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Characterisation of two distinct HKT1-like potassium transporters from *Eucalyptus camaldulensis*

David J. Fairbairn[1,5,*], Weihong Liu[2], Daniel P. Schachtman[2], Sara Gomez-Gallego[1,3], Sharon R. Day[1,4] and Robert D. Teasdale[1]

[1]*ForBio Research Pty Ltd, PO Box 2256, Milton, BC 4064, Australia (*author for correspondence; e-mail: fairbairn@comcen.com.au); [2]CSIRO Plant Industry, PO Box 350, Glen Osmond, SA 5064, Australia; present addresses: [3]GADI Research Centre, University of Canberra, Canberra, ACT, Australia; [4]Department of Veterinary Microbiology and Parasitology, School of Veterinary Medicine, Louisiana State University, LA 70803, USA; [5]Department of Botany, University of Queensland, Brisbane QLD 4072, Australia*

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Abstract

Potassium is an essential macronutrient in higher plants. It plays an important physiological role in stoma movements, osmoregulation, enzyme activation and cell expansion. The demand for potassium can be substantial, especially when the plant concerned is a *Eucalyptus* tree in excess of 50 m tall. We have isolated two cDNAs, *EcHKT1* and *EcHKT2*, from *Eucalyptus camaldulensis* (river red gum) which are expressed in leaves, stems and roots. These encode potassium transporter polypeptides with homology to the wheat K^+-Na^+ symporter, HKT1. *EcHKT1* and *EcHKT2* both complemented the K^+-limited growth of an *Escherichia coli* K^+-uptake-deficient triple mutant. *EcHKT1* and *EcHKT2* also mediated Na^+ and K^+ uptake when expressed in *Xenopus* oocytes. A comparison of the *EcHKT1* and *EcHKT2* sequences and their transport properties indicated that these cDNAs represent two K^+ transporters with distinct functional characteristics. The functional and structural conservation between these two *E. camaldulensis* genes and the wheat *HKT1* suggests that they play an important, albeit elusive, physiological role.

Introduction

Potassium is the most abundant cellular cation, with cytoplasmic concentrations of ca. 100 mM (Clarkson and Hanson, 1980). This is much higher than the concentration of K^+ in soil, which is normally in the micromolar to low millimolar range. The importance and high demand for this cation by plants could explain why some plants have numerous genes involved in K^+ transport. For example, several genes encoding K^+ channels have been cloned from *Arabidopsis* including *KAT1* (Anderson *et al.*, 1992), KAT2 (GenBank accession number U25694), *ATK1* (Sentenac *et al.*, 1992), *ATK2/3* (Cao *et al.*, 1995; Ketchum and Slayman, 1996) *KCO1* (Czempinski *et al.*, 1997) and *SKOR* (Gaymard *et al.*, 1998). In addition, *Arabidopsis* has several transporters thought to be responsible for high-affinity K^+ transport *AtKUP1-4* (Fu and Luan, 1998; Kim *et al.*, 1998) and *AtKT1-2* (Quintero *et al.*, 1997). Recent studies using a reverse genetic approach have provided insights into the physiological roles of two *Arabidopsis* potassium channels (Gaymard *et al.*, 1998; Hirsch *et al.*, 1998). Other plants are also likely to contain many genes for K^+ transport. The physiological relevance of the numerous genes involved in K^+ uptake is likely to be related to the fact that most plants are sessile organisms and must be able to adapt to changing environmental conditions.

The nucleotide sequence data reported will appear in the GenBank Nucleotide Sequence Database under the accession numbers AF176035 (*EcHKT1*) and AF176036 (*EcHKT2*).

The wheat HKT1 was the first plant K^+ transporter to be cloned (Schachtman and Schroeder, 1994) and is a member of a large family of transporters from plants, bacteria and fungi (Schachtman and Liu, 1999). The HKT1 protein represents one class of at least four families of putative K^+ symporter proteins from prokaryotes and eukaryotes believed to have evolved from bacterial K^+ channel proteins (Durell *et al.*, 1999). Localisation studies showed that the *HKT1* gene was expressed in root cortical cells and in cells adjacent to the vascular tissue in leaves of wheat seedlings (Schachtman and Schroeder, 1994). Potassium starvation of barley and wheat roots resulted in a rapid and strong up-regulation of *HKT1* mRNA level (Wang *et al.*, 1998). Re-supply of 1 mM K^+ was sufficient to strongly reduce *HKT1* transcript levels. Expression levels of *HKT1* were correlated with increases of high-affinity K^+ uptake and preceded any detectable changes of shoot or root [K^+] (Wang *et al.*, 1998). These results demonstrate that HKT1 plays a role in high-affinity K^+ uptake in barley and wheat.

Functional characterisation in yeast and oocytes demonstrated that HKT1 mediates Na^+-coupled high-affinity K^+ uptake, functioning as a high-affinity K^+-Na^+ cotransporter at micromolar concentrations of external Na^+, and as a low-affinity Na^+ transporter at millimolar external Na^+ (Rubio *et al.*, 1995). Functionally similar Na^+-coupled K^+ transporters have been described in certain aquatic angiosperms (Maathuis *et al.*, 1996) and algae (Smith and Walker, 1989). However, electrophysiological evidence suggests that high-affinity K^+ uptake by terrestrial plant roots is energised by H^+ (Maathuis and Sanders, 1994, 1996). Other HKT1 homologues that are found in fungi and bacteria are also K^+ transporters, but may be energised by H^+ rather than Na^+ (Schachtman and Liu, 1999).

