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Cloning and functional characterization of the high-affinity K^+ transporter HAK1 of pepper

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Abstract

High-affinity K^+ uptake in plants plays a crucial role in K^+ nutrition and different systems have been postulated to contribute to the high-affinity K^+ uptake. The results presented here with pepper (*Capsicum annum*) demonstrate that a HAK1-type transporter greatly contributes to the high-affinity K^+ uptake observed in roots. Pepper plants starved of K^+ for 3 d showed high-affinity K^+ uptake (K_m of 6 μM K^+) that was very sensitive to NH_4^+ and their roots expressed a high-affinity K^+ transporter, CaHAK1, which clusters in group I of the KT/HAK/KUP family of transporters. When expressed in yeast (*Saccharomyces cerevisiae*), CaHAK1 mediated high-affinity K^+ and Rb^+ uptake with K_m values of 3.3 and 1.9 μM, respectively. Rb^+ uptake was competitively inhibited by micromolar concentrations of NH_4^+ and Cs^+, and by millimolar concentrations of Na^+.

Introduction

Potassium is an essential macronutrient for plants. It is the most abundant cation in plant cells and fulfils important functions related to enzyme activity, osmoregulation, movement, anionic counterbalance and growth. The K^+ concentration of the cytosol of plant cells is maintained constant near 100 mM (Walker *et al.*, 1996), in contrast with the highly variable concentrations of K^+ in the soil solution, which can range from micromolar to millimolar concentrations. To maintain K^+ homeostasis the plasma membrane of plant cells is furnished with high-affinity K^+ transporters (Epstein, 1973), which may mediate active transport (Rodríguez-Navarro, 2000), and low-affinity K^+ transporters, which may be channels (Maathuis and Sanders, 1992).

The first plant K^+ transporters cloned were shaker-like inward rectifying channels (Anderson *et al.*, 1992; Sentenac *et al.*, 1992) and, since then, several families of channels and transporters have been identified in various plant species (Very and Sentenac, 2003). Some of these families are composed of a large number of members. In *Arabidopsis* there are 9 shaker-like K^+ channels and 13 KT/HAK/KUP K^+ transporters (Rubio *et al.*, 2000; Maser *et al.*, 2001) and in rice (*Oryza sativa*) there are at least 17 transporters of the latter family (Banuelos *et al.*, 2002). The KT/HAK/KUP family of transporters includes high- and low-affinity K^+ transporters, as shown by heterologous expression in yeast (*Saccharomyces cerevisiae*) or bacteria (*Escherichia coli*) (Rodríguez-Navarro, 2000; Very and Sentenac, 2003), and is made up of four phylogenetic groups (Rubio *et al.*, 2000). All HAK transporters of group I that have been functionally characterized mediate high-affinity K^+ influx (Santa-María *et al.*, 1997; Rubio *et al.*, 2000; Banuelos *et al.*, 2002). By expressing these transporters in yeast it was found that the barley (*Hordeum vulgare*) and rice HAK1 transporters showed the hallmark characteristics of the high-affinity K^+ influx observed in plant roots, similar

K_m values, no discrimination between K^+ and Rb^+ and inhibition by NH_4^+. Interestingly, the inhibition by NH_4^+ of HAK1-type transporters is not related to NH_4^+ transport capacity (Santa-María *et al.*, 2000). These results indicated that the HAK1 transporters were major contributors to high-affinity K^+ transport in barley and rice (Santa-María *et al.*, 1997; Banuelos *et al.*, 2002) and this notion was further reinforced by expression studies that demonstrated that the genes encoding these transporters are strongly induced by K^+ starvation, which is in agreement with the induction of the high-affinity K^+ influx observed in barley and rice roots deprived of K^+ (Glass, 1975; Banuelos *et al.*, 2002). Although the mechanism operating in HAK1 K^+ transporters has not been reported, they may be K^+–H^+ symporters as has been suggested for the *Neurospora crassa* HAK1 (Haro *et al.*, 1999).

