

A new family of K⁺ transporters from *Arabidopsis* that are conserved across phyla

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Abstract Transport of K⁺ in higher plants, as in bacteria and fungi, is mediated by two broad classes of transport proteins that operate in the millimolar and micromolar K⁺ concentration ranges. A search of the Expressed Sequence Tag database using amino acid consensus sequences for the K⁺ transporters HAK1 from *Schwanniomyces* and Kup of *Escherichia coli* yielded two homologous sequences for *Arabidopsis*. Cloning and sequencing of these genes gave single open reading frames for the putative transporters, AtKT1 and AtKT2, with predicted molecular weights of 79 and 88 kDa. The predicted gene products showed a high degree of homology at the amino acid level (56% identity) and exhibited significant hydrophobic stretches in their N-terminal halves, consistent with 12 membrane-spanning, α -helical domains. Database searches using AtKT1 and AtKT2 identified 10 additional sequences in *Arabidopsis* as well as additional homologous sequences in the plant species *Oryza* and *Allium*, the bacterium *Lactococcus lactis*, and in *Homo sapiens*. Expression of AtKT2 rescued growth on low millimolar [K⁺] in *Saccharomyces cerevisiae* carrying deletions for the genes encoding the K⁺ transporters TRK1 and TRK2. Rescue was associated with a 2-fold stimulation of Rb⁺ uptake and was sensitive to competition with external Na⁺ but not to extracellular pH, indicating that the gene encodes a low-affinity K⁺ transporter. These and additional results suggest that AtKT1 and AtKT2 belong to a superfamily of cation transporters that have been conserved through evolution.

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Key words: K⁺ uptake, low affinity; K⁺ channel; Multigene family; Yeast complementation; Gene expression

1. Introduction

Potassium is the most important inorganic plant nutrient and is involved in important processes at the cellular and whole plant level, such as turgor maintenance, enzyme activation, tropism or stoma movements [1–4]. Physiological studies have suggested the existence of different transport systems involved in potassium transport. Two general classes of uptake mechanisms have been described [5], the first comprising high-affinity systems, operative at K⁺ concentrations in the micromolar range, and the second including K⁺ channels and other low-affinity systems effective at concentrations near 1 mM and above.

The variety of transporters within each of these general classes is likely to be substantial, and knowledge of these systems at the molecular level remains very poor. Even in a simple microorganism like *Escherichia coli*, several K⁺ transporters with different characteristics have been described:

high-affinity K⁺ uptake is mediated by the Kdp system [6] and low-affinity uptake occurs via three different transporters, TrkG, TrkH and Kup [7]. This diversity allows the cell to transport K⁺ in different environmental conditions. A much more complex organisation can be expected in multicellular organisms such as higher plants in which distribution and internal transport of potassium is needed as well as uptake from soil.

In the last few years several K⁺ channels have been isolated from *Arabidopsis* [8,9] and other higher plants [10]. The contributions of these channels to low-affinity K⁺ flux have been discussed extensively [11–13]. More recently, a high-affinity K⁺ transporter coupled to Na⁺ was isolated from wheat [14,15]. We report here the cloning of two K⁺ transporters from *Arabidopsis thaliana*. These genes, AtKT1 and AtKT2, belong to a multigene family and are homologous to K⁺ transporters previously described from *Schwanniomyces occidentalis* [16] and *E. coli* [17].

2. Materials and methods

2.1. Plant material, strains and media

A. thaliana var. Columbia was grown in MS medium in sterile conditions. For the K⁺ starvation experiments MS medium without K⁺ salts was used. The strains W303-1A (*MATa*, *leu2*-13/112, *ura3*-1, *trp1*-1, *his3*-11/15, *ade2*-1, *can1*-100) and its derivative WΔ3 (*MATa*, *leu2*-13/112, *ura3*-1, *trp1*-1, *his3*-11/15, *ade2*-1, *can1*-100, *trk1*::*LEU2*, *trk2*::*HIS3*) were a gift from Dr. A. Rodríguez-Navarro. YPD and synthetic media were prepared as described [18], with the only exception that KCl 50 mM was added. K⁺ growth tests were performed in arginine-phosphate medium [19] with different KCl concentrations. *E. coli* XL1-blue (Stratagene, La Jolla, CA) was used for selection and amplification of recombinant DNA.

