

Characterization of a HKT-type transporter in rice as a general alkali cation transporter

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Summary

We report the characterization of rice *OsHKT1* (*Oryza sativa* ssp. *indica*) homologous to the wheat K⁺/Na⁺-symporter HKT1. Expression of *OsHKT1* in the yeast strain CY162 defective in K⁺-uptake restored growth at mM and μM concentrations of K⁺ and mediated hypersensitivity to Na⁺. When expressed in *Xenopus* oocytes, rice *OsHKT1* showed uptake characteristics of a Na⁺-transporter but mediated transport of other alkali cations as well. *OsHKT1* expression was analysed in salt-tolerant rice Pokkali and salt-sensitive IR29 in response to external cation concentrations. *OsHKT1* is expressed in roots and leaves. Exposure to Na⁺, Rb⁺, Li⁺, and Cs⁺ reduced *OsHKT1* transcript amounts in both varieties and, in some cases, incompletely spliced transcripts were observed. By *in situ* hybridizations the expression of *OsHKT1* was localized to the root epidermis and the vascular tissue inside the endodermis. In leaves, *OsHKT1* showed strongest signals in cells surrounding the vasculature. The repression of *OsHKT1* in the two rice varieties during salt stress was different in various cell types with main differences in the root vascular tissue. The data suggest control over HKT expression as a factor that may distinguish salt stress-sensitive and stress-tolerant lines. Differences in transcript expression in space and time in different lines of the same species appear to be a component of ion homeostasis correlated with salt sensitivity and tolerance.

Keywords: HKT1, Rice, Salinity tolerance.

Introduction

Physiological evidence for different K⁺ uptake systems in higher plant roots has been presented long ago, based on the kinetics of K⁺-influx which showed both high- and low-affinity components (Epstein *et al.*, 1963). More recently, these K⁺ uptake systems have become amenable to molecular analysis by the isolation and functional characterization of cDNAs for transmembrane proteins involved in plant K⁺ homeostasis (Anderson *et al.*, 1992; Cao *et al.*, 1995; Fu and Luan, 1998; Kim *et al.*, 1998; Maathuis *et al.*, 1997; Müller-Röber *et al.*, 1995; Santa-Maria *et al.*, 1997; Schachtman and Schroeder, 1994; Schachtman *et al.*, 1992;

Sentenac *et al.*, 1992; Su *et al.*, 2001; Zimmermann *et al.*, 1998). The existence of several types of K⁺-transporting membrane proteins has been documented, among them AKT/KAT-type channels, HKT-type transporters, and HAK/AT/KUP-like transporters (Maser *et al.*, 2001).

Based on their kinetic behaviour, the AKT/KAT family of inwardly rectifying K⁺ channels seems to represent a low-affinity component of the uptake machinery although this assignment has recently been questioned (Hirsch *et al.*, 1998; Spalding *et al.*, 1999). Expression of the root-specific K⁺ channel AKT1 was reported for both μM and mM

external K^+ concentrations (Lagarde *et al.*, 1996). Also, growth of an *Arabidopsis* AKT1-mutant was reduced at μM K^+ concentrations indicating that AKT-type K^+ channels are not only involved in low- but also in high-affinity root K^+ uptake (Spalding *et al.*, 1999). The selectivity of these channels for K^+ over other alkali metal ions is variable, but most are characterized by efficient Na^+ exclusion and their contribution to sodium uptake seems to be minimal (Amtmann and Sanders, 1999; Blumwald *et al.*, 2000; Maathuis *et al.*, 1997).

Another family of K^+ transporters is represented by the HAK/AT/KUP-type proteins with homology to bacterial KUP and HAK1 from *Schwanniomyces occidentalis* (Banelos *et al.*, 1995). Homologues have been isolated from barley, ice plant and *Arabidopsis thaliana* (Fu and Luan, 1998; Kim *et al.*, 1998; Santa-Maria *et al.*, 1997; Su, 2001). These transporters show low- as well as high-affinity K^+ uptake characteristics when expressed in yeast (Quintero and Blatt, 1997).

A member of a presumably high-affinity K^+ -uptake system has been described in the wheat HKT1 protein (Rubio *et al.*, 1995; Schachtman and Schroeder, 1994). It could also be a major route for the entry of Na^+ into roots, according to its specificity as a K^+/Na^+ symporter (Rubio *et al.*, 1995). Because many conclusions about HKT1 derive from functional analysis in yeast and *Xenopus* oocytes, HKT's action in cells of the root may be different, may be restricted to specific cells, or its activity may be masked or counteracted by the presence of other transport systems (Maathuis and Sanders, 1995, 1997; Maathuis *et al.*, 1997; Tanner and Caspari, 1996). The presence of wheat HKT1 leads to Na^+ uptake in high- Na^+ media and, if expressed and active in roots, could significantly increase Na^+ toxicity. Thus, a characterization of HKT-type transcripts and proteins in additional models is appropriate.

With the characterization of three K^+ -uptake systems, views about ion uptake are changing. Several protein subfamilies of the AKT and KUP families exist, each with different isoforms, and seemingly with gradual transitions in their kinetic and biochemical characteristics. In addition, ion specificity varies not only between the members of a family in one species, but also for orthologous family members among different species.

