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The *Arabidopsis HKT1* Gene Homolog Mediates Inward Na^+ Currents in *Xenopus laevis* Oocytes and Na^+ Uptake in *Saccharomyces cerevisiae*[1]

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The Na^+-K^+ co-transporter HKT1, first isolated from wheat, mediates high-affinity K^+ uptake. The function of HKT1 in plants, however, remains to be elucidated, and the isolation of HKT1 homologs from Arabidopsis would further studies of the roles of HKT1 genes in plants. We report here the isolation of a cDNA homologous to HKT1 from Arabidopsis (*AtHKT1*) and the characterization of its mode of ion transport in heterologous systems. The deduced amino acid sequence of AtHKT1 is 41% identical to that of HKT1, and the hydropathy profiles are very similar. *AtHKT1* is expressed in roots and, to a lesser extent, in other tissues. Interestingly, we found that the ion transport properties of AtHKT1 are significantly different from the wheat counterpart. As detected by electrophysiological measurements, AtHKT1 functioned as a selective Na^+ uptake transporter in *Xenopus laevis* oocytes, and the presence of external K^+ did not affect the AtHKT1-mediated ion conductance (unlike that of HKT1). When expressed in *Saccharomyces cerevisiae*, AtHKT1 inhibited growth of the yeast in a medium containing high levels of Na^+, which correlates to the large inward Na^+ currents found in the oocytes. Furthermore, in contrast to HKT1, AtHKT1 did not complement the growth of yeast cells deficient in K^+ uptake when cultured in K^+-limiting medium. However, expression of *AtHKT1* did rescue *Escherichia coli* mutants carrying deletions in K^+ transporters. The rescue was associated with a less than 2-fold stimulation of K^+ uptake into K^+-depleted cells. These data demonstrate that AtHKT1 differs in its transport properties from the wheat HKT1, and that AtHKT1 can mediate Na^+ and, to a small degree, K^+ transport in heterologous expression systems.

The interaction between the two related alkali cations, Na^+ and K^+, in the maintenance of membrane potential, osmoregulation, and salt sensitivity is very complex. Rains and Epstein (1965) reported that Na^+ uptake in excised roots was affected by K^+ availability, which indicated that K^+ transporters may contribute to Na^+ uptake in plants. However, the molecular basis of the Na^+ uptake into plant cells remained unknown.

HKT1, isolated from wheat by a method exploiting its ability to complement a defect in K^+ transport in yeast, encodes a high-affinity K^+ transporter that is expressed in roots and leaves (Schachtman and Schroeder, 1994). Furthermore, detailed analyses of HKT1 using tracer flux experiments performed in *Saccharomyces cerevisiae* and electrophysiological studies in *Xenopus laevis* oocytes revealed that HKT1 functions as a Na^+-coupled K^+ transporter (Rubio et al., 1995, 1999; Gassmann et al., 1996). A bacterial homolog of HKT1, KtrB, has recently been shown to be part of the Na^+-dependent K^+ transporter KtrAB (Tholema et al., 1999). Studies exploiting selected or site-directed point mutations of HKT1 have shown that substitutions of single amino acids within HKT1 could dramatically change the selectivity of either a predicted K^+ binding site (Rubio et al., 1995, 1999) or a predicted Na^+ binding site (Diatloff et al., 1998).

HKT1 mRNA levels in wheat and barley roots rise rapidly upon withdrawal of external K^+ (Wang et al., 1998). These results fit a model that predicts that HKT1 contributes to K^+ uptake under K^+-limiting conditions. It has been proposed that multiple transport systems functioning in the roots of terrestrial plants could limit the resolution of individual gene products (Rubio et al., 1996). In barley and

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Arabidopsis, mRNA levels of individual members of the HAK/KUP family of K^+ transporters have been found to be induced under K^+-limiting conditions (Santa-María et al., 1997; Kim et al., 1998), which indicates that multiple transport systems may contribute to the inducible high-affinity uptake component. The apparent complexity of the K^+ uptake systems may therefore account for the difficulties encountered in detecting a Na^+ coupled K^+ transport activity in plants (Maathuis et al., 1996). Still, several aquatic plant species have been shown to possess a dominant high-affinity Na^+-K^+ co-transport system in vivo (Smith and Walker, 1989; Walker and Sanders, 1991; Maathuis et al., 1996).

Among the K^+ transporters, *HKT1* (Schachtman and Schroeder, 1994), *AKT1* (Sentenac et al., 1992; Hirsch et al., 1998), and members of the HAK/KUP family (*AtKT1-2/HvHAK1/AtKUP1–AtKUP1–4*) are expressed in root cells (Quintero and Blatt, 1997; Santa-María et al., 1997; Kim et al., 1998; Fu and Luan, 1998). Plant mutants with a T-DNA insertion in *AKT1* grew poorly on medium containing micromolar K^+ concentrations when millimolar NH_4^+ was added to the medium. These plants lacked inward-rectifying K^+ channel activity in their roots, indicating that AKT1 contributed to the K^+ uptake by the roots in the presence of NH_4^+ and low K^+ in the medium (Hirsch et al., 1998). Interestingly, a recent study has shown that, in *akt1-1* disruption mutants of Arabidopsis, a high-affinity Na^+-K^+ uptake mechanism is likely to exist as a component of the K^+ uptake mechanism (Spalding et al., 1999).

In our effort to better understand the physiological role of HKT1-like genes, we isolated and functionally characterized a homologous HKT1 gene from Arabidopsis. The activity of the encoded protein was analyzed by heterologous expression in *X. laevis* oocytes, *S. cerevisiae*, and *Escherichia coli*. The protein differs in several interesting aspects from the wheat HKT1.

MATERIALS AND METHODS

Cloning and Sequencing of AtHKT1

The Arabidopsis (Landsberg *erecta*) cDNA library used in this study was a kind gift from Dr. Minet (Minet et al., 1992). Degenerate PCR primers were designed based on the deduced HKT1 amino acid sequence. The primers of successful combinations were as follows: 5′-GGIAA(C/T) ACI(C/T) TITT(C/T) CC-3′ and 5′-(A/G/C)(A/G/T) IGGIA(A/G)(A/G) TACATCAT-3′. Plasmid libraries were screened by replicating colonies on Hybond-N^+ nylon membranes (Amersham, Buckinghamshire, UK) and probing with ^{32}P-labeled PCR products. Hybridization was performed at 65°C for 16 h in 5× SSPE (1× SSPE: 0.72 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.0), 5× Denhardt's solution (Sambrook et al., 1989), 0.5% (w/v) SDS, and 20 mg/mL denatured salmon sperm DNA. After hybridization, filters were washed in 0.1× SSPE, 0.1% (w/v) SDS.

