



Expression of the cation transporter McHKT1 in a halophyte

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Abstract

From the ice plant, *Mesembryanthemum crystallinum*, *McHKT1* was isolated encoding a protein 41–61% identical to other plant HKT1-like sequences previously described as potassium or sodium/potassium transporters. *McHKT1* acts as a potassium transporter in yeast with specificity similar to that of wheat HKT1. In *Xenopus* oocytes it transports cations with a specificity $Rb^+ > Cs^+ > [K^+ = Na^+ = Li^+]$. *McHKT1* is exclusively localized to the plasma membrane. The isoform isolated is most highly expressed in leaves and is present in stems, flowers and seed pods but absent from the root where, according to immunological data, a second isoform exists which does not cross-hybridize with the leaf form in RNA blots at high stringency. *McHKT1* transcript amounts increase during the first 6–10 h of stress and then decline to pre-stress levels with kinetics reminiscent of the initial influx of sodium into this halophyte. Immunocytological localization showed strong signals in the leaf vasculature and surrounding mesophyll cells but low-intensity signals are also detected in other cell types. In roots, *McHKT1* is mainly confined to endodermis and stele. Possible functions of *McHKT1* in ion homeostasis in the halophytic ice plant are discussed.

Introduction

In saline environments, high external Na^+ , $[Na^+]_{ext}$, favors sodium entry down the electrochemical gradient across the plasma membrane. In addition to out-right exclusion and vacuolar compartmentalization of internal Na^+ , gaining control over Na^+ influx might improve plant salinity tolerance. Control might, for example, be coordinated with protective reactions, such as the synthesis of compatible solutes, or the development of salt glands or storage organs. Entry pathways for Na^+ into plant cells are still unclear, because the evidence suggests that different families or spe-

cies might possess dissimilar entry ports (Rubio *et al.*, 1995; Uozumi *et al.*, 2000; Rus *et al.*, 2001). Generally accepted is that Na^+ enters cells via one or several cation passage ways, through potassium transporters and/or voltage-independent non-selective cation channels (Amtmann and Sanders, 1999; Schachtman and Liu, 1999; Blumward *et al.*, 2000; Maathuis and Sanders, 2001; Rus *et al.*, 2001; Demidchik and Tester, 2002).

Inward rectifying K^+ channels (IRC), such as *Arabidopsis* AKT1, display high K^+/Na^+ selectivity under physiological conditions. They are not likely to mediate significant Na^+ uptake (Amtmann and

Sanders, 1999; Blumwald *et al.*, 2000) yet it is possible that other IRC with much higher Na^+ permeability exist (Amtmann and Sanders, 1999). HAK transporters mediate low-affinity Na^+ uptake, suggesting they could be a way for Na^+ to enter under saline conditions (Santa-Maria *et al.*, 1997). Finally, high-affinity K^+ transporters of the HKT type could be a major sodium entry pathway, at least in some plants, at high external Na^+ concentrations, unless their expression or activity in the plasma membrane is reduced under such conditions (Rubio *et al.*, 1995; Gassman *et al.*, 1996; Uozumi *et al.*, 2000; Golldack *et al.*, 2002).

The first plant HKT-type transporter, with homology to yeast TRK, was isolated from wheat by complementation of a yeast mutant (Schachtman and Schroeder, 1994). A 'four-MPM' model has been proposed for the HKT-type transporter proteins (Durell *et al.*, 1999; Durell and Guy, 1999). Each MPM motif consists of two transmembrane domains, M1 and M2, connected by a pore-forming region P that is embedded in but does not transect the membrane. HKT1 proteins contain four repeats of the MPM motif. This topology has been proved for *Arabidopsis* HKT1 by alkaline phosphatase fusion protein expression in *Escherichia coli* and FLAG-epitope tagging (Kato *et al.*, 2001).

The wheat HKT1 is functionally distinguished from fungal TRK transporters in that it mediates Na^+ -energized high-affinity K^+ transport and low-affinity Na^+ uptake when the external Na^+ concentration is high (Rubio *et al.*, 1995; Gassmann *et al.*, 1996). In yeast the TRK transporters have been proposed to function as K^+ - H^+ co-transporters (Rodríguez-Navarro, 2000). Furthermore, *Arabidopsis* HKT1 is primarily a Na^+ transporter when expressed in *Xenopus* oocytes and yeast cells (Uozumi *et al.*, 2000). Mutations that eliminate the expression of At-HKT1 confer increased salinity tolerance to plants (Rus *et al.*, 2001), indicating that HKT1 could be a major port for the entry of sodium into *Arabidopsis* roots. Point mutations altering low-affinity Na^+ binding of wheat HKT1 lead to improved salt tolerance of yeast cells expressing HKT1 (Rubio *et al.*, 1995, 1999; Diatloff, 1998). Recently, an analysis based on site-directed mutagenesis identified a glycine residue in the first of four pore-forming domains to affect ion specificity in yeast (Mäser *et al.*, 2002a, b). A HKT transporter in the monocot rice is an alkali cation transporter with high affinity to Na^+ (Horie *et al.*, 2001). A second HKT-type transporter in *indica* rice

cv. Pokkali has been reported as a general alkali transporter (Golldack *et al.*, 2002). The recently completed DNA sequences of rice genomes revealed the presence of at least seven HKT-type transporters (F. Quigley, unpublished data).

In previous studies, the expression of some potassium channels and HAK transporters from the ice plant, *Mesembryanthemum crystallinum*, has been investigated (Su, 2001; Su *et al.*, 2001, 2002). Regulation of their expression suggests roles in the maintenance of potassium homeostasis under salt stress conditions, and suggests that channels and HAK transporters may have little to do with sodium uptake. In this study, we describe the expression of a HKT1-type potassium transporter in the ice plant under salt stress conditions.