Our interest in K^+ uptake systems is based on the goal of understanding the mechanisms that confer salinity tolerance to land plants. We have concentrated on the rice homologue of wheat HKT1 because of the large dataset available on rice salinity stress responses based on physiological experiments (Flowers and Yeo, 1995; Garcia *et al.*, 1995; Yeo *et al.*, 1990). Also, a number of varieties and breeding lines are available that are characterized with respect to K^+ uptake and how salinity stress tolerance is acquired. We decided to utilize the Na^+ uptake characteristics of two rice cultivars, the salt-tolerant line

Pokkali and the salt-sensitive line IR29, for a comparative analysis of the function of rice HKT1. We have characterized a transcript, *OsHKT1*, encoding a plasma membrane protein with homology to the wheat high-affinity K^+ transporter HKT1. As we will show, Pokkali and IR29 exhibit significant differences in their ability to take up or exclude Na^+ . Expression of *OsHKT1* in both lines is affected by the presence of high concentrations of alkali ions, which repress the HKT transcript. However, repression was less pronounced in the root and leaf vascular tissues of the salt-sensitive IR29 compared to amounts present in the salt tolerant Pokkali. A similar conclusion has recently been drawn by Horie *et al.* (2001) in a comparison between lines Nipponbare and Pokkali. Taking their analyses and our results, that include different characterizations, it seems that *HKT1* gene expression characteristics in the two varieties are significantly correlated with ion homeostasis.

Results

Rice varieties with different K^+ -uptake characteristics

K^+ and Na^+ accumulation was analysed in two lines of indica rice (*Oryza sativa*): IR29 (salt-sensitive) and Pokkali (salt-tolerant), adapted to either 4 mM K^+ , 0.1 mM K^+ , or medium without K^+ added (referred to as 0 mM K^+ in the following) that contained 10–16 μM K^+ from impurities in other salts (Figure 1). Under control conditions, leaf Na^+ and K^+ concentrations between the varieties were not significantly different when the plants were grown at 0, 0.1, and 4 mM of K^+ , respectively. Pokkali and IR29 showed, however, different K^+ and Na^+ uptake characteristics when the plants were salt-stressed at different external K^+ concentrations. Both lines accumulated Na^+ very efficiently in leaves but not roots when salt-stressed. When adapted to 4 mM and 0.1 mM external K^+ , IR29 plants accumulated more than 1000 $\mu mol g^{-1}$ FW Na^+ in leaves following 72 h of salt treatment whereas Pokkali plants excluded Na^+ and had a leaf Na^+ contents of about 30% compared to IR29. In the absence of K^+ in the medium Pokkali accumulated less Na^+ than IR29 during short-term treatment of 24 h NaCl. After 72 h of salt treatment, however, the leaf Na^+ contents were similar in both rice lines with approximately 700 $\mu mol g^{-1}$ FW. Na^+ uptake was generally less in the salt tolerant rice line Pokkali, an effect that was dependent on the presence of external K^+ in the nutrition medium.

A rice HKT1-homologue

Based on the nucleotide sequence of a partial rice cDNA clone (Cornell Collection RZ405) homologous to the wheat high-affinity K^+ transport protein HKT1 (Accession No.

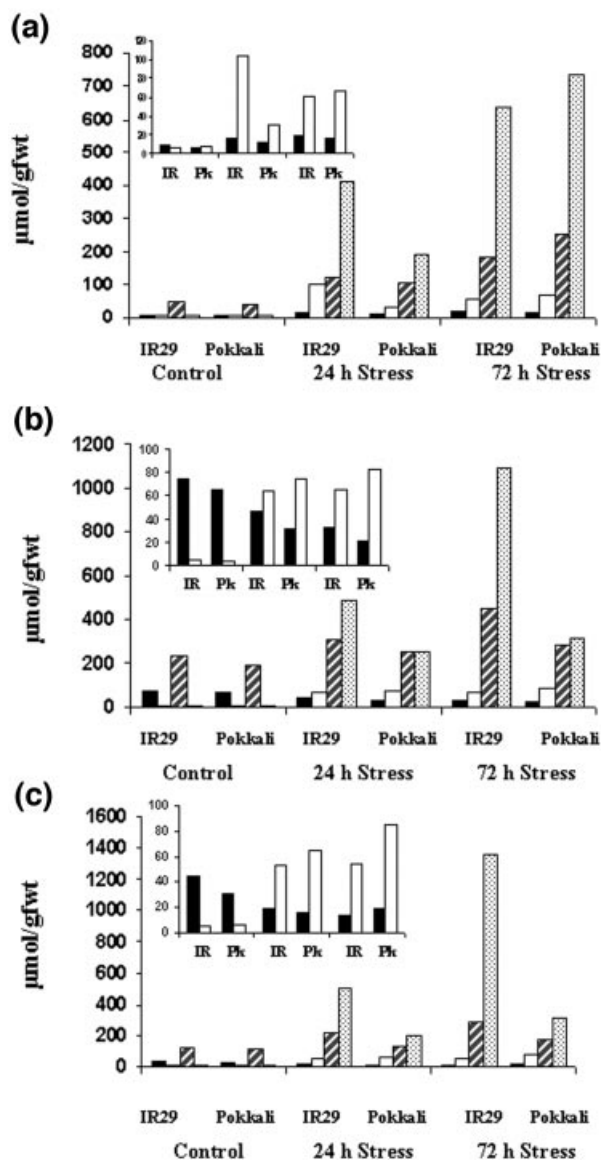


Figure 1. K^+ and Na^+ uptake in different varieties of rice. Plants were grown in hydroponic tanks for 3 weeks and were then stressed by the addition of 150 mM NaCl for 24 and 72 h. Plants were grown without (a), 100 μ M (b), 4 mM K^+ (c). Filled bars, K^+ in roots; open bars, Na^+ in roots; striped bars, K^+ in leaves; stippled bars, Na^+ in leaves. The second oldest leaf was used in all cases with standard deviation ($n = 3$). Media were controlled for the amount of ions by HPLC. The medium to which no K^+ was added contained according to HPLC analyses (Adams *et al.*, 1992) between 10 and 16 μ M K^+ from impurities in the other ions used. Inserts show amounts of potassium (filled bars) and sodium (open bars) in the roots drawn to a different scale.

