HAK Transporters from *Physcomitrella patens* and *Yarrowia lipolytica* Mediate Sodium Uptake

Begoña Benito, Blanca García de las and Alonso Rodriguez-Navarro

The widespread presence of Na⁺-specific uptake systems across plants and fungi is a controversial topic. In this study, we identify two HAK genes, one in the moss *Physcomitrella patens* and the other in the yeast *Yarrowia lipolytica*, that encode Na⁺-specific transporters. Because HAK genes are numerous in plants and are duplicated in many fungi, our findings suggest that some HAK genes encode Na⁺ transporters and that Na⁺ might play physiological roles in plants and fungi more extensively than is currently thought.

**Keywords:** Bacteria • HAK transporters • *Physcomitrella* • Sodium uptake • *Yarrowia*.

**Abbreviations:** RT-PCR, reverse transcription-PCR.

The nucleotide sequences reported in this paper have been submitted to the GenBank database under accession numbers: HE798566.

**Introduction**

The current paradigm of K⁺ and Na⁺ homeostasis in living cells holds that K⁺ is the most abundant cation in all living cells and that high cytoplasmic Na⁺/K⁺ ratios are toxic. This model of alkali cation cellular distribution was originated through studies with animal cells (Steinbach 1962, and references therein) and later applied to plant and bacterial cells (Evans and Sorger 1966, Suelter 1970). In eukaryotic organisms, the model is convincingly correct in mammalian cells, which are permanently bathed by a medium that contains 150 mM Na⁺ and 5 mM K⁺. In plant and fungal cells, however, the evidence is less clear. Both types of organisms evolved over a long period of time in an extremely oligotrophic environment as they colonized the continental rocks and primitive soils (Heckman et al. 2001); in the absence of K⁺, the accumulation of Na⁺ instead of K⁺ is likely to be more advantageous than a complete lack of any monovalent cation (Rodríguez-Navarro and Rubio 2006). Considering these differences, it is unlikely that Na⁺ serves the same functions in animal, plant and fungal cells.

The application of the knowledge of the role of Na⁺ in animals to other organisms may have influenced the acceptance of the current notion of Na⁺ toxicity and the absence of Na⁺-specific transporters in plants and fungi. Na⁺ cannot completely replace K⁺ in living cells, and some enzymes in particular species may be highly sensitive to Na⁺. Neither of these observations, however, implies that Na⁺ could not replace a substantial amount of cellular K⁺. In fact, there is evidence that Na⁺ may be well tolerated by plant (Greenway and Osmond 1972, Flowers and Läuchli 1983) and fungal (Benito et al. 2009) cells. Previous studies have also shown that high- and medium-affinity Na⁺ transporters are Na⁺ specific (Benito et al. 2004, Rodríguez-Navarro and Rubio 2006, Horie et al. 2007, Haro et al. 2010), suggesting that low-affinity Na⁺ uptake may also be mediated by specific transporters (Wang et al. 2007, Kronzucker et al. 2008) and not by K⁺ transporters with low K⁺/Na⁺ specificity (Blumwald et al. 2000). The Na⁺-specific transporters described in those previous studies belong to the Trk-HKT family of cation transporters. This transporter family is widely present in plants, fungi and bacteria, and displays a large diversity in terms of ionic permeability (Corrêa-Faillé et al. 2010), which suggests that the plant low-affinity Na⁺ transporters might belong to this family. However, the obvious candidates of HKT appear to be more involved in Na⁺ transport in internal tissues (Mäser et al. 2002, Rus et al. 2004) than in root Na⁺ uptake (Berthomieu et al. 2003, Essah et al. 2003) at least in Arabidopsis. Alternatively, the putative low-affinity Na⁺ transporter might belong to the Kup-HAK family of K⁺ transporters, which is also widely present in plants, fungi and bacteria (Grabov, 2007). The problem in this case is that no HAK transporter has been shown to be Na⁺ specific.