2.2. Recombinant DNA techniques and hybridisations

Standard protocols for nucleic acid manipulations were used [20]. High molecular weight genomic DNA was isolated from leaves of *Arabidopsis* [21]. DNA samples were digested with restriction enzymes, electrophoretically separated through 0.8% agarose gels, denatured and transferred to nylon membrane. The filters were hybridised with ³²P-labelled DNA probe following standard protocols [20]. Total RNA was isolated from *Arabidopsis* [22] and was electrophoretically separated on 1% denaturing formaldehyde agarose gel and blotted onto nylon membranes. The hybridisation protocol was the same as for Southern blots.

cDNAs encoding the potassium transporters AtKT1 and AtKT2 were amplified from an *Arabidopsis* cDNA library [23] by PCR using specific primers of the 5' region (*AtKT1*: GCCATGAACCAATCAC-CATCTCTTATCG; *AtKT2*: ACCATGGATCTCAATCTCGGAA-AAATGCT) and the *PGK* 3' region (AAGCTTTTTCGAAACGCA-GAATTTTCGA). The fragments were cloned in vector pCR2.1 (Invitrogen Co., San Diego, CA). The sequence of the clones was determined by automatic sequencing using an Applied Biosystems Model 310 sequencer (Perkin Elmer Corporation). The cDNAs were subcloned in a yeast expression vector under the control of the *PMAl* promoter [24]. Yeast was transformed by the Li-acetate/PEG method [25]. RT-PCR analyses of gene expression were carried out using

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MoMuLV reverse transcriptase with 5 µg samples of total RNA. The reaction product was amplified by PCR using the same 5' primers as were used for gene cloning and with the 3' primers GCGCAGAGAC-TATACGCGGGTT (*AtKT1*) and TCCCGCTAGAGACATC-CATCC (*AtKT2*).

2.3. Transport analysis

Yeast was grown in arginine phosphate medium supplemented with 50 mM KCl and was K⁺-starved for 4–6 h before the Rb⁺ uptake experiments in the same medium without KCl. Cells were resuspended in 10 mM Ca²⁺-MES buffer, pH 6.0 ([Ca²⁺] ~ 2 mM), 0.1 mM MgCl₂ and 2% glucose. RbCl was added at time zero, samples were taken at

intervals, filtered through 0.8 µm Millipore membranes, washed with 20 mM MgCl₂, and finally extracted with acid. The Rb⁺ content of the samples was determined by atomic emission spectrophotometry.

3. Results

3.1. The *Arabidopsis* genes *AtKT1* and *AtKT2* are members of a superfamily of transport proteins

A search in the EST database for sequences homologous to the potassium transporter HAK1 of *S. occidentalis* and Kup