U16709), we used RT-PCR amplification, cDNA library screening and 5'RACE extension to obtain a full-length transcript of the rice homologue. The cDNA sequence of *OshKT1* (AF313388) isolated from the rice line IR36 is 1827 nucleotides in length. The 5'UTR of *OshKT1* is 60 nucleotides in length and contains a consensus sequence for eukaryotic translation start sites (Kozak, 1984). The

3'-end is 173 nucleotides in length and includes several putative polyadenylation sites. *OshKT1* includes an open reading frame encoding a protein of 530 amino acids for which a location in the plasma membrane is predicted (<http://psort.ims.u-tokyo.ac.jp>). The theoretical pI of *OshKT1* is 9.43 and the molecular weight is 59.3 kDa (<http://www.expasy.ch>). An analysis of the reading frame predicted a protein characterized by 12 hydrophobic domains, which fits the four-MPM model proposed by Durell *et al.* (1999). Kato *et al.* (2001) have convincingly shown this membrane topology for *AtHKT1*. A comparison with the wheat *HKT1* deduced protein sequence indicated co-linearity of the two proteins with high sequence identity in the hydrophobic regions. The deduced amino acid sequence of rice *OshKT1* is 65% identical to that of wheat *HKT1* (Schachtman and Schroeder, 1994), with an overall similarity of 77%. *OshKT1* and the *Arabidopsis* homologue *AtHKT1* (Uozumi *et al.*, 2000) show 39% sequence identity and 56% sequence homology. Recently, DNA sequences of *OshKT1* have been obtained from japonica rice Nipponbare and indica line Pokkali (Horie *et al.*, 2001). The deduced amino acid sequences of both are identical to *OshKT1* isolated from line IR36 reported here. Between Nipponbare and the two indica lines four nucleotides are different in the coding region and the 5' non-coding region of Nipponbare shows a four-nucleotide deletion. All rice *HKT* sequenced so far contains two short introns.

Southern-type hybridizations (Figure 2) indicated three different DNA fragments with sequence homology to the cDNA of the isolated *OshKT1* in the rice Pokkali and IR29 genomes. Identical hybridization patterns were obtained from the genomes of both rice cultivars. Based on a genome size of 560 Mb for rice, a copy number reconstitution also indicated a signal strength equivalent to at least two copies. Also, restriction digests with several enzymes, the sites of which are not present in the sequenced cDNA, highlighted gene-size fragments whose intensity indicated at least two *HKT1*-homologous sequences in the rice genome. In fact, two different cDNAs with 91% identity to each other have been isolated from line Pokkali (Horie *et al.*, 2001).

Rice HKT1 complements yeast mutants defective in K^+ uptake

Yeast complementation was used to monitor the potential function of *OshKT1* in K^+ transport (Figure 3). A mutant, CY162, which is deficient in *TRK1* and *TRK2* (Ko and Gaber, 1991) grows at 100 mM KCl. This strain, as well as a strain transformed with vector only, is unable to grow on 10 mM KCl. When the *OshKT1* coding region under control of the inducible *Gal1*-promoter was transformed into CY162, cells were unable to grow at low K^+ in the absence of galactose (Figure 3a). Induction of the promoter by

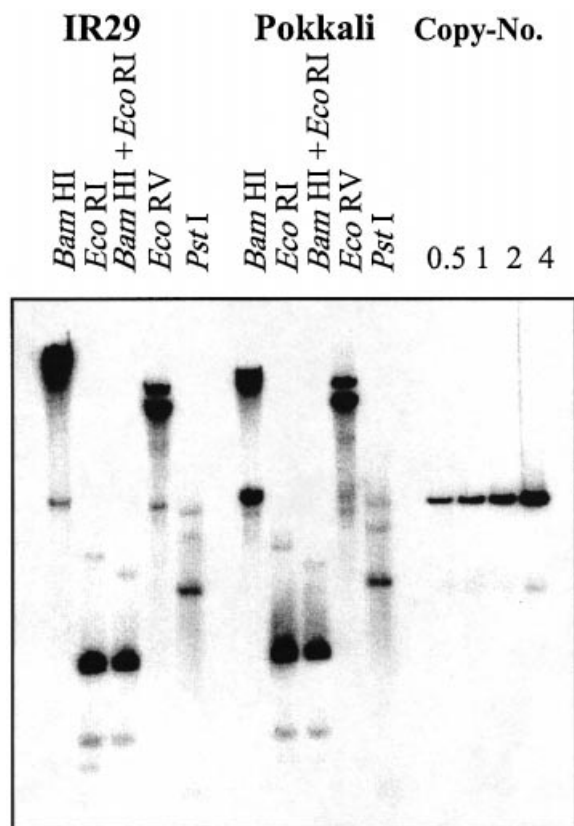


Figure 2. Southern-type hybridizations of rice *OshKT1* to rice genomic DNA.

Total rice DNA restricted as indicated was hybridized with a probe including the 3' region of the coding region and 3'UTR under high stringency conditions of washing. A difference in the *Bam*HI digestions of the IR29 and Pokkali DNAs is due to incomplete digestion of the IR29 DNA. A copy number reconstitution based on a rice genome size of 560 Mb was used. The results indicated the presence of more than 2 copies of *OshKT1*-related sequences.

galactose (arginine/phosphate-based medium; Rodriguez-Navarro and Ramos, 1984) allowed growth in as low as 300 μ M KCl (Figure 3a). Addition of 150 mM NaCl inhibited growth on plates and in liquid culture at 0.3 mM KCl (Figure 3b). CY162 transformed with the empty pYES2 vector did not grow in the presence of galactose on agar plates and in liquid culture when KCl in concentrations of 7 mM or less was present (not shown).