Amplification of 5′ cDNA

Total RNA was purified by phenol/chloroform extraction and lithium chloride precipitation as described by Verwoerd et al. (1989). To obtain the 5′ end of the *AtHKT1* cDNA, RACE was performed essentially as described by Frohman (1993). Polyadenylated RNA was reverse transcribed using three different *AtHKT1*-specific antisense primers 5′-AGGGAACAAAGTGTTTCC-3′, 5′-GAAATA GGAGACGTAGAGG-3′, and 5′-TTGGAGAAGACTTCCA TGTCGAC-3′ (HKTSAL) with RACE amplification primers (Frohman, 1993).

Genomic DNA Isolation and DNA-Blot Analysis

Approximately 5 μg of Arabidopsis genomic DNA was digested with *Eco*RI, *Bam*HI, and *Hin*dIII, and separated on a 0.6% (w/v) agarose gel. DNA gel-blot hybridization was performed as described by Sambrook et al. (1989) for high stringency in the presence of 50% (w/v) formamide or for low stringency in the presence of 20% (w/v) formamide at 42°C. For the isolation of the *AtHKT1* promoter region, thermal asymmetric interlaced (TAIL) PCR was performed as described by Liu et al. (1993, 1995). Three nested primers hybridizing to the *AtHKT1* cDNA were used: the HKTSAL primer, 5′-GTGATCTTGAGTGCCAAAAACCC-3′, and 5′-GAACGTAATTTAGTAAGCTGCG-3′. For the confirmation of the DNA sequence of the *AtHKT1* promoter region in the genomic DNA, a 5′-ACTCCATGTGTCAATACC-3′ primer and the HKTSAL primer were used.

RNA Expression and Competitive Reverse Transcriptase (RT)-PCR

Arabidopsis plants were grown for about 6 weeks on agarose medium containing 0.8% (w/v) agarose, 3% (w/v) Suc, 2 mM $MgSO_4$, 1 mM KCl, 1 mM $CaCl_2$, 5 mM $Ca(NO_3)_2$, 1 mM H_3PO_4, 0.1 mM Fe-EDTA, 7 μM H_3BO_3, 1.4 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.2 μM Na_2MO_4, 0.01 μM $CoCl_2$, and 5 mM MES-Ca(OH) $_2$, pH 5.7at 20°C in a growth chamber under constant light. For the K^+ and Na^+ induction studies, RNA was isolated from roots grown in liquid culture medium containing 1 mM KCl for 10 d. Then, the medium in the individual culture flasks was replaced with the same medium containing various concentrations of K^+ and Na^+ (K^+/Na^+ mM: 0/0, 1/0, 1/100, and 100/0). Root tissues were harvested after 4 d. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Analysis of *AtHKT1* expression was performed using an *AtHKT1*-specific primer pair (5′-TGACGTTGAGACTGTTACTG-3′ and 5′-CTTTGGGTGATTGAAATGAG-3′), which yielded a RT-PCR product of 843 bp. A 432-bp heterologous competitor DNA fragment, competing for the same set of primers, was obtained by deletion of a 411-bp *Sna*BI-*Spl*I internal fragment. Reverse transcription and PCR cycling were performed using a kit (Super Script One-Step RT-PCR System Kit, Gibco-BRL, Rockville, MD), 80 pg of total RNA, and the indicated amounts of competitor DNA.

Recordings in *X. laevis* Oocytes

The *Not*I site located downstream of the *AtHKT1* stop codon on the plasmid obtained from the cDNA library was converted to a *Pst*I site by oligonucleotide (5′-GGCCTG-CA-3′) insertion. The 0.3-kb *Xho*I-*Sal*I fragment corresponding to the N-terminal region of AtHKT1 from the RT-PCR product and the 1.4-kb *Sal*I-*Pst*I fragment from the plasmid corresponding to the C terminus of AtHKT1 were ligated into the *Sal*I and *Pst*I sites of a plasmid constructed previously for *KAT1* expression (Uozumi et al., 1995). This resulted in a construct for *AtHKT1* expression under the control of a T7 promoter and a GAL1 promoter. Capped complementary RNA was injected into *X. laevis* oocytes prepared as described previously (Schachtman et al., 1992). The oocytes were kept for 1 to 2 d at 18°C in standard Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, and 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)-NaOH, pH 7.4, before we recorded ionic currents by two-electrode voltage clamping. Voltage-pulse protocols, data acquisition, and data analysis were performed with an 80,386-based microcomputer using a voltage clamp amplifier (Cornerstone model TEV-200, Dagan, Minneapolis) (Schachtman and Schroeder, 1994). Experiments were performed in a solution containing 6 mM $MgCl_2$, 1.8 mM $CaCl_2$, the indicated concentrations of K^+ and Na^+, 10 mM 2-(N-morpholino)-ethanesulfonic acid (MES)- 1,3-bis(Tris[hydroxymethyl]methylamino) propane (BTP), pH 5.5, and osmolality of 240 to 260 mosmol/kg with D-mannitol. Water- and *HKT1* cRNA-injected oocytes were tested in parallel to ensure the quality of oocytes.

Expression of AtHKT1 in *Saccharomyces cerevisiae*

Expression plasmids containing *AtHKT1*, *KAT1* (Uozumi et al., 1995), and *HKT1* (Rubio et al., 1995) under the control of the GAL1 promoter in the pYES2 vector (Invitrogen, Carlsbad, CA) were used for yeast complementation assays. *S. cerevisiae* strain G19 (MATa, his3, leu2, ura3, trp1, ade2, and ena1::HIS3::ena4; Quintero et al., 1996) and CY162 (MATa, trk1Δ, trk2::pCK64, his3, leu2, ura3, trp1, and ade2; Anderson et al., 1992) were transformed. Ura^+ transformants were selected on Ura-selective medium (0.67% [w/v]) yeast nitrogen base, 2% [w/v] Glc, 100 mM KCl, and 1.5% [w/v] agar). For growth tests, a medium containing 0.67% (w/v) yeast nitrogen base, 2% (w/v) Suc, 2% (w/v) Gal, 1.5% (w/v) agar, and the indicated concentrations of NaCl were used.

Expression of AtHKT1 in *Escherichia coli*

AtHKT1 was ligated into the multicloning sites in pPAB404 (Buurman et al., 1995), resulting in the plasmid pPAB-AtHKT1. The plasmid was introduced into the *E. coli* strain LB2003, which lacks the three K^+ uptake systems Trk, Kup, and Kdp (Stumpe et al., 1996; Stumpe and Bakker, 1997). Growth tests of the plasmid-containing *E. coli* LB2003 at different K^+ concentrations were carried out as described previously (Uozumi et al., 1998). For K^+ influx measurement, cells were pre-cultured in synthetic medium containing 0.5 mM KH_2PO_4, 50 mM Tris-HCl (pH 7.0), 5 mM $(NH_4)_2SO_4$, 10 mM Glc, 6 μM $FeSO_4$, and 0.4 mM $MgCl_2$ in the presence of 30 mM KCl, 0.5 mM isopropylthio-β-galactoside (IPTG), and 50 μg/mL ampicillin. The pre-cultured cells were then collected by centrifugation and resuspended in synthetic medium with varying concentrations of KCl. Tris-EDTA treated cells were loaded with Na^+ (Bakker and Mangerich, 1981; Nakamura et al., 1998) or with triethanolamine (Tholema et al., 1999). Net uptake of K^+ by these cells was measured by the silicone filtration technique. Contents of K^+ in the cell pellets were determined by flame photometry (Bakker and Mangerich, 1981). To correct for extra cytoplasmic cations in the cell pellet, the extra cytoplasmic space in this fraction was taken to amount to 1.25 μL/mg dry weight (Bakker and Mangerich, 1981), and the concentration of K^+ in this space was assumed to be equal to that in the medium. With this correction, the net uptake of K^+ was approximately linear with time and could be extrapolated to about zero at $t = 0$ (see Fig. 7). We therefore concluded that these assumptions were valid.

