Na^+ were further compared by using bath solutions containing Na^+ in the 0–100 mM concentration range and either K^+ or Cs^+ in the 100–0 mM range, the total concentration of Na^+ and of the other cation being 100 mM in every condition. Solutions containing NMDG and either

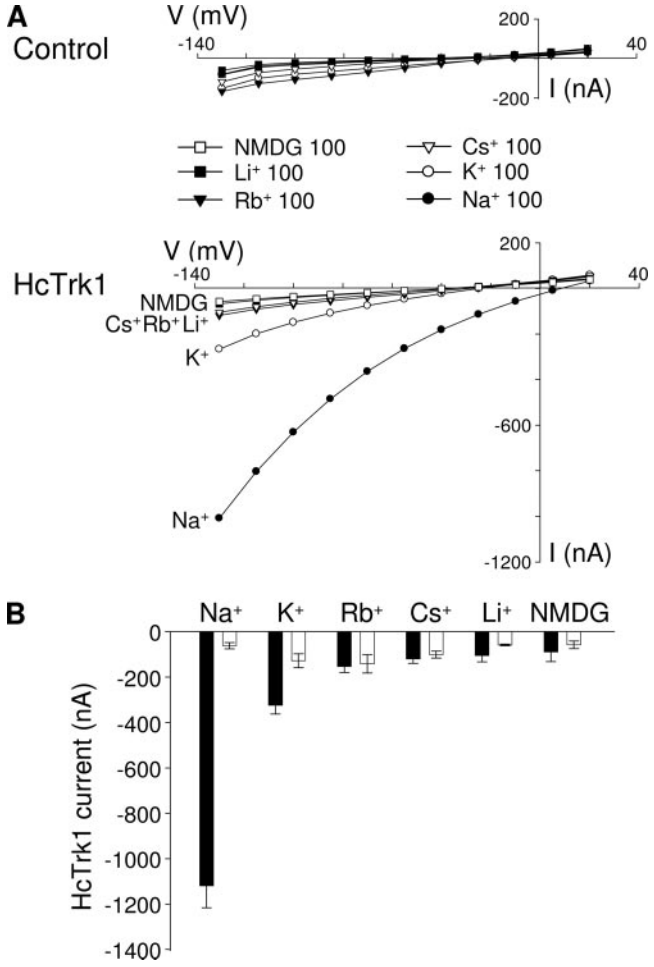

FIGURE 4. **HcTrk1 selectivity for alkali cations.** The bath solution contained 100 mM concentrations of either Li^+, Na^+, K^+, Rb^+, Cs^+, or NMDG in the standard background medium. *A*, representative current-voltage (I-V) curves recorded in a control oocyte injected with water (*upper panel*) or in a *HcTrk1*-expressing oocyte (*lower panel*). *B*, mean steady state current recorded at −130 mV in *HcTrk1*-expressing oocytes (*black bars*) compared with water-injected (control) oocytes (*white bars*). Bath solutions are as in *A*. Data are mean values ± S.D. for four oocytes.