We report here the cloning of two K^+ transporters from *Eucalyptus camaldulensis* (river red gum). These genes, *EcHKT1* and *EcHKT2*, are homologous to the HKT1 class of K^+ transporters, complement an *E. coli* K^+-uptake-deficient triple mutant and transport both Na^+ and K^+ in *Xenopus* oocytes.

Materials and methods

Strains

The *Escherichia coli* K^+-uptake-deficient mutant TK2463 (*F⁻ thi lacZamx82 rha D[trkA] trkD1 D[kdpFABJ5 endA*) was made available by W. Epstein (University of Chicago; Epstein *et al.*, 1993).

Gene isolation and sequence analysis

Two ESTs with homology to *HKT1* were identified from BLASTX searches (Altschul *et al.*, 1990) of the GenBank database during a *Eucalyptus* Gene Discovery Program. The cDNAs were isolated from pGEM-T (Promega, Madison, WI) plasmid libraries of root (*EcHKT2*) and stem (*EcHKT1*) tissues of micropropagated *Eucalyptus camaldulensis* (river red gum). Rooted plants were maintained in tissue culture on hormone-free KG medium (Laine and David, 1994) containing 2.5 mM K^+ at 23 °C with a 16 h photoperiod (20 μmol m^{-2} s^{-1} PAR, white fluorescent light) and subcultured every 6–8 weeks.

Complete DNA sequencing of the full-length clones was carried out on an Applied Biosystems DNA sequencer model 377 (Applied Biosystems, Foster City, CA) using ABI PRISM Dye Terminator Cycle Sequencing methods (Perkin-Elmer, Foster City, CA) according to the manufacturers' instructions and using a primer-based strategy. Sequence information was analysed using the University of Wisconsin Genetics Computer Group (UWGCG) programs version 8.1 (Devereux *et al.*, 1984).

Extraction of total DNA and genomic Southern hybridization

For Southern blot analysis, total genomic DNA was prepared from micropropagated shoots of *E. camaldulensis* and digested (5 μg/digestion) for 16 h with restriction enzymes (New England Biolabs), which had no sites in the *EcHKT1* cDNA. The digests were size-fractionated by agarose gel electrophoresis, depurinated in 0.25 M HCl and then denatured and blotted onto positively charged nylon (Qiabrane, Qiagen, Hilden, Germany) using 0.4 M NaOH. A radiolabelled [α-^{32}P]dCTP *EcHKT1* probe was hybridized to the nylon membrane by standard procedures (Sambrook *et al.*, 1989).

Extraction of total RNA and RT-PCR analysis

Steady-state *EcHKT1* and *EcHKT2* message levels were estimated in samples by RT-PCR. Total RNA was prepared from leaf, stem and root tissue of soil-grown *E. camaldulensis*. First-strand cDNA products were prepared from ca. 10–20 μg of total

RNA with Superscript II reverse transcriptase (Gibco-BRL) according to the manufacturer's recommendations. The cDNAs were diluted ten-fold and used for PCR. Message levels of the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene were used as an internal control to assess cDNA yield between the RNA samples. The *GAPDH* PCR conditions were optimised separately from those for *EcHKT*. Time course experiments estimated the range of cycles when the PCR products were being amplified in a linear manner and therefore were proportional to the amount of target. Primers for the specific amplification of *Eucalyptus GAPDH*, *EcHKT1* and *EcHKT2* were designed.

GAPDH	5′-TAGCCATTTCCAGAACCCTCG-3′
	5′-CGGAGATGACAACCTTCTTAG-3′
EcHKT1	5′-GCCCTTCCGCCCCAACAAATG-3′
	5′-CTACAAGAGTATCCAAGCTCG-3′
EcHKT2	5′-CTTGCCGGGCTTCACTTCAGG-3′
	5′-GAAAATCATGACGGCAATGAG-3′

A 5 μl aliquot of the cDNAs was used for *GAPDH* PCR (94 °C 30 s, then 30 cycles of 94 °C 30 s, 50 °C 30 s, 72 °C 30 s, followed by 72 °C for 6 min) and *EcHKT* PCR (94 °C 30 s, then 38 cycles of 94 °C 30 s, 54 °C 30 s, 72 °C 1 min, followed by 72 °C for 6 min) with AmpliTaq DNA polymerase (Perkin Elmer Cetus). The PCR products were separated on a 1% TAE agarose gel containing ethidium bromide. The amount of PCR product was then quantified by comparison to mass standards (Mass Ladder Marker, Gibco-BRL) using GelPro v2.0 software (Media Cybernetics) and the *EcHKT1* and *EcHKT2* transcript levels normalised to those of *GAPDH*. Normalising the amount of *EcHKT* PCR product to that of *GAPDH* for a given volume had the effect of standardising the cDNA yield across the various RNA samples. This enabled a direct comparison of the relative levels of *EcHKT1* or *EcHKT2* in stem, leaf or root tissues. The gene-specific primers gave larger bands with genomic DNA as template. This helped identify potential genomic contamination of the RT-PCR products.