Materials and methods

Plant materials

Mature plants were grown according to Adams *et al.* (1998). Plants were transferred to hydroponic tanks with $0.5\times$ Hoagland's nutrition solution 3 weeks after germination. Aeration of the hydroponic cultures was started a week after transferring the plants. For RNA blot analysis and RT-PCR, plants were treated with 400 mM NaCl in $0.5\times$ Hoagland's solution for the time periods indicated. For fluorescence localization, potassium was omitted from the nutrition solution 7 days before starting the experiments. Unstressed control plants were grown in parallel and harvested at the same time.

cDNA library screening

For *McHKT1*, a PCR product covering conserved region of HKT/TRK proteins was used to screen a leaf cDNA library. For further screening, the entire coding sequence of *McHKT1* was used to screen cDNA libraries with procedures according to Sambrook *et al.* (1989).

Sequence analysis and nucleic acid hybridization

DNA and RNA isolations, sequence alignments and hydropathy analyses were performed as described (Su, 2001; Su *et al.*, 2001). For RNA blots, a 3' UTR fragment of 500 bp was used. For genomic Southern blot, the entire coding sequence of *McHKT1* (1.6 kb) was used as probe. The gene copy number reconstitution

experiment is based on an ice plant genome size of 390 Mb (Adams *et al.*, 1998).

Yeast complementation

The coding sequence of McHKT1 was inserted into the yeast expression vector pYES2, between a GAL1 promoter and a CYC1 terminator. CY162 cells were transformed with a McHKT1 construct, a wheat HKT1 construct (also in pYES2), or a pYES2 plasmid. Transformants were selected on YNB medium containing 100 mM K⁺ lacking uracil, then adapted in YNB liquid medium with 20 mM K⁺, 2% galactose and 2% sucrose for two days. The liquid culture was diluted to equal density and dropped on LS medium with 3 mM KCl and the same amount of galactose and sucrose, and incubated at 28 °C for 5 days.

Semi-quantitative RT-PCR

Oligonucleotide primers used for McHKT1 were 5'-TCTTCTCCCTCTTCGTCACC-3' (primer 1) and 5'-GCCCTCCCTCACAAAATTTC-3' (primer 2) (Gibco-BRL). The concentration gradient control was a plasmid carrying a partial cDNA of McHKT1 deleted by *Nco*I. A 1–5 µg portion of total RNA or 50–500 ng poly(A)⁺-RNA was used for reverse transcription. RNA and 50 ng of oligo(dT)_{12–18} in a volume of 12 µl were incubated at 70 °C for 10 min. After quenching on ice, 4 µl of 5× first-strand buffer, 2 µl 0.1 M DTT, 1 µl 10 mM dNTP mix, were added and tubes were incubated at 42 °C for 2 min. A 1 µl aliquot of Superscript II (Life Technologies, Rockville, MD) was added and the incubation was continued for 50 min. The reaction was stopped by heating (70 °C, 15 min), and 1 µl RNase A (2 units) was added to remove RNA complementary to the cDNA. Amplification reactions were carried out with 1 µl first-strand cDNA, 1 µl plasmid DNA (concentration gradients of 0.1, 1, 10, 100 ng/µl), 5 µl 10× PCR buffer, 1.5 µl 50 mM MgCl₂, 1 µl 10 mM dNTP mix, 2 µl primer 1 (5 µM), 2 µl primer 2 (5 µM), and 2.5 units *Taq* DNA polymerase in a volume of 50 µl (Life Technologies). After denaturation (3 min, 94 °C), samples were subjected to 32 cycles of 1.5 min at 94 °C, 1.5 min (55 °C or temperature gradient), and 2 min at 72 °C. PCR products were separated on 0.8% agarose gels (0.5× TBE). The results were analyzed with GelExpert Software (Nucleotech, San Carlos, CA).

Two-electrode voltage clamp of Xenopus oocytes

Template DNA and *McHKT1* cRNA were synthesized *in vitro* as previously described (Golldack *et al.*, 2002). Oocytes were used 3 to 5 days after injecting 50 ng mRNA for recording currents induced by the expression of McHKT1 with the two-electrode voltage clamp technique as described by Golldack *et al.* (2002). Initially, the voltage electrode was introduced into the oocyte; after recording a stable membrane potential for more than 2 min, the current electrode was inserted. The experiments were started once the two electrodes read the same membrane potential. The electrodes were filled with 1 M KCl and the oocytes bathed with a solution containing 6 mM MgCl₂, 1.8 mM CaCl₂, 10 mM Tris/MES pH 5.5, with osmolality adjusted to 240–260 mOsmol/kg with D-mannitol. K⁺ and Na⁺ were added as gluconate salts in experiments where only these two cations were studied. The selectivity of HKT1 was assayed for the alkali cations by employing chloride solutions. Recordings were obtained with a GeneClamp 500B amplifier (Axon Instruments, Foster City, CA). Data acquisition and analysis were done with the programs Clampex and Clampfit included in pCLAMP6 (Axon Instruments). Current levels are raw data without correction for leak currents, which were in the order of 20 nA at –120 mV. Results were obtained from more than three oocytes from each of three different frogs.

Antibody and immunolocalization

An oligopeptide corresponding to the sequence of the carboxyl terminus of the deduced McHKT1 protein (LKRFFKGGKAWK) was synthesized and used to generate antibodies (Alpha Diagnostic International, San Antonio, TX). Serum of the second bleeding was used for all immunological studies. The specificity of the antiserum was checked in initial assays; no signal was obtained with the preimmune serum. For western blotting, a 1:500 dilution was used. Membrane fractionations and protein blotting were conducted by standard procedures (Vera-Estrella *et al.*, 1999).

For fluorescence cytolocalization, root and leaf tissue were fixed with FAA, dehydrated, infiltrated with xylene and embedded with Paraplast Plus (Fisher Scientific) according to McKhann and Hirsch (1993). Immunolocalization was according to Kirch *et al.* (2000). Antibody was used in a 1:200 dilution. Signal detection was performed with an alkaline phosphatase conjugated goat anti-rabbit IgG and naphthol-AS-phosphate and Fast Red TR as substrates (Roche

Diagnostics, Germany). Microscopic images were obtained with a cooled CCD camera coupled to an Axioskop fluorescence microscope and processed by Axiovision (Zeiss, Germany).