The *Arabidopsis* HAK5, also belonging to group I, has been functionally characterized in yeast and shares with the barley and rice homologs all the characteristics described above (Rubio *et al.*, 2000). Microarray and RT-PCR analyses have recently shown that *AtHAK5* is up-regulated in response to K^+ deprivation and down-regulated upon K^+ re-supply (Ahn *et al.*, 2004; Armengaud *et al.*, 2004; Shin and Schachtman, 2004). In tomato, the expression of *LeHAK5* is also induced by K^+ starvation (Wang *et al.*, 2002). Interestingly, the identification of an *Arabidopsis* mutant disrupted in the gene encoding the K^+-channel AKT1 has allowed the demonstration of a role for K^+ channels in K^+ nutrition from low micromolar external concentrations of the cation (Hirsch *et al.*, 1998; Spalding *et al.*, 1999). The characterization of this mutant by membrane potential measurements in root cells demonstrated that the K^+ permeability was the sum of an NH_4^+-insensitive AKT1 component and an NH_4^+-sensitive non-AKT1 component. The AKT1 component accounted for 63% of the wild-type K^+ permeability when external K^+ was between 10 and 100 μM and NH_4^+ was absent. In barley, an NH_4^+-sensitive or NH_4^+-insensitive pathway dominates high-affinity K^+ uptake depending on the absence or presence of NH_4^+ in the solution employed for plant growth, respectively. The K^+ transporter HvHAK1 has been postulated to provide a major route for the NH_4^+-sensitive component (Santa-María *et al.*, 2000).

From the results with barley, rice and *Arabodpsis* it can be concluded that several systems, including K^+ transporters and K^+ channels, may be operating in parallel at the root to mediate high-affinity K^+ uptake. The contribution of each system may vary depending on the species, the growth conditions or the developmental stages. Characterization of the systems involved in K^+ absorption in other plant species will provide valuable information for establishing a solid model for K^+ acquisition in plants. To gain insight into the molecular mechanisms involved in high-affinity K^+ uptake in plants we selected pepper (*Capsicum annum*) because it is an important crop, in which preliminary studies showed an extraordinary capacity to deplete external K^+ to very low concentrations.

Materials and methods

Plant growth

Seeds of pepper, (*Capsicum annum*, cv. california), were pre-hydrated with an aerated 0.5 mM $CaSO_4$ solution for 72 h and germinated in vermiculite at 28 °C. After 2 d the seedlings were placed in 15 l containers filled with modified one-fifth Hoagland's solution, which consists of the following macronutrients (mM): 1.2 KNO_3, 0.8 $Ca(NO_3)_2$, 0.2 KH_2PO_4, 0.2 $MgSO_4$ and the following micronutrients (μM): 50 $CaCl_2$, 12.5 H_3BO_3, 1 $MnSO_4$, 1 $ZnSO_4$, 0.5 $CuSO_4$, 0.1 H_2MoO_4, 0.1 $NiSO_4$ and 10 Fe-EDDHA and transferred to a controlled-environment chamber with a 16 h light – 8 h dark cycle and air temperatures of 25 and 20 °C, respectively. The relative humidity was 65% (day) and 80% (night) and the photon flux density 550 μmol m^{-2} s^{-1}. Modest aeration was provided. The nutrient solution was replaced with fresh solution after weeks 2, 3, and 4.

Potassium depletion experiments in plants

Four-week-old plants grown as described above were employed for K^+ depletion experiments. For K^+ starvation, plants were transferred for 0, 24, 48 and 72 h to a nutrient solution deprived of K^+ which contained (mM) 1.4 $Ca(NO_3)_2$, 0.1 $Ca(H_2PO_4)_2$, 0.2 $MgSO_4$ and the micronutrients described above. Control plants were maintained

in the K^+-containing nutrient solution described above. Plants were rinsed in a cold K^+-free nutrient solution and at time zero transferred to 250 ml containers with a K^+-free nutrient solution supplemented with 50 μM KCl. Samples of 1 ml were taken every 10 min for 2 h and their K^+ concentrations determined by atomic emission in a Perkin-Elmer (Boston, MA) 5500 spectrophotometer. To study the effect of NH_4^+ on K^+ uptake the plants were transferred either directly to the solution with 50 μM K^+ plus NH_4^+ or after incubating them for 30 min in the presence of NH_4^+. Similar results were obtained with the two sets of plants excluding that the inhibition of K^+ uptake by NH_4^+ originated from a transient depolarization of the membrane potential induced by NH_4^+. Data were fitted to a Michaelis–Menten function as described elsewhere (Banuelos *et al*., 2002). At the end of the experiment, plants were separated into roots and shoots and the fresh weight determined. Then, roots and shoots were dried at 65 °C for 4 days and the dry weight determined.