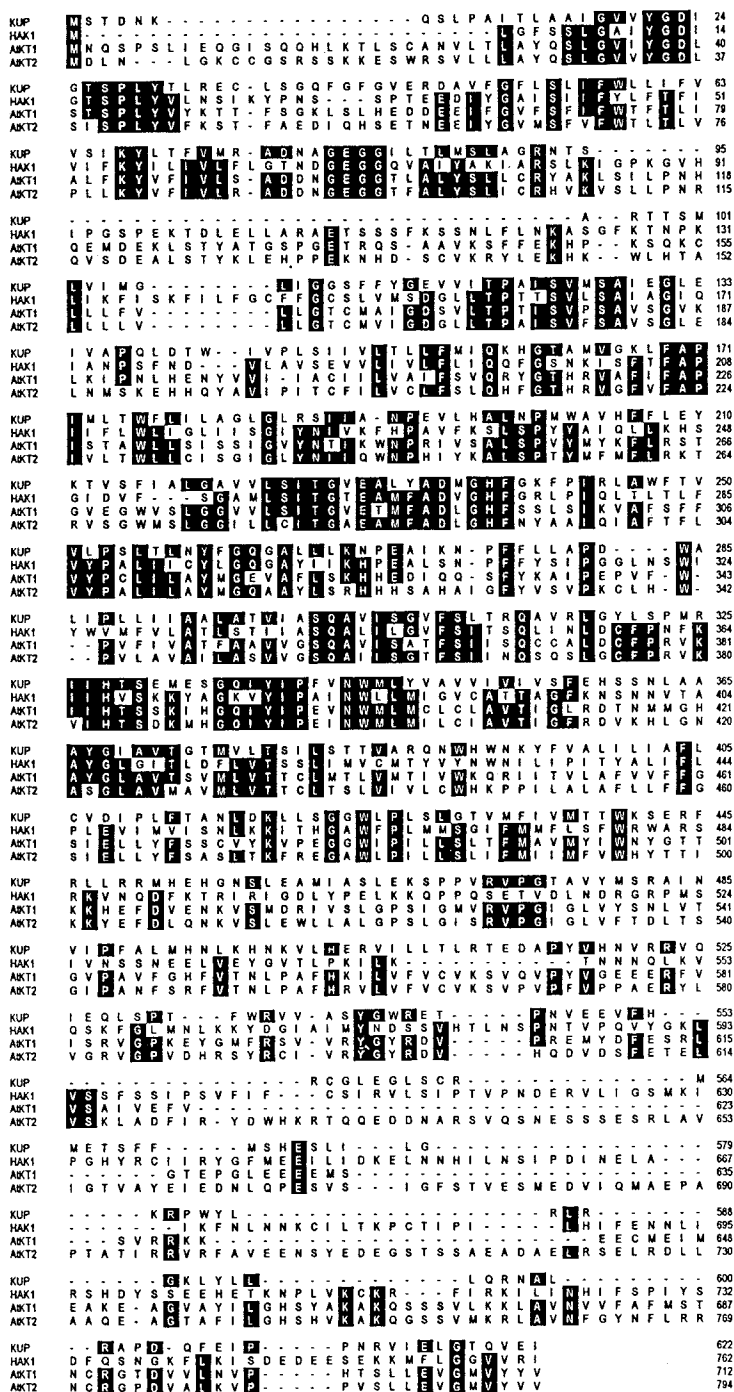


Fig. 1. Alignment of protein sequences of the *Arabidopsis* transporters *AtKT1* and *AtKT2*, the high-affinity K⁺ transporter HAK1 (*S. occidentalis*) and the low-affinity K⁺ transporter Kup (*E. coli*). Identical residues are shown in boxes. The alignment by the Clustal method was carried out using the program Megalign (DNASTAR Inc).

from *E. coli* led to the identification of several *Arabidopsis* sequences (accession numbers T20469, N96203, W43758 and W43757). All except T20469 appeared to be full-length. Comparisons showed that sequences N96203 and W43757 are almost identical, with some differences in the 5' region upstream of the putative ATG, while W43758 proved to be completely different. Two full-length clones, designated *AtKT1* and *AtKT2* (accession numbers AF012656, AF012657), were isolated by PCR from an *Arabidopsis* cDNA library using 5' primers to the N-terminal regions of N96203 and W43758 and 3' primers corresponding to the *PGK1* terminator. The *AtKT1* fragment contained a single putative open reading frame of 2136 bp interrupted by three introns, probably corresponding to an unspliced mRNA template. A minor PCR fragment was cloned using *AtKT1*-specific primers and was sequenced to confirm the absence of introns. The complete open reading frame encoded a putative 712 amino acid polypeptide of 79.04 kDa. The spliced sequence matched this predicted open reading frame with the exception that the coding region for amino acid residues 16–31 was absent. Putative splicing sequences [26] were found around this region, indicating that the missing sequence probably is not a cloning artifact. Interestingly, the original sequences N96203 and W43757 contain the region encoding the amino acids 16–31 and probably do not contain the introns found in the unspliced cDNA.