Results

Isolation of McHKT1 cDNA and sequence analysis

A PCR product based on degenerate primers of conserved regions of wheat and rice HKT1 was used to screen ice plant cDNA libraries. A full-length cDNA (*McHKT1*, AF367366) was isolated from a leaf library. *McHKT1* encodes an open reading frame of 505 amino acids with a predicted molecular mass of about 55 kDa. Figure 1A shows the deduced amino acid sequence of *McHKT1* aligned with other plant HKT-type proteins. According to the 'four-MPM' model, the transmembrane domains (M1 and M2) and pore regions (P) of the four repeats (a–d) are marked. The glycine residue in the third position before the end of the last three P-regions is conserved, while in the first region, [P(a)], the glycine residue is substituted by serine, like in the other dicots (Figure 1A). This replacement has now been related to HKT1 ion specificity in as far as the presence of a glycine seems to signify potassium as the ion transported most efficiently while a serine changes specificity such that HKT1 functions primarily as a sodium transporter (Mäser *et al.*, 2002a). In all K⁺ channels, the GYG residues in the P-region act as a selective filter that is essential for K⁺ selectivity (Yool and Schwarz, 1991; Uozumi *et al.*, 1995). This glycine might be necessary for K⁺ binding, but HKT transporters may not be as selective as K⁺ channels that show the GYG filter (Rodriguez-Navarro, 2000).

McHKT1 shares 51%, 46% and 44% identity and 61%, 55% and 55% amino acid similarity to *Arabidopsis*, rice and wheat HKT1, respectively. Clustal-X analysis indicated that plant HKT-type transporters are distantly related to fungal TRK transporters, and to transporters present in prokaryotes. HKT-type transporters of monocots (wheat, rice) are separated from those of dicots (*Arabidopsis*, gum tree, ice plant) (Figure 1B). A standard protein-protein BLAST search in the GenBank nr database revealed 39 entries for HKT/TRK-like proteins (cut-off e-value 0.001), including 19 from higher plants (including *McHKT1*). The *Arabidopsis thaliana* (Columbia) genome includes one *Hkt* gene, from *Eucalyptus* two transcripts

are known and Southern-type hybridizations with ice plant genomic DNA suggest two copies of *McHKT1*-like sequences (data not shown). In the *japonica* and *indica* rice genomes a large number of HKT1-like sequences have been revealed. The *japonica* sequence indicates at least 7 *Hkt* genes, and possibly 8 genes. The *indica* cultivar Pokkali includes possibly an even larger number of *Hkt* genes due to the detection of *Ospokhkt2* (Figure 1B), which does not have a counterpart in *japonica* rice.

Complementation of a yeast mutant by McHKT1

The yeast strain CY162 ($\Delta trk1trk2$) deficient in K⁺ uptake, which could be rescued by the expression of wheat HKT1 (Schachtman and Schroeder, 1994), was used to test the function of *McHKT1*. The coding sequence of *McHKT1* was inserted into the yeast expression vector pYES2 (Su, 2001). CY162 cells were transformed with *McHKT1*, TaHKT1, or the empty yeast vector. The transformants were selected on YNB medium containing 100 mM KCl but without uracil, adapted in YNB liquid medium containing 20 mM K⁺, 2% sucrose and 2% galactose to induce the expression of HKT1 genes. Yeast cells expressing *McHKT1* grew better than the mutant cells on medium containing 3 mM KCl, but not as well as cells transformed with wheat HKT1 (data not shown; see Su, 2001).

Transport properties of McHKT1

Transport properties of *McHKT1* were studied by its heterologous expression in *Xenopus* oocytes following established methods (Gollmack *et al.*, 2002). The presence of either 0.3 mM K⁺ or 1 mM Na⁺ alone, or added together in the bathing solution of an oocyte and held at the resting potential (−90 mV to −50 mV), did not elicit a measurable current (data not shown). Increasing the concentration of both cations up to 50 mM only caused the development of small inward currents (data not shown). The magnitude of the inward currents was not affected by the addition of both cations together in a combination 1 mM K⁺/20 mM Na⁺ (data not shown). When the concentrations of these cations were increased to 100 mM it was possible to record inward currents close to 200 nA (Figure 2A, and data not shown). Among the different alkali cations, rubidium was the most permeable with current levels close to 400 nA at −120 mV, with the other cations eliciting smaller currents (Figure 2A). From these and similar data the permeability sequence Rb⁺ > Cs⁺ > [K⁺ =

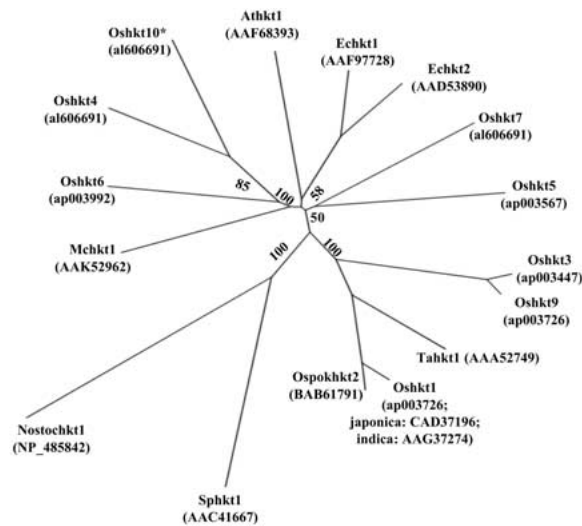


Figure 1. Continued.