Yeast and bacterial strains and media

The K^+ uptake deficient yeast (*Saccharomyces cerevisiae*) strain WΔ3 (*MAT***a**, *ade2*, *ura3*, *trp1*, *trk1*Δ::LEU2, *trk2*Δ::HIS3) (Haro *et al*., 1999) and the NH_4^+ uptake deficient yeast strain 31019b (*Mat***a**, *ura3, mep1*Δ, *mep2*Δ*::LEU2, mep3*Δ*::Kan-MX2*) (Marini *et al*., 1997) were employed for growth tests and cation uptake experiments. Yeast strains were routinely grown on SD medium (Sherman, 1991) which was supplemented with 100 mM K^+ for growing WΔ3. For growth experiments at low K^+, the NH_4^+-free AP medium (Rodriguez-Navarro and Ramos, 1984) was employed and at low NH_4^+, minimal mineral media with NH_4^+ as the sole nitrogen source (Marini *et al*., 1997). *Escherichia coli* DH5α was employed for plasmidic DNA propagation.

Plant cDNA isolations

Plants were grown and starved of K^+ as described above, separated into roots and shoots and frozen in liquid nitrogen. Total RNA from pepper roots was isolated and reversed transcribed (RT) by using an anchored oligo-dT primer and avian myeloblastosis virus transcriptase (Amersham Pharmacia Biotech, Uppsala) following standard protocols (Sambrook *et al*., 1989). The reverse transcription products were amplified by polymerase chain reaction (PCR) with the *Taq* polymerase (Amersham Pharmacia Biotech) using the degenerate sense primer 5′-GAYAAYGGNGA NGGNGGNACNTTYGC-3′ and the degenerate antisense primer 5′-AANTGNCCNARRTCNG CRAA-3′ deduced from the conserved regions DNG[D/E]GGTFA and FADLGHF, respectively present in HvHAK1 and HvHAK2 (Rubio *et al*., 2000). PCR products were cloned into the PCR2.1 vector by using the TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced. A full-length cDNA was obtained for *CaHAK1* by using the 5′/3′ rapid amplification of cDNA ends kit (Roche Biochemicals, Summerville, NJ) following manufacturer's instructions. The full-length cDNA was cloned into the pYPGE15 plasmid (Brunelli and Pall 1993) for cDNA expression in yeast.

Nucleic acid gel blot hybridizations

DNA and RNA gel blot hybridizations were carried out by the Southern and Northern techniques, respectively, as described elsewhere (Sambrook *et al*., 1989). The probe was prepared from the cDNA fragment that was obtained by PCR and the degenerate primers described above. Probe labeling, hybridization and detection were performed by using the DIG high prime DNA labeling and detection starter kit II (Roche Biochemicals) following manufacturer's instructions. For RNA gel blot hybridization, 30 μg of total RNA from roots or shoots were separated by electrophoresis in a formaldehyde–1.1% agarose gel and transferred to a nylon membrane.

Cation uptake experiments in yeast

Yeast cells were grown overnight at 28 °C in AP medium supplemented with either 30 mM K^+ (for WΔ3 transformed with empty pYPGE15) or 3 mM K^+ (for WΔ3 transformed with CaHAK1 or for 31019b) and then were K^+ starved for 6 h in K^+-free AP medium for K^+ depletion or Rb^+ uptake experiments or used directly for NH_4^+ depletion experiments. For K^+ depletion experiments, cells were suspended in AP medium containing 70 μM K^+ and incubated at 28 °C. Samples were taken at different time points and