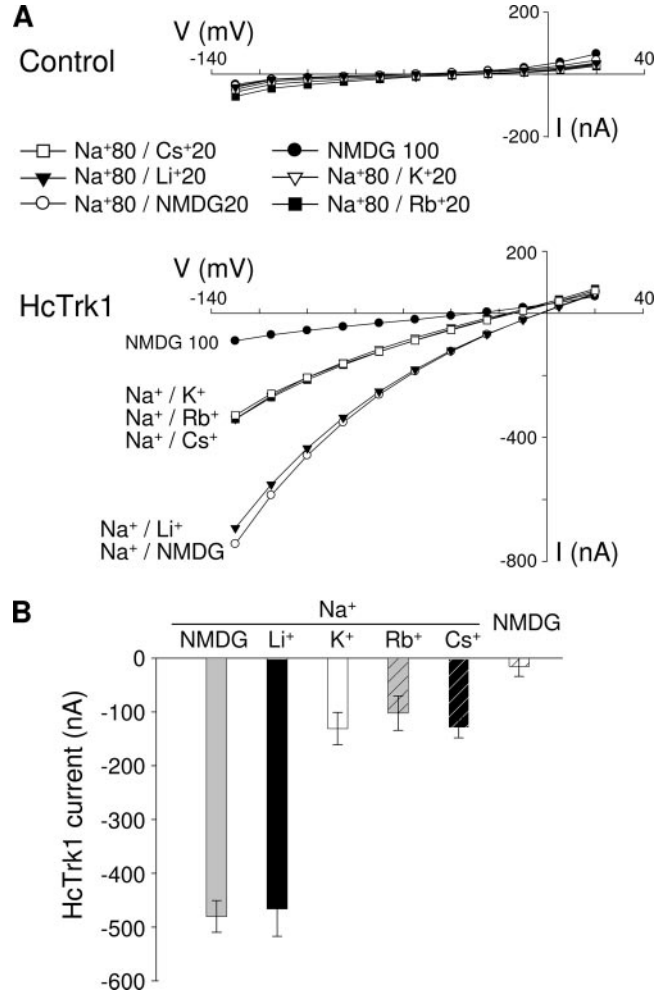

FIGURE 5. **Inhibition by K^+, Rb^+, and Cs^+ of HcTrk1 currents in presence of Na^+.** The bath solution contained a total monovalent cations concentration of 100 mM: 80 mM Na^+ and 20 mM *X* (*X* is either K^+, Rb^+, Cs^+, Li^+, or NMDG) or 100 mM NMDG in the standard background medium. *A*, representative I-V curves recorded in a control oocyte injected with water (*upper panel*) or in a *HcTrk1*-expressing oocyte (*lower panel*). *B*, mean HcTrk1 current at −115 mV. Bath solutions as in *A*. Each *bar* represents the mean HcTrk1 current (±S.D.; $n = 4$). *HcTrk1 current*, total current in *HcTrk1*-expressing oocyte minus mean control current obtained from three water-injected oocytes in parallel experiments.

K^+ or Na^+ were used for comparison. In the case of the Na^+-K^+, Na^+-Cs^+, and Na^+-NMDG solutions, HcTrk1 current was plotted against the concentration of Na^+ (in the 0–100 mM range) and thereby against the mole fraction of Na^+ (expressed in %) in the corresponding solutions. In the case of the NMDG-K^+ solutions, the current was plotted against the concentration of NMDG (against the mole fraction of NMDG). From these four experimental curves (Fig. 6*A*), the one corresponding to the Na^+-K^+ mixtures displayed a unique pattern characterized by a non-monotonic variation; the HcTrk1 current decreased when the mole fraction of Na^+ was raised from 0 to 0.25 (Na^+ concentration from 0 to 25 mM, Fig. 6, *A* and *B*) and then increased when the Na^+ mole fraction was further increased (Fig. 6*A*). Although the decrease in HcTrk1 current upon increase of the Na^+ mole fraction from 0 to ~0.25 appeared to be within the error bars of mean currents (which integrate the differences in HcTrk1 expression levels among the individual oocytes), this decrease was systematically observed in every oocyte (Fig. 6*B*; 12 oocytes of 12) and was, thus, statistically significant ($p < 0.01$; Wilcoxon signed-rank test). Thus, HcTrk1 displays an anomalous behavior; that is, a decrease in the current upon an increase in the mole fraction (from 0 to ~0.25) of the more permeant ion, Na^+. By analogy with similar behavior displayed by ion channels (46, 47), this so-called anomalous mole fraction effect supports the hypothesis that both K^+ and Na^+ can be simultaneously present and interact in the conduction pathway, since the conductance of a single ion pore is expected to monotonically increase (or decrease) with the mole fraction of the more (or less) permeant ion. Indeed, the three other experimental curves, corresponding to the Na^+-Cs^+, Na^+-NMDG, and NMDG-K^+ mixtures displayed a monotonic variation (Fig. 6*A*).