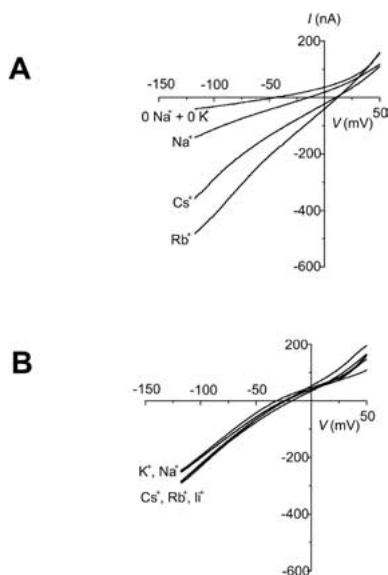


Figure 2. MchHKT1 mediates low-affinity alkali cation uptake. **A.** Inward currents were recorded from an oocyte injected with 50 ng of the ice plant cRNA only in the presence of 100 mM concentrations of the different alkali cations. The reversal potentials were: $E_{Rb} = 10$ mV; $E_{Cs} = 7$ mV; $E_{Na} = -16$ mV; $E_K = -18$ mV; $E_{Li} = -25$ mV. Results for Li^+ and K^+ are not shown. **B.** Exposure of a control oocyte (injected with water) to 100 mM solutions of the alkali cations failed to induce large inward currents or to cause changes in E_r . Representative results of at least three oocytes obtained from four different frogs.

$Na^+ = Li^+$] was derived for MchHKT1 (Figure 2A), similar to the results reported for OsHKT1 (Golldack *et al.*, 2002). The selectivity of MchHKT1 was obtained from changes in the reversal potential (E_r) caused by

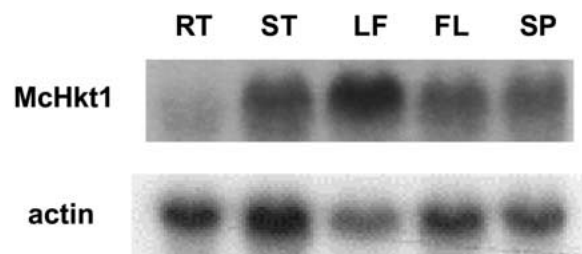


Figure 3. Tissue-specific expression of MchHKT1 detected by northern blot analysis. Total RNAs (20 μ g) extracted from unstressed plants (except flower and seed pod) were loaded. Tissues were specified as RT (root), ST (stem), LF (leaf), FL (flower) and SP (seed pod). Actin was used as a loading control. Shown are representative experiments from a total of three experiments.

the presence of the different alkali cations at 100 mM. Figure 2A clearly shows how E_r was shifted to positive values in the presence of Rb^+ and Cs^+ , with Na^+ , K^+ and Li^+ causing smaller displacements in E_r . These results indicate a higher selectivity of MchHKT1 towards Rb^+ and Cs^+ with respect to Na^+ , K^+ or Li^+ . In control oocytes injected with DEPC-water and exposed to 100 mM solutions of the different alkali cations, negative potentials induced inward currents around -200 nA at -120 mV (Figure 2B). In Figure 2B it is also clearly shown that the different cations did not shift E_r away from that recorded in the absence of any cation.

Tissue-specific expression of MchHKT1

RNA blot analysis was conducted to monitor tissue specificity of MchHKT1 with total RNA from root,

stem, leaf, flower and seedpods of mature plants. A 500 bp 3'-UTR fragment was used as a probe. When comparing the signal obtained with actin, the *McHKT1* transcript was primarily detected in leaf tissue (Figure 3). Signals were also detected in stems, flower and seedpods. A very weak signal was obtained with root RNA after hybridization at high stringency (washed in $0.1\times$ SSC and 0.1% w/v SDS, at 65 °C). In wheat, rice, and *Arabidopsis*, *HKT1* transcripts were primarily detected in root tissues (Schachtman and Schroeder, 1994; Uozumi *et al.*, 2000; Gollmack *et al.*, 2002) but in rice multiple HKT genes have been described (Horie *et al.*, 2001) or are predicted by analyzing the draft of the genome sequence. These multiple forms could constitute tissue-specific isoforms of HKT1, possibly with different transport or regulatory functions. From the ice plant we obtained a second full-length HKT cDNA (*McHKT2*; accession number AY231175) that appears to be a root-specific isoform (F. Quigley and H.J. Bohnert, unpublished results).

Regulation of *McHKT1* expression in ice plant

Evidence from other plant species and data presented here (Figure 2) suggest that HKT-type transporters are involved in Na^+ uptake, so it was important to learn how these transporters might behave in a halophyte. The regulation of *McHKT1* expression was investigated by RNA blot analysis as well as by quantitative RT-PCR (Figure 4A, B). Five-week old ice plants were exposed to 400 mM NaCl for the time periods indicated and total RNA from leaf was probed with a *McHKT1* 3'-UTR fragment. *McHKT1* transcripts increased shortly after the stress started and reached the highest levels 6 h after the start of salt treatment. Later, the transcript decreased to a level even lower than that found in unstressed plants. This expression pattern was confirmed by quantitative RT-PCR (Figure 4B). At 6 h, accumulation of *McHKT1* was 2.5 times of that at 0 h. When the plants were stressed for 12 h, the amount of transcript was 50% of that before stress.

McHKT1 protein localization

Antibodies for *McHKT1* were generated against an oligopeptide corresponding to the carboxyl terminus of the deduced protein (-LKRFNFKGGKAWK). Plasma membrane and tonoplast fractions were prepared from leaf and root tissue by sucrose gradient centrifugation. The immunological blot showed specific recognition of a 56 kDa polypeptide that resided

