Comparative Functional Features of Plant Potassium HvHAK1 and HvHAK2 Transporters*

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Plant K⁺ transporters of the HAK family belong to four rather divergent phylogenetic clusters, although most of the transporters belong to clusters I or II. A simple phylogenetic analysis of fungal and plant HAK transporters suggests that an original HAK gene duplicated even before fungi and plants diverged, generating transporters that at present fulfill different functions in the plant. The HvHAK1 transporter belongs to cluster I and mediates high-affinity K⁺ uptake in barley roots, but no function is known for the cluster II transporter, HvHAK2, which is not functional in yeast. The function of HvHAK2 was investigated by constructing HvHAK1-HvHAK2 chimeric transporters, which were not functional even when they included only short fragments of HvHAK2. Then, amino acids characteristic of cluster II in the N terminus and in the first transmembrane domain were introduced into HvHAK1. All of these changes increased the Rb⁺ Km, introducing minimal changes in the Na⁺ Kₘ, which suggested that HvHAK2 is a low-affinity, Na⁺-sensitive K⁺ transporter. Using a K⁺-defective Escherichia coli mutant, we functionally expressed HvHAK2 and found that the predicted characteristics were correct, as well as discovering that the bacterial expression of HvHAK2 is functional at pH 5.5 but not at 7.5. We discuss whether HvHAK2 may be a tonoplast transporter effective for vascular K⁺ depletion in K⁺ starved plants.

Potassium is the most abundant cationic component in all living cells, and in plants it plays important roles related to osmoregulation, enzyme activity, and movement. Plant roots take up K⁺ from the soil solution where the cation concentration is highly variable, at several orders of magnitude lower than in the cytoplasm of plant cells. Thus, in most cases K⁺ absorption at plant roots is an “active” process mediated by high-affinity K⁺ transporters.

Two types of K⁺ transporters, TRK/HKT1 and KT/HAK/KUP, have been universally described in non-animal cells, and genes or cDNAs encoding these two types of transporters have been identified in several plant species (1). Recent reports and sequencing projects indicate that plants have a low number of genes encoding HKT⁺ transporters and a high number of genes encoding KT/HAK/KUP transporters. In Arabidopsis, only one HTK gene has been identified (2), but 13 genes belonging to the KT/HAK/KUP family exist (3). The high number of genes encoding KT/HAK/KUP transporters highlights the complexity of this family and suggests that these K⁺ transporters may have different functions in the plant. They operate in roots as well as in shoots (3, 4) and possibly in the plasma membrane and in the tonoplast (1, 5).

Structure-function relationship studies on the wheat HTK1 transporter have contributed to the understanding of the function in plants of the TRK/HKT family of transporters (6–11). However, studies of this type on HAK transporters have not been carried out. HAK transporters are probably K⁺⁻H⁺ symporters (1) that have been included in the 2.A.72 family of the transmembrane solute transporters (12). The most thorough study on the structure-function relationships of H⁺-coupled transporters has been carried out on the Na⁺-, Li⁺-, or H⁺-coupled transporters MelB (13) and LacY (14, 15) of Escherichia coli. However, these bacterial transporters belong to families 2.A.1 and 2.A.2, respectively (12), which are different from the HAK family of transporters.

Plant KT/HAK/KUP transporters form four phylogenetic clusters (3). Representatives of cluster I are the barley HAK1 and the Arabidopsis HAK5, which have been characterized in yeast as high-affinity K⁺ transporters. The HvHAK1 gene is expressed exclusively in roots and is induced when plants are starved for K⁺ (16). The AtHAK5 gene is constitutively expressed in roots and it is induced in shoots of K⁺-starved plants (3). Representatives of cluster II are the Arabidopsis KT1/KUP1, KT2/KUP2, and KT3/TRH1 and the barley HAK2. The characterization of all of these transporters presents serious problems in yeast mutants defective for the endogenous K⁺ transporters, and the results from different laboratories do not always coincide (4, 17). Assuming that the technical problems in the study of these transporters (1) have not produced misleading results, this cluster would include low-affinity transporters KT2/KUP2 (18), high-affinity transporters KT3/TRH1 (19), and biphasic (high- and low-affinity) transporters KT1/KUP1 (4, 17).