Comparison of the two curves describing the effect on HcTrk1 current of the Na^+-K^+ and Na^+-NMDG mixtures (Fig. 6*A*) indicated that the interaction between K^+ and Na^+ during the permeation process resulted in a strong decrease of HcTrk1 current by low K^+ concentrations, mainly occurring when the external concentration of K^+ was raised from 0 to 10 mM (Na^+

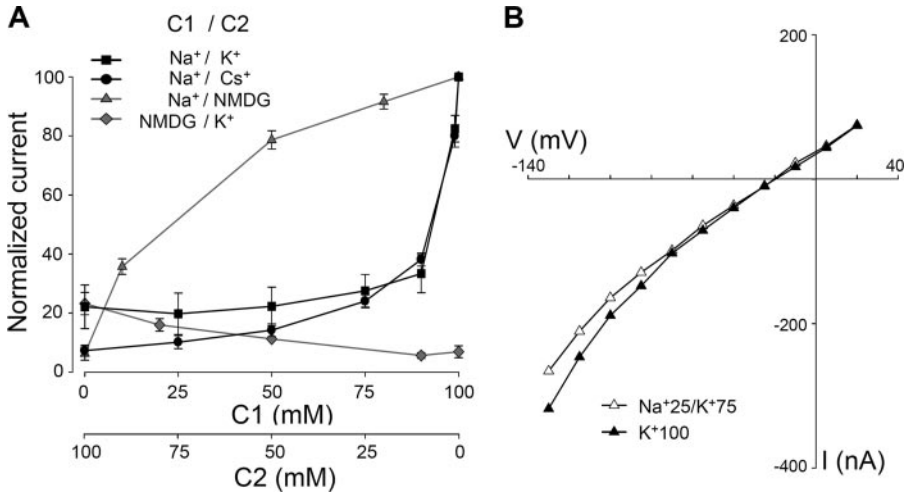

FIGURE 6. **Interaction between Na^+ and K^+ in HcTrk1 revealed by anomalous mole fraction effect.** *A*, the bath solution contained in the standard background medium a mix of two monovalent cations, C1 and C2, for a total concentration of 100 mM: Na^+ and NMDG (*gray triangles*; data from the same experiments as in Fig. 3), Na^+ and K^+ (*black squares*), Na^+ and Cs^+ (*black circles*), and NMDG and K^+ (*gray diamonds*; data from the same experiments as in Fig. 3), as C1 and C2 respectively. For each oocyte in each bath solution, the HcTrk1 current was obtained by subtracting from the total current the mean control current recorded in three water-injected oocytes in parallel experiments. Normalized HcTrk1 current is HcTrk1 current at −115 mV in a given bath expressed as a percentage of the HcTrk1 current recorded at the same potential in 100 mM Na^+. Data of the means (±S.D.) obtained from 4–8 *HcTrk1*-expressing oocytes. *B*, HcTrk1 current magnitude is lower in 25 mM Na^+/75 mM K^+ than in 100 mM K^+. Current/voltage curves in 100 mM K^+ or 25 mM Na^+/75 mM K^+ were obtained in a representative HcTrk1-expressing oocyte. Independent experiments performed on 12 oocytes displaying different levels of HcTrk1 currents systematically revealed that the increase in Na^+ mole fraction from 0 (100 mM K^+ solution) to 0.25 (25 mM Na^+/75 mM K^+ solution) resulted in decreased current magnitudes at every membrane potential. Wilcoxon signed-rank test indicates that this decrease in current magnitude upon the increase in Na^+ mole fraction was statistically significant ($p \ll 0.01$).