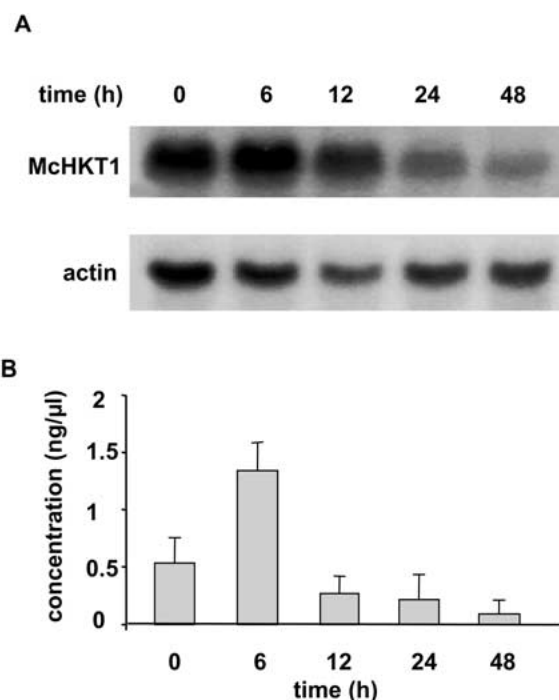


Figure 4. *McHKT1* expression in leaf tissue during a time course of NaCl stress. A. Northern blot analysis. Five-week old plants grown in $0.5\times$ Hoagland's solution were stressed with 400 mM NaCl for the time period (hours) indicated. Total RNA (20 μg) from leaf tissues was probed with the 3'-UTR region of *McHKT1*. B. Quantitative RT-PCR of the same samples of RNA. The oligonucleotide primers used were 5'-TCTTCTCCCTCTTCGTCACC-3' and 5'-GCCCTCCCTCACAAAATTTC-3'.

exclusively in the plasma membrane, as indicated by the co-localization of *McHKT1* and P-ATPase in the same membrane fraction. The signal from the roots weaker was than the signal in leaves (Figure 5). The isolation of the ice plant *HKT2* cDNA (accession number AY231175) indicated that the antibody should cross-react because of the similarity of the C termini (-KKFNMKGGRAWKLL). So far, this is the first substantiation for the subcellular localization of a HKT1-type transporter in plants.

To learn more about the location of *McHKT1* in specific tissues or cell types, fluorescence cellular localization was conducted with the same antibody as in the protein blots. Signals were detected with an alkaline phosphatase conjugated goat anti-rabbit IgG, using naphthol-AS-phosphate and Fast Red TR as substrates. In Figure 6, the red color indicates the signal of the *McHKT1* protein, and background fluorescence is shown by green color. In cross-sections of leaves, *McHKT1* was detected primarily in vascular bundles and surrounding mesophyll cells (Figure 6A, C). In the

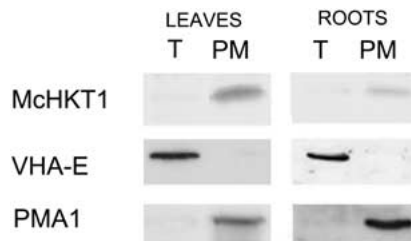


Figure 5. Membrane location of McHKT1. Membranes were isolated from *M. crystallinum* leaves or roots after sucrose density gradient fractionation as described in Materials and methods. Western blot analysis of membrane proteins (15 μ g) from adult *M. crystallinum* plants probed with the antibody raised against a synthesized oligopeptide corresponding to the sequence of the carboxyl terminus of the McHKT1 protein (56 kDa). Membrane markers for the tonoplast and plasma membrane were VHA-E (31 kDa) and PMA1 (100 kDa), corresponding to subunit E of the vacuolar ATPase and for the plasma membrane ATPase, respectively. The blots are representative of three independent experiments.

vasculature, all phloem and phloem-associated cells were highlighted, as well as cells of the xylem. Signals were strongest in xylem parenchyma cells. After stress (400 mM NaCl, 72 h), the signal decreased in vascular bundles and mesophyll cells, but increased or at least was maintained in the epidermal bladder cells (Figure 6B, D).

High-stringency RNA blot analysis detected a weak signal in roots but immunolocalization led to the detection of HKT protein also in roots, indicating that the C termini of the leaf and root forms should be conserved (and also indicating high specificity in the RNA blot hybridizations). In cross-sections of root tips, the protein signals were most concentrated in epidermal cells and were weaker in the vascular cylinder (Figure 6E). In cross-sections of older roots, highest expression was seen in the vascular cylinder (Figure 6F). In older roots under stress (72 h stress), signals decreased in the vasculature and in cortex cells but increase in the epidermis (Figure 6F and H), while in root tips the change was not significant or marginal (Figure 6E and g). In images taken at the same scale, where a direct comparison of fluorescence intensity is possible, the overall signal strength seemed to be lower in roots than in leaves (data not shown).

Discussion

We are interested in effects of high salinity on potassium homeostasis and the roles that potassium transport systems play in salt stress adaptation. Physiological and molecular studies have begun to ad-

dress the question about which channels and transporters mediate sodium entry into the plant cell (Amtmann and Sanders, 1999; Schachtman and Liu, 1999; Blumwald *et al.*, 2000). In contrast, only a few studies investigated how sodium entry might interfere with potassium uptake and how potassium transport systems are regulated in adaptation to a saline environment.

After sodium shock by, for example, 400 mM NaCl of the ice plant, severe wilting of the leaves occurs within 6 h yet full turgor is established, and no tissues are sacrificed within ca. 36 h (Adams *et al.*, 1998). This rapid osmotic adjustment in the halophytic ice plant is at least partially dependent on sodium influx (Adams *et al.*, 1992) and McHKT1 could be involved in this process. The capacity of HKT to mediate Na⁺ uptake, at least in some species, makes it a candidate that could have a major function for maintaining or breaking ion homeostasis under saline conditions.