Complementation tests in E. coli

An *Nco*I site was introduced into the translation initiation codon of both *EcHKT1* and *EcHKT2* with custom PCR primers and *Pfu* DNA polymerase (Stratagene, La Jolla, CA). The PCR products were digested with *Nco*I and *Not*I to give sticky ends and subcloned into pSE420 (Invitrogen, Carlsbad, CA). Confirmed pSE420 constructs were transformed into cells of the *E. coli* K^+-uptake-deficient mutant TK2463 (Epstein *et al.*, 1993) and selected on carbenicillin-containing medium. Transformants were then tested for their ability to grow in medium containing a low (2 mM) level of K^+ (TYM; 10 g tryptone, 2 g yeast extract and 100 mmol mannitol per litre) at pH 7.0 and 37 °C. Cultures were initiated by inoculation to a density of $OD_{600} = 0.05$ with TYM washed cells from an overnight culture grown in KML medium (10 g tryptone, 5 g yeast extract and 10 g KCl per litre) with carbenicillin selection when appropriate.

Xenopus *oocyte expression and electrophysiology*

The *EcHKT2* cDNA (*Not*I fragment) was subcloned from pGEM-T into pYES2 (Invitrogen) in a desired orientation. The vector containing *EcHKT1* was linearised with *Not*I and *EcHKT2* with *Xba*I, cRNA was synthesised with the Ambion mMessage mMachine kit using T7 RNA polymerase. Oocytes were isolated from six different frogs by standard techniques (Schroeder, 1995), and expression data were gathered on injected and uninjected oocytes. The two-electrode voltage clamp was performed using microelectrodes containing 3 M KCl, and with a Dagan Cornerstone TEV-200 voltage clamp amplifier (Dagan, Minneapolis, MN). Electrical responses were measured and analysed with pClamp5 and pClamp6 software (Axon Instruments, Foster City, CA). The bath solution used in these experiments contained 1 mM $CaCl_2$, 10 mM MES plus Na^+ and/or K^+ added as glutamate salts to the desired concentration. D-sorbitol (200 mM) was added to balance the osmotic potential of the solution when necessary. The pH of the bath solutions was adjusted with Tris base to 7.5. Statistical analysis was performed with Excel 97 either as paired or unpaired *t*-tests.

Results

Eucalyptus *contains two distinct* HKT1 *homologues*

Two ESTs (*EcHKT1* and *EcHKT2*) with homology to *HKT1* were identified during a *Eucalyptus* Gene Discovery Program. Complete sequencing revealed extensive homology at the protein level to both HKT1 and AtHKT1 K^+ transporters (Figure 1). The *Arabidopsis* homologue (AtHKT1) of the wheat HKT1 high-affinity K^+ transporter was recently identified in genome sequencing projects (accession num-

Table 1. Binary comparison scores for members of the HTK1 family of proteins; identity (%), with similarity (%) in parenthesis. Calculated with the GCG BESTFIT program (Devereux *et al.*, 1984).

Protein	EcHKT1	EcHKT2	AtHTK1
EcHTK1	76.0 (85.4)		
EcHKT2	53.1 (72.8)	50.1 (71.4)	
AtHTK1	44.4 (66.4)	41.0 (63.4)	41.2 (60.8)

ber AF096373, gene T9A4.5 and accession number AL049488.1). The *Eucalyptus* cDNAs were isolated from plasmid libraries of root (*EcHKT2*) and stem (*EcHKT1*) tissues from the same *Eucalyptus camaldulensis* (river red gum) plants growing in tissue culture medium initially supplied with 2.5 mM K^+.

The *EcHKT1* cDNA (1986 bp) contains an open reading frame of 1653 bp that encodes a 62.0 kDa polypeptide of 550 amino acid residues. The *EcHKT2* cDNA (1909 bp) contains an open reading frame of 1650 bp that encodes a 61.7 kDa polypeptide of 549 amino acid residues. These cDNAs represent distinct K^+ transporters sharing only 73.1% and 76.0% identity at the nucleotide and protein levels respectively. Binary comparisons indicate that the *Eucalyptus* proteins have high homology to both HKT1 and AtHKT1, but are more similar to the *Arabidopsis* protein than the wheat protein (Table 1).

The SOSUI program (http://www.genome.ad.jp/SIT/SIT.html; Hirokawa *et al.*, 1998) predicted a membrane protein with 9 transmembrane helices for both EcHKT1 and EcHKT2. Between 8 and 10 transmembrane-spanning domains were predicted for HKT1 using 5 different modelling programs (Diatloff *et al.*, 1998). A structural analysis of putative K^+ symporter proteins, based upon the crystal structure of the bacterial K^+ channel KcsA from *Streptomyces lividans*, has suggested the presence of four sequential membrane-pore-membrane (MPM) motifs resulting in eight transmembrane-spanning domains (Durell and Guy 1999; Durell *et al.*, 1999). The MPM motif consists of two transmembrane helices with an intervening loop segment that determines the ion selectivity. The *Eucalyptus* proteins contain an extra 12 or 17 residues around position 171 when compared to HKT1 and AtHKT1 respectively. Using standard membrane topology predictor methods, this region is predicted to be a loop between the 3rd and 4th transmembrane-spanning domains and located exofa-

Figure 1. An alignment of the deduced amino acid sequences of EcHKT1 (accession number AF176035), EcHKT2 (accession number AF176036), AtHKT1 (J. Schroeder and N. Uozumi, personal communication), and HKT1 (accession number U16709). Amino acids common to three or four sequences are boxed. Residues marked with an asterisk or bar are discussed in the text.

cially if the N-terminal region is endofacial. However, a new model for this symporter family puts this loop in the cytoplasm of the cell (Durell *et al.*, 1999).