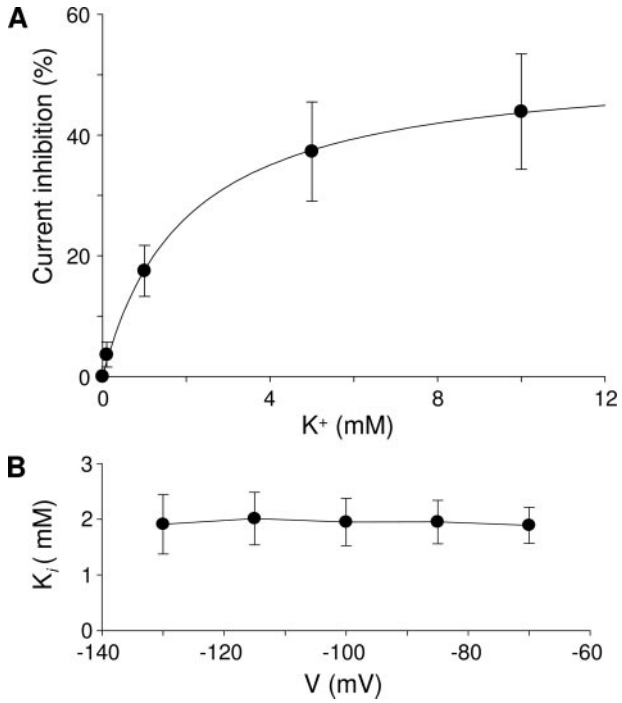

FIGURE 7. **K^+ inhibition of HcTrk1 currents.** The bath solution contained 80 mM Na^+ and either 0, 0.1, 1, 5, or 10 mM K^+ in the standard background medium. *A*, HcTrk1 current inhibition was expressed as a percentage of HcTrk1 current recorded at −115 mV in 80 mM Na^+. These percentages were fitted to a Michaelis function to derive a K_i value. Mean values ± S.D. for four oocytes. The ionic strength of the solutions was kept constant to 100 mM by adding *N*-methyl-D-glucamine to compensate for the change in K^+. *B*, K^+ inhibition of HcTrk1 current in presence of Na^+ is not sensitive to voltage. Michaelis hyperbolic functions were used to derive inhibition constants K_i as described in *A* at −70, −85, −100, −115, and −130 mV. Data are the means ± S.D. for four oocytes.

being decreased from 100 to 90 mM). To further analyze the effect of K^+ on HcTrk1 current in the presence of a high Na^+ concentration, the concentration of K^+ in the bath solution was varied in the 0–10 mM range, whereas that of Na^+ was kept constant at 80 mM (Fig. 7*A*). The corresponding data could be fitted with a hyperbolic function, yielding an apparent inhibition constant (K_i) close to 2 mM. Interestingly, this inhibition constant did not depend on the membrane potential (Fig. 7*B*), and the inhibition of HcTrk1 current by Cs^+ or Rb^+ (in the presence of 80 mM Na^+; Fig. 5*B*) was also found to be independent of the membrane potential (data not shown).

HcTrk1 Is Expressed in External Hyphae of Ectomycorrhizal Roots—Northern hybridization analyses of *HcTrk1* expression were performed in *H. cylindrosporum* mycelia cultivated in liquid cultures. A single transcript of ~2.5 kilobases, a size consistent with the *HcTrk1* open reading frame, was detected. As previously reported for other *Trk* genes (*SoTrk1* (48), *NcTrk1*, and *ScTrk2* (43)), *HcTrk1* expression was barely detected by Northern blot (data not shown). Decreasing the availability of K^+ in the external medium down to nominal concentrations, increasing Na^+ in the external medium, or varying the external pH from 4.5 to 8 were without any significant effect on *HcTrk1* transcript accumulation (data not shown).

In ectomycorrhizal symbiosis, external hyphae that spread out from mycorrhizal roots efficiently explore the soil, taking up nutrient ions, whereas fungal cells within the root cortex secrete the ions toward the host plant. External hyphae collected from mycorrhizal roots grown either in soil or on agar plates were shown by RT-PCR analyses to express *HcTrk1* whatever the experimental growth conditions (Fig. 8).

DISCUSSION

H. cylindrosporum as a Model Ectomycorrhizal Fungus—K^+ is the most abundant inorganic cation in the cytoplasm of plant cells where it plays different crucial roles, *e.g.* in regulation of cell turgor and control of cell membrane polarization (49, 50). Large amounts of K^+ are, therefore, required for plant growth, and the availability of this cation is often limited in the absence of fertilization. In forest ecosystems, ectomycorrhizal symbiosis improves the whole mineral nutrition of the tree, including K^+ nutrition (3, 51). The fungal partner can explore large soil volumes and be very efficient in mobilizing and taking up K^+ ions, which can thereafter be secreted toward the host plant.

H. cylindrosporum is an attractive model, since it is so far the only ectomycorrhizal species for which the whole life cycle can