Sequence alignment and phylogenetic analysis of McHKT1 show high homology with other plant HKT proteins, especially those from dicot plants. Topology predictions and the experimentally determined membrane structure are available (Durell and Guy, 1999; Kato *et al.*, 2001) and can be used to investigate inter- and intra-species variation in function. Specifically, the conserved glycine residue in the first P region that may be important for K⁺ (or other cation) binding is substituted by a serine in McHKT1, as in other dicot HKT proteins. Recently, this residue has been shown to be critical for ion selectivity in HKT (Mäser *et al.*, 2002a). The point mutation S68G restores K⁺ permeability in AtHKT1, which in its wild type form is a Na⁺ transporter, while the corresponding reverse mutation, G91S, in wheat HKT1 abolishes the K⁺ transport capacity. Moreover, four out of the five point mutations that alter Na⁺-binding affinity of wheat HKT1, A240V, L247F, N365S and E464Q, are in P-regions (Rubio *et al.*, 1995, 1999). Among them, L247F exists in McHKT1 and other dicot HKT proteins, including *Arabidopsis* HKT1 and *E. camaldulensis* (river red gum) HKT1 and HKT2 (Figure 1A) (e.g. ECHKT1, Liu *et al.*, 2001). The substitution L247F reduced Na⁺ inhibition of K⁺ uptake and decreased Na⁺ accumulation in yeast and thus improved salt tolerance of yeast grown in a high-Na⁺ environment (Rubio *et al.*, 1995). These amino acid differences reflect not only divergence between monocot and dicot HKT, but they also seem to indicate functional adaptation to specific environments in terms of ion selectivity or cation binding affinity. Thus, the presence of single residues may not be sufficient to

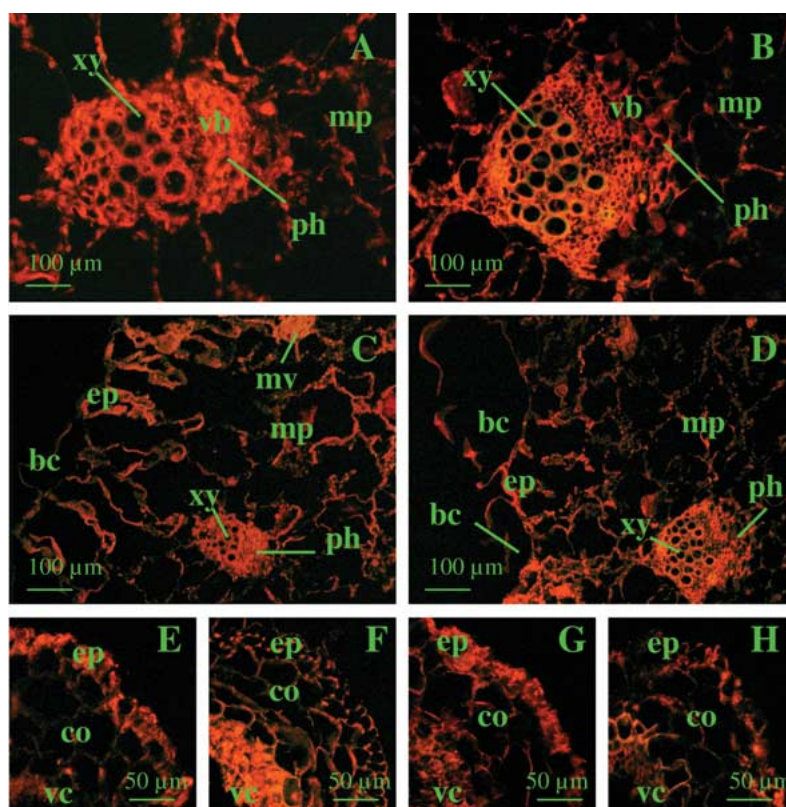


Figure 6. Fluorescence immunolocalization of McHKT1 protein. For the experiments 5-week old plants were used that were transferred to nutrition medium without potassium a week before the experiment. The following treatments are shown: A, cross-section through a leaf focusing on a vascular bundle, control; B, cross-section through a leaf from a plant treated with 400 mM NaCl for 72 h focusing on a vascular bundle; C, leaf cross-section, control; D, cross-section through a leaf from a plant treated with 400 mM NaCl for 72 h; E, cross-section through a root tip about 200 μm from the apex, control; F, cross-section through a root about 8 cm from the apex, control; G, cross-section through a root tip about 200 μm from the apex from a plant treated with 400 mM NaCl for 72 h; H, cross-section through a root about 8 cm from the apex from a plant treated with 400 mM NaCl for 72 h. vb, vascular bundle; ph, phloem; xy, xylem; mp, mesophyll; mv, minor vein; ep, epidermis; bc, epidermal bladder cell; vc, vascular cylinder; co, cortex. The signal is presented in red and the background in green.

unequivocally assign transport properties of a HKT1 protein. For example, the dicot McHKT1, AtHKT1 and EcHKTs mediate Na^+ and K^+ uptake, yet kinetic differences exist among them, which seem not to be determined by those particular residues.

So far the *HKT* transcripts from wheat, rice and *Arabidopsis* are mainly expressed in roots. *EcHKT1* and *EcHKT2* from river red gum are expressed in stem, leaf and root (Fairbairn *et al.*, 2000). Possibly, based on the small size of the family, tissue specificity might not be identical in different species, as is the case for AKT and KAT channels. Ice plant RNA blot analysis of *McHKT1* detected a strong signal in leaf but a very weak signal in root tissue. The increased sensitivity imparted by immunodetection (Figures 5 and 6) detected HKT protein in both tissues. The overall intensity

was lower in roots than in leaves when the two tissues were compared (Figures 5 and 6).

McHKT1 protein was detected in the plasma membrane fraction in leaf and roots, and this is the first clear indication about its location. This location of McHKT1, together with the oocyte transport data, suggest a role for McHKT1 in the uptake of cations into cells. McHKT1 expression seems to be present in every cell although at vastly different amounts. Fluorescence cytolocalization further illustrated that McHKT1 is expressed in tissues and cells that are responsible for primary cation uptake and long-distance transport. For example, in root tips the signal is strongest in epidermal cells, while in older root its signal highlights primarily cells of the vascular cylinder. McHKT1 may be important in cation uptake from soil, in sodium loading to vascular tissue, which ulti-