Electrophysiological characterization of *OshKT1*

The electrophysiological characterization of *OshKT1* was performed following methods established for wheat HKT (Gassmann *et al.*, 1996; Rubio *et al.*, 1995). Figure 4a shows that perfusion of the solutions containing 1.0 mM Na^+ , 0.3 mM K^+ plus 1.0 mM Na^+ or 0.3 mM K^+ over an oocyte injected with water and held at the resting potential of -40 mV, did not cause any appreciable changes in the current levels. In contrast, perfusion of the same solutions

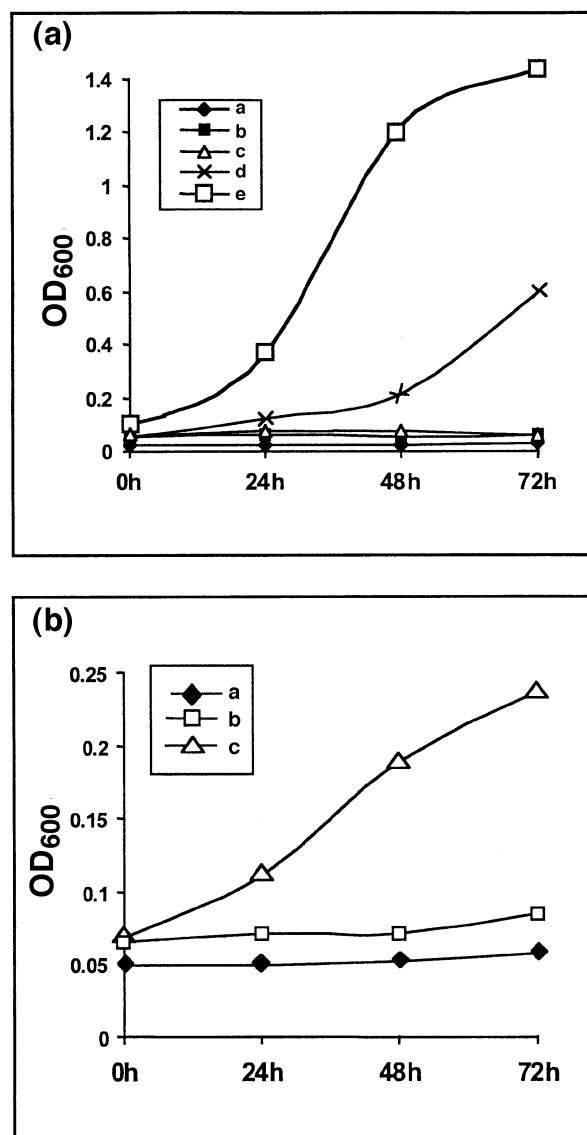


Figure 3. Rice *OshKT1* complements a yeast TRK1/TRK2 mutant.

The *OshKT1* sequence cloned into a yeast expression vector (pYES2) was transferred into yeast strain CY162 (Ko and Gaber, 1991). Growth of CY162 required 100 mM KCl.

(a) Growth rate at different K^+ concentrations. (a) CY162, glucose-AAP medium, 7 mM K^+ (b) CY162::*OshKT1*, glucose-AAP, 7 mM K^+ (c) CY162::*OshKT1*, galactose-AAP, 0.1 mM K^+ (d) CY162::*OshKT1*, galactose-AAP, 1 mM K^+ (e) CY162::*OshKT1*, galactose-AAP, 7 mM K^+ .

(b) Effect of salt stress. (a) CY162::*OshKT1*, glucose-AAP medium, 0.3 mM K^+ (b) CY162::*OshKT1*, galactose-AAP, 0.3 mM K^+ , 150 mM NaCl; (c) CY162::*OshKT1*, galactose-AAP, 0.3 mM K^+ .

over an oocyte injected with *OshKT1* and held at the resting potential of -160 mV induced the activation of inward currents. These currents were larger in the presence of 1.0 mM Na^+ plus 0.3 mM K^+ than in the presence of 0.3 mM K^+ or 1.0 mM Na^+ alone. (Figure 4b). The expression of *OshKT1* caused polarization of the oocytes towards more negative potentials in the absence of Na^+ and K^+ . This is probably due to the release of Na^+ and/or K^+ that

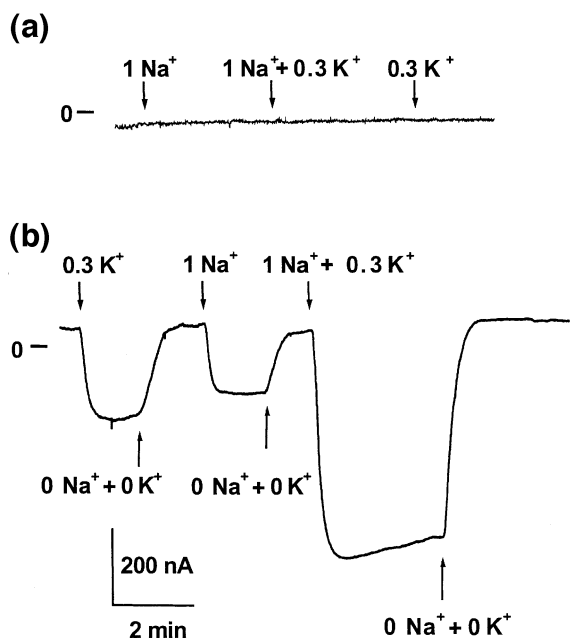


Figure 4. Expression of rice HKT1 in *Xenopus* oocytes induces inward currents in the presence of Na^+ and K^+ .

(a) Exposure of water-injected oocytes to 1 mM Na^+ , 1 mM Na^+ plus 0.3 mM K^+ or 0.3 mM K^+ did not cause any current changes.