RESULTS

Isolation of AtHKT1

By aligning the deduced amino acid sequences of the homologous K^+ transporters HKT1 from wheat and TRK1 and TRK2 from yeast, we designed degenerate oligonucleotides for use in PCR amplification from an Arabidopsis cDNA library. PCR yielded amplification products of the expected lengths. Because the deduced amino acid sequence of the PCR product was similar to the primary structure of HKT1, we used this product as a probe to isolate a full-length cDNA from the same library. We isolated one clone that contained the identical sequence as the initial PCR product and characterized it further. A sequence of 16 consecutive adenine residues was located at one end of the clone, identifying the polyadenylation site and the 3′ end of the mRNA (Fig. 1A). However, based on homology comparisons with the wheat *HKT1*, we concluded that the clone was likely truncated at the 5′ end. Using the RACE procedure (Frohman 1993), we were able to extend the 5′-end by an additional 36 bp. Since an in-frame stop codon preceded the first probable Met, we concluded that we now had the complete protein coding sequence (Fig. 1A).

A chimeric construct consisting of the original cDNA and the RACE-amplified 36-bp sequence was generated and designated *AtHKT1*, and this construct was used in all subsequent studies. The context of the ATG codon, AAAATGG, conforms closely to the consensus sequence for eukaryotic translation start sites, A/GXXATGG (Joshi, 1987; Kozak, 1987). To confirm the amplified 5′-cDNA sequence and to isolate the promoter region of AtHKT1, we isolated the 5′ flanking sequence of the *AtHKT1* from the genomic DNA of Arabidopsis using the TAIL PCR method (Liu et al., 1993, 1995). The longest genomic PCR product extended about 850 bp beyond the putative translation

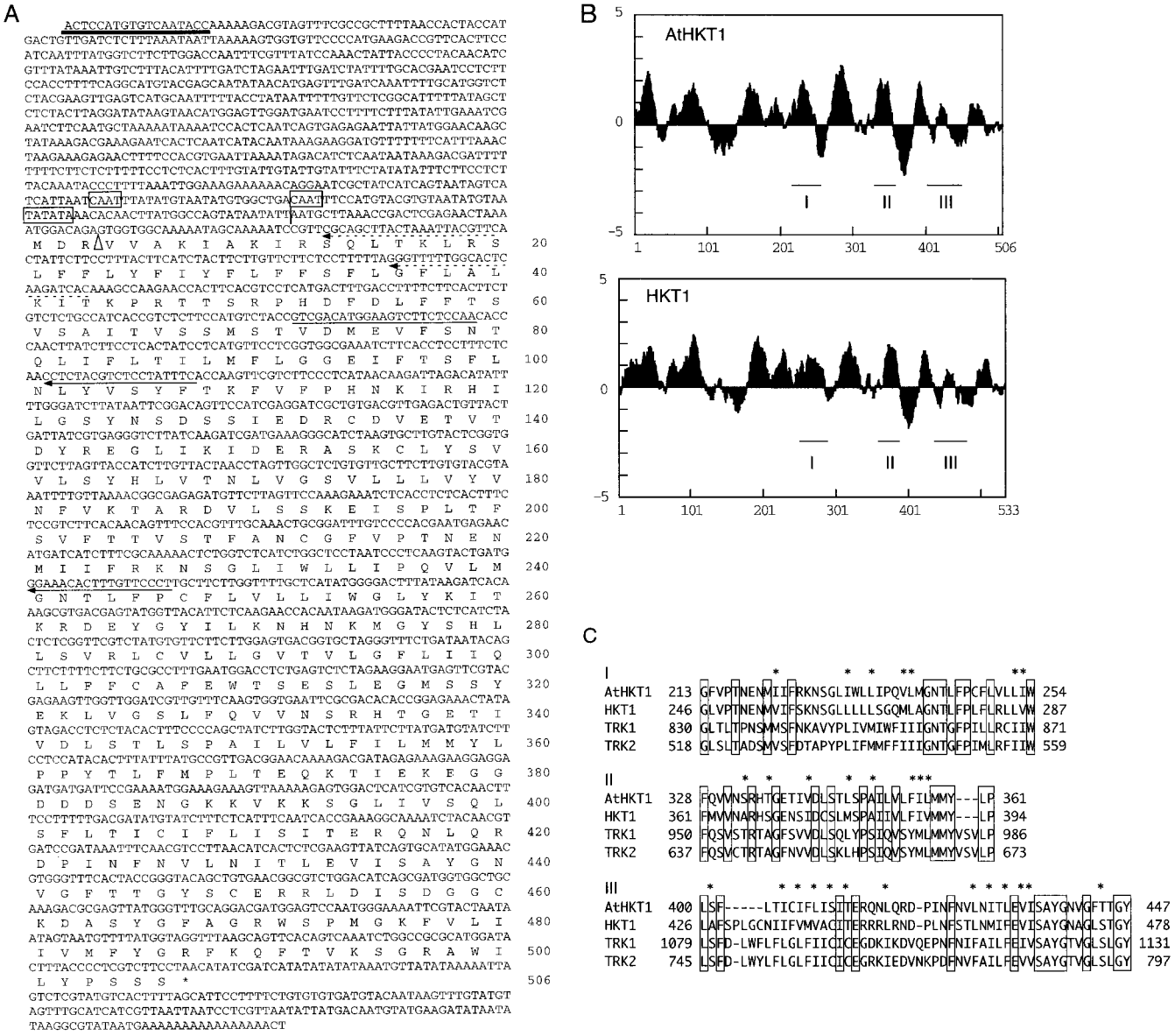


Figure 1. AtHKT1 sequence analysis. A, Nucleotide sequence of *AtHKT1*. The deduced amino acid sequence of AtHKT1 is shown below the nucleotide sequence. Putative TATA and CAAT boxes are boxed. The primer HKTSAL used for RACE-PCR and TAIL-PCR is underlined. The dashed underlines with arrowheads mark other primers used for RACE-PCR. The arrows indicate primers for TAIL-PCR. The thick underline shows the primer used to confirm the promoter sequence of *AtHKT1*. The end of the cDNA isolated from the cDNA library is indicated by a white arrowhead. The vertical bar indicates the end of nucleotides extended by RACE-PCR. B, Hydropathy plots of AtHKT1 and HKT1. The plots were performed according to the method of Kyte and Doolittle (1982). Hydrophobic amino acids have positive values. The homologous regions (I, II, and III in C) are underlined. C, Homologous regions in the deduced amino acid sequences of AtHKT1, HKT1, TRK1, and TRK2. The first amino acid residue of the translation start site is designated position 1. Identical amino acids are boxed. Conserved amino acids are indicated by asterisks.

start codon (Fig. 1A). The DNA sequence was confirmed independently by the isolation of the corresponding genomic DNA using PCR primers corresponding to the 5′ end of the sequence (thick underline in Fig. 1A) and the HKTSAL primer (thin underline in Fig. 1A). The sequence of the RACE-extended cDNA sequence matched the genomic DNA. There was no intron between the initiation codon and the HKTSAL primer sequence. A putative TATA box and two putative CAAT boxes were identified in the promoter region, as indicated in Figure 1A (Joshi, 1987).