There are many areas of conservation between these four plant K^+-Na^+ symporters including a motif of 16 amino acid residues (XEV(I/V)SAYGN(V/A)G (L/F)(T/S)(T/I)GY; marked with a bar, Figure 1) near the C-terminal end and predicted to be located exofacially in HKT1, which is conserved across a range of putative K^+ transport proteins from different phyla (Diatloff *et al.*, 1998). Gassmann and colleagues (Gassmann *et al.*, 1996) suggested that HKT1 has distinct and separate binding sites for Na^+ and K^+. Mutational analysis within this 16 amino acid motif in HKT1 suggested that F463 and E464 play a role in the binding and transport of Na^+ (Diatloff *et al.*, 1998). The glutamic acid residue (E464 in HKT1) but not the phenylalanine residue (F463 in HKT1) is conserved across the 4 K^+-Na^+ symporters. Additional mutational analysis of HKT1 in yeast (Rubio *et al.*, 1995, 1999) identified four substitutions which enhanced salt tolerance (positions marked with an asterisk, Figure1). Only two of these residues are conserved. Interestingly, one of the substitutions (L247F), which improved salt tolerance in yeast over-expressing HKT1, is already present in the *Arabidopsis* and *Eucalyptus* proteins.

Southern blot analysis of *E. camaldulensis* genomic DNA (Figure 2) identified a number of *EcHKT1* hybridising bands under high-stringency wash conditions (0.1× SSPE, 0.1% SDS, 65 °C). This suggested that *EcHKT1* belongs to a small gene family, which includes *EcHKT2*. Southern blot analysis has indicated that the HKT1 class of Na^+-coupled K^+-uptake transporters in *Arabidopsis* and diploid wheat are represented by single genes (Schachtman and Schroeder, 1994).

Rescue of the potassium transport-deficient mutant TK2463

Transformation of the *E. coli* mutant TK2463 with *EcHKT1, EcHKT2* and *AtKUP1* resulted in complementation of the potassium-sensitive phenotype (Figure 3). This confirms that these function as K^+-uptake mechanisms. AtKUP1 was used as a positive control and is an *Arabidopsis* homologue of the *E. coli* KUP1 potassium transporter that complements this mutant (Kim *et al.*, 1998). AtKUP1 is not structurally similar to the EcHKT1 and EcHKT2 transporters and only shares about 18% identity at the protein level. Expression of both the *AtKUP1* and *EcHKT1* genes were controlled by IPTG-inducible promoters but IPTG was not necessary for complementation of the TK2463 mutant by these genes. Apparently, enough transporter molecules were produced as the result of promoter leakage in the absence of IPTG to provide sufficient potassium uptake for growth of the cells. This sug-

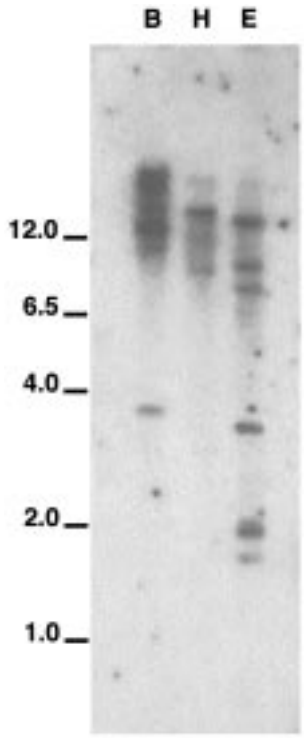

Figure 2. EcHKT1 belongs to a small multigene family in *Eucalyptus*. A Southern blot of *Eucalyptus camaldulensis* genomic DNA digested with the restriction endonucleases *Eco*RI (E), *Hin*dIII (H) and *Bam*HI (B) and hybridised to an *EcHKT1* radiolabelled probe.

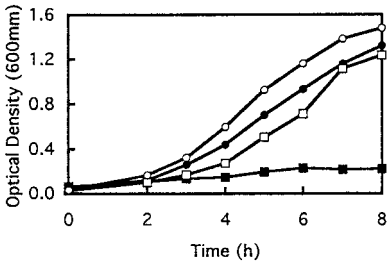

Figure 3. Complementation of TK2463 cells by *EcHKT1* and *EcHKT2*. The *E. coli* TK2463 mutant is defective in potassium uptake (Trk, Kup, and Kdp) (Epstein *et al.*, 1993) and was transformed with plasmids containing the *EcHKT1* (●), *EcHKT2* (□) and *AtKUP1* (○) (Fu and Luan, 1998; Kim *et al.*, 1998) genes or with an empty vector (■). Saturated overnight cultures grown in KML medium were washed once and added to low potassium medium to a starting density of OD_{600} 0.05.