The apparent simplicity of obtaining functional analysis in cluster I HAK transporters makes it possible to predict a rapid understanding of the in planta functions of transporters in this cluster. On the contrary, functional understanding of cluster II transporters may be slow if a simple heterologous expression is not found. It is worth noticing that for low-affinity transporter characterization, as in the case of KT2/KUP2 (18), yeast mutants may not be used because of their large ectopic transport (16). The difficulties with cluster II would also be explained if...
all or some of the transporters in this cluster were tonoplast transporters, mediating the transfer of K\(^+\) from the vacuole to the cytoplasm (1, 5). This would interfere seriously with the typical tests of complementing yeast transport mutants, because of the difficulty in targeting tonoplast transporters to the plasma membrane.

To gain insight into the physiological roles of the plant HAK transporters in cluster II, we have undertaken a structure-function relationship study on the barley transporters HvAK1 and HvAK2. Functional analyses in yeast of chimeric proteins and amino acid changes that are involved in K\(^+\) and Na\(^+\) binding and expression in bacterial mutants suggest that many transporters in cluster II are low-affinity K\(^+\) transporters, very sensitive to physiological Na\(^+\) concentrations, and that their cellular functions may be considerably different from those fulfilled by cluster I transporters.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, Plasmids, and Growth Conditions**—The E. coli strain DH5A was routinely used for plasmidic DNA propagation. The bacterial strain TKW4205 (thi rha lacZ nagA recA 5::Tn10 hslABC5 trkA455 kup1) provided by E. P. Bakker (deficient in the three K\(^+\) uptake systems Kdp, TrkA, and Kup) was used for complementation assays. The yeast strain W3 (Mat a ade2 ura3 trpl1 K13::LEU2 trk2::HIS3) (20) deficient in the endogenous K\(^+\) uptake systems TRK1 and TRK2 was used for functional complementation assays in yeast. Bacterial TKW4205 strains were grown in LB medium supplemented with 30 mM K\(^+\). For expression of HvHAK2 in bacteria, the HvHAK2 cDNA was cloned into plasmid pBAD24 (21) and transformed into bacterial strain TKW4205. For bacterial growth tests at low K\(^+\), serial dilution drops of strains grown in LB supplemented with 30 mM K\(^+\) were inoculated on a solid medium containing 5 mM PO₄, 0.4 mM MgSO₄, 6 mM FeSO₄, 1 mM citric acid, 1 mg/liter thiamine, 0.2% glycerol, 8 mM asparagine, 20 mM CaCl₂, and oligo-elements brought to pH 7.5 with arginine and supplemented with the indicated K\(^+\) and arabinose concentrations (this medium is based on that described in Ref. 22). A similar medium was used at pH 5.5, adding to the described medium 10 mM MES\(^-\) and adjusting the pH with HCl. Yeast strains were grown in SD medium (23) supplemented with 50 mM K\(^+\). For expression in yeast, the cDNAs were cloned into the shuttle vector pYPEG15 (24). For yeast growth experiments at low K\(^+\) concentrations, serial dilution drops of strains were inoculated on arginine phosphate medium (25) supplemented with the indicated K\(^+\) concentrations.

**DNA Manipulation**—Manipulation of nucleic acids was performed by standard protocols or, when appropriate, according to the manufacturer’s instructions. PCR's were performed in a PerkinElmer thermocycler using the Expand-High-Fidelity PCR system (Roche Molecular Biochemicals). Some of the PCR fragments were first cloned into the PCRII-Topo vector using the TOPO TA cloning kit (Invitrogen).

The HvHAK1-HvHAK2 cDNA for expression in bacteria was obtained by PCR using specific primers including the start and stop codons. The PCR product was cloned into the EcoRI site of pBAD24 situating the ATG triplet at 7 bp from the Shine-Dalgarno box present in the plasmid.