These different cDNAs corresponding to the same gene may reflect differential mRNA splicing during transcription.

The second gene, designated *AtKT2*, was predicted to encode a 794 amino acid polypeptide of 88.64 kDa. Alignments of *AtKT1* and *AtKT2* showed that the predicted proteins are very similar, with 56.3% identity at the amino acid level, and are strongly hydrophobic. The proteins also share significant homology with the yeast and bacterial K^+ transporters HAK1 and Kup. Alignments yielded 23–24% and 27% identity overall, respectively (Fig. 1), and within the central portion of the *Arabidopsis* proteins these figures were higher. Hydropathy analyses indicated that *AtKT1* and *AtKT2* share a common structure with those proposed for HAK1 and Kup proteins. The N-terminal halves of both *Arabidopsis* proteins contained extensive hydrophobic domains that aligned closely with the 12 putative transmembrane domains of the HAK1 and Kup proteins, followed by long hydrophilic tails at the end of the proteins. Indeed, all four proteins are remarkably homologous in the hydrophobic region, and much more divergent in the hydrophilic tail (Fig. 2). The C-terminus may be important for specific functional characteristics. In *E. coli*, the C-terminus is known to play an essential role in the activity of the Kup transporter [17]. No significant homology was found with any other K^+ transporter identified to date.