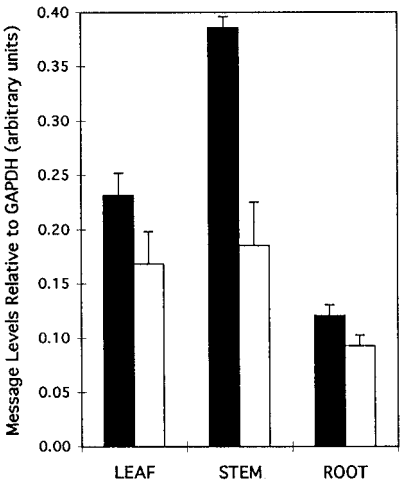

Figure 4. Expression pattern of *EcHKT1* and *EcHKT2* in *Eucalyptus*. RT-PCR estimated *EcHKT1* (■) and *EcHKT2* (□) message levels relative to *GAPDH* in leaf, stem and root tissues of soil grown *E. camaldulensis*. The error bars represent standard error of the mean ($n = 12$).

gests that these transporters are very efficient carriers of K^+. We then compared the effect of various potassium channel and transporter inhibitors on the growth of TK2463 cells expressing EcHKT1, EcHKT2 or AtKUP1 (Table 2). The two *Eucalyptus* transporters showed different sensitivities to these inhibitors. The growth of EcHKT1-expressing cells showed a greater sensitivity to sodium (1 and 10 mM), ammonium (1 and 10 mM) and tetraethyl ammonium (TEA; 10 mM) than EcHKT2- and AtKUP1-expressing cells, whereas the growth of EcHKT2-expressing cells was sensitive to inhibition by calcium (5 and 10 mM).

Expression analysis

Plant K^+ transporters are often expressed at very low levels (Quintero and Blatt, 1997); therefore, we used RT-PCR to investigate the expression levels of *EcHKT1* and *EcHKT2* relative to *GAPDH* message levels in leaf, stem and root tissues of soil-grown *E. camaldulensis* (Figure 4). Both K^+ transporters were expressed in all three tissues with *EcHKT1* transcripts being more abundant than those of *EcHKT2*. *EcHKT1* expression was highest in stem as compared to leaf or root tissues, while *EcHKT2* expression was higher in stem and leaf than root tissues.

Table 2. The effect of various inhibitors on the growth of control (psSE420) and potassium transporter expressing TK2463 cells. Saturated overnight cultures grown in KML medium were washed once and added to low potassium medium to a starting density of OD_{600}=0.05. The optical density of the culture was measured 9 h later and expressed as a percentage of a control culture with no inhibitors added. The results are means of duplicate cultures.

Treatment	(mM)	EcHKT1	EcHKT2	AkKUP1
None		100.0	100.0	100.0
CaCl2	1	118.8	100.4	95.0
	5	110.2	35.4	93.4
	10	86.9	10.7	86.8
NaCl	1	73.6	94.7	93.8
	10	60.5	85.8	83.8
	100	12.4	8.7	12.6
NH4Cl	1	72.1	99.1	108.7
	10	52.7	91.2	91.8
	100	16.5	9.7	9.3
CsCl2	1	68.5	83.3	83.2
	5	50.5	42.8	28.2
	10	29.6	25.8	24.9
BaBl2	1	27.3	18.1	31.6
	5	17.9	8.9	9.5
	10	16.0	9.9	9.4
TEA	1	109.5	94.0	99.4
	5	91.5	95.4	105.3
	10	61.7	96.2	92.6

Electrophysiological characterization of EcHKT1 *and* EcHKT2 *expressed in* Xenopus *oocytes*

Voltage clamp recording was performed within one to three days after oocytes had been injected with mRNA. Only oocytes with resting membrane potentials less than −30 mV were used. The bath chamber containing oocytes was perfused with K^+, Na^+ and K^+ plus Na^+ containing solutions. The oocytes expressing either *EcHKT1* or *EcHKT2* were permeable to both K^+ and Na^+ (Figure 5A). In the range of 1 to 10 mM Na^+ or K^+ concentration, the induced currents increased as the external ion concentration increased (Figure 5B). At −120 mV and 10 mM Na^+ or K^+, the difference in current amplitude between 0 mM and 10 mM Na^+ or K^+ was -62 ± 13 nA ($n = 4$, 10 mM K^+) and -56 ± 12 nA ($n = 4$, 10 mM Na^+) for