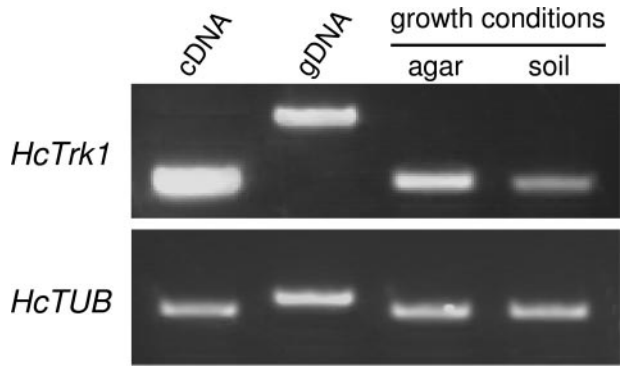

FIGURE 8. **HcTrk1 transcripts are present in external hyphae of *H. cylindrosporum* grown in symbiotic conditions.** The symbiotic plant partner of *H. cylindrosporum* is *P. pinaster*. The symbionts were grown either on thin layer of forest soil or agar plates. External hyphae were collected for RT-PCR analyses of *HcTrk1* transcripts. Reverse transcription was performed using 0.5 μg of total RNA. PCR amplifications were performed with either *HcTrk1* primers (*top panel*) or *HcTUB* primers (*bottom panel*; control). The number of cycles was 40 for both *HcTrk1* and *HcTUB*, respectively. For both genes the size of the amplified fragments was checked by comparison with DNA fragments obtained in parallel experiments by PCR on the corresponding cDNA or on genomic DNA (*first two lanes*).

be achieved *in vitro*, from spore to spore, allowing genetic approaches. Regarding its ecology, *H. cylindrosporum* has been qualified as a pioneer species, which thrives in newly established forests, where organic matter accumulation is low, or in disturbed habitats. This species is frequently found in forest stands developing on poor sandy soils along the Atlantic coast. In such biotopes most of the K^+ ions available in the soil for the ecosystem can be provided by sea sprays, which also bring Na^+. Thus, it is tempting to speculate that the transport mechanisms responsible for K^+ uptake in *H. cylindrosporum* can efficiently cope with soils displaying low K^+ availability in the presence of Na^+.

Molecular Identification of a K^+ Transporter from the Trk Family in H. cylindrosporum—As a first step in molecular analysis of K^+ transport in ectomycorrhizal symbiosis, a sequence encoding a transporter belonging to the Trk transporter family, identified in an EST resource from *H. cylindrosporum*, was cloned and analyzed. Very few genes have been characterized in *H. cylindrosporum* and more generally in ectomycorrhizal fungi. Interestingly, all genes already identified in *H. cylindrosporum* display an original structure with many introns of small and homogenous size (9). *HcTrk1* displays a similar structure, harboring 20 introns, all of them having a size around 60 bp. Intron gain has been suggested to be a significant driving force in the evolution of genes in fungi (52), but the actual biological significance of this type of structure is still unknown.

Like its homologues ScTrk1 and ScTrk2 in *S. cerevisiae*, HcTrk1 is able to transport K^+ since it can complement *S. cerevisiae* mutants defective for K^+ uptake (Fig. 2). When the fungus grows in symbiotic conditions, in association with *P. pinaster*, *HcTrk1* is expressed in external hyphae that explore the soil. Thus, it could play a role in the K^+ nutrition of the fungus and, thereby, of the host plant.

Electrophysiological Characterization of a Trk Transporter—HcTrk1 can be expressed in *Xenopus* oocytes, where it is targeted to the plasma membrane in a functional state, giving rise to macroscopic currents that can be recorded using the two-electrode voltage-clamp technique. The present study provides the first detailed analysis of the functional properties of a member of the Trk family. The results can be summarized as follows; (i) HcTrk1 mediates instantaneously activating inwardly rectifying currents, (ii) HcTrk1 is permeable to K^+, (iii) among alkali cations, HcTrk1 is more permeable to Na^+ than to K^+, and (iv) K^+ and Na^+ interact during permeation through HcTrk1. The subsequent discussion mainly focuses on the last two points and the possible significance for the physiological role of HcTrk1 in the fungus.