Recently, two BAC clones (BAC T9A4, accession no. AF096373; BACF24G24, accession no. AL049488) that contain the AtHKT1 gene have become available. Sequence information from these BAC clones revealed that the AtHKT1 gene is located on chromosome 4 between mark-

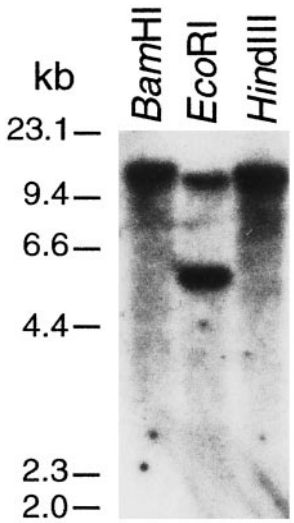


Figure 2. Genomic DNA gel-blot analysis of *AtHKT1*. Arabidopsis genomic DNA digested with the indicated restriction enzymes was probed with the *AtHKT1* cDNA. DNA size markers are indicated on the left.

ers less than 50 kb from DET1 on chromosome 4 and near the marker CIC9G5. However, the BAC entries predict proteins of 458 and 490 amino acids, respectively, which is very likely due to errors in splicing predictions. There seem to be two substitutions, Leu to Val at position 453 and Val to Ala at position 493, when comparing the sequence of our cDNA isolated from a cDNA library with that in the database. However, the BAC clones were derived from a different ecotype (Columbia) than the cDNA library (Landsberg) we used, so the discrepancies may represent ecotypic variation.

The *AtHKT1* cDNA contains an open reading frame that predicts a protein of 506 amino acid residues with a molecular mass of 56 kD. The deduced amino acid sequence of AtHKT1 is 41% identical (63% similarity) to that of HKT1 (Schachtman and Schroeder, 1994). The hydrophobicity profiles of HKT1 and AtHKT1 are very similar from the N terminus all the way to the C terminus. The hydrophobicity analysis indicates that the predicted AtHKT1 protein is very hydrophobic in that it contains eight to 12 hydrophobic domains (Fig. 1B). Alignments revealed three relatively highly conserved regions (Fig. 1C). The AtHKT1 protein shares 23% to 24% amino acid sequence identity (48%–49% similarity) with the high-affinity K^+ uptake transporters TRK1 and TRK2 of *S. cerevisiae.*

AtHKT1 Is a Single-Copy Gene in the Arabidopsis Genome

The AtHKT1 cDNA was used as a hybridization probe for Southern-blot analysis of Arabidopsis genomic DNA digested with three different restriction enzymes. As seen in Figure 2, after relatively high-stringency washes, a single hybridizing band was detected when the genomic DNA was digested with *Bam*HI or *Hin*dIII. As expected from the restriction map of the cloned AtHKT1, two hybridization bands were detected when the DNA was digested with *Eco*RI. Moreover, a low-stringency hybridization analysis gave the same results (data not shown). Therefore, the data indicate that *AtHKT1* is a single-copy gene.

Expression of AtHKT1 in Plants

The expression of *AtHKT1* mRNA in different tissues was examined using competitive RT-PCR. The transcript was most abundant in roots (Fig. 3), whereas only approximately one-third to one-tenth of the amount was detected in flowers, leaves, and stems (data not shown). The expression of *AtHKT1* mainly in roots is consistent with the pattern observed for wheat *HKT1* (Schachtman and Schroeder, 1994; Wang et al., 1998).

To determine whether expression of the *AtHKT1* is modulated by K^+ and/or Na^+ levels, total RNA was collected from roots of plants grown for 4 d in media containing varying concentrations of K^+ and Na^+ (K^+/Na^+, in mM: 0/0, 1/0, 1/100, and 100/0). No significant effect of K^+/Na^+ addition or removal on induction or suppression of *AtHKT1* transcription was observed under the four different culturing conditions. This indicates a difference in *HKT1* expression in the monocots wheat, barley, and rice, in which K^+ withdrawal enhanced transcript levels (Golldack et al., 1997; Wang et al., 1998.

Na^+ Currents in AtHKT1-Expressing *X. laevis* Oocytes

HKT1 isolated from wheat shows K^+/Na^+ co-transport (Schachtman and Schroeder, 1994; Rubio et al., 1995; Gassmann et al., 1996). To determine the effect of K^+ and Na^+ on the AtHKT1 transporter, AtHKT1-mediated currents were recorded under similar experimental conditions (Rubio et al., 1995; Gassmann et al., 1996). When 1 mM Na^+ was added to the bath solution, *AtHKT1*-expressing oocytes showed inward currents (Fig. 4B). No significant currents appeared in uninjected control oocytes under the same conditions (Fig. 4A). Exposure of *AtHKT1*-expressing oocytes to 0.3 mM K^+ elicited no inward current, and the combined addition of 1 mM Na^+ and 0.3 mM K^+ did not

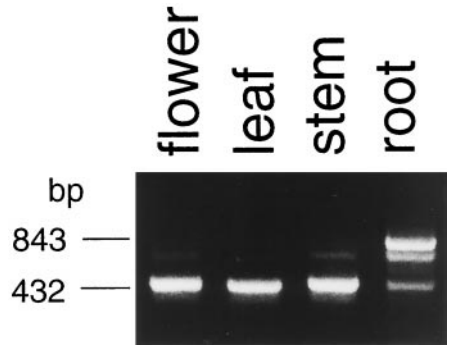
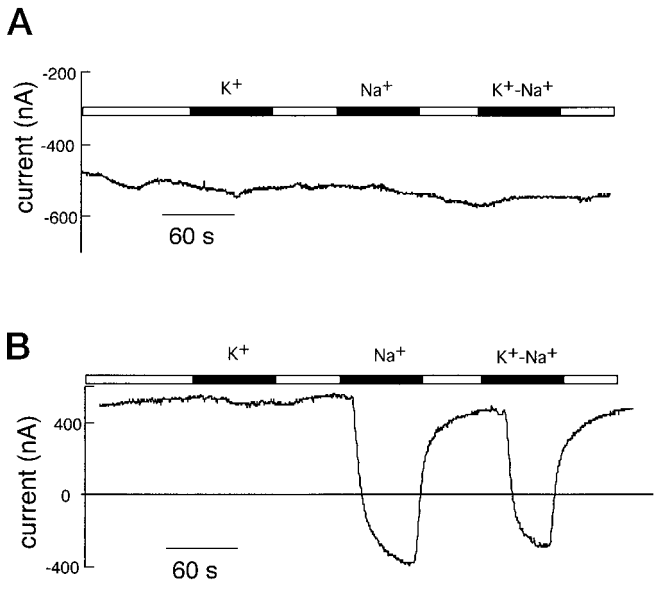