(b) Exposure of an oocyte injected with 25 ng of rice HKT1 mRNA to solutions of 1 mM Na^+ or 0.3 mM K^+ caused the development of inward currents; presence of 1 mM Na^+ plus 0.3 mM K^+ caused an stimulation in the magnitude of the inward currents.

has accumulated previously, as reported (Gassmann *et al.*, 1996; Rubio *et al.*, 1995), thus explaining the more negative holding potential for the injected oocyte (Figure 4b). We also characterized the HKT-induced currents by determining the reversal potential under two conditions, maintaining extracellular K^+ constant at 1 mM, while varying Na^+ , and *vice versa*, keeping external Na^+ at 1 mM while changing K^+ (Rubio *et al.*, 1995). With constant Na^+ (1 mM), increases in K^+ positively shifted the reversal potential by 21 mV when K^+ was changed from 1 mM to 10 mM (Figure 5a). In contrast, when K^+ was kept constant at 1 mM and Na^+ varied between 1 mM and 10 mM the reversal potential shifted positively by 15 mV (Figure 5b). The wheat HKT1 co-transporter is highly selective towards K^+ and Na^+ , allowing the movement of these two cations when present together, but it also mediates the movement of Na^+ in the absence of K^+ when Na^+ is found at millimolar levels (Gassmann *et al.*, 1996; Rubio *et al.*, 1995). The *Arabidopsis* homologue, AtHKT1 was shown to function as a Na^+ uptake transporter independent of external K^+ when expressed in *Xenopus* oocytes (Uozumi *et al.*, 2000). The AtHKT1-mediated current was not affected by the external K^+ concentration (Uozumi *et al.*, 2000). Figure 5(c) shows the current-voltage relationships of a rice HKT1-injected oocyte exposed to alkali cations at concentrations

of 100 mM. This figure demonstrates that in the presence of any of these cations, hyperpolarization of the oocyte induced inward currents in all cases, with a permeability sequence of $\text{Rb}^+ > \text{Cs}^+ > \text{Na}^+ > \text{K}^+ > \text{Li}^+$. In contrast, the currents induced by the alkali cations in water injected oocytes were below 50 nA with a permeability sequence $\text{Na}^+ > \text{K}^+ > \text{Li}^+ > \text{Rb}^+ > \text{Cs}^+$ (data not shown). These results are in contrast to reports on wheat HKT1 (Gassmann *et al.*, 1996; Rubio *et al.*, 1995) where inward currents were recorded only in the presence of 100 mM Na^+ (however, see Schachtman and Schroeder, 1994). These results are also different from those reported by Horie *et al.* (2001) for japonica rice, cv. Nipponbare (Ni-*OshKT1*) who reported a permeability sequence of $\text{Na}^+ > \text{Li}^+ > \text{K}^+ \geq \text{Rb}^+ > \text{Cs}^+$.

HKT1 transcript abundance in different rice lines

Expression of *OshKT1* in IR29 and Pokkali depending on K^+ nutrition and salt stress was analysed by RT-PCR amplification with gene-specific oligonucleotide primers that were designed for the coding region and the non-coding region of the gene, respectively (Figure 6). In plants adapted to nutrition medium without K^+ , added expression of *OshKT1* was detected in leaves and roots. Following treatment with 150 mM NaCl for 24 h, transcription of *OshKT1* was reduced in leaves and roots of both rice lines.

To study effects of increasing external K^+ on the expression of *OshKT1*, the plants were adapted to medium containing 4 mM K^+ . By RT-PCR, amplification signals of *OshKT1* were consistently detected in leaf (not shown) and root tissue of both rice lines (Figure 7). Two signals were obtained in transcripts from IR29 roots. Sequencing of the additional (slightly larger) cDNA-fragment revealed this band as a differently spliced *OshKT1* that included an intron of 291 bp inserted between bp 1388 and 1389 of the cDNA (not shown; accession: AF450299, AF313388). The presence of this intron is confirmed by the analysis of the rice genome sequence available of NCBI (HKT located on PAC clone #AP003726). The altered transcript includes two in-frame stop codons indicating that no functional *OshKT1* protein will be translated. By exposure to 150 mM NaCl for 24 h *OshKT1* signals were reduced in roots of both rice lines.

Cell-specific expression of rice HKT1

Cell-specificity of expression of *OshKT1* was studied by *in situ* hybridizations in the rice lines IR29 and Pokkali adapted to 0 mM K^+ . In root tips, IR29 showed signals in the epidermis and exodermis, less in the cortex and strong expression in the vascular cylinder (Figure 8). In the vascular cylinder, signals were of approximately similar strength in endodermis, pericycle, phloem and xylem parenchyma cells. Pokkali showed little expression of