HcTrk1 Ionic Selectivity—The transport activity of two members of the Trk family present in *S. cerevisiae*, ScTrk1 and ScTrk2, has been extensively analyzed mainly by comparing K^+ (Rb^+) transport in wild type and in mutant strains displaying homozygous disruptions of *ScTrk1* and/or *ScTrk2* genes (21). The whole set of data provided by yeast uptake experiments indicates that the two genes encode selective K^+ transporters or H^+:K^+ symporters that do not significantly transport Na^+. A similar conclusion can be drawn from the data available for other fungal Trk members characterized so far in the yeasts *S. pombe* and *S. occidentalis*, and in the filamentous ascomycete *N. crassa* (43, 45, 48). Electrophysiological analyses of ScTrk1 and ScTrk2 activities have also been performed by yeast patch-clamp experiments. They have confirmed that ScTrk1 and ScTrk2 are endowed with K^+ transport activity, identifying small fast-activating K^+ inward currents in wild type yeast cells that were absent in mutant cells deleted for *Trk1* and *Trk2* (53). Intriguingly, further patch-clamp analyses have revealed that ScTrk1 and ScTrk2 can also mediate large outward chloride currents in addition to their K^+ transport properties (54, 55). The physiological significance of this chloride transport activity is, however, still unclear (54). When compared with these fungal Trk systems, the *H. cylindrosporum* Trk transporter displays a unique ionic selectivity, being more permeable to Na^+ than to K^+. Thus, functional diversity exists in terms of ionic selectivity within the fungal Trk family.

Cation transporters displaying structural and sequence similarities with fungal Trk have been identified in plants, where they form a family named HKT. The HKT transporters characterized so far either mediate selective Na^+ transport or are permeable to both Na^+ and K^+ (56–59). A glycine residue that is conserved in the selectivity filter of the pore domains of HKT transporters as well as of bacterial KtrB transporters permeable to both Na^+ and K^+ has been identified as a crucial determinant of the permeability to K^+ (60–62). This glycine residue is conserved also in HcTrk1, ScTrk1, and ScTrk2 (see the *arrow* in Fig. 1*B*). Thus, also in fungal Trk transporters, this glycine residue could be part of the selectivity filter-controlling ion permeation and K^+ transport. On the other hand, the available information provides no clue regarding the molecular basis of the permeability to Na^+ (in HcTrk1) or H^+ (in ScTrk1 and ScTrk2).

A Transporter Endowed with Channel-like Permeation Mechanisms—In the classical view, two classes of secondary transport systems, named channels and transporters, can be distinguished (63). A channel, when open, allows permeation of millions of ions through the pore without any conformational change of the protein. Conversely, a transporter is thought to

undergo a global conformational change per each transport event, as described by the classical alternating access model (63–65). As a consequence of this difference in permeation process, the transport velocity is much higher (at least 10^2–10^3 times) in channels than in transporters. Besides this mechanistic distinction, another difference between channels and transporters based on a thermodynamic point of view is that only the latter systems can mediate active transport against the electrochemical gradient of the transported species. However, this rigid dichotomization is increasingly proving to be too simplistic to describe the functional diversity of these proteins (66–69).

Trk transporters have probably evolved from ancestors related to K^+ channels, as suggested by their K^+ channel-like structure (14). The present electrophysiological data indicate that HcTrk1 does display K^+ channel-like functional properties. (i) The isotherm describing the effect of the concentration of the permeant cation (Na^+ or K^+) on HcTrk1 current displays only a weak saturation (Na^+) or no saturation at all (K^+), being quasi-linear up to a concentration of 100 mM, whereas a transporter is expected to typically display saturation kinetics in a range of physiological concentrations. (ii) HcTrk1 current is strongly inhibited by Cs^+, a classic inhibitor of K^+ channels, acting by occluding the channel pore. (iii) The magnitude of the macroscopic currents mediated by HcTrk1 in oocytes in the presence of Na^+ is rather high and, although single-transporter currents have not been recorded herein, such current amplitudes are poorly compatible with a permeation process that would occur according to the alternating access model (70, 71). (iv) Finally, Na^+-K^+ interaction in HcTrk1 results in a phenomenon reminiscent of anomalous mole fraction effect in ion channels. Interactions between permeant ions giving rise to an anomalous mole fraction effect have been extensively analyzed in numerous ion channels (46, 47). They are thought to indicate that permeation occurs through a narrow pore where several ions move simultaneously in a single-file, hopping from one binding site to the next, each ion pushing the one ahead of it along (63). Interactions between two permeant ionic species are ascribed to the fact that at least one of the binding sites along the pore differs in its affinity for the two species, *i.e.* binds one of the two species more tightly than the other, the more tightly bound ion hindering the passage of the other. Thus, both the channel-like structure and functional properties of HcTrk1 support the hypothesis that a single-file pore is present in this transport protein. Because the inhibition by K^+ (or Cs^+) of the HcTrk1 current in the presence of Na^+ is poorly dependent on the transmembrane electric potential difference, the main region(s) of the permeation pathway where ions interact/bind might be outside the transmembrane electric field, at the external mouth of the transporter. Non-permeant ions as Cs^+ or Rb^+ that inhibit HcTrk1 current would remain bound at this place, blocking the conduction pathway.