To increase our knowledge about Na⁺ uptake systems, we conducted a systematic study to isolate genes that encode these systems in plants and fungi. We report here the identification of two Na⁺-specific HAK transporters, one in *Physcomitrella patens* and the other in *Yarrowia lipolytica*. We also extended our study to bacteria and found that, unlike many plants and fungi, all of the tested bacterial species showed high-affinity K⁺ uptake but not high-affinity Na⁺ uptake.
Results

PpHAK13 mediates Na⁺ uptake in yeast cells

Assuming that the expression of an Na⁺ transporter might be repressed in the presence of high Na⁺ concentrations, we identified the PpHAK13 gene by microarray analysis (unpublished results). We confirmed this result by reverse transcription–PCR (RT–PCR) experiments, which showed that the PpHAK13/Act5 transcript abundance ratio for plants grown in normal medium, with 100 mM NaCl, and after 15 d under K⁺ starvation were 0.87 ± 0.02, 0.13 ± 0.02 and 1.7 ± 0.04, respectively. These figures indicated that PpHAK13, which is expressed in normal conditions similarly to the ACT5 gene, was slightly induced under K⁺ starvation and repressed during salt treatment. In summary, PpHAK13 was a good candidate to be a Na⁺ transporter. Furthermore, the phylogenetic analysis of PpHAK13 located it in group IV of HAK transporters for which there is no functional information. Remarkably, we found that transporters in this group exist in most plants species for which the whole genome sequence is available, but not in Arabidopsis species or Thellungiella halophila (Fig. 1). Altogether these results prompted us to clone the PpHAK13 cDNA. This cDNA encodes a protein of 299 amino acids and a mol. wt. of 88,571 kDa, which showed a typical HAK structure, with 12 transmembrane fragments and a long hydrophilic C-terminus (Garcia-deblas et al. 2002a).

We then expressed the PpHAK13 cDNA in a trk1 trk2 yeast mutant (Haro and Rodriguez-Navarro 2003). In this transformed strain, neither K⁺ uptake nor improved growth at low K⁺ was observed. In contrast, the PpHAK13 transformants showed high-affinity Na⁺ uptake (Fig. 2). The influx kinetics exhibited a low Vₘₐₓ (1.1 ± 0.2 mmol mg⁻¹ min⁻¹; n = 4) and a Kₘ that varied from 5 to 12 μM Na⁺. This Na⁺ uptake does not exist in wild-type strains of Saccharomyces cerevisiae.

Function of PpHAK13 in P. patens

To study the function of the PpHAK13 transporter, we disrupted the PpHAK13 gene. We isolated five ΔPpHak13 lines, in which the zeocin-resistant cassette substituted for the coding region of PpHAK13. These mutant lines grew normally at high or low K⁺ but we observed that the Na⁺ uptake normally induced in wild-type plants under K⁺ starvation (Haro et al. 2010) did not take place in the ΔPpHak13 lines (Fig. 3).

Physcomitrella patens plants with a normal K⁺ content require from 10 to 15 d under K⁺ starvation to develop high-affinity Na⁺ uptake (Fig. 3 shows an example of the process). Therefore, finding that the increase of the expression of the PpHAK13 gene under K⁺ starvation was low and of doubtful biological relevance prompted us to test the role of PpHAK13 in low-affinity Na⁺ uptake. The experiments were carried out at 10 mM Na⁺, with wild-type and ΔPpHak13 plants, and it was found that Na⁺ uptake was not abolished in ΔPpHak13 plants. This result proved that the PpHAK13 gene did not encode the low-affinity Na⁺ uptake system of P. patens. However, a minor contribution of PpHAK13 to this uptake could not be ruled out because net Na⁺ uptake was low and exhibited a significant overall variability between different plant batchs. We recently found that this variability was due to the activity of the PpSOS1 and PpENa1 Na⁺ efflux systems because it disappeared in a ΔSOS1 ΔENa1 double mutant.