The copy numbers of *AtKT1* and *AtKT2* in the *Arabidopsis*

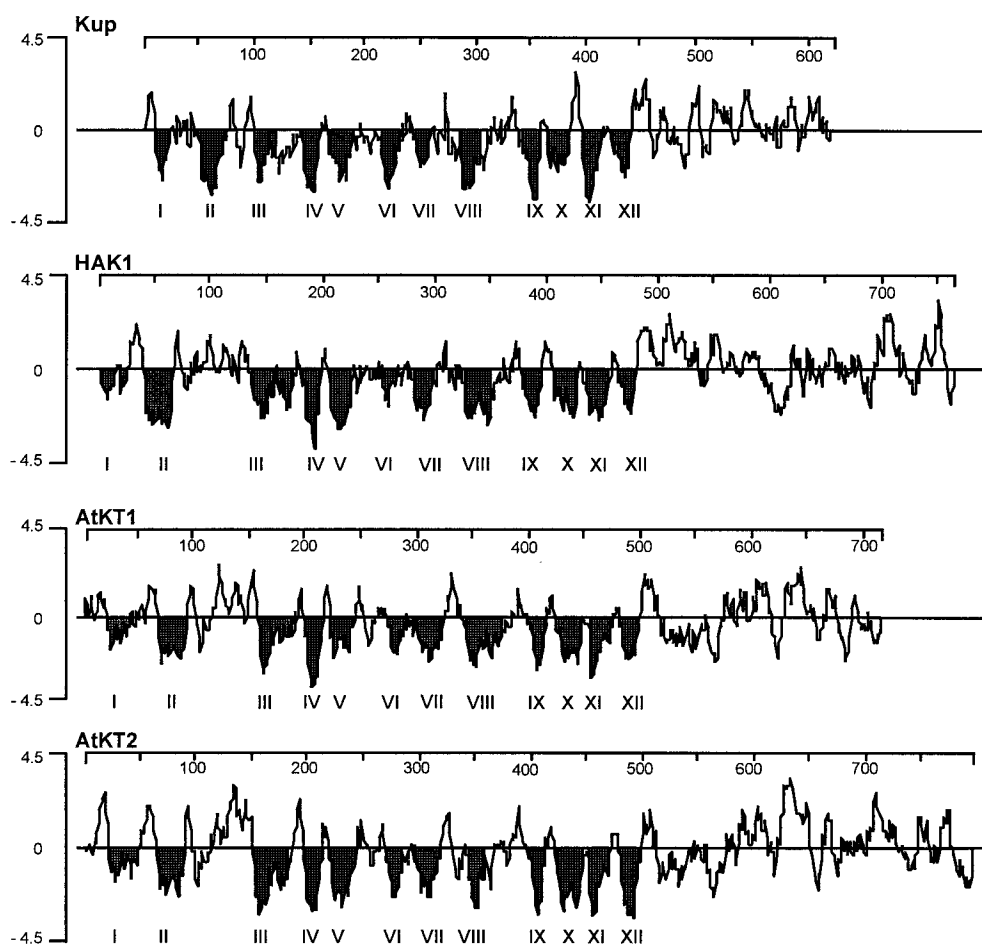


Fig. 2. Hydrophobicity plot comparison of proteins encoded by *Kup* (*E. coli*), *HAK1* (*S. occidentalis*), *AtKT1* and *AtKT2* (*A. thaliana*). Predicted α -helical transmembrane domains are in black. Structural alignments are centred on the highly conserved fourth transmembrane domain in each protein.

genome were determined by Southern blot. Genomic DNA was isolated from *Arabidopsis* leaves, digested with *Eco*RI or *Bam*HI and hybridised under high-stringency conditions using the corresponding 32 P-labelled cDNA. From the sequence *Eco*RI was predicted to cut *AtKT1* at one site but not *AtKT2*; *Bam*HI was not expected to cut either cDNA. In fact only two and one positive bands were detected, respectively, for *AtKT1* and only a single band in each case for *AtKT2*, indicating that these genes are present in single copy (Fig. 3). Nonetheless, a database search using the deduced *AtKT1* and *AtKT2* amino acid sequences identified 10 further *Arabidopsis* expressed sequence tags (accession numbers AA042476, T04361, T13770, W43598, W43749, B08446, B09415, B09607, B10149, B10459) that were predicted to encode highly homologous peptides. The alignments were confirmed by partial sequencing of some of these clones (accession numbers AF012658, AF012659, AF012660), generously provided by the Arabidopsis Biological Resource Center (Ohio State University). The findings suggest the existence of a multigene family of transporters in this plant. In addition, sequences encoding other putative homologues were identified in rice (*Oryza sativa*, accession numbers D40091 and D23315), onion (*Allium cepa*, accession number AA451555), the bacterium *Lactococcus lactis* (accession number U74322) and in mammals (*Homo sapiens*, accession numbers Z13060 and Z15484). The presence of similar proteins in all of these organisms implies that these transporters may play an important role and have been conserved during evolution.

3.2. *AtKT2* mediates low-affinity K^+ transport

To test the ability of the *AtKT1*, *AtKT2*-encoded polypeptides to mediate K^+ transport, the cDNAs were subcloned in a yeast expression vector and transformed into the strain WΔ3. This yeast strain is deleted for the K^+ transporter genes *TRK1* and *TRK2*. Compared with the wild type, the WΔ3 strain needs in excess of 100-fold higher K^+ concentrations in the medium to sustain growth. The K^+ requirements of transformants were tested by growing the yeast colonies in defined arginine phosphate medium supplemented with different KCl concentrations. Both the WΔ3 strain and yeast transformed with a control vector, containing an empty cassette, were unable to grow at K^+ concentrations of 10 mM and below. By contrast, transformants expressing *AtKT2* grew

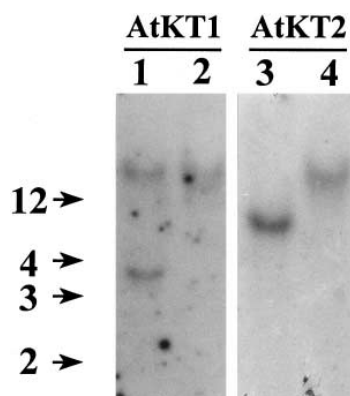


Fig. 3. Southern analysis of the *AtKT1* and *AtKT2* genes. Genomic DNA from *Arabidopsis* was digested with *Eco*RI (lanes 1 and 3) and *Bam*HI (lanes 2 and 4). The blot was hybridized with the 32 P-radiolabelled *AtKT1* and *AtKT2* cDNAs. Numbers and arrows indicate the size in kbp and the position of the DNA standards.