Mechanistic models that allow an understanding of how transport proteins can be endowed with both channel-like properties and co-transport activity have been developed based on the assumption that permeation occurs through a multisubstrate single-file pore (71, 72). Simply speaking, because the transporter pore is too narrow to allow the ions to pass one another, different permeant ionic species can interact during the permeation step, a slowly moving species (strongly attracted by a region in the pore) inhibiting the flux of a rapidly moving one, producing an anomalous mole fraction effect analogous to that occurring in some ion channels. Furthermore, in transporters having such a narrow pore, the exergonic flow of an ionic species in the pore, down its electrochemical gradient, can energize the endergonic flux of another ionic species by pushing it against its own gradient. According to this type of transporter model, channel-like properties of HcTrk1 could underlie co-transport activity. Interestingly, similar models employing concepts derived from ion channels that possess multiple binding sites within a single pore have been proposed for the plant Na^+-coupled K^+ transporter TaHKT1 (73) as well as for the bacterial transporter VaKtrB (62).

Physiological Role of HcTrk1—*S. cerevisiae* possesses two transport systems dedicated to K^+ uptake, both belonging to the Trk family, ScTrk1 and ScTrk2 (74). However, at least two other types of systems can contribute to K^+ uptake in fungal cells, K^+ transporters from the HAK family (21) and K^+ pumps from the P-type ATPase family (75). In *N. crassa* and in the soil yeast *S. occidentalis*, two types of K^+ transport systems, Trk and HAK, have been identified (48, 43). In *Laccaria bicolor*, the first symbiotic fungus genome to be sequenced (79), two Trk and one HAK genes can be found. Thus, HcTrk1 is probably not the only system responsible for K^+ uptake in *H. cylindrosporum*.

The absence of sensitivity to external K^+ availability of *HcTrk1* transcript levels suggests that this transporter is a constitutive system that might play a role in K^+ uptake in a large concentration range. The hypothesis that it might mediate K^+ uptake in the low concentration range is not ruled out by the fact that HcTrk1 expression in *S. cerevisiae* mutant cells deficient for K^+ uptake does not restore growth on low K^+ media (<1 mM; Fig. 2). The absence of complementation on low K^+ media could result from poorly efficient targeting or regulation of HcTrk1 in the heterologous context. Such problems are indeed quite common in this type of experiment.

HcTrk1 can also mediate Na^+ transport, unlike the other fungal Trk systems characterized so far. Two non-exclusive hypotheses can be proposed concerning the significance of this Na^+ transport activity. As discussed above, it could correspond to K^+:Na^+ co-transport activity, with variable stoichiometry. Within the framework of this hypothesis, Na^+ excretion systems such as Na^+-ATPases or Na^+:H^+ antiporters already identified in *S. cerevisiae* (76, 77) would build the Na^+ electrochemical gradient and prevent toxic Na^+ accumulation. The second hypothesis is that HcTrk1 could allow the fungus, growing in nutrient-poor soils, to take up Na^+ and use it instead of K^+ for osmotic adjustment after compartmentalization in the vacuoles. Fungal cells can indeed substitute Na^+ for K^+ in this unspecific role of osmoticum (75, 78).

In conclusion, the identification of HcTrk1 opens the way to molecular analysis of K^+ transport in ectomycorrhizal symbiosis. Functional expression of this system in *Xenopus* oocytes has provided the first electrophysiological characterization of a transporter from the Trk family. HcTrk1 is permeable to both K^+ and Na^+. The whole set of data is consistent with the

hypothesis that channel-like permeation through a single-file pore underlies transport activity and could also allow K^+:Na^+ co-transport activity. HcTrk1 is likely to play a role in both K^+ and Na^+ nutrition, the fungus using the latter cation as vacuolar osmoticum in nutrient-poor soils.

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Molecular and Functional Characterization of a Na^+-K^+ Transporter from the Trk Family in the Ectomycorrhizal Fungus *Hebeloma cylindrosporum*

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