Six chimeric HvHAK1-HvHAK2 transporters were expressed in yeast (see Fig. 1) from the expression vector pYPGE15 and constructed as follows. The partial HvHAK1 or HvHAK2 cDNA fragments, which were necessary to obtain the full-length cDNA chimeras, were obtained by PCR from plasmid pYPEG15 containing the HvHAK1 or HvHAK2 cDNAs. For this purpose, four primers, two sense and two antisense, were designed based on the amino acid sequence GGTFAVSL (S1), which is present in HvHAK1 and HvHAK2, and on the amino acid sequence GQVYIPE (S2) present in HvHAK1 (the corresponding sequence of HvHAK2 is GQYIPE). In addition, two primers corresponding to the promoter sequence 5′-CTATTTCCTTAAAGTTCTAG-3′ (YPG5) and terminator sequence 5′-TTTCTCTCTTCAAACACCAA-3′ (YPG3) of plasmid pYPGE15 were used. Five PCR fragments from each cDNA were obtained by combining YPG5 and the antisense primers corresponding to S1 or to S2, a sense primer corresponding to S1 and an antisense primer corresponding to S2, and sense primers corresponding to S1 or S2 and YPG3. Full-length cDNA fragments for the chimeric transporters were finally obtained by using two or three of the PCR fragments described above and performing a final PCR with primers YPG5 and YPG3. Single point mutations in HvHAK1 were obtained by PCR using mutagenic primers as described elsewhere (26). All constructs were sequenced by using an automated ABI PRISM 377 DNA sequencer (PerkinElmer Life Sciences).

**Transport Assays**—Yeast cells were grown in the arginine medium supplemented with 30 mM K\(^+\) and then K\(^+\) starved for 2 h in K\(^+\)-free arginine medium. For Rb\(^+\) uptake experiments, cells were suspended in a 2% glucose, 10 mM MES buffer brought to pH 6.0 with Ca(OH)\(_2\). At intervals after the addition of Rb\(^+\), samples were taken, filtered through 0.45-μm pore nitrocellulose membrane filters (Millipore), and washed with 20 mM MgCl₂. Filters were incubated overnight in 0.1 μM HCl, and Rb\(^+\) was determined by atomic emission spectrophotometry in the acid extracts. The initial rates of Rb\(^+\) uptake were determined from the time courses of the cellular Rb\(^+\). For the experiments on external K\(^+\) depletion, cells were suspended in K\(^+\)-free arginine phosphate medium supplemented with micromolar K\(^+\) concentrations. Cell samples were taken at intervals and centrifuged at 5000 rpm for 5 min, and the K\(^+\) concentration in the supernatant was determined by atomic emission spectrophotometry. For transport assays in bacteria, cells were grown at 37°C in LB medium supplemented with 100 μg/ml ampicillin and 30 mM K\(^+\) to an optical density of 1. Arabinose was then added to a final concentration of 15 mM, and cells were kept in this medium for 15 min. Afterward, the cells were centrifuged, transferred, and kept for 30 min in K\(^+\)-free minimal medium, pH 5.5, of the composition described above with 13 mM arabinose. After this K\(^+\)-starving period, the cells were transferred to fresh K\(^+\)-free minimal medium at pH 5.5 or 7.5 to which the indicated amounts of Rb\(^+\) were added to initiate the uptake experiment. Cell samples were taken at intervals, filtered through 0.45-μm pore membrane filters (Millipore), and washed with a 25 mM MgCl₂ solution. Cells were acid-extracted overnight in an 0.1 M HCl solution, and the Rb\(^+\) in the supernatant was determined by atomic emission spectrophotometry. The initial rates of Rb\(^+\) uptake were determined as described above for yeast experiments.

**RESULTS**

**Plant HAK Transporters May Have Different Functions**—HAK transporters belonging to different phylogenetic clusters keep a low level of identity (3). For example, in Arabidopsis, AtKT1/KUP1 and AtHAK5 are only 41% identical. As a comparison, animal H\(^+\), K\(^+\), and Na\(^+\)-ATPases keep 63% identity and are enzymes with different functions. The phylogenetic tree of fungal and plant HAK transporters (3) suggests that before plants and fungi diverged, an ancestral HAK gene duplicated and gave rise to the present plant HAK clusters, in which there are monocot and dicot representatives. The preservation up to the present of transporters belonging to divergent clusters suggests that they may have different functions. HvHAK1-HvHAK2 Chimeras Are Not Functional—This similarity, phylogenetic analysis suggests that HAK transporters in different clusters have different functions. To test for this possibility we selected two barley transporters, HvHAK1 and HvHAK2, that belong to clusters I and II, respectively. HvHAK1 has been expressed in yeast and is characterized as the system mediating the high-affinity K\(^+\) uptake described by Epstein et al. (27), whereas no function has been determined for HvHAK2 (3, 16). By Northern blot analysis we verified that the