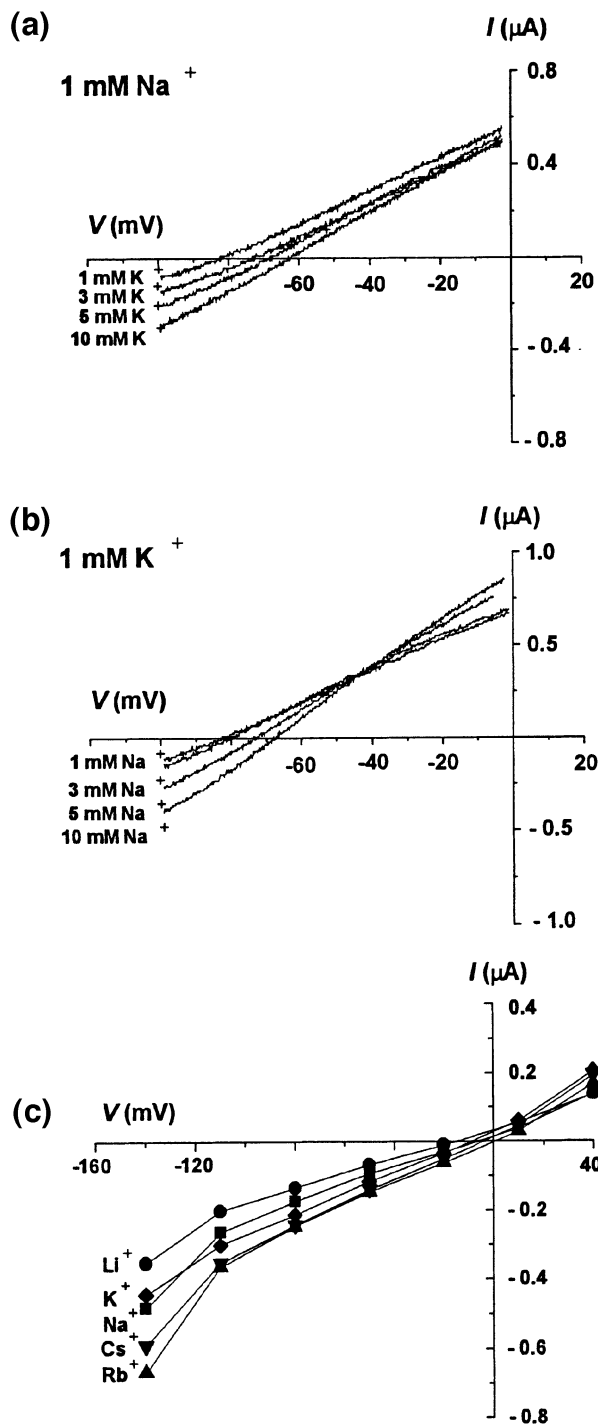


Figure 5. Expression of rice HKT1 in *Xenopus* oocytes mediates alkali cation uptake. (a) In the presence of 1 mM Na⁺, the reversal potential of the HKT1-induced currents changed by 21 mV per decade of extracellular K⁺ concentration. (b) While maintaining extracellular K⁺ at 1 mM, a 10-fold variation in external Na⁺ caused a change in the reversal potential of 15 mV (c) I-V relationships from Os-HKT-injected oocytes exposed to 100 mM solutions of different alkali cations. The results are representative traces. Similar results were obtained with >5 oocytes from three different frogs.

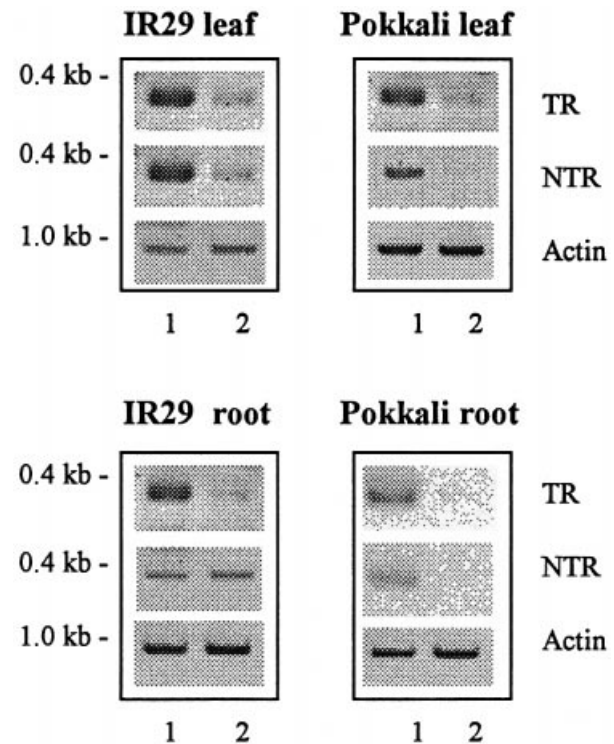


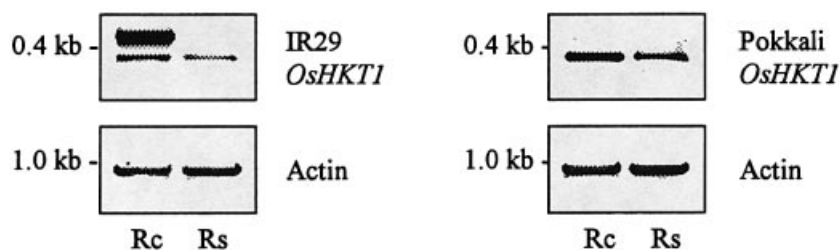
Figure 6. Quantification of rice *OsHKT1* transcript amounts by RT-PCR in plants grown in nutrition medium without K⁺ added. Quantification of *OsHKT1* by reverse transcription PCR. Left hand lanes are controls with RNA from seedlings, right hand lanes contain RNA from plants stressed for 24 h in the presence of 150 mM NaCl. TR, transcribed region of *Hkt1*; NTR, probe including the extreme 3'-end of the coding region and part of the 3'UTR; actin is a control amplification with a rice actin gene probe.

OsHKT1 in the vascular cylinder, but patchy expression in root epidermis, exodermis and adjacent cells of the cortex. In mature roots, expression in IR29 was observed in epidermis, exodermis and the developing sclerenchymatic cells, as well as in the endodermis and the cells inside. Pokkali showed hybridization to the exodermis and weak signals in selected cells neighbouring the xylem vessels.

For *in situ* hybridizations in leaves the region around the midrib of the second youngest leaf from 3-week-old plants was used. Signals were similar in IR29 and Pokkali, with strongest expression in regions between the phloem and xylem and the transition from vasculature to mesophyll cells.

In root tips of both varieties, when adapted to medium without K⁺ added, salt stress of 150 mM NaCl decreased the signal to background levels within 24 h (Figure 9). Salt stress did not abolish *OsHKT1* signals in the mature root as it did in the root tip. In IR29, signals in the epidermis and in part also in the exodermis disappeared or became weaker, but strong signals were still observed with sclerenchymatic cells, which still contain cytoplasm. In the vasculature, the endodermis stained weakly, but signals

Figure 7. Expression of *OshKT1* in rice adapted to 4 mM K^+ . *OshKT1* transcription was monitored by reverse transcription PCR. Rc, root, control. Rs, roots from plants stressed with 150 mM NaCl for 24 h.



persisted in xylem parenchyma cells. *OshKT1* expression in Pokkali was nearly exclusively restricted to the sclerenchymatic cells subtending the exodermis, and to xylem parenchyma cells in the centre of the vascular stele. In leaves, *OshKT1* signals increased in the phloem of IR29 plants under salt stress but not in Pokkali (not shown).