mately leads to sodium accumulation in leaves of the ice plant under salt stress, for example, in sequestering Na^+ in epidermal bladder cells where the signal persists even after declining in the rest of the plant (Figure 6). Sodium concentrations exceeding 1 M in the vacuolar space have been detected in these bladder cells (Adams *et al.*, 1992). An involvement of this transporter in the recirculation of sodium by the phloem has recently been reported for the *Arabidopsis* AtHKT1 (Berthomieu *et al.*, 2003). What function McHKT1 exactly fulfills in various tissues and cells depends largely on its transport properties in relation to the size of the electrochemical gradient in different cells. Transporters in the HKT family display different ion selectivity and transport mechanisms. Wheat, rice, and the gum tree HKT transporters have been shown to mediate both K^+ and Na^+ currents when expressed in *Xenopus* oocytes or in *E. coli* (Rubio *et al.*, 1995; Gassman *et al.*, 1996; Uozumi *et al.*, 2000; Fairbairn *et al.*, 2000; Liu *et al.*, 2001; Gollmack *et al.*, 2002). *EcHKT1* and *EcHKT2*, the two orthologous members from *Eucalyptus*, differ in Na^+ permeability relative to their K^+ permeability and are sensitive to external osmotic changes. Genetic selection in yeast allowed identification of a few of amino acids in the wheat HKT1 that are important for Na^+ binding and transporter function, at least in yeast and oocytes (Rubio *et al.*, 1995, 1999; Diatloff *et al.*, 1998). A clearer role for the HKT transporters has been obtained from knockout mutants and anti-sense plants (Rus *et al.*, 2001; Mäser *et al.*, 2002b). Screening a T-DNA tagged population of *Arabidopsis* mutants (Col-0 *gll sos3-1*), Rus *et al.* (2001) were able to identify a T-DNA insertion in AtHKT1 that suppressed the Na^+ hypersensitivity of the *sos3-1* mutants, thus confirming the role of AtHKT as a Na^+ transporter (Uozumi *et al.*, 2000). An HKT1 deletion demonstrated lower root Na^+ content that increased short-term salt tolerance of mutant plants compared to wild type but the *athkt1* knockout resulted in higher shoot amounts rendering the plant Na^+ -hypersensitive in the long term (Mäser *et al.*, 2002b). Similar results have recently been reported by Berthomieu *et al.* (2003). Also, Laurie *et al.* (2002) produced antisense HKT1 wheat plants that grew better than the controls under high salinity, and showed lower Na^+ influx into the roots, indicating that wheat HKT functions as a Na^+ transporter rather than a high-affinity K^+ transporter.

Unlike the HAK transporters, HKT-type proteins consist of a small family. The HKT-type proteins may not play major roles in K^+ acquisition because no

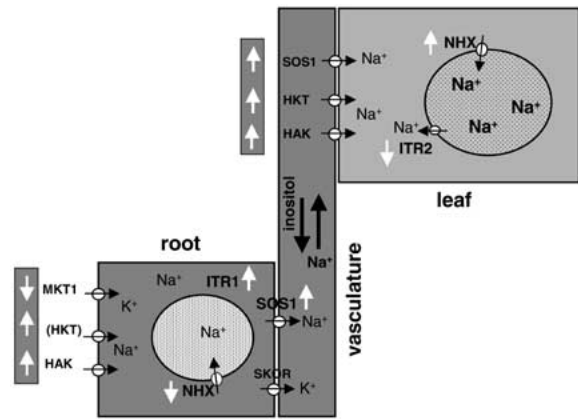


Figure 7. Schematic presentation of ion uptake in salt stressed *M. crystallinum*. Black arrows indicate the direction of ion transport; white arrows indicate regulation of the transporter gene expressions under salt stress. The scheme summarizes data presented here and in previous studies (Vera-Estrella *et al.*, 1999, 2000; Chauhan *et al.*, 2000; Kirch *et al.*, 2000; Su, 2001; Su *et al.*, 2001, 2002; Yokoi *et al.*, 2002; Barkla *et al.*, 2002; see Table 1 and Discussion).

direct evidence for Na^+ -coupled K^+ uptake has been found in roots of terrestrial plants (Maathuis *et al.*, 1996) or for the particular cases of McHKT1 (Figure 2A) and AtHKT1 (Uozumi *et al.*, 2000). Instead, they may be important for ion homeostasis under abnormal growth conditions (Adams *et al.*, 1992). In the ice plant several alkali ion transport systems have been characterized under similar conditions during the initial phase of salt stress (Table 1). Measurements of the ion content in *Mesembryanthemum* and the regulation of potassium channel and transporter transcripts, including the transient up-regulation of *McHKT1*, suggest that the salt stress-affected K^+ uptake function of the MKT1 channel in the roots becomes replaced by other K^+ transport systems, including the McHAK transporters. An indication of this may be seen in the dramatic up-regulation of *McHAK3* transcript at 12 h, and by the continuous increase of *McHAK1* and *McHAK2* for up to 48 h after stress (Su *et al.*, 2002). Should McHKT1 mediate sodium uptake throughout the plant, it may be that the short-term increased expression provides a signal for the long-term stress adaptation for this halophyte and/or that the increase of sodium serves osmotic purposes as it increases the osmotic potential in the plant (Figure 7, Table 1). The McHKT1 protein expressed in epidermal bladder cells (EBC) may be an important conduit for sodium uptake and storage that allows the ice plant to grow based on the osmotic potential that is provided by the increase of sodium in distal plant parts (Adams

Table 1. Expression of ion transporters under salinity stress in ice plant.