Yarrowia lipolytica has a HAK high-affinity Na⁺ transporter

HAK transporters are abundantly present in fungal species, many of which have two or more transporters that belong to this group (Benito et al. 2011). Therefore, our results with PpHAK13 raised the possibility that some fungal HAK transporters might be Na⁺ transporters. To explore this possibility, we chose the yeast Y. lipolytica; according to its complete genome sequence, it has no ACU ATPases, which are P-type ATPases that mediate high-affinity K⁺ and Na⁺ uptake in fungi (Benito et al. 2004). The genome also encodes two HAK transporters and one TRK transporter (Benito et al. 2011). Simple uptake experiments revealed that K⁺-starved Y. lipolytica cells showed high-affinity K⁺ and Na⁺ influxes (Fig. 4A).

To investigate whether any of the two HAK genes from Y. lipolytica encodes an Na⁺ transporter, we cloned the corresponding cDNAs, naming them YIHA1 and YIHA2, and expressed them in the trk1 trk2 yeast mutant. The YIHA2 transformant grew well at low levels of K⁺ (50 μM), but the YIHA1 transformant did not grow under these conditions (Fig. 4B). In independent K⁺ or Na⁺ uptake experiments, the YIHA1 transformant showed very rapid high-affinity uptake for both cations (compare Fig. 5A and B), while the YIHA2 transformant took up only K⁺ (Fig. 5C). The capacity of YIHA1 to transport K⁺ and its incapacity to suppress the growth defect of the yeast mutant strain at low K⁺ appeared to be contradictory results; we hypothesized that the K⁺ uptake by YIHA1 might have been inhibited by the Na⁺ content of the agar medium (750 μM). Therefore, we repeated the K⁺ uptake experiment in liquid medium in the presence of 750 μM Na⁺, and it was found that K⁺ uptake was completely inhibited in these conditions (Fig. 5B). This result indicates that the physiological function of YIHA1 is Na⁺ uptake. The K⁺ uptake activity of this transporter that we observed when Na⁺ was absent is unlikely to be physiological because Na⁺-free environments are probably very rare.

High-affinity Na⁺ uptake may not exist in bacteria

High-affinity Na⁺ uptake has not been described in bacteria, but its existence cannot be ruled out; bacterial Kup transporters show high sequence homology to the high-affinity HAK transporters of plants and fungi. Therefore, our demonstration that PpHAK13 and YIHA1 are Na⁺ transporters raised the question of whether some bacterial Kup transporters, which are widely present in Proteobacteria, are Na⁺ transporters.

To answer the question of the existence of high-affinity Na⁺ uptake in bacteria, we selected seven proteobacterial species with kup genes and two Gram-positive species, which do not have kup genes (Table 1). When cells of these species were
suspended in a medium with 50–100 μM of both K⁺ and Na⁺, the cells depleted K⁺ very rapidly but failed to deplete Na⁺. This pattern was found even in long-term experiments when the cells started to lose K⁺, indicating that they were dying. Fig. 6 displays the results of one experiment with Azotobacter vine landii, which is representative of the results for all the bacterial species tested.

**Discussion**

The current results raise the number of gene families of cation uptake systems with Na⁺-specific members to three: TRK-HKT (Rodríguez-Navarro and Rubio 2006), ACU ATPases (Benito et al. 2011) and HAK (this report). Because these families do not show phylogenetic relationships, their convergent evolution to produce Na⁺ uptake systems supports the notion that Na⁺ uptake is an important function in both plants and fungi. Originally, high-affinity Na⁺ uptake might appear in response to the selective pressure of an oligotrophic environment (Benito et al. 2004, Rodríguez-Navarro and Rubio 2006). Remarkably, our results suggest that this function is mostly or completely absent in bacteria. We did not detect high-affinity Na⁺ uptake in bacterial species that grow well in soils, which is the environment in which plants and fungi putatively developed the function. This finding suggests the possibility that bacteria might have played a relatively minor role in the colonization of rocks and primitive soils. However, more bacterial species must be studied before a general conclusion can be reached.