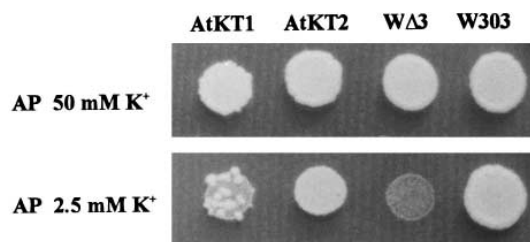


Fig. 4. Complementation test in the mutant yeast WΔ3. *S. cerevisiae* was transformed with the cDNAs *AtKT1*, *AtKT2* or the empty vector. Diluted drops from saturated cultures were grown on arginine phosphate medium with KCl concentrations as indicated.

successfully in the presence of K^+ concentrations as low as 2.5 mM KCl. Growth of the transformants was appreciably reduced at 1 mM K^+ , suggesting that *AtKT2* mediates low-affinity potassium transport. Yeast expressing *AtKT1* failed to complement the mutation, although some isolated colonies grew in low- K^+ medium (Fig. 4).

The transport characteristics of the *AtKT2*-transformed yeast were also examined using Rb^+ as a tracer for K^+ . Yeast expressing the *AtKT2* protein showed a significant increase in Rb^+ uptake when compared to the WΔ3 mutant strain (Fig. 5). Kinetic analyses as a function of Rb^+ concentration yielded very similar Rb^+ affinities (K_m 40 ± 10 mM) for the mutant and *AtKT2*-transformed yeast. However, estimated values for J_{max} of 21 ± 4 were roughly 2-fold higher in yeast expressing the *Arabidopsis* protein. Uptake was found to be insensitive to Na^+ concentrations below 10 mM and was suppressed at higher concentrations of Na^+ (not shown). No significant differences in Rb^+ uptake rates were detected when the experiments were carried out at pH 7.5.

3.3. Expression of *AtKT1* and *AtKT2* in *Arabidopsis*

Standard Northern blot analysis of *AtKT1* and *AtKT2* gene expression failed to uncover expression of the genes either in roots and leaves of mature plants, K^+ -starved plants, or in whole 7-day-old germlings grown in the presence or absence of K^+ when total RNA was probed with the full-length cDNAs. The results suggest that the genes are normally expressed at very low levels. Indeed, using RT-PCR both *AtKT1* and *AtKT2* could be detected in the same RNA preparations from mature leaves, roots and whole germlings (not shown). In this case, similar results were obtained with germlings grown on normal MS medium and on MS medium without K^+ . A direct comparison of RT-PCR experiments is difficult, but the fact that the mRNAs could not be demonstrated in either of these samples by Northern blot suggests that the expression of the genes is not dramatically influenced by K^+ starvation.

4. Discussion

Potassium is a very important element for higher plants from the point of view of nutrition and cellular functions, including osmotic homeostasis and growth [1–4]. Potassium transport has been studied extensively using electrophysiological methods, but very little is known about the molecular identity of the proteins involved in the process. In the last years significant progress has been made with the isolation of different K^+ inward channels from *Arabidopsis* [8–10] and one K^+ transporter from wheat [14,15]. Nevertheless, these

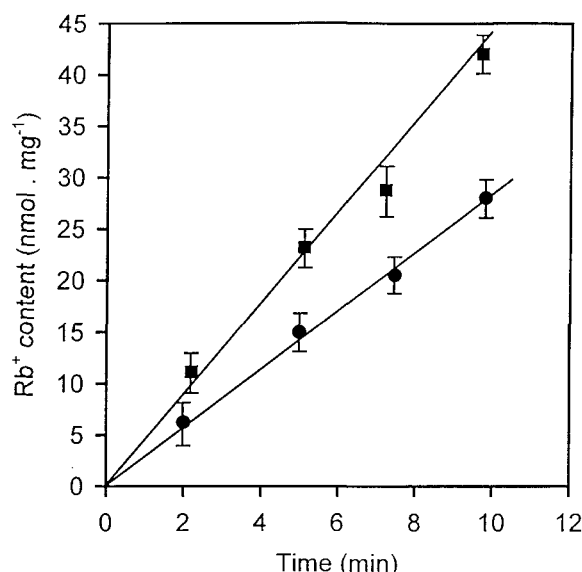


Fig. 5. Rb^+ uptake of the strain WΔ3 (●) and WΔ3 expressing *AtKT2* (■). Exponentially growing cells were K^+ -starved for 6 h before the start of the uptake experiments. 10 mM RbCl was added at time zero, and the Rb^+ content of the yeast was determined by taking aliquots at intervals thereafter.

probably represent only a small fraction of the elements involved in the transport of potassium in plants.