centrifuged at 5000 rpm for 10 min. K^+ in the supernatant was determined by atomic emission spectrophotometry. Data were fitted to a Michaelis-Menten function as described elsewhere (Banuelos *et al.*, 2002). For NH_4^+ depletion experiments cells were suspended in minimal mineral medium supplemented with 100 μM NH_4^+, samples were taken and processed as described previously and the NH_4^+ in the supernatant determined by a modified Berthelot method (Searle, 1984) by using the Ammonium Cell Test Kit (Merck, Darmstadt) following the manufacturer's instructions. For Rb^+ uptake experiments, cells were suspended in 10 mM MES brought to pH 6.0 with $Ca(OH)_2$ supplemented with 2% glucose. At time zero, Rb^+ was added and samples were taken at intervals, filtered through a 0.8-μm pore nitrocellulose membrane filter (Millipore, Bedford, MA) and washed with 20 mM $MgCl_2$. Filters were incubated overnight in 0.1 M HCl. Rb^+ was determined by atomic emission spectrophotometry of acid-extracted cells (Rodriguez-Navarro and Ramos, 1984). Results are expressed on a cell dry weight basis, and the parameters of the concentration dependence of the initial rates of Rb^+ uptake were obtained by fitting the rates to a Michaelis–Menten function. Control experiments were performed with the WΔ3 strain transformed with plasmid pYPGE15 without an insert. The reported initial rates of uptake are the means of at least three independent experiments. Error bars denote standard deviations.

Protein alignments and phylogenetic tree generation

Protein sequence alignments and phylogenetic trees were obtained by using the Clustal X program (Thompson *et al.*, 1997).

Results

High-affinity K^+ influx in pepper plants

Experiments were designed to study the capacity of pepper plants to deplete external K^+ from solutions containing micromolar concentrations of the cation. Plants were grown hidroponically as described in Materials and methods and transferred to a K^+-free solution, whereas control plants remained in the K^+-containing solution. K^+ depletion experiments from a 50 μM K^+ external solution were performed at days 1, 2 and 3 after transferring the plants to the K^+-free solution. The rate of K^+ depletion increased as the time of K^+ starvation increased. Plants starved of K^+ for 3 d showed the highest rates of K^+ depletion and were able to deplete external K^+ from the 50 μM K^+ solution to concentrations bellow 1 μM (Figure 1). On the contrary, non-starved control plants did not produce K^+ depletion (Figure 1). The K^+ depletion data of K^+-starved plants from three independent experiments were fitted to a Michaelis–Menten equation as described elsewhere (Banuelos *et al.*, 2002) and a K_m of 6.0 ± 0.4 μM K^+ was calculated. To further characterize the high-affinity K^+ transport system its sensitivity to NH_4^+ was studied. The presence of 250 μM NH_4^+ in the external solution greatly reduced the capacity to deplete K^+ and at 1 mM NH_4^+ the depletion of K^+ was almost completely abolished (Figure 1).

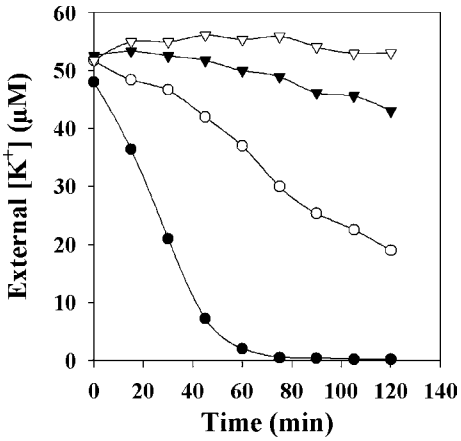

Figure 1. Uptake of K^+ in pepper plants and the effect of NH_4^+. Plants starved of K^+ for 72 h (closed circles, open circles and closed triangles) or non-starved plants (open triangles) were employed for K^+ depletion experiments. At time zero, plants were transferred to one-fifth Hoagland solution containing 50 μM KCl (closed circles and open triangles), 50 μM KCl plus 250 μM NH_4^+ (open circles) or 50 μM KCl plus 1 mM NH_4^+ (closed triangles). At different time points samples of the external solution were taken and the external K^+ determined. A representative experiment is shown. The data from three independent experiments with K^+-starved plants in 50 μM KCl were fitted to a Michaelis–Menten equation (see Materials and methods) and a K_m of 6.0 ± 0.4 μM K^+ was calculated.