Figure 3. Competitive RT-PCR analysis of *AtHKT1* expression in Arabidopsis. The ethidium bromide-stained agarose gel shows the products obtained by competitive RT-PCR products after amplification of 80 pg of total RNA and 16 pg of heterologous competitor DNA using *AtHKT1*-specific primers. Competitive DNA, 432-bp heterologous competitor DNA; *AtHKT1*, 843-bp DNA fragment amplified from the *AtHKT1* cDNA.



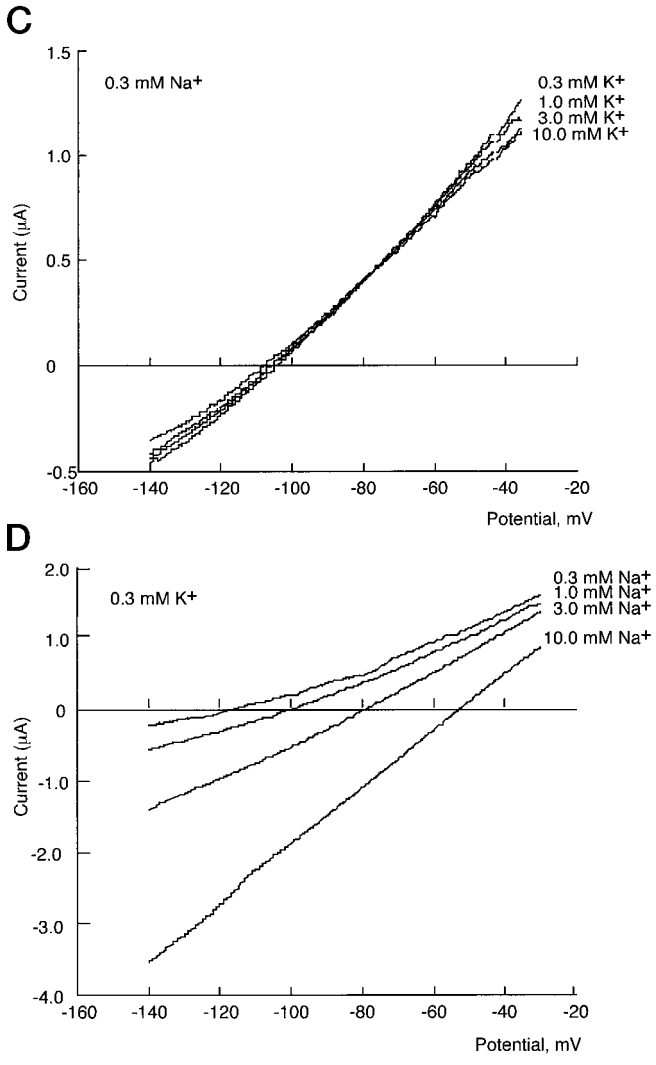


Figure 4. AtHKT1 expressed in oocytes exhibits selectivity for Na^+. A and B, AtHKT1 expressed in oocytes causes inward Na^+ currents. cRNA-injected oocytes and uninjected oocytes were maintained in standard Barth's solution (containing 88 mM NaCl) and later used for recordings. The membrane potential of uninjected oocytes (A) and *AtHKT1*-expressing oocytes (B) was held at −120 mV. The bath solution contained 0.3 mM K^+ Glu, 1 mM Na^+ Glu, or 0.3 mM K^+Glu plus 1 mM Na^+ Glu with Tris-Glu to balance varying K^+ and Na^+ concentrations. C and D, The Na^+ conductance of AtHKT1 does not depend on external K^+, but on external Na^+. Steady-state current-voltage curves were obtained from an *AtHKT1*-expressing oocyte exposed to 0.3 mM Na^+ plus varying concentrations of K^+ (0.3, 1, 3, and 10 mM) (C) and to 0.3 mM K^+ plus varying concentrations of Na^+ (0.3, 1, 3, and 10 mM) (D).

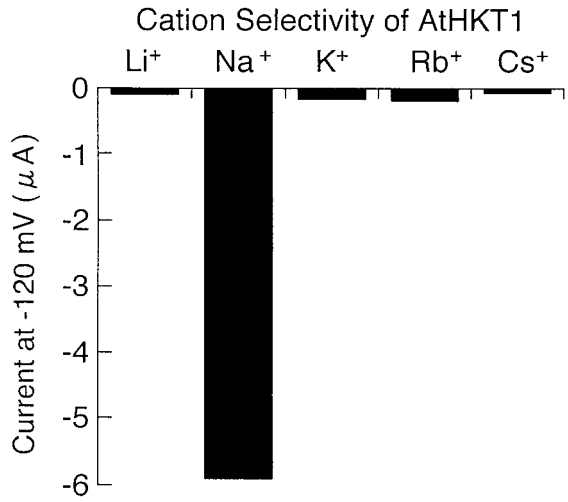


Figure 5. Average steady-state current magnitudes recorded at −120 mV from *AtHKT1*-expressing oocytes with 100 mM Li^+, Na^+, K^+, Rb^+, or Cs^+ in the bath solution.

enhance the current amplitude achieved with 1 mM Na^+ alone (Fig. 4B). Evidence for Na^+-coupled K^+ transport was, therefore, not found for AtHKT1 under the imposed experimental conditions (Fig. 4B). In control experiments performed in parallel with HKT1 from wheat, typical Na^+-coupled K^+ currents were observed (data not shown), as has been reported previously (Rubio et al., 1995, 1999). The positive background current appeared (Fig. 4B), since the *AtHKT1*-expressing oocytes had accumulated internal Na^+ through AtHKT1 during the incubation in standard Barth's solution (Rubio et al., 1995, 1999; Gassmann et al., 1996).