Name	Direction of change in expression after NaCl	Time course of induction	Localization	Reference
MKT1	down	rapid	root	Su <i>et al.</i> , 2001
MKT2	down	slow	leaf	Su <i>et al.</i> , 2001
KMT1	up	rapid, transient	leaf	Su <i>et al.</i> , 2001
McHAK1	up	rapid, transient	root	Su <i>et al.</i> , 2002
	up	slow	stem, leaf	
McHAK2	similar to McHAK1		weakly expressed	Su <i>et al.</i> , 2002
McHAK3	up	slow	primarily in root	Su <i>et al.</i> , 2002
McHAK4	no significant change	N/A	all tissues	Su <i>et al.</i> , 2002
McHKT1	up	rapid, transient	leaf	this study
ITR1	up	rapid	root	Chauhan <i>et al.</i> , 2000
ITR2	down	rapid	leaf	Chauhan <i>et al.</i> , 2000
NHX	up (in seedling)	N/A	stem; slightly in root	Chauhan <i>et al.</i> , 2000
	down (in seedling)	N/A	leaf	
SOS1	up	rapid	root, leaf	Chauhan <i>et al.</i> , 2000

et al., 1992). In synchrony with the tonoplast NHX-like Na^+/H^+ antiporters (Apse *et al.*, 1999; Gaxiola *et al.*, 1999; Barkla *et al.*, 2002; Yokoi *et al.*, 2002), McHKT1 might contribute to the path for Na^+ into the vacuole. The signal of McHKT1 protein in stressed EBCs is stronger than in the unstressed leaf (Figure 6C and D), consistent with such a role in Na^+ uptake. *McNhx* transcripts in ice plants also increase after salt stress, most drastically in stem tissue (Chauhan *et al.*, 2000), and an increase in Na^+/H^+ antiport activity, particularly in the epidermal bladder cells has been reported (Barkla *et al.*, 2002). Also, McHKT1 transcripts respond like those of *SOS1* in ice plants during the initial 6–12 h after stress (Chauhan *et al.*, 2000). *Arabidopsis* *SOS1* protein, a plasma membrane Na^+/H^+ antiporter, is primarily expressed in cells surrounding the xylem (Shi *et al.*, 2000, 2002). *SOS1* may be involved in xylem loading of Na^+ or K^+ for long-distance translocation, and its function is probably significantly related to Na^+ accumulation in leaves (Serrano and Rodriguez-Navarro, 2001). The expression patterns of the *SOS1*, *HKT1* and *NHX* transporters in stressed ice plants are consistent with a role in xylem loading, uptake and compartmentalization of Na^+ , which constitute a survival strategy for this halophyte.

A generally accepted notion is that high external Na^+ triggers the switch of K^+ uptake from systems less selective for K^+ over Na^+ to those more selective (Niu *et al.*, 1995), but the assumption may not

describe halophytic mechanisms. In glycophytes such as *Arabidopsis*, Na^+ is primarily excluded, and this limitation of Na^+ uptake at the root level is an efficient strategy for salt tolerance as long as the osmotic potential can be adjusted such that water influx continues. A switch in the K^+ -uptake machinery improves K^+/Na^+ selectivity and reduces the translocation of Na^+ to shoots. In halophytes such as *M. crystallinum*, however, Na^+ is accumulated in shoot tissues and sequestered in vacuoles, where it functions as an osmoticum, which stimulates or maintains cell growth (Adams *et al.*, 1998). AKT-type IRCs are highly selective for K^+ over Na^+ (Schachtman *et al.*, 1992; Amtmann and Sanders, 1999; Blumwald *et al.*, 2000), while the HAK/KUP- and HKT-type transporters mediate low-affinity Na^+ uptake (Figure 2A) (Rubio *et al.*, 1995; Santa-Maria *et al.*, 1997; Fu and Luan, 1998; Kim *et al.*, 1998). If the MKT1 channel is as selective as other IRCs, a switch from the channel to McHKT1, and possibly McHAK, in the ice plant would facilitate short-term Na^+ accumulation and trigger long-term responses. The short-term facilitated uptake of sodium would induce synthesis of methylated inositols, which would accumulate in the cytosol and balance the osmotic potential of the vacuole (Nelson *et al.*, 1998). Turgor would be restored after less than 2 days when osmolyte synthesis and other adaptive mechanisms were established (Adams *et al.*, 1998).

The McHKT1 transcripts are reduced rapidly in leaf after a short-term increase (Figure 4). The expression patterns of other ice plant channels and transporters under salt stress are summarized in Table 1. We speculate that other pathways, such as non-selective cation channels, function to fulfill Na^+ entry after that period of time. Another possibility is that McHKT1 proteins are still active although their transcription is reduced. In fact, in comparison with unstressed tissue, the fluorescence signal from McHKT1 was slightly stronger in the EBC (Figure 6D vs. 6C), which function as Na^+ storage organs in the ice plant. Taken together, the analysis of transcript behavior, protein location, protein cellular distribution, and stability and persistence of the protein argue for a function of the HKT1 cation transporter in short- and long-term sodium accumulation.

A model proposing how ion homeostasis is achieved in the ice plant under salt stress is outlined in Figure 7. In the plasma membrane of root epidermal cells, down-regulation of MKT1 and up-regulation of the HKT and HAK facilitate Na^+ uptake from the soil. Down-regulation of tonoplast NHX and up-regulation of ITR1, a Na^+ /inositol symporter, removes extra Na^+ from the vacuole of root cells (Chauhan *et al.*, 2000). Together with the up-regulated SOS1, HKT and HAK function to translocate Na^+ from root to shoot tissues (Chauhan *et al.*, 2000; Nelson *et al.*, 1998; Shi *et al.*, 2000, 2002; Su *et al.*, 2001, 2002) in the halophyte. In leaves, up-regulation of SOS1, HKT and HAK in the plasma membrane and of the NHX antiporter in the tonoplast facilitate uptake and accumulation of Na^+ into the vacuole of leaf cells (including the epidermal bladder cells in the ice plant) (Adams *et al.*, 1992; Barkla *et al.*, 2002; Yokoi *et al.*, 2002). ITR2, a leaf-specific Na^+ /inositol symporter expressed in the tonoplast, is down-regulated thus reducing Na^+ leakage from vacuoles (Chauhan *et al.*, 2000). Coordinate regulation of this multiplicity of channels, transporters, symporters and antiporters, and possibly including other not yet analyzed transporters, results in irreversible transport from root to shoot, accumulation in leaves, and sequestration of Na^+ into the vacuoles of cells in the leaves and shoot in the halophytic *M. crystallinum*.

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Note

A second cDNA, McHKT2, encoding a root-specific isoform, has been isolated and deposited in GeneBank under the accession number AY231175.

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