Cloning of CaHAK1

The fact that the high-affinity K^+ influx described above was induced by K^+ starvation, mediated K^+ depletion to concentrations below 1 μM and showed high sensitivity to NH_4^+ suggested to us that it could be mediated by a K^+ transporter of the HAK family. Therefore we undertook the molecular cloning of a pepper HAK gene that could encode such a transport system.

Fragments of cDNA corresponding to genes that putatively encoded transporters of the HAK family were amplified by RT-PCR. Total RNA was isolated from roots of pepper plants starved of K^+ for 3 d which, as described above, showed high-affinity K^+ influx. The degenerate primers used for the PCR have been described previously (Rubio *et al*., 2000) and corresponded to conserved regions among HAK transporters of several plant species. Several cDNA fragments from pepper were isolated and sequenced and their translated sequences aligned with representative sequences of HAK transporters of other plant species. The translated sequence of one of the cDNAs clustered within group I of HAK transporters (Rubio *et al*., 2000), which includes several representatives of high-affinity K^+ transporters that are mainly expressed in roots (Santa-María *et al*., 1997; Rubio *et al*., 2000; Banuelos *et al*., 2002). A full-length cDNA that contained the original fragment was obtained, sequenced and named *CaHAK1*. Southern blot hybridizations under high-stringency conditions demonstrated that the isolated cDNA strongly hybridized to fragments of pepper DNA (not shown) and that probably only one copy of the *CaHAK1* gene was present in the pepper genome. The translated sequence of *CaHAK1* could encode a protein of 805 amino acids with 12 putative membrane-spanning domains and the conserved regions characteristic of HAK transporters (Rodríguez-Navarro, 2000). A phylogenetic analysis with HAK transporters representative of the four groups previously described (Rubio *et al*., 2000) showed that CaHAK1 clustered in group I (Figure 2).

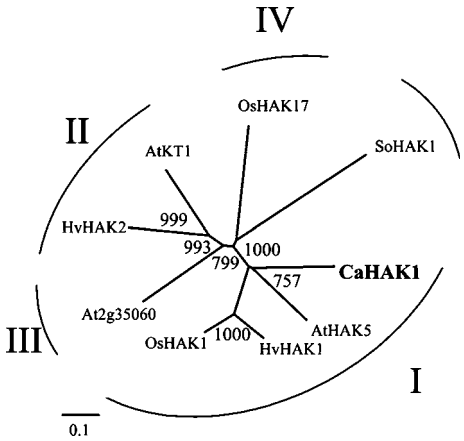

Figure 2. Phylogenetic tree of representative KT/HAK/KUP transporters. Alignment of the sequences were performed with the Clustal X program. The scale bar corresponds to a distance of 10 changes per 100 amino acid positions. Values indicate the number of times of 1000 bootstraps that each branch topology was found during bootstrap analysis. Accession numbers: CaHAK1, AY560009; AtHAK5, AF129478; HvHAK1, AF025292; OsHAK1, AJ427970; HaHAK2, AF129479; AtKT1, AF012656; OsHAK17, AJ427975; SoHAK1, U22945.

CaHAK1 *is exclusively expressed in* K^+*-starved Roots*

To determine the organs and conditions of *CaHAK1* expression Northern blot hybridizations were performed on total RNA extracted from pepper plants grown under different conditions. Plants were grown hydroponically for 1 month in one-fifth Hoagland solution and then transferred to a K^+-free nutrient solution for several periods of time, whereas control plants remained in the same one-fifth Hoagland solution. *CaHAK1* was strongly expressed in roots of plants subjected to K^+ deprivation for 3 d, although a weak expression could be detected 2 d after plants were transferred to the K^+-free solution (Figure 3). *CaHAK1* expression was not detected either in the shoots of the K^+-starved plants or in any part of the plants grown in the control one-fifth Hoagland solution.