We also evaluated the effect of K^+ and Na^+ on AtHKT1-mediated currents by perfusing oocytes with varying concentrations of K^+ and Na^+ (Fig. 4, C and D). First, AtHKT1-mediated currents were measured in the presence of 0.3 mM Na^+ with increasing K^+ concentrations of 0.3, 1, 3, and 10 mM (Fig. 4C). The current-voltage relationship stayed almost the same under these conditions. K^+ did not show a clear effect on the AtHKT1-mediated inward current (Fig. 4C). For the control, wheat HKT1 was expressed in oocytes. As has been reported previously for wheat HKT1, the addition of external K^+ produced a positive shift in the reversal potentials of the HKT1-mediated steady-state currents when the Na^+ concentration was kept constant (Rubio et al., 1995). Secondly, we exposed oocytes to

0.3 mM K^+ and increasing Na^+ concentrations of 0.3, 1, 3, and 10 mM. As depicted in Figure 4D, increasing the Na^+ concentration led to positive shifts in the reversal potentials. The amplitude of the inward current at hyperpolarized membrane potential, such as −120 mV, increased with the increasing external Na^+ concentrations. Based on the results depicted in Figure 4, C and D, we concluded that the AtHKT1-mediated inward current strongly depended on the presence of external Na^+ but not K^+. In the presence of both cations, the Na^+ uptake by AtHKT1 occurred without being greatly affected by the presence of K^+. The reversal potential shown in Figure 4C was about −105 mV, which indicates that AtHKT1 mediates Na^+ uptake during incubation in Barth's solution.

Average steady-state inward cation currents mediated by AtHKT1 were then analyzed at −120 mV, making only one alkali cation available (Fig. 5; Table I). Large Na^+ currents were recorded with *AtHKT1*-expressing oocytes. Li^+, K^+, Rb^+, or Cs^+ (100 mM) produced currents into *AtHKT1*-expressing oocytes that were almost comparable to the levels of uninjected oocytes (data not shown). In previous reports, HKT1-mediated low-affinity alkali cation uptake into oocytes was highly selective for Na^+ when oocytes were exposed to only one cation (Rubio et al., 1995; Gassmann et al., 1996). The ion selectivity of AtHKT1 thus was similar to that of HKT1 when only one cation was present.

To analyze the effect of protons on AtHKT1-mediated currents, we changed the pH of the external bathing solution of the oocytes from 5.5 to 7.5 in the presence or absence of K^+ and/or Na^+. Under the imposed conditions, no shifts in the reversal potential were detected upon changing the pH of the bath solution (data not shown).

AtHKT1 Fails to Rescue Yeast Mutants Defective in K^+ Transport and Causes Hypersensitivity to Na^+

Expression of wheat *HKT1* in yeast causes Na^+ hypersensitivity due to increased Na^+ uptake (Rubio et al., 1995, 1999). We analyzed the Na^+ sensitivity of yeast cells expressing *AtHKT1*, *HKT1*, *KAT1*, and pYES2 using a medium supplemented with 250 mM Na^+ (Fig. 6, A and C). *S. cerevisiae* G19 was more sensitive to Na^+ than the wild-type strain, because the Na^+-extruding ATPase genes *ENA1* to *ENA4* had been deleted. AtHKT1 conferred increased Na^+ sensitivity to the yeast cells. Interestingly, yeast cells expressing *HKT1* were less sensitive to Na^+ than those expressing *AtHKT1* under the imposed conditions (Fig. 6C). Note that the G19 yeast strain had functional K^+ transporters encoded by TRK1 and TRK2, and is thus distinct from those used in previous studies of HKT1-induced Na^+ toxicity (Rubio et al., 1995, 1999). Our data, therefore, suggest that AtHKT1 functioned as a Na^+ transporter in the yeast cells.

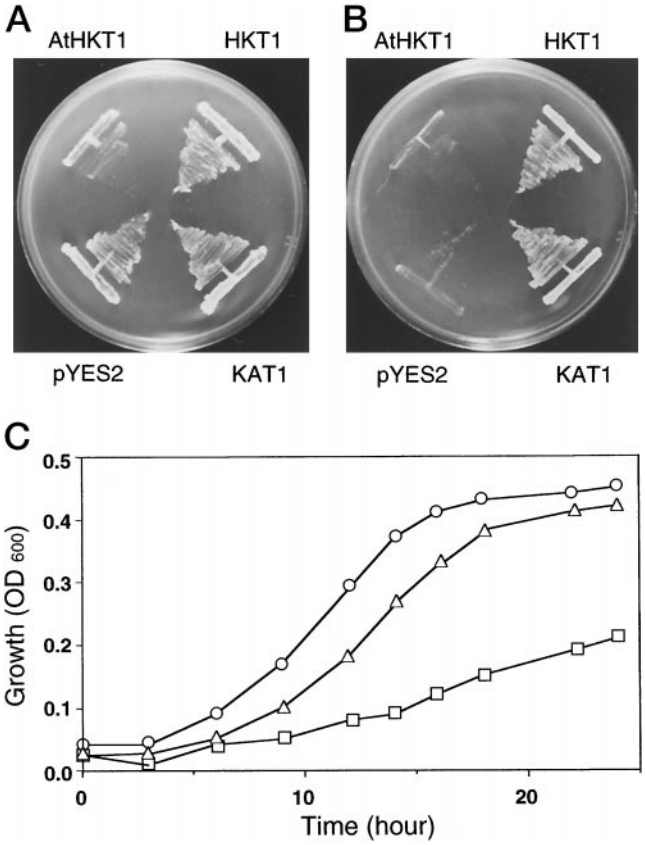


Figure 6. Na^+-induced growth inhibition of *S. cerevisiae* G19 expressing *AtHKT1*. A, Growth inhibition of *S. cerevisiae* G19 expressing *AtHKT1*, *HKT1*, *KAT1*, and empty vector. The agar medium contained 250 mM Na^+. B, Complementation of the K^+ uptake-deficient yeast mutant (*S. cerevisiae* strain CY162) with *AtHKT1*, *HKT1*, *KAT1*, and empty vector. C, Growth curve of the *S. cerevisiae* G19 expressing *AtHKT1* (□), *HKT1* (△), and pPAB404 (○) in liquid culture containing 250 mM Na^+. Note that higher Na^+ concentrations are needed to cause Na^+ toxicity via wheat HKT1 in the G19 line.

A K^+-uptake-deficient yeast strain was used in a complementation assay to analyze whether AtHKT1 can mediate K^+ uptake. *S. cerevisiae* strain CY162, which is deficient in the TRK1 and TRK2 K^+ transporters, cannot grow on yeast nitrogen base (YNB) medium that contains approximately 7 mM K^+ (Fig. 6B) (Anderson et al., 1992). In control experiments, HKT1 and the Arabidopsis hyperpolarization-activated K^+ channel KAT1 both conferred robust growth to the mutant strain in that medium (Fig. 6B). In contrast, AtHKT1 did not restore growth under limiting K^+ concen-

Table I. *Current amplitude ratios for AtHKT1 of monovalent cations relative to Na^+*

Values represent means ± SD of currents measured at −120 mV and relative to the Na^+ current amplitude, which was defined as 100% for each oocyte. Data obtained from four oocytes were averaged.

Cation	Percentage of Na^+ Current Amplitude
	%
Li^+	1.6 ± 0.25
Na^+	100
K^+	3.3 ± 0.66
Rb^+	3.5 ± 0.42
Cs^+	1.6 ± 0.60

trations (Fig. 6B). The results, therefore, suggest the uptake of Na^+ but not of K^+ via AtHKT1 expressed in yeast, which is consistent with our observations during the electrophysiological measurements with oocytes (Figs. 4 and 5).