The simplest interpretation of the data shown in Fig. 3 is that PpHAK13 is the high-affinity Na⁺ transporter of *P. patens*. However, the situation may be more complex because high-affinity Na⁺ uptake in *P. patens* appears after a long
period of K⁺ starvation (Haro et al 2010; Fig. 3) and PpHAK13 transcripts were present at similar levels in plants growing both in K⁺-sufficient and under K⁺-starvation conditions. The high-affinity Na⁺ uptake mediated by PpHAK13 might therefore result from the interaction of PpHAK13 with another HAK transporter or after its activation by an unknown mechanism that increases the affinity of the system under K⁺ starvation. However, our results demonstrated that PpHAK13 is not the low-affinity Na⁺ uptake system of *P. patens* although it might mediate a minor part of this uptake.

The primary results of our research are the findings that two HAK genes, one from *P. patens* and the other from *Y. lipolytica*, encode Na⁺ uptake systems. This finding and the extended presence of HAK transporters in plants and fungi suggest that root Na⁺ uptake may be more frequently mediated by HAK than by HKT transporters; in fact, the HKT transporter of Arabidopsis does not mediate root Na⁺ uptake (Berthomieu et al. 2003, Essah et al. 2003). Previous studies have shown that high-affinity Na⁺ uptake in rice is mediated by OsHKT2;1 (Garciaebelas et al. 2003, Horie et al. 2007). However, this uptake mechanism might be unique to rice because the characteristic sensitivity of the OsHKT2;1-mediated Na⁺ uptake to K⁺ or Ba⁺ suggests that Na⁺ uptake is not mediated by HKT transporters in many other plant species (Haro et al. 2010).

It has been proposed that root Na⁺ uptake occurs through specific Na⁺ transporters in conditions of salinity (Wang et al. 2007, Kronzucker et al. 2008), as opposed to the idea that it is mediated by K⁺ transporters (Blumwald et al. 2000). In addition to their biological implications, the current results open up a new line of research involving HAK transporters in the field of crop salt tolerance.

### Materials and Methods

#### Plant material and growth conditions

The moss *P. patens* (Ashton et al. 1979) was maintained axenically in BCDAT medium (Nishiyama et al. 2000) supplemented with 5 g l⁻¹ agar as necessary. K⁺-starved plants were prepared by transferring moss protonema samples to biofermenters or jars containing K⁺- and Na⁺-free modified KFM medium (Garciaebelas et al. 2007b), which originally contained 3–5 μM K⁺ and Na⁺. Plants were grown under air bubbling in a 25°C phytochamber with continuous white light at a quantum irradiance of 200 μmol m⁻² s⁻¹ for 3 weeks. All media were inoculated with aliquots of moss suspensions that were fragmented with a Polynor PT2100 homogenizer. The plants exhausted the original supply of K⁺ in 4–5 d.

#### Bacterial and yeast strains, and growth conditions

*Escherichia coli* strain DH5α was routinely used for plasmid DNA propagation. *Yarrowia lipolytica* CCL122 and *S. cerevisiae* WΔ6 (Mat a ade2 ura3 trpl1 trk1 Δ1 LEU2 trk2 Δ1 H153), which is deficient in the endogenous K⁺ uptake systems TRK1 and TRK2 (Haro and Rodriguez-Navarro 2003), were normally grown...
Fig. 5 High-affinity K\(^+\) or Na\(^+\) uptake mediated by YIHAK1 and YIHAK2. (A) Absence of high-affinity K\(^+\) or Na\(^+\) uptake in the trk1 trk2 yeast strain transformed with the empty vector used to clone YIHAK1 and YIHAK2. (B) Time courses of the decrease of the external Na\(^+\) or K\(^+\) concentrations in K\(^+\)-starved trk1 trk2 yeast cells transformed with YIHAK1 and exposed to only K\(^+\) or Na\(^+\) and time course of the variation of K\(^+\) in the presence of 750 \(\mu\)M NaCl. (C) Time courses of the variation of external K\(^+\) or Na\(^+\) concentrations in K\(^+\)-starved trk1 trk2 yeast cells transformed with YIHAK2.