Effects of alkali cations on the expression of *OshKT1*

Plants adapted to 0 mM K^+ were treated with 150 mM each of RbCl, LiCl, and CsCl, for 24 h followed by RT-PCR amplification of *OshKT1*. The *OshKT1* transcript level was reduced by treatment with all three cations in roots (Figure 10) and leaves (not shown) of Pokkali and by Li^+ -treatment alone in roots (Figure 10) and leaves (not shown) of IR29. Rb^+ and Cs^+ induced alternate splicing of *OshKT1* in roots and leaves of IR29 as it was similarly observed in roots of IR29 plants adapted to 4 mM K^+ (compare with Figure 7). Comparison of ion contents showed slightly increased K^+ , Li^+ and Rb^+ amounts in leaves of IR29 treated with 150 mM LiCl and 150 mM RbCl, respectively, for 24 h. In Pokkali, the K^+ content was not changed in comparison to control plants and the amounts of Li^+ and Rb^+ accumulated were lower than the concentrations in IR29 (Figure 11).

Discussion

Rice HKT1

We have isolated and characterized a homologue of wheat HKT1 from the indica rice, IR36. *OshKT1* expression in the salt-sensitive rice line IR29 and the salt-tolerant line Pokkali was compared to begin to understand the possible role of this transporter in the homeostasis of monovalent alkali cations. *OshKT1* shares 65% identity with the wheat homologue, 39% identity with *Arabidopsis* AtHKT1, 42% with *Eucalyptus* EchKT1, and 46.5% with ice plant McHKT1 (Su, 2001). Recently, sequences of *OshKT1* have been isolated from two rice varieties, the japonica rice, Nipponbare, and Pokkali (Horie *et al.*, 2001). Both are identical to the IR36 HKT1 at the amino acid level, and only 4 nucleotide differences exist in the coding region between

HKT1 of the lines Nipponbare, Pokkali and IR29. *OshKT1* appears to be encoded by a single gene in Nipponbare but a second transcript, *OshKT2*, with 91% identity to *OshKT1*, has been isolated from a Pokkali cDNA library (Horie *et al.*, 2001). Our hybridizations indicated the existence of a small gene family of 2–3 HKT-type sequences in both Pokkali and IR29. Possibly, HKT1 copy number distinguishes indica and japonica rice.

Functional analyses of HKT-type transporters have been performed by heterologous expression in yeast and bacterial strains deficient in K^+ -uptake and in *Xenopus* oocytes. The wheat HKT1, for example, is highly selective for K^+ and Na^+ acting as a Na^+ -coupled K^+ uptake transporter. At high millimolar concentrations of Na^+ , low-affinity Na^+ -uptake is mediated and high-affinity K^+ -uptake is blocked (Gassmann *et al.*, 1996; Rubio *et al.*, 1995). For *Arabidopsis* AtHKT1 expressed in *Xenopus* oocytes, large Na^+ currents were observed but no significant uptake of K^+ , Li^+ , Rb^+ , or Cs^+ occurred (Uozumi *et al.*, 2000). Conversely, the *Eucalyptus* homologues EchKT1 and EchKT2 mediated alkali cation uptake with a higher permeability shown towards Na^+ and K^+ when heterologously expressed in *Xenopus* oocytes (Liu *et al.*, 2001). Horie *et al.* (2001) reported *OshKT1* identified from rice Nipponbare and from indica Pokkali to be a Na^+ transporter and *OshKT2* in Pokkali to be a Na^+/K^+ -coupled transporter based on functional analyses in *Xenopus* oocytes and yeast. Interestingly, in the present study we found that the expression of *OshKT1* conferred not only Na^+ hypersensitivity to a yeast mutant deficient in the TRK1/TRK2 K^+ transport systems but also K^+ uptake was restored enabling the mutant to grow on μM and mM K^+ concentrations. In contrast to the experiments on yeast complementation with *OshKT1* (Horie *et al.*, 2001) we used a medium lacking NH_4^+ but containing arginine as the nitrogen source because competition of NH_4^+ with K^+ transport has been reported in yeast (Rodriguez-Navarro and Ramos, 1984). These results indicate that the *OshKT1*-mediated K^+ -transport in yeast is NH_4^+ -sensitive and may be inhibited by the ion. This is in contrast to the insensitivity to external NH_4^+ of wheat HKT1 when the transporter is expressed in yeast (Santa-Maria *et al.*, 2000).