We report in this work the isolation of two *Arabidopsis* cDNAs, *AtKT1* and *AtKT2*, encoding K^+ transporters homologous to the K^+ transporters HAK1 from *S. occidentalis* [16] and Kup from *E. coli* [17]. The predicted protein sequences in each case exhibit a common structure characterised by a highly hydrophobic stretch of 12 putative, membrane-spanning α -helical domains. Identification of additional DNA sequences in the EST database encoding homologous proteins to *AtKT1* and *AtKT2* in *Arabidopsis* suggests the presence of a large multigene family in this species. Such genomic organisation is often found in plant membrane transporters, and probably underlies a differential expression of the isoforms that contributes to a functional or regulatory diversity in planta [27–29]. Database searches showed that similar proteins are probably also present in different species across all the major phyla, indicating that the protein has been conserved during evolution.

Of the proteins derived from the two *Arabidopsis* clones, *AtKT2* behaves as a low-affinity transporter, apparently facilitating passive diffusion of K^+ . The *Arabidopsis* gene complemented the WΔ3 strain of yeast that lacks the endogenous TRK1 and TRK2 K^+ transporters; it was found to mediate low-affinity Rb^+ uptake under these conditions; and uptake of Rb^+ could be suppressed in the presence of high concentrations of Na^+ but was completely insensitive to external pH. The Kup transporter also mediates low-affinity uptake in *E. coli* [17]. However, the eukaryotic homologue HAK1 is known to transport K^+ with a high affinity in the low micromolar range [18]. At present, we cannot rule out the possibility that the *Arabidopsis* protein function is altered when expressed in *Saccharomyces* or that its characteristics depend on further, unknown ancillary subunits. Similar factors may explain the fact that *AtK1* was unable to complement the K^+ transport-deficient yeast strain. Alternatively, this second isoform might not be correctly expressed in yeast, as has been

observed in comparable circumstances with other plant transport proteins [28–30]. Another explanation is that amino acids 16–31, which were not present in *AtKT1* (as compared with the original EST cDNA), might have been important for functionality. Finally, it may be that the characteristics of *AtKT1* are so close to the endogenous low-affinity K^+ uptake system of the WΔ3 yeast strain that it could not be detected [31].

It is intriguing to speculate on possible internal transport functions within the plant for *AtKT1* and *AtKT2*. Conventional Northern blot analyses were unsuccessful in identifying and localising transcription of the genes in any tissue, whether K^+ -starved or not, suggesting that the expression of these genes is very low and is probably not dramatically affected by external K^+ conditions. The results of RT-PCR analyses support this assessment and are consistent with the presence of the cDNAs in the CD4-16 *Arabidopsis* hypocotyl cDNA library. Moreover, the sequences found in rice and onion belong to cDNA libraries from etiolated shoots, callus and bulb. This combination of low expression and broad tissue distribution might be understood if the proteins function in background cation movement between cells within the body of the plant, or contribute to related K^+ conductances in the plasma membrane [32]. Alternatively, they could serve cation transport across internal membranes.

In conclusion, *AtKT1* and *AtKT2* appear to be members of a multigene family in *Arabidopsis*. *AtKT2* is expressed in yeast where it mediates low-affinity cation uptake. The expression of both genes is very low in planta, apparently distributed throughout most tissues and between developmental stages. Taken together, these results suggest that *AtKT1* and *AtKT2* may be involved in the internal transport of K^+ in *Arabidopsis*.

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