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1. The abbreviations used are: MES, 4-morpholinolinesulfonylic acid; PCR, polymerase chain reaction.
yeast cells transformed with HvHAK2 expressed correctly the corresponding transcript and that the lack of functional expression was not a problem of a defective mRNA expression of our construct. Therefore, our first approach was to construct chimeras of the two proteins. The rationale of this strategy was that the exchange of fragments of proteins with similar functions should produce functional proteins, thus allowing a causal analysis of the lack of function of HvHAK2 in yeast. Chimeras have been successfully used for studying transporters of the same family, such as the melibiose carriers of E. coli and Klebsiella pneumoniae (28), the animal Ca$^{2+}$-ATPases (29), or the K$^+$ channels KAT1 from Arabidopsis and Xsha2 from Xenopus (30), as well as many others.

The amino acid sequences of HvHAK1 and HvHAK2 show two regions that are highly conserved and could be used to fuse fragments of HvHAK1 and HvHAK2 without altering the transporter topology in the fusion region; we called them S1 and S2 (see “Experimental Procedures” and Fig. 1). The N and C terminus and the two conserved regions defined three fragments in each transporter, to which corresponded six cDNA fragments. Ligating these fragments in the correct order, we constructed six chimeric cDNAs. These were then used to transform the W33 strain. Growth analyses of the six transformants at low K$^+$ were negative, which showed a complete lack of activity of the chimeric cDNAs. In the light of these negative results, we concentrated our attention on the fragment preceding the first transmembrane domain, which is much shorter in all transporters of cluster II, lacking an aspartic acid residue that is conserved in cluster I (Fig. 2). An additional chimera, in which the fragment preceding the first transmembrane domain of HvHAK2 substituted for the corresponding fragment of HvHAK1, was then tested. Again, this chimera did not complement W33, showing that a small fragment of HvHAK2 completely abolished the function of HvHAK1.

These results suggested that relevant information on the functions of HvHAK1 and HvHAK2 is located in several domains of the proteins and highlighted the complexity of the function-structure relationships of the HAK transporters. We then decided to identify single amino acid residues important to function by obtaining single point mutants in HvHAK1.

**Introduction in HvHAK1 Characteristic Amino Acids of HvHAK2**—Sequence analysis of clusters I and II, to which HvHAK1 and HvHAK2 belong, failed to show striking specific sequence signatures. However, two differences were clear: the N-terminal fragment up to the first transmembrane domain GXXXGDXGDSPLY in cluster I changes to GXXXGDLS/T/DSPLY in cluster II. The mutated amino acids are shown in bold.

Because the substitution of the N-terminal HvHAK1 fragment for the corresponding one from HvHAK2 abolished the function, our next step was to study the significance of the conserved Asp$^{21}$ in HvHAK1 and the great number of negative charges occurring in this fragment in cluster I transporters by introducing the D21N or E60Q/D77N mutations in HvHAK1. The D21N substitution resulted in a strong change in the transport properties. Yeast cells expressing the D21N mutant neither grew at micromolar K$^+$ (Fig. 3) nor were they able to deplete external K$^+$ at micromolar concentrations (Fig. 4). The affinity for Rb$^+$ and the $V_{\text{max}}$ of Rb$^+$ influx were reduced (Fig. 5), whereas the $K_i$ for Na$^+$ was not greatly affected (Table I). In contrast, the E60Q/D77N mutations had no effect at all on the transport properties of HvHAK1 (not shown).

In the first transmembrane domain, we introduced three different mutations in HvHAK1, G61S, T62I, and G61S/T62I, which are the variant amino acids in HvHAK2. As shown in Fig. 3, the three mutations had a negative effect on HvHAK1 function. The G61S mutation had the mildest effect, followed by the T62I and G61S/T62I mutations. The mutations reduced the capacity to grow at micromolar K$^+$ concentrations, and suspensions of yeast cells expressing the HvHAK1 mutants depleted external K$^+$ more slowly, down to a higher K$^+$ concentration than cells expressing wild type HvHAK1 (Fig. 4). All of the mutations produced a decrease in the affinity for Rb$^+$ and variable reduction of the $V_{\text{max}}$ (Fig. 5 and Table I), which could explain the described defects of the mutants. HvHAK1 mediates millimolar Na$^+$ influx (16), and millimolar concentrations of Na$^+$ competitively inhibited Rb$^+$ influx via HvHAK1 and the HvHAK1 mutants. As described above for the D21N mutation, the notable effect of the G61S, T62I, and G61S/T62I mutations on the $K_i$ of Rb$^+$ influx contrasted with the mild effect on the Na$^+$ $K_i$ (Table I).