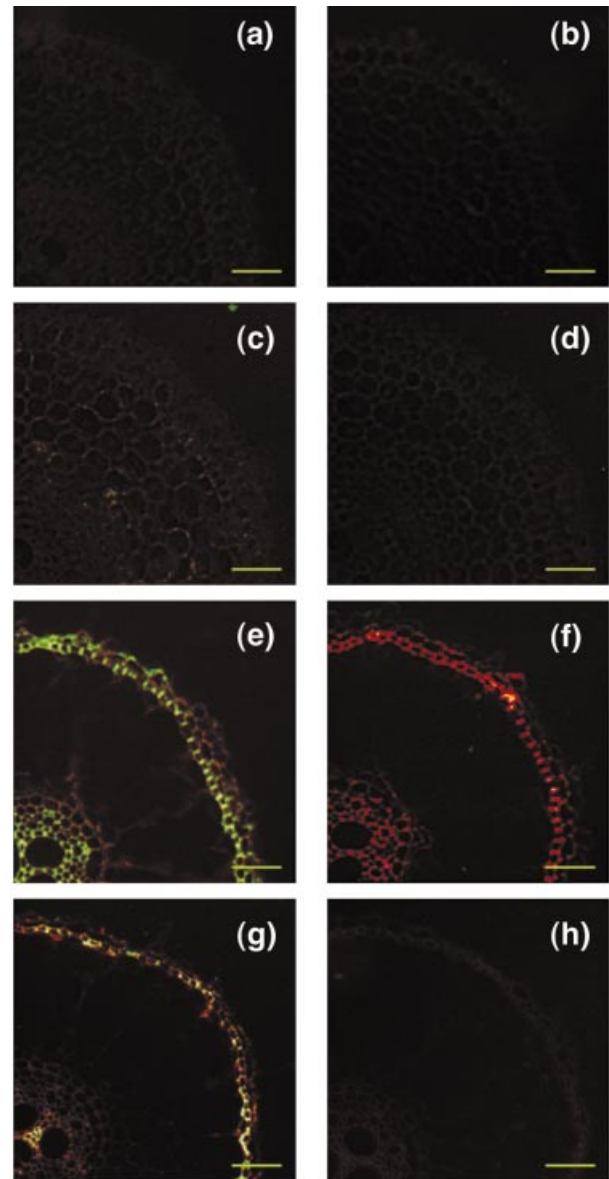
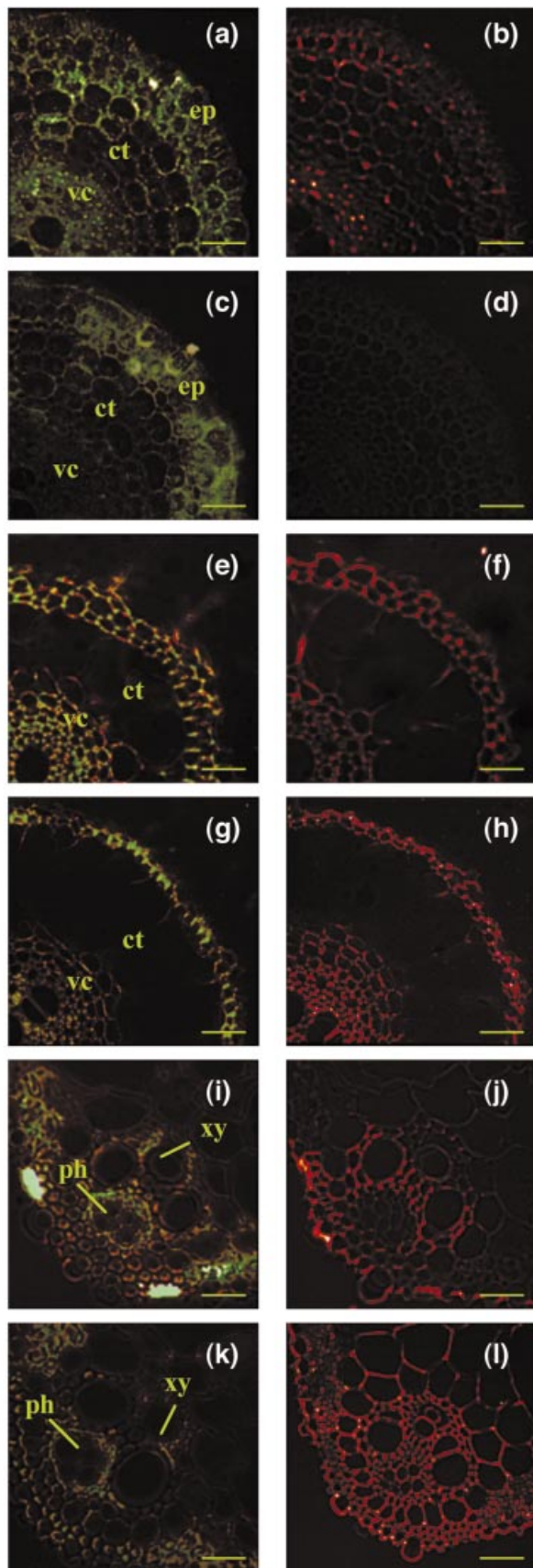


Figure 9. *In situ* hybridization of *OsHKT1* cDNA to tissue sections of salt-stressed rice.

a, c, e, and g antisense; b, d, f, and h sense hybridizations.

(a, b) IR29, root tip, 24 h 150 mM NaCl.

(c, d) Pokkali, root tip, 24 h 150 mM NaCl.

(e, f) IR29, mature root, 24 h 150 mM NaCl.

(g, h) Pokkali, mature root, 24 h 150 mM NaCl.

The rice plants were adapted to nutrition solution without K^+ added. The bars equal 50 μ m.

Figure 8. *In situ* hybridization of *OsHKT1* cDNA to tissue sections.

a, c, e, g, i, and k antisense; b, d, f, h, j, and l sense hybridizations.

(a, b) IR29, root tip.

(c, d) Pokkali, root tip.

(e, f) IR29, mature root.

(g, h) Pokkali, mature root.

(i, j) IR29, leaf.

(k, l) Pokkali, leaf.

The plants were adapted to nutrition solution without K^+ added. The bar in all prints represents 50 μ m. Abbreviations are: ep, epidermis; ct, cortex; vc, vascular cylinder; ph, phloem; xy, xylem.

Figure 10. Effects of Rb⁺, Li⁺, and Cs⁺ on the expression of *OsHKT1*.

Transcript amounts were quantitated by RT-PCR. Rice plants were adapted to nutrition solution without K⁺ added and treated for 24 h with 150 mM Rb⁺, Li⁺, or Cs⁺. Rc, root, control.

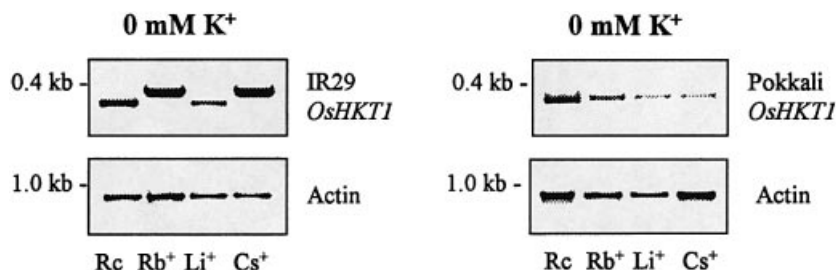