Expression of AtHKT1 in *E. coli*

We had previously described the functional expression of plant K^+ transporters in *E. coli* (Kim et al., 1998; Uozumi et al., 1998). Furthermore, in control experiments using the K^+-uptake-deficient *E. coli* with non-functional, point-mutated KAT1, the K^+ channel was not able to compliment the defect (Uozumi et al., 1998). In contrast to yeast cells, *E. coli* cells are known to require only small amounts of K^+ for cell growth (Epstein and Kim, 1971). To study AtHKT1 in *E. coli*, we transformed *E. coli* strain LB2003, which is deficient in all three major K^+ uptake systems, with pPAB-AtHKT1 or an empty vector. We then tested for growth at limiting K^+ concentrations. Figure 7A shows that pPAB-AtHKT1 allowed growth of *E. coli* at low millimolar K^+ concentrations on solid medium. In liquid medium containing 8.7 mM K^+, *AtHKT1*-expressing *E. coli* cells grew

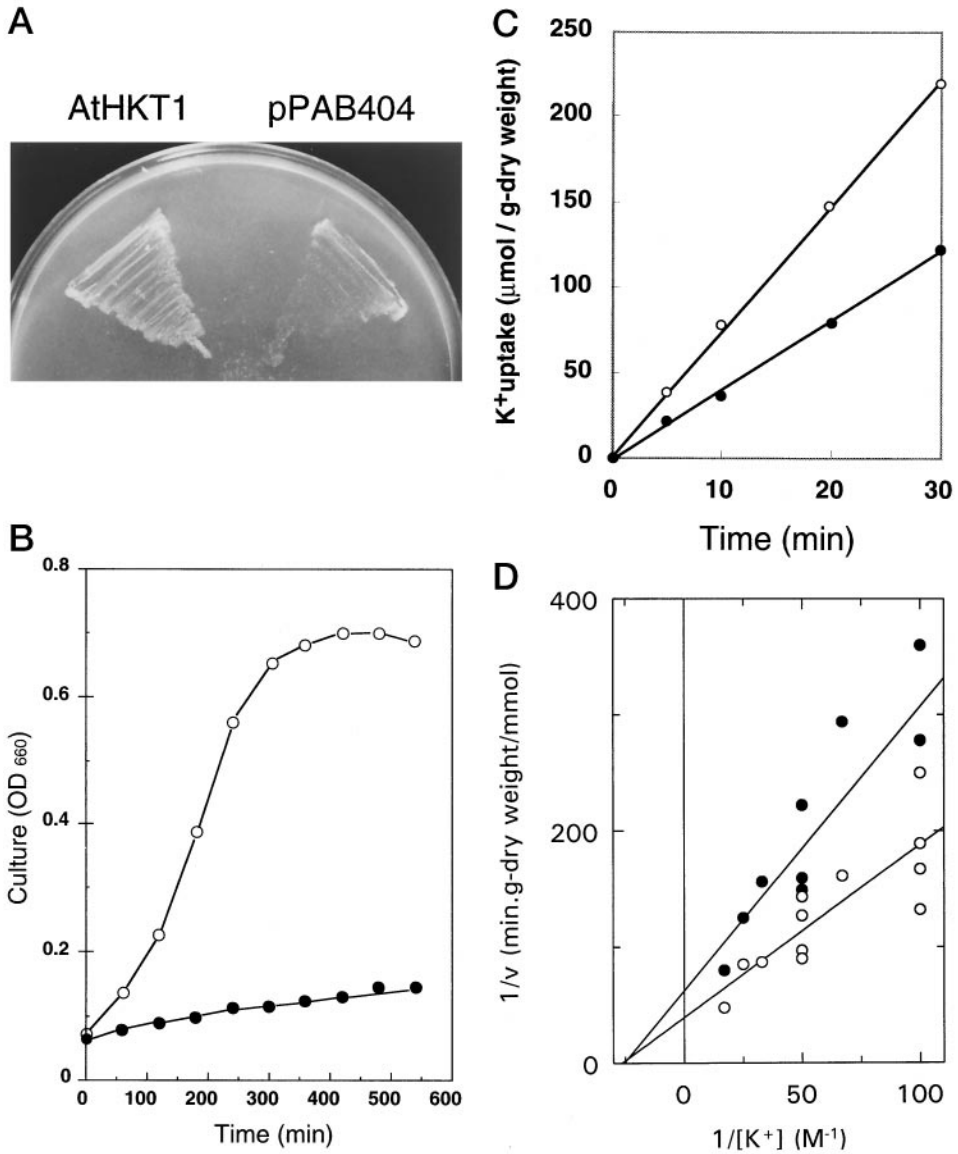


Figure 7. Complementation of the K^+-uptake-deficient *E. coli* strain by AtHKT1 and K^+ uptake rate of *AtHKT1*-expressing *E. coli* cells. A, Complementation of LB2003 cells by AtHKT1. *E. coli* LB2003 was transformed with plasmids containing *AtHKT1* or with the empty vector. Transformants were grown on medium supplemented with 10 mM K^+. B, Growth curve of transformants containing *AtHKT1* (○) or the empty vector (●) in liquid medium. The initial K^+ concentrations were measured by flame photometry (8.7 mM). C, K^+ uptake by K^+-depleted *E. coli* containing *AtHKT1*. Net K^+ uptake by *AtHKT1*-expressing LB2003 (○) and control cells with empty vector (●) were measured at 20 mM KCl. D, Lineweaver-Burk plot of K^+ uptake data. The results of experiments with six different cell preparations of *AtHKT1*-expressing cells and control cells are compiled. The lines represent linear regression of the data given in D. Experimental conditions and symbols as in C.

well, in contrast to the vector controls, which increased turbidity at a much lower rate (Fig. 7B). These data suggest that AtHKT1 may mediate K^+ uptake when expressed in *E. coli*.

To obtain more information on this effect, we measured net K^+ uptake into Na^+-loaded (and therefore K^+-depleted) cells in a Na^+-containing medium. In the experiment shown in Figure 7C, the AtHKT1-expressing *E. coli* cells took up K^+ approximately 1.8 times faster than the control cells. However, the extent of the stimulation varied among the cell preparations, ranging from 1.1 to 1.9. The data from six independent experiments with K^+ uptakes between 10 and 60 mM K^+ were pooled and represented in a Lineweaver-Burk diagram (Fig. 7D). The V_{max} values for K^+ uptake were 26 μmol min^{-1} g^{-1} dry weight for the *AtHKT1*-expressing cells and 17 μmol min^{-1} g^{-1} dry weight for the control cells (Fig. 7D). In contrast, the apparent K_m (approximately 40 mM) was not significantly different between *AtHKT1*-expressing cells and the control cells. The above data thus suggest that AtHKT1 in *E. coli* mediates K^+ uptake at a low rate.