CaHAK1 *encodes a high-affinity* K^+ *transporter*

To determine whether CaHAK1 was a K^+ transporter, the *CaHAK1* cDNA was inserted into the yeast expression vector pYPGE15 (Brunelli and Pall, 1993) and the construct transformed into the K^+-uptake deficient yeast strain WΔ3 (Haro *et al*., 1999). Growth tests on the NH_4^+-free medium AP (Rodriguez-Navarro and Ramos, 1984) showed that the yeast strain WΔ3 transformed with the plasmid containing *CaHAK1* could grow with

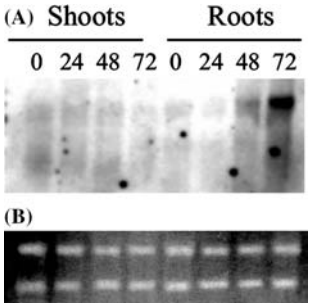

Figure 3. Northern blot analysis of CaHAK1 transcript. Total RNA (30 μg) was isolated from roots or shoots of plants exposed to K^+ starvation for 0, 24, 48 or 72 h. (A) Detection of *CaHAK1* mRNA by hybridizing to a DIG-labeled *CaHAK1* probe. (B) Ethidium bromide staining of the gel containing the RNA used for the Northern analysis.

100 μM K^+ whereas the same strain transformed with the empty plasmid did not grow (Figure 4A). When NH_4^+, Cs^+ or Na^+ were added to the above medium yeast growth was inhibited (Figure 4B). Suspensions of WΔ3 yeast cells expressing CaHAK1 were able to deplete external K^+ to less than 1 μM and this capacity was inhibited by the presence of micromolar concentrations of NH_4^+ (Figure 5). The data of the K^+ depletion from three independent experiments were fitted to a Michaelis–Menten equation as described elsewhere (Banuelos *et al.*, 2002) and a K_m of 3.3 ± 0.2 μM was calculated.

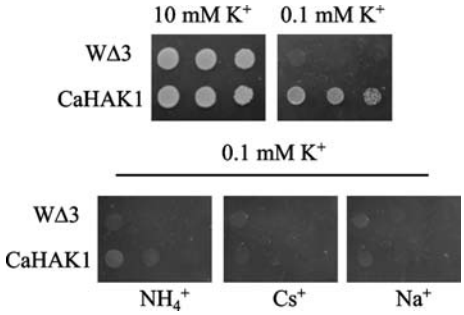

Figure 4. Functional complementation of a yeast strain defective in K^+ uptake by CaHAK1. The *trk1, trk2* yeast strain WΔ3 was transformed with the empty plasmid pYPGE15 or with the plasmid containing *CaHAK1*. Serial dilution drops of both strains were plated on the AP minimal medium. (A) Medium supplemented with 10 or 0.1 mM K^+. (B) Medium supplemented with 0.1 mM K^+ plus 20 mM NH_4^+, or 0.1 mM K^+ plus 5 mM Cs^+ or 0.1 mM K^+ plus 50 mM Na^+.

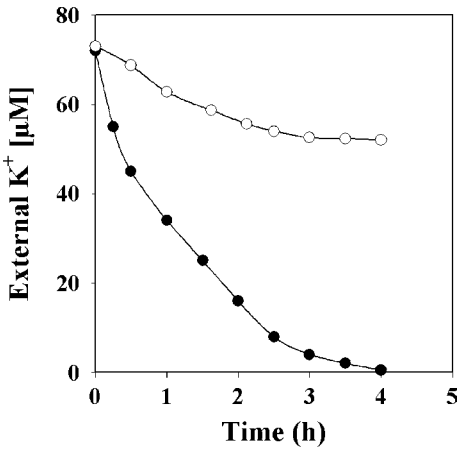

Figure 5. CaHAK1-mediated K^+ uptake in yeast and its inhibition by NH_4^+. The yeast strain WΔ3 expressing CaHAK1 was starved of K^+ for 6 h. At time zero, cells were transferred to AP medium containing 70 μM K^+ with no NH_4^+ (closed circles) or with 250 μM NH_4^+ added (open circles). At different time points samples were taken, centrifuged and the K^+ in the supernatant determined. A representative experiment is shown. Data from the K^+ depletion from three different experiments were fitted to a Michaelis–Menten equation and a K_m of 3.3 ± 0.2 μM was calculated (see Materials and methods).