**HvHAK1 Mutant Suppressor Isolation—**To investigate whether the mutations described above could be compensated with other mutations, we tried to isolate spontaneous suppressors of the HvHAK1 mutants. A large number of cells of the W33 strain expressing the HvHAK1 mutants were plated on low K$^+$ media, where the HvHAK1 mutants do not grow. Suppressors of the D21N mutation were easily isolated, but no suppressors could be isolated for the rest of the mutations. One of the best suppressors of D21N was R397S. The double mutant D21N/R397S exhibited wild type Rb$^+$ influx kinetics and normal K$^+$ uptake (not shown). Despite the notable effect of the R397S mutation on the D21N mutation, the R397S single mutation produced a mild effect increasing the Rb$^+$ $K_i$ up to 28 μM.

**HvHAK2 Expression in an E. coli Mutant—**One of the most important impediments to progress in the understanding of the function of cluster II KT/HAK/KUP transporters is that only very few of them can be expressed functionally in yeast. In our hands, HvHAK2 did not show any transport capacity in yeast mutants (3), and as mentioned above, two reasons might explain this problem, either because it is a low affinity transporter or because it is a tonoplast transporter in the plant.

Several expression systems have been proposed for the study of the KT/HAK/KUP transporters (4, 18), but in case of non-plasma-membrane transporters only bacterial mutants offer
clear potential for successful use. However, even in this case, two sources of trouble can be predicted, the toxicity of the transporter and the deleterious effects of Na\(^+\), if it is used at relatively high concentrations, as has been traditional in bacterial media (31, 32). To solve these problems we used an expression vector with an inducible promoter and formulated a synthetic medium in which neither K\(^+\) nor Na\(^+\) was used in its basic formulation. The HvHAK2 cDNA was cloned into the bacterial expression vector pBAD24 (21) under the control of the arabinose-responsive promoter PBAD, and the construct was transformed into strain TKW4205, deficient in the endogenous K\(^+\) uptake systems. In solid medium tests at pH 7.5, the suppression of the bacterial mutation was practically non-existent, probably because the HvHAK2 transporter mediated a low rate of uptake below 1 mM K\(^+\), and at this concentration the bacterial mutant started growing. Therefore, we performed the tests at pH 5.5, finding that, at this pH, HvHAK2 suppressed the defect of the mutant but only in a set of conditions for which the combination of the expression level of HAK2, which determines the \(V_{max}\), and the K\(^+\) concentration resulted in a sufficient but not excessive K\(^+\) uptake. For example, at pH 5.5, the transformant grew at 2.5 mM K\(^+\) (the mutant does not grow at 5 mM) at any arabinose concentration from 0.01 to 13 mM; but at 50 mM K\(^+\), it grew well at 0.01 but not at 13 mM arabinose (Fig. 6).

To further characterize in bacteria the transport mediated by HvHAK2, we tried to determine the kinetics of Rb\(^+\) influx. To perform these experiments we fully induced the transporter at 13 mM arabinose for 15 min (this induction was carried out to...

| HvHAK1 mutants | \(K_m\) (\(\mu\)M) | \(V_{max}\) (nmol mg\(^{-1}\) min\(^{-1}\)) | Na\(^+\) \(K_i\) (mM) |
|--------------|-----------------|-----------------|-----------------|
| HvHAK1       | 11              | 9               | 5               |
| D21N         | 300             | 2               | 6.3             |
| G61S         | 83              | 5.5             | 15              |
| T62I         | 132             | 2               | 12              |
| G61S/T62I    | 800             | 2.4             | 25              |

Fig. 3. Growth of yeast cells expressing HvHAK1 or the mutants of HvHAK1. The WΔ3 yeast mutant, deficient in the endogenous K\(^+\) transport systems, was transformed with the empty plasmid pYFGE15 or with the plasmid containing the wild type HvHAK1 cDNA or the cDNAs corresponding to mutants G61S, T62I, G61S/T62I, or D21N. The arginine phosphate medium supplemented with 3 mM or 50 \(\mu\)M K\(^+\) was inoculated with serial dilution drops of yeast cell suspensions.