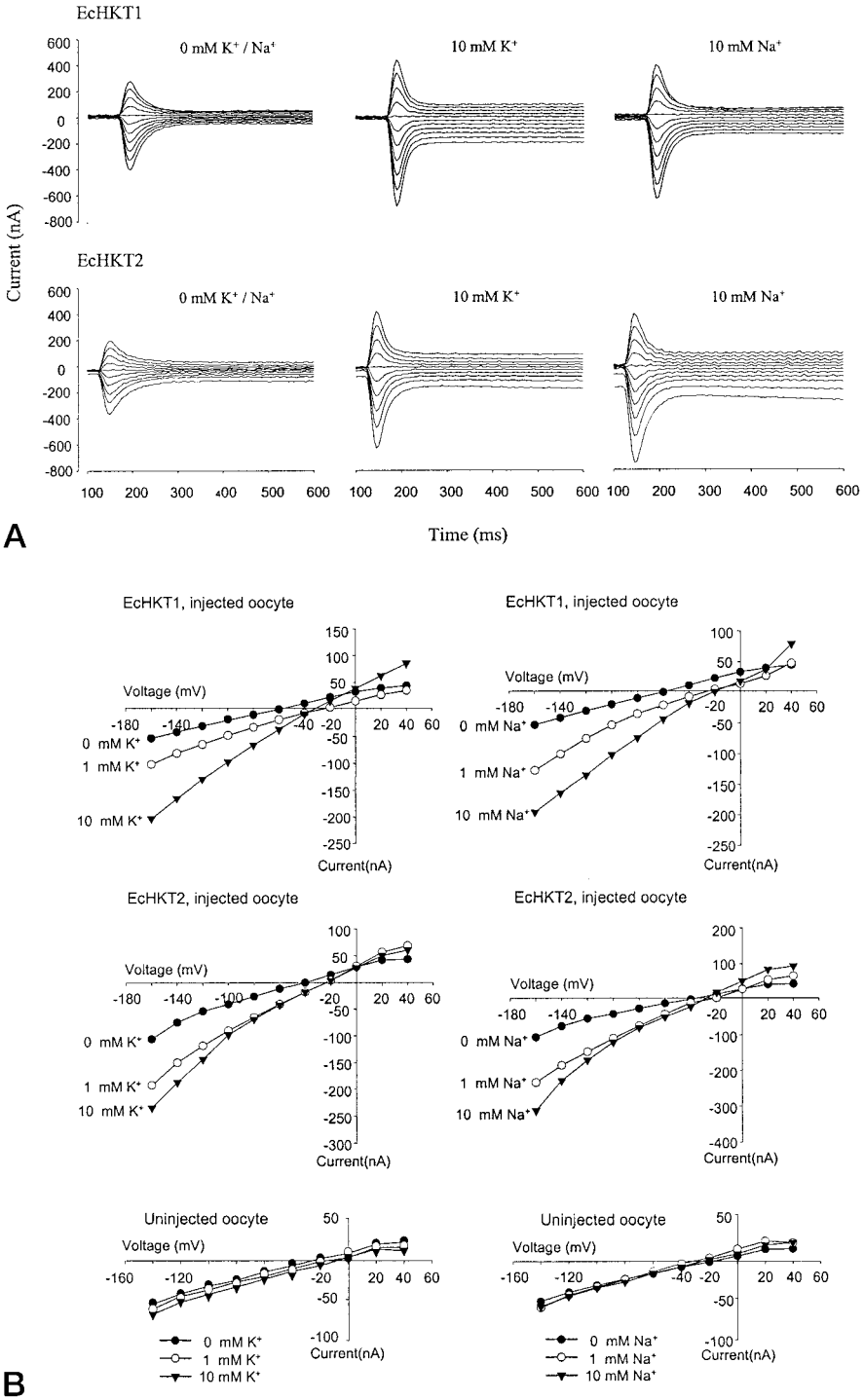

Figure 5. A. Currents induced in oocytes injected with *EcHKT1* or *EcHKT2* mRNA when the bath chamber was perfused with K^+- or Na^+-containing solutions. Holding potential was −40 mV, step pulses from −160 mV to +40 mV with 20 mV increment applied to oocytes. Transient capacitance currents and steady-state currents were observed. The steady-state currents were used to construct current-voltage curves. B. I-V plot from two representative injected oocytes (*EcHKT1* and *EcHKT2* mRNA injection, respectively) and one uninjected oocyte.

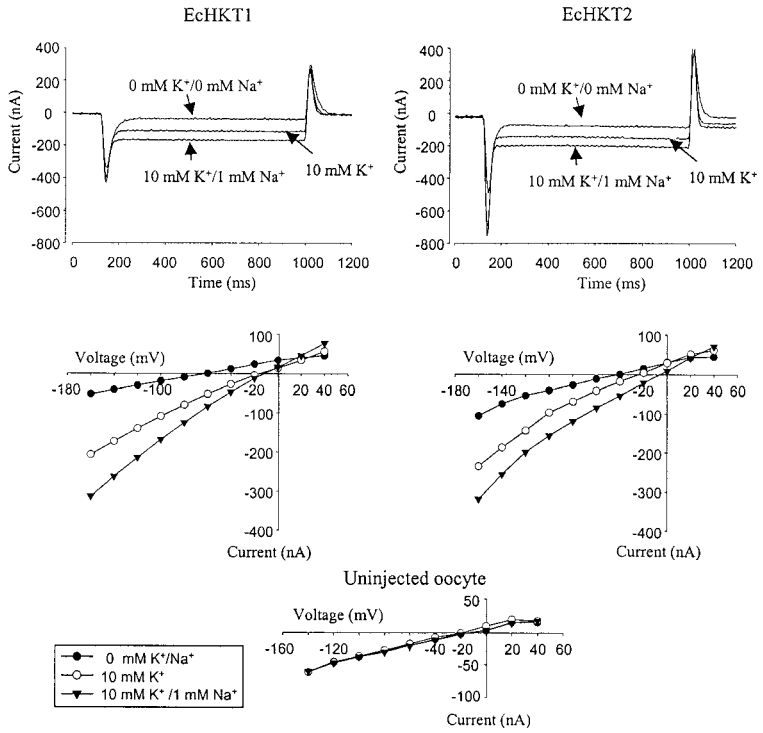

Figure 6. The currents carried by K^+ in both *EcHKT1* and *EcHKT2* mRNA-injected oocytes increased upon addition of 1 mM Na^+. The upper panel shows current traces at membrane potentials −120 mV. The lower panel is the I-V plot from the same oocytes in the upper panel and an uninjected oocyte.