Table 1 Bacterial species that were used for high-affinity Na\(^+\) uptake tests; the numbers of KUP genes in each species are recorded

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Strain</th>
<th>KUP</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>K12</td>
<td>1</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>Dickeya dadantii</td>
<td>Ech20</td>
<td>2</td>
<td>P. Rodriguez-Palenzuela</td>
</tr>
<tr>
<td>Sinorhizobium meliloti</td>
<td>1021</td>
<td>1</td>
<td>T. Ruiz-Argecio</td>
</tr>
<tr>
<td>Rhizobium etli</td>
<td>CFM42</td>
<td>2</td>
<td>T. Ruiz-Argecio</td>
</tr>
<tr>
<td>Agrobacterium tumefaciens</td>
<td>CS8</td>
<td>2</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>Azotobacter vinelandii</td>
<td>D1</td>
<td>1</td>
<td>D. Dean</td>
</tr>
<tr>
<td>Pseudomonas putida</td>
<td>KT2440</td>
<td>1</td>
<td>J. L. Ramos</td>
</tr>
<tr>
<td>Pseudomonas syringae</td>
<td>DC3000</td>
<td>1</td>
<td>A. Collmer</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>168</td>
<td>0</td>
<td>R. Perez-Mellado</td>
</tr>
<tr>
<td>Streptomyces lividans</td>
<td>TK21</td>
<td>0</td>
<td>R. Perez-Mellado</td>
</tr>
</tbody>
</table>

* BLAST searches were carried out with the Escherichia coli KUP protein sequence against the translated genomic sequences in the database http://www.ncbi.nlm.nih.gov.

either in complex YPD or in mineral SD media (Sherman 1991) supplemented with 50 mM K\(^+\) as indicated. Growth tests of S. cerevisiae were performed on arginine phosphate medium at pH 6.5 (Rodriguez-Navarro and Ramos 1984) supplemented with KCl or NaCl at the indicated K\(^+\) and Na\(^+\) concentrations. High-affinity Na\(^+\) uptake was tested in the nine bacterial species recorded in Table 1.

Recombinant DNA techniques

The full-length PpHAK13, YIHAK1 and YIHAK2 cDNAs were amplified from P. patens and Y. lipolytica total RNA by standard RT–PCR methods using specific forward and reverse primers, which included the ATG and STOP codon triplets. The resulting PCR fragments were first cloned into the PCR2.1-Topo vector using the TOPO TA Cloning Kit (Invitrogen). For expression in yeast cells, the full-length cDNAs were cloned into the vector pYPGE15 (Brunelli and Pall 1993). In all cases most of the polyclinker sequences preceding the translation initiation codon were eliminated and the sequence environment around the AUG was as similar as possible to (A/U)A(A/C)/(A/C)A AUG/C(U/C) (Hamilton et al. 1987).

Generation of the ΔPphak13 knockout lines

The PpHAK13 knockout fragment was constructed in the p35S-Zeo vector by inserting two fragments of the HAK13 gene 5’ and 3’ non-coding regions such that they flankled the zeocin resistance cassette. The 5’ fragment extended from the −989 to the −44 positions and the 3’ fragment from the 164 to the 947 positions downstream of the STOP codon. The 5’ fragment was inserted between the KpnI and SalI restriction sites and the polyclinkers in the p35S-Zeo vector, while the 3’ fragment was placed between the XhoI and SalI restriction sites of the polyclinkers. Knockout mutants were generated by transforming P. patens protoplasts as described in Hohe et al. (2004).
with 25 μg of the linear DNA fragment obtained by digesting the knockout vector with the KpnI and SacI restriction enzymes. Stable antibiotic-resistant clones were selected after two rounds of incubation in BCDAT medium supplemented with 50 μg ml⁻¹ zeocin (Invitrogen). The integrated fragments were sequenced in putative mutants to check that integration occurred as designed. The basic defects of the hak13 mutants were studied in five independent lines designated pphak13- (1–5), which were identical.