Fig. 4. Depletion of external K\(^+\) in yeast cell suspensions expressing HvHAK1 or HvHAK1 mutants. The external K\(^+\) of suspensions of yeast cells of strain WΔ3 expressing wild type HvHAK1 (open circles) or the mutant transporters G61S (closed circles), T62I (open squares), G61S/T62I (closed squares), D21N (open triangles) was recorded at time intervals after the addition of 120 \(\mu\)l. Tests were done at 0.7 mg cell dry weight ml\(^{-1}\).

Fig. 5. Concentration dependence of Rb\(^+\) influx in yeast expressing HvHAK1 or HvHAK1 mutants. WΔ3 yeast cells expressing wild type HvHAK1 (open circles) or the transporter mutants G61S (closed circles), T62I (open squares), G61S/T62I (closed squares), D21N (open triangles) were used.

Table I

**Km, \(V_{max}\), and Na\(^+\) \(K_i\) values of Rb\(^+\) influx mediated by HvHAK1 and HvHAK1 mutants**
get a \( V_{\text{max}} \) as large as possible; however, because it was carried out at 30 mM K\(^+\), some cells were probably killed) and then K\(^+\)-starved the cells for 30 min. Under these conditions, the bacterial mutant expressing HvHAK2 showed a Rb\(^+\) uptake clearly distinguishable from that found in the mutant (Fig. 7). Moreover, at increasing Rb\(^+\) concentrations in the range of 0.5 to 5 mM, the HvHAK2-mediated influx increased notably. We then performed net K\(^+\) uptake experiments and found K\(^+\) uptake rates very similar to the Rb\(^+\) influxes. These results suggest that HvHAK2 is a low-affinity K\(^+\) transporter, which could exhibit a Rb\(^+\) \( K_m \) of \( \sim 5 \) mM.

The effects of the D21N, G61S, and T62I mutations in HvHAK1 suggested that HAK transporters in cluster II could be Na\(^+\)-sensitive, because they increased the K\(^+\) \( K_m \)'s, slightly affecting the Na\(^+\) \( K_m \)'s. Therefore, we tested for this possibility in the bacterial expression system with HvHAK2, finding that 100 mM Na\(^+\) inhibited Rb\(^+\) influx at 5 mM by more than 90% (Fig. 8). (As a comparison, 100 mM Na\(^+\) did not show any effect on HvHAK1-mediated Rb\(^+\) influx in yeast at 5 mM Rb\(^+\), as expected from its low K\(^+\) \( K_m/Na^+ K_m \) ratio (16).) Consequently, the HvHAK2-transformed strain was sensitive to Na\(^+\), even more so than the bacterial strain transformed with the empty plasmid, which is already Na\(^+\)-sensitive (results not shown in 0.25-strength LB containing 50 or 100 mM NaCl).

The incapacity of HvHAK2 to suppress the defect of the TKW4205 strain at pH 7.5 suggested that HvHAK2 does not function at that pH. Therefore, we tested Rb\(^+\) influx at pH 7.5 and found that this pH enhanced Rb\(^+\) influx in the TKW4205 mutant transformed with an empty plasmid (2-fold at 1 mM Rb\(^+\)) but inhibited the HvHAK2-mediated uptake to the point that it was undetectable.

**DISCUSSION**

Duplications of \( KT/HAK/KUP \) genes probably took place early in evolution, leading to a large number of now rather divergent plant transporters, which can be grouped into several phylogenetic clusters (3). HvHAK1 and HvHAK2 (41% of identity) belong to clusters I and II respectively; HvHAK1 can be functionally expressed in yeast but not HvHAK2 (3, 16), although the corresponding mRNA is correctly expressed. Now we have found that chimeric \( HvHAK1-HvHAK2 \) cDNAs do not generate functional transporters in yeast. This result can be expected if HvHAK1 and HvHAK2 have very different functions, and relevant information on these functions is located in several domains of the proteins. As a consequence, the protein chimeras for both functions would be defective, misallocated, or rapidly degraded. The two obvious functional differences between the two transporters could be in the ions that they transport and the membrane in which they function. Regarding the latter, all HAK transporters are probably K\(^+\)H\(^-\) symporters (1), and it is worth observing that a tonoplast K\(^+\)H\(^-\) symporter is required for the control of the vacuolar K\(^+\) content in K\(^+\)-starved plants (33). A similar requirement may exist when the vacuolar K\(^+\) content is low, as in Na\(^+\)-stressed plants (34) or when the tonoplast potential is reversed as may occur in hyperacidic vacuoles (35).