A more complete kinetic characterization of the transport mediated by CaHAK1 was performed by using Rb^+ as a K^+ analog in uptake experiments in K^+-starved yeast cells. In these experiments CaHAK1 mediated high affinity Rb^+ uptake with a K_m of 1.9 ± 0.4 μM and a V_{max} of 0.25 ± 0.01 nmol mg^{-1} min^{-1} (Figure 6). The effects of K^+, NH_4^+, Cs^+ and Na^+ on Rb^+ uptake were studied by using several concentrations of the cations and it was shown that micromolar concentrations of K^+, NH_4^+ and Cs^+ and millimolar concentrations of Na^+ competitively inhibited Rb^+ uptake. The K_i values obtained from experiments with several concentrations of the inhibitors were (μM) 3.2 ± 0.2 for K^+, 4.3 ± 2.7 for NH_4^+, 7 ± 4.9 for Cs^+ and (mM) 10.3 ± 1.6 for Na^+.

The competitive inhibition exerted by micromolar concentrations of NH_4^+ on micromolar- K^+ and Rb^+ uptake suggested that CaHAK1 could mediate NH_4^+ transport with high affinity. To check this possibility, the yeast strain 31019b (Marini *et al.*, 1997) deficient in the NH_4^+ transporters MEP1, MEP2 and MEP3, and expressing CaHAK1 was employed for complementation and NH_4^+ uptake experiments. CaHAK1 expression did not improve growth of strain 31019b in

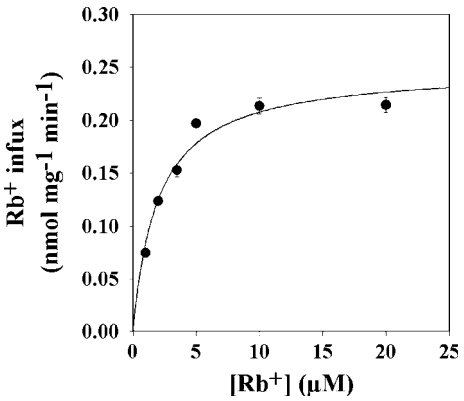

Figure 6. Concentration dependence of Rb^+ influx in yeast cells expressing CaHAK1. Yeast cells of the strain WΔ3 expressing CaHAK1 were starved of K^+ for 6 h and suspended in 10 mM MES brought to pH 6 for the influx experiments. Reported are the initial rates of uptake for Rb^+ on a mg dry weight basis. Data from three different experiments were fitted to a Michaelis–Menten equation and a K_m of 1.9 ± 0.4 μM and a V_{max} of 0.25 ± 0.01 nmol mg^{-1} min^{-1} were calculated.

comparison with the same strain transformed with the empty plasmid at any of the NH_4^+ concentrations tested (20 mM to 20 μM). Moreover, suspensions of 31019b strain expressing CaHAK1 did not deplete external NH_4^+ from a 100 μM NH_4^+ solution (not shown).

Discussion

Here we report on the isolation of a cDNA from pepper, *CaHAK1*, that encodes a high-affinity K^+ transporter. CaHAK1 clusters in group I of HAK transporters (Rubio *et al.*, 2000) and transporters of this group such as HvHAK1 and OsHAK1 have been postulated as being major contributors to the high-affinity K^+ influx observed in the roots of barley and rice (Santa-María *et al.*, 1997; Banuelos *et al.*, 2002). In these two species the conclusion is drawn firstly from the kinetic coincidences between K^+ transport in roots and in the yeast expressing the transporters and secondly from the coincidences between the expression patterns of the genes encoding these transporters and the occurrence of high-affinity K^+ influx in roots. The same conclusion can be drawn also for the here characterized CaHAK1. The expression in yeast of CaHAK1 showed that it mediated high-affinity K^+ transport with a K_m of 3.3 μM (Figure 5) and high-affinity Rb^+ transport with a K_m of 1.9 μM (Figure 6), and that K^+ competitively inhibited Rb^+ uptake with a K_i of 3.2 μM. Micromolar concentrations of NH_4^+ and Cs^+ also produced competitive inhibition of Rb^+ uptake with K_i values of 4.3 and 7 μM for NH_4^+ and Cs^+, respectively. Na^+ competitively inhibited Rb^+ uptake in the millimolar range of concentrations and showed a K_i value of 10.3 mM Na^+. All together these results demonstrated that *CaHAK1* encoded a high-affinity K^+ uptake system (K_m = 3.3 μM K^+) that did not discriminate between K^+ and Rb^+, and was very sensitive to NH_4^+, characteristics that are entirely coincident with those determined in the roots of pepper (Figure 1). In addition, the similar reduction of high-affinity K^+ uptake by NH_4^+ (250 μM NH_4^+) in roots and in yeast expressing CaHAK1 suggested that in our experimental conditions CaHAK1 was the only system mediating high-affinity K^+ uptake in pepper roots. Moreover the gene encoding CaHAK1 was strongly induced in roots of K^+-starved pepper plants, and both the expression of transcripts and high-affinity K^+ influx increased almost in parallel with the time of K^+ starvation (Figure 3). Therefore, we conclude that CaHAK1 dominates the high-affinity K^+ influx of pepper.