EcHKT1-expressing oocytes, -53 ± 13 nA ($n = 4$, 10 mM K^+) and -84 ± 13 nA ($n = 4$, 10 mM Na^+) for *EcHKT2*-expressing oocytes. Statistical analysis showed that *EcHKT2*-expressing oocytes are more permeable to Na^+ than to K^+ at 10 mM external ion concentrations ($P<0.05$). No significant difference between Na^+ and K^+ permeability was found for *EcHKT1*-expressing oocytes. Voltage-dependent endogenous currents were recorded in both injected and uninjected oocytes at 0 mM Na^+/K^+, which become larger at potentials more negative than −140 mV. At −120 mV, the steady currents at 0 mM K^+/Na^+ within the first 500 ms were in the range of 50–100 nA for the injected and uninjected oocytes. These currents were not significantly different between injected and uninjected oocytes. In contrast to the *EcHKT1*-injected oocytes, the currents recorded in uninjected oocytes did not increase upon addition of 10 mM K^+ or Na^+ (Figure 5B). Since HKT1 from wheat root exhibits Na^+-coupled K^+ uptake when expressed in yeast and oocytes, experiments were carried out to study the effects of Na^+ plus K^+-containing solution on EcHKT1 and EcHKT2 transporter currents. The data demonstrated that when the external K^+ concentration was 10 mM, addition of 1 mM Na^+ in the perfusion solution significantly increased the inward currents for both *EcHKT1*- and *EcHKT2*-expressing oocytes (Figure 6). The increase in inward currents at −120 mV and 10 mM K^+ plus 1 mM Na^+ was -117 ± 15 nA ($n = 4$) for *EcHKT1*-expressing oocytes and 94 ± 4 nA ($n = 4$) for *EcHKT2*-expressing oocytes. The currents at −120 mV increased significantly ($P<0.05$) in solutions containing 10 mM K^+ and 1 mM Na^+ as compared with solutions containing 10 mM K^+ alone for both *EcHKT1*- and *EcHKT2*-injected oocytes. No significant change in current amplitude was found in uninjected oocytes in all observed ion concentrations. The electrophysiological properties exhibited by EcHKT1 and EcHKT2 suggest that EcHKT1 and EcHKT2 are most likely Na^+-coupled K^+-uptake transporters, because the increased current upon addition of 1 mM Na^+ was greater than what was observed if 1 mM K^+ or Na^+ was added to the bath solutions that solely contained that ion.

Discussion

In this paper we report the isolation and characterisation of two homologues (EcHKT1 and EcHKT2) of the wheat K^+-Na^+ symporter HKT1 from *E. camaldulensis*. Complementation of the growth of an *E. coli* K^+-uptake-deficient mutant under K^+-limiting conditions by these *Eucalyptus* genes demonstrated that they encode functional K^+ transporters. *Xenopus* oocytes were used to confirm the function of EcHKT1 and EcHKT2. These studies showed that oocytes expressing either *EcHKT1* or *EcHKT2* are permeable to both K^+ and Na^+. The correct assembly of functional EcHKT1 and EcHKT2 K^+ transporters in *E. coli* will facilitate future structure-function studies and topological analysis (Uozumi *et al.*, 1998).

The *EcHKT1* and *EcHKT2* genes encode two distinct Na^+-coupled K^+-uptake transporters. They are structurally distinct, sharing just 76% identity at the protein sequence level. They are also functionally distinct because the growth of *E. coli* TK2463 cells over-expressing *EcHKT1* or *EcHKT2* showed different sensitivities to Na^+, NH_4^+, Ca^{2+} and TEA^+ ions added to the growth medium. For example, supplementation of a K^+-limited growth medium with Ca^{2+} (10 mM) greatly reduced the growth of TK2463 cells over-expressing *EcHKT2* but not cells over-expressing *EcHKT1*. This suggests that Ca^{2+} ions block the EcHKT2 transporter. Na^+, NH_4^+, Ca^{2+} and TEA^+ ions are all known to interact with plant cation channels (Maathuis *et al.*, 1997). In addition, oocytes expressing *EcHKT1* or *EcHKT2* demonstrated a difference in Na^+ permeability relative to K^+ permeability. The inward currents for both *EcHKT1* and *EcHKT2* expressing oocytes in a bath chamber perfused with 10 mM K^+ significantly increased upon the addition of 1 mM Na^+. Thus EcHKT1 and EcHKT2 are likely to be Na^+-coupled K^+-uptake transporters as has been shown for HKT1 (Rubio *et al.*, 1995). Interestingly, *EcHKT2*-expressing oocytes are more permeable to Na^+ than to K^+ at 10 mM ion concentration ($P<0.05$), but no significant difference between Na^+ and K^+ permeability was found for *EcHKT1*-expressing oocytes. This result was unexpected because *E. coli* cells expressing *EcHKT1* grew more slowly in medium supplemented with NaCl compared to the growth of *EcHKT2*-expressing cells. However, until the physiological role of Na^+-coupled K^+-uptake transporters in higher plants becomes clearer, it is difficult to draw conclusions on the physiological relevance from the different functional properties of EcHKT1 and EcHKT2.