Sequence analyses of clusters I and II show two significant differences: the N-terminal fragment is shorter in all transporters of cluster II, lacking an aspartic acid residue that is conserved in cluster I (Fig. 2); and the amino acid sequence in the first transmembrane domain, GXXXGD\(X\)GTP\(X\)S\(\text{P}\)\(\text{S}\) in cluster I, changes to GXXXGD\(X\)T\(\text{J}\)P\(\text{S}\)\(\text{P}\)\(\text{P}\)\(\text{P}\) in cluster II (mutated amino acids are shown in bold). When the HvHAK2-characteristic residues were introduced in HvHAK1, growth experiments and kinetic studies proved that mutations D21N, G61S, T62I, and

**FIG. 6.** HvHAK2-mediated bacterial growth at low K\(^+\). The \( E. \text{ coli} \) strain TKW4205, deficient in the K\(^+\) transport systems Kdp, TrkA, and Kup, was transformed with the empty plasmid pBAD24 or with the plasmid containing the HvHAK2 cDNA under the control of an arabinose-responsive promoter. Two drops with serial dilutions of the bacteria were inoculated on a minimal medium (see “Experimental Procedures”) containing 5 or 50 mM K\(^+\) and 0.01 or 13 mM arabinose.

**FIG. 7.** HvHAK2-mediated Rb\(^+\) influx in bacteria. Shown is Rb\(^+\) uptake in the \( E. \text{ coli} \) strain TKW4205 transformed with the empty plasmid (open symbols) or with the plasmid containing the HvHAK2 cDNA (closed symbols). Rb\(^+\) influx was measured at 0.5 (squares), 1 (circles), 2 (triangles), and 5 (diamonds) mM Rb\(^+\). Assays in bacterial minimal medium were done at pH 5.5.
bacterial mutants rule out the first possibility and demonstrate that HvHAK2 is a K⁺ transporter, although it exhibits a much higher $K_m$ than HvHAK1. The clarity of these results indicates that a secondary but notable contribution of our research on KT/HAK/KUP transporters has been the finding of a simple procedure for studying the kinetics of cluster II transporters in an *E. coli* mutant. However, three main points should be considered when using this system: (i) the expression of the plant transporter must be under the control of an inducible promoter, because an excessive expression can be toxic; (ii) Na⁺ should not be used in buffers or testing media, because it can be toxic when the bacterium expresses the plant transporter; and (iii) for kinetic experiments, the promoter can be fully expressed by exposing the cells to the inductor for a short period of time. The fidelity of the characteristics of a transporter when expressed in a heterologous system with reference to the physiological ones cannot be predicted easily if a direct comparison with the physiological function is not possible. This comparison was possible for HvHAK1 (16) but not for the characteristics of HvHAK2 reported herein. However, the low affinity and the high Na⁺ sensitivity found for HvHAK2 when expressed in bacteria were already predicted from the results obtained when we introduced the D21N, G61S, T62I, and G61S/T62I mutations in HvHAK1. The consistency of the results obtained by such different procedures lends additional support to the accuracy of our conclusions.

Finally, the pH dependence of HvHAK2, active at pH 5.5 and inactive at pH 7.5, suggests that this transporter depends on $\Delta$pH but is independent from the membrane potential, which is low in bacteria at pH 5.5. This is what could be expected for a tonoplast transporter. Although demonstrating that some of the KT/HAK/KUP transporters locate to the tonoplast requires physical evidence, probably with specific antibodies, our results warn against taking for granted that all of these transporters locate to the plasma membrane as suggested by the earlier reports (4, 16–18).