The results presented here with pepper and the previous results with *Arabidopsis* and barley may suggest that the contribution of NH_4^+-sensitive and -insensitive components to high-affinity K^+ uptake may differ among species. In *Arabidopsis*, 50% of the membrane depolarization of roots of wild-type plants grown in the absence of NH_4^+ resulting from an increase in the external concentration of K^+ from 10 to 100 μM was inhibited by 2 mM NH_4^+ (Spalding *et al.*, 1999). However we observed in pepper plants that K^+ uptake from a 50 μM solution was inhibited almost 90% by the presence of 1 mM NH_4^+ (Figure 1) and in barley plants grown in the absence of NH_4^+, the uptake of Rb^+ from a 100 μM solution was inhibited 84% by 5 mM NH_4^+ (Santa-María *et al.*, 2000). Further studies will be needed to determine the contribution of different components to high-affintiy K^+ uptake in plants.

An important applied characteristic of the K^+ transport mediated by CaHAK1 is its inhibition by NH_4^+ and by Na^+. Both are cations that interact with K^+ nutrition in many agricultural situations. NH_4^+ is used as a nitrogen source in fertilizers and it is known that it causes toxicity (Gerendas *et al.*, 1997), and high concentrations of

Na^+, which also produce toxic effects, are found in saline soils.

NH_4^+ toxicity has been explained by the displacement of crucial cations such as K^+ (Xu *et al.*, 2002). In agreement with this we observed inhibition of high-affinity K^+ influx by micromolar concentrations of NH_4^+ in pepper plants and in yeast cells expressing CaHAK1. These results suggested that CaHAK1 might also transport NH_4^+. However, as demonstrated for HvHAK1 (Santa-María *et al.*, 2000), CaHAK1 neither complements a yeast mutant defective in NH_4^+ uptake nor mediates any detectable NH_4^+ influx. These results lend support to the hypothesis that HAK1 transporters are inhibited by NH_4^+ but do not mediate NH_4^+ transport.

Regarding Na^+ toxicity it is accepted that in saline soils K^+ acquisition is impaired by the presence of high Na^+ concentrations (Greenway and Munns, 1980), Na^+ substitutes for K^+ and the plant suffers both K^+ nutritional imbalance and Na^+ toxicity (Flowers and Läuchli, 1983). The here described pepper HAK1 transporter shows competitive inhibition of high-affinity K^+ uptake by millimolar Na^+ concentrations as has been described for other members of the family (Santa-María *et al.*, 1997; Rubio *et al.*, 2000; Banuelos *et al.*, 2002) and may be a determinant of Na^+ tolerance. It has been proposed that increasing the K^+ over Na^+ selectivity of K^+ transporters might improve plant salt tolerance (Rubio *et al.*, 1995; Rubio *et al.*, 1999) and this might be especially feasible in salt-sensitive plants such as pepper (Maas and Hoffman, 1977). In agreement with this, supplying K^+ as KNO_3 or KH_2PO_4 improves salt tolerance in bell pepper plants (Kaya and Higgs, 2003; Kaya *et al.*, 2003).

Further characterization of the transport systems involved in K^+ acquisition in pepper plants will help understanding of K^+ nutrition and the factors affecting it in this and other crops. The information obtained from these molecular approaches will prove valuable for developing improved new varieties and for designing new strategies for crop management in the future.

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