**Real-time PCR assays**

Real-time PCR assays were performed as described previously (García-Deblas et al. 2003), except that the standard DNA solutions corresponded to the genes studied in this report, namely the HAK13 and Act5 genes of *P. patens*. Total RNA preparations were treated with RNase-free DNase I (40 U 100 μl⁻¹, Roche Applied Science) for 1 h at 37°C. After treatment, RNA was purified using the methods described in the RNEasy plant kit (Qiagen). Real-time quantitative PCR of the derived cDNA was carried out based on the TaqMan technology using the Universal ProbeLibrary system (Roche Diagnostics). Primers and probes for each gene assay were designed using the Universal ProbeLibrary Assay Design Center (https://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). For HAK13 the primers and probe were: forward, CAGCCGTATGCCTCA AAGTA; reverse, ACATTGATGCTATTCGGAAG; probe number, 83. The corresponding primers and probe for ACT5 were: forward, CAAGGAGATCCGGCAGCTG; reverse, CCTCG ATCCACACACTGTA; probe number, 55. Quantitative PCR assays were carried out in duplicate using the FastStart TaqMan Probe Master (Rox) kit and the Applied Biosystems 7500 real-time PCR system. For each growth condition, two independent batches of plants were PCR tested. Because the differences between the two batches were low, we report the mean of the four PCR results.

**Na⁺ or K⁺ uptake in yeast, bacteria and *P. patens***

Cation uptake tests at micromolar K⁺ or Na⁺ concentrations were carried out by following the decrease of the cations in the external medium. The experiments were started by the addition of the selected cation; at intervals, yeast or bacterial cells were removed by centrifugation and plants by filtration; the K⁺ and Na⁺ concentrations in the cell-free medium were determined by atomic emission spectrophotometry. The time courses of cation depletions can be used for kinetic analyses, considering that the cation influx at any given concentration is the slope of the tangent to the depletion curve at that point. The procedure for fitting the curves and computing the Vmax and Km values, as well as the limits of the method, have been previously described (Bañuelos et al. 2002). All uptake tests were repeated three or four times; in all experiments we checked that the medium pH had not changed significantly. *Physcomitrella patens* experiments were performed in KFM with K⁺-starved plants that were prepared by growing the plants for 10–15 d in KFM medium, as described above. Yeast experiments were performed with K⁺-starved cells. First, cells were grown in AP medium supplemented with 3 mM K⁺, washed in water and then inoculated in K⁺- and Na⁺-free AP medium. The starvation time was 4 h. Uptake tests were performed in 10 mM MES-Ca²⁺ pH 6.0 supplemented with 2% glucose. The experiments with all bacterial species were initiated with cultures grown in 0.5% glucose LB medium (1% tryptone, 0.5% yeast extract and 87 mM NaCl). Then the cells were washed, and transferred to K⁺-free testing medium, which contained: 5 mM H₃PO₄, 2 mM MgSO₄, 6 μM FeSO₄, 0.1 mM CaCl₂, 1 mM citric acid, 0.2% glycerol, 8 mM l-asparagine, 8 mM serine and 8 mM glutamic acid. The medium was brought to pH 7.5 with l-arginine, and oligoelements and vitamins were added as described in Rhoads et al. (1976). This medium was supplemented with 50–100 μM K⁺ and Na⁺ as shown in Fig. 6.

Low-affinity Na⁺ uptake tests in *P. patens* were performed at 10 mM Na⁺ in plants growing in 1 mM K⁺ KFM medium. After the addition of Na⁺, the Na⁺ content of the plants was determined in washed, dried and weighed plants which were extracted with 0.1 M HCl, as described previously (Fraile-Escanciano et al. 2010). Plants were exposed to 10 mM Na⁺ for 24 h.

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