DISCUSSION

Differences in Transport Properties between AtHKT1 and HKT1

AtHKT1 and HKT1 show 41% sequence identity and share conserved hydrophobic domains (Fig. 1B). HKT1 and AtHKT1 share at least three kinds of conserved regions with the yeast high-affinity K^+ uptake transporters TRK1 and TRK2 (Fig. 1C). Recent alignment studies between the prokaryote K^+ transporter subunits KtrB (Nakamura et al., 1998) and TrkH (Schlösser et al., 1995), the yeast transporters TRK1 and TRK2, and the wheat HKT1 suggest that the number of transmembrane domains is approximately eight in this family of K^+ symporters, and that, in addition, these proteins all contain four loops that are homologous to the selectivity filter-forming P loops of K^+ channels (Durell and Guy, 1998; Durell et al., 1998; Tholema et al., 1999). Recent studies have shown that K^+ uptake by KtrAB is also Na^+ dependent (Tholema et al., 1999).

Several differences were observed in the transport properties of AtHKT1 and HKT1. Since TRK1 and TRK2 have been identified as plasma membrane high-affinity K^+ transporters in *S. cerevisiae* (Gaber et al., 1988; Ko and Gaber, 1991; Ramos et al., 1994), we expected that AtHKT1 would rescue a K^+-uptake-deficient yeast mutant. However, AtHKT1 did not complement K^+ transporters in yeast (Fig. 6), in spite of the fact that expression of AtHKT1 caused Na^+ hypersensitivity in yeast, which suggests that the gene was expressed (Fig. 6). The characterization experiments with wheat HKT1 using *X. laevis* oocytes and yeast showed that HKT1 mediates Na^+-K^+ symport into cells (Rubio et al., 1995; Gassmann et al., 1996). As shown in Figure 4C, K^+ did not cause a large shift in the voltage dependence of AtHKT1-mediated Na^+ currents regardless of the concentrations of external K^+. Likewise, the pH of the external solution also did not affect the AtHKT1-mediated current in the pH range of 5.5 to 7.5. These data suggest that AtHKT1 does not functionally couple Na^+ and K^+ or Na^+ and H^+.

Several amino acids in wheat HKT1 have been identified as being involved in Na^+ transport by random genetic selection of mutants (Rubio et al., 1995, 1999) or by site-directed mutagenesis (Diatloff et al., 1998). The amino acids predicted to affect the K^+-binding site of HKT1 were located close to or in the proposed P-loops. Q270, N365, and E464 in HKT1 are conserved in AtHKT1, whereas A240, L247, and F463 in HKT1 are not. One possible explanation for differences between AtHKT1 and HKT1 is that the cation selectivity of HKT1 or AtHKT1 could be altered by structural alterations. Maathuis and Sanders (1993) reported that millimolar Na^+ concentrations increased growth of Arabidopsis by 1.5-fold in the absence of K^+ from the medium. If Na^+ uptake is beneficial for plants under certain conditions, it is possible that AtHKT1 represents a pathway for Na^+ uptake under these conditions. Furthermore, at high millimolar Na^+ concentrations, *AtHKT1* is a candidate gene for constitutive low-affinity Na^+ uptake, which results in Na^+ toxicity during salt stress (Rains and Epstein, 1965). Molecular physiological analyses can now directly test these hypotheses in Arabidopsis.

Although AtHKT1-mediated K^+ uptake could not unequivocally be observed in oocytes and yeast, the *E. coli* expression system exhibited enhanced growth at limiting concentrations of K^+ and increased K^+ uptake activity in *AtHKT1*-expressing cells (Fig. 7). These data therefore show that AtHKT1 can mediate both Na^+ and (to a lesser degree) K^+ transport in the heterologous expression system. The physiological backgrounds are different in these systems in terms of membrane composition, resting membrane potential, and post-translational modification mechanisms (for example, glycosylation and phosphorylation). Parameters like these have been shown to affect the functioning of many ion transporters (Bibi et al., 1993; Schwalbe et al., 1995; Pei et al., 1996; Piotrowiski et al., 1998; Baunsgaard et al., 1998). We cannot exclude the possibility that it was just the low level of K^+ uptake required to complement the K^+-deficient *E. coli* mutants that allowed us to resolve the small K^+ uptake activity of AtHKT1.

AtHKT1 in Arabidopsis

The present study utilizing heterologous expression systems has clearly revealed different ion transport properties of AtHKT1 compared with HKT1. The physiological role of AtHKT1 in ion transport, though, remains to be elucidated. *AtHKT1* expression is higher in roots than in shoots, leaves, and flowers. In wheat *HKT1* mRNA was found in roots (Wang et al., 1998). In situ RNA hybridization using wheat seedlings showed that *HKT1* was expressed in root cortical cells and in cells adjacent to the vascular tissue in leaves (Schachtman and Schroeder, 1994). Rapid up-regulation of *HKT1* in barley occurred in response to K^+ limitation conditions within 1 d, and the expression level remained high for 5 d (Wang et al., 1998). In a preliminary analysis, total RNA from Arabidopsis roots grown for 4 d under K^+-limiting conditions was quantified by competitive RT-PCR. A significant up-regulation of *AtHKT1* expression was not

observed. Since AtHKT1 functions as a Na^+-selective transporter in yeast and oocytes, its expression may differ from that of *HKT1*. In this regard, it is interesting that differences have been observed in root K^+ channel inductions between wheat and *Brassica napus*. Whereas *AKT1* expression was not affected by K^+ starvation in *B. napus* (Lagarde et al., 1996), in wheat roots, K^+ starvation enhanced mRNA levels of a wheat *AKT1* homolog (Buschmann et al., 2000). Precise and detailed characterization of the cellular localization of AtHKT1 and its gene expression will be required to understand the role of AtHKT1 in plant cells.

It has previously been shown that AKT1 can account for a significant proportion of total root high-affinity K^+ uptake in the presence of supramillimolar ammonium concentrations, as the *akt1-1* mutant was found to exhibit poor growth in medium supplemented with 10 or 50 μM K^+ (Hirsch et al., 1998; Spalding et al., 1999). The apparent dominant activity of AKT1 in terms of K^+ uptake by roots under such conditions may explain the difficulty of revealing individual K^+ uptake mechanisms in wild-type roots. Consistent with this possibility is that a Na^+-stimulated K^+ depolarization was observed when roots of the *akt1-1* mutant were examined (Spalding et al., 1999). Taking into consideration the properties of HKT1, as well as those of AtHKT1 presented in the current study, some modification of AtHKT1 would be required to account for such an activity. It cannot be excluded that additional subunits or other modifications contribute to AtHKT1 function in vivo. With the increasing complexity of genes found to encode K^+ and Na^+ transporters, genetic approaches will be required to unravel the multiple ion transport pathways. To identify the role of AtHKT1 in plant cells, localization of AtHKT1 and disruption experiments with *AtHKT1* and/or other transporter genes will have to be performed.

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