The finding that HKT1 may be one of the pathways for Na^+ entry into plants has important implications for salt toxicity. The wheat *HKT1* gene is expressed throughout the cortical cells of the root (Schachtman and Schroeder, 1994). Presumably, HKT1 is involved in the uptake of Na^+ into root cortical cells. The precise contribution of HKT1 to the total Na^+ influx into root cells is unknown. However, non-selective cation channels are currently believed to be responsible for at least 50% of Na^+ influx into root cells (White, 1999; Davenport and Tester, 2000). Once inside the cortical cell, the Na^+ that is not sequestered into the vacuole is carried in the transpiration stream to the xylem and then distributed to the aerial parts of the plant. The exclusion of Na^+ from leaves is one strategy used by non-halophytes to improve their tolerance to salinity. As EcHKT1 and EcHKT2 are likely to be K^+-Na^+ symporters, they could be considered as transporters of Na^+ as well as K^+ transporters. A physiological role for *EcHKT1* and *EcHKT2* may be to control or limit the transport of Na^+ around the plant by excluding Na^+ from the transpiration stream. Compartmenting Na^+ into specific cells could contribute to salinity tolerance. *Eucalyptus* is known to sequester Na^+ in bark and wood tissues under salt-stress conditions (Lambert and Turner, 1999). Interestingly, stem tissues contained the highest level of *EcHKT1* transcripts of the tissues examined in this study.

In plants, the cytosolic ratio of K^+ and Na^+ is an important determinant of Na^+ toxicity. Studies with the salt-sensitive mutant *sos1* have shown that *Arabidopsis* salt sensitivity is not closely related to Na^+ tissue content (Wu *et al.*, 1996; Ding and Zhu, 1997; Zhu *et al.*, 1998). Indeed, the *sos1* plants take up less Na^+ and consequently have a lower Na^+ content than wild-type plants. A study of three *E. camaldulensis* seedling samples, differing in their tolerance to salt, also had no significant differences in their Na^+ tissue content (Sands, 1981). On the other hand, the level of salt tolerance of *Arabidopsis sos1* plants closely correlated with K^+ content (Zhu *et al.*, 1998). The *kna1* locus, which partly controls K^+/Na^+ selectivity in wheat, has been shown to be important in plant productivity under saline conditions (Dvorak and Gorham, 1992). Therefore, maintaining a high cellular K^+ content in the presence of excess Na^+ is critical for plant growth under saline conditions. This suggests that K^+ transporters are important determinants of salinity tolerance. Modifying the properties

of a K^+ transporter such as HKT1 may be a means of improving salt tolerance in plants.

HKT1 mediates Na^+-coupled high-affinity K^+ uptake at micromolar concentrations of external Na^+ but at millimolar external Na^+ it functions as a low-affinity Na^+ transporter (Rubio *et al.*, 1995). It has been proposed that at high Na^+ concentrations Na^+ displaces K^+ from the HKT1 K^+-binding site (Gassmann *et al.*, 1996). Studies in yeast have shown that single-base mutations of *HKT1* enable HKT1 to continue mediating Na^+-coupled high-affinity K^+ uptake at high millimolar external Na^+. This resulted in an increase in the ratio of K^+ to Na^+ of yeast cells and enhanced salt tolerance in yeast over-expressing *HKT1* mutants (Rubio *et al.*, 1995, 1999). Interestingly, one of these positive effect mutations (L247F) is already present in the *Eucalyptus* orthologues. If the results in yeast with HKT1 mutant genes are transferable to plants, then small changes in the HKT1 protein structure can be expected to produce profound alterations in cellular K^+ to Na^+ ratios. Consequently, the natural variation in HKT1 orthologues and paralogues amongst higher plants may be expected to produce a class of proteins with a wide range of transport properties. For example, EcHKT1 has over 300 changes in amino acid residues compared to HKT1. It is likely that EcHKT1 will exhibit some differences in transport properties when compared to HKT1. Root cortical cells with an HKT1 orthologue, which is resistant to Na^+ inhibition of high-affinity K^+ transport, would be expected to have a higher K^+ to Na^+ ratio under conditions of high salinity. This could result in improved salt tolerance. An understanding of the structure and function of specific transporters may allow K^+/Na^+ uptake characteristics and salt tolerance to be engineered into important crop and plantation species.

The expression patterns of HKT1 orthologues may provide some clues as to their physiological role. The wheat *HKT1* gene is expressed in root cortical cells and several layers of mesophyll cells surrounding vascular tissue in the leaf (Schachtman and Schroeder, 1994). These tissues are involved in the transfer of K^+ from the roots to leaves. The expression level of *EcHKT1* and *EcHKT2* transcripts was higher in stems and leaves than roots, which suggests some role for these K^+ transporters other than in the primary uptake of K^+ from soil solutions. In most plants K^+ is readily re-translocated, moving freely from older tissues towards the growing tips (Grove *et al.*, 1996). The expression pattern for *EcHKT1* and *EcHKT2* is indicative of a role in the transport or compartmentalisation of K^+ within the plant. Indeed, the wide distribution of this class of K^+ transporters among plant, fungal, eubacterial and archaebacterial genomes has led some authors to suggest that HKT1 orthologues are involved in the ionic homoeostasis of the cells in which they are expressed (Schachtman and Liu, 1999).

In conclusion, this study has demonstrated that *Eucalyptus* contains at least two HKT1 homologues, which are more highly expressed in shoot tissues than in roots. This is in contrast to diploid wheat, which has a single HKT1 gene expressed mainly in root cortical cells (Schachtman and Schroeder, 1994). These differences suggest that this intriguing class of K^+-Na^+ transporters may not necessarily play the same physiological role in wheat and eucalypts. Furthermore, it is tempting to speculate that the differences between EcHKT1 and EcHKT2 observed in this study represents specialised roles for these transporters in *Eucalyptus* trees.

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