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REFERENCES

1. Rodriguez-Navarro, A. (2000) *Biochim. Biophys. Acta* 1469, 1–30
2. Uozumi, N., Kim, E. J., Rubio, F., Yamaguchi, T., Muto, S., Tsuboi, A., Bakker, E. P., Nakamura, T., and Schroeder, J. I. (2000) *Plant Physiol.* 122, 1249–1259
3. Rubio, F., Santa-Maria, G. E., and Rodriguez-Navarro, A. (2000) *Physiol. Plant.* 109, 44–49
4. Kim, E. J., Kwon, K. J., Uozumi, N., and Schroeder, J. I. (1998) *Plant Cell* 10, 51–62
5. Serrano, R., and Rodriguez-Navarro, A. (2001) *Curr. Opin. Cell Biol.* 13, 399–404
6. Diatloff, E., Kumar, R., and Schachtman, D. P. (1998) *FEBS Lett.* 432, 31–36
7. Rubio, F., Gassmann, W., and Schroeder, J. I. (1995) *Science* 270, 1660–1663
8. Rubio, F., Schwarz, M., Gassmann, W., and Schroeder, J. I. (1999) *J. Biol. Chem.* 274, 6839–6847
9. Schachtman, D. P. (2000) *Biochim. Biophys. Acta* 1465, 127–139
10. Liu, W., Schachtman, D. P., and Zhang, W. (2000) *J. Biol. Chem.* 275, 27924–27932
11. Kato, Y., Sakaguchi, M., Mori, Y., Saijo, K., Nakamura, T., Bakker, E. P., Sato, Y., Goshima, S., and Uozumi, N. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 6488–6493
12. Saier, M. H. (2000) *Microbiol. Mol. Biol. Rev.* 64, 354–411
13. Pourcher, T., Deckert, M., Bassilana, M., and Leblanc, G. (1991) *Biochim. Biophys. Res. Commun.* 178, 1176–1181
14. Poolman, B., and Konings, W. N. (1993) *Biochim. Biophys. Acta* 1183, 5–39
15. Kabaek, H. R. (1992) *Int. Rev. Cytol.* 137, 97–125
16. Santa-Maria, G. E., Rubio, F., Dubcovsky, J., and Rodriguez-Navarro, A. (1997) *Plant Cell* 9, 2281–2289
17. Fu, H.-H., and Luan, S. (1998) *Plant Cell* 10, 63–73
18. Quintero, F. J., and Blatt, M. R. (1997) *FEBS Lett.* 415, 206–211
19. Bigas, S., Debrussares, G., Haralampidis, K., Vicent-Aguila, F., Feldmann, K., Grabov, A., Dolan, L., and Hatzopoulos, P. (2001) *Plant Cell* 13, 139–151
20. Haro, R., Sainz, L., Rubio, F., and Rodriguez-Navarro, A. (1999) *Mol. Microbiol.* 31, 111–120
21. Guzman, L. M., Belin, D., Carson, M. J., and Beckwith, J. (1995) *J. Bacteriol.* 177, 4121–4130
22. Rhoads, D. B., Waters, F. B., and Epstein, W. (1976) *J. Gen. Physiol.* 67, 325–341
23. Sherman, F. (1991) Methods Enzymol. 194, 3–21
24. Brunelli, J. P., and Pall, M. L. (1993) Yeast 9, 1309–1318
25. Rodriguez-Navarro, A., and Ramos, J. (1984) J. Bacteriol. 150, 940–945
26. Clarkson, T., Gussow, D., and Jones, P. T. (1991) in PCR: A Practical Approach (McPherson, M. J., Quirke, P., and Taylor, G. R., eds) pp. 187–213, Oxford University Press, New York
27. Epstein, E., Rains, D. W., and Elzam, O. E. (1963) Proc. Natl. Acad. Sci. U. S. A. 49, 684–692
28. Ham, H., and Wilson, T. H. (1993) J. Biol. Chem. 268, 10060–10065
29. Summila, C., Lu, L., Lewis, D. E., and Inesi, G. (1993) J. Biol. Chem. 268, 21185–21192
30. Cao, Y., Crawford, N. M., and Schroeder, J. I. (1995) J. Biol. Chem. 270, 17697–17701
31. Epstein, W., and Davies, M. (1969) J. Bacteriol. 101, 836–843
32. Epstein, W., and Kim, B. S. (1971) J. Bacteriol. 106, 639–644
33. Walker, D. J., Leigh, R. A., and Miller, A. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10510–10514
34. Blumwald, E., Aharon, G. S., and Apse, M. P. (2000) Biochim. Biophys. Acta 1465, 140–151
35. Muller, M. L., Irkens-Kiesecker, U., Rubinstein, B., and Taiz, L. (1996) J. Biol. Chem. 271, 1916–1924
36. Durell, S. R., and Guy, H. R. (1999) Biophys. J. 77, 789–807
37. Durell, S. R., Hao, Y., Nakamura, T., Bakker, E. P., and Guy, H. R. (1999) Biophys. J. 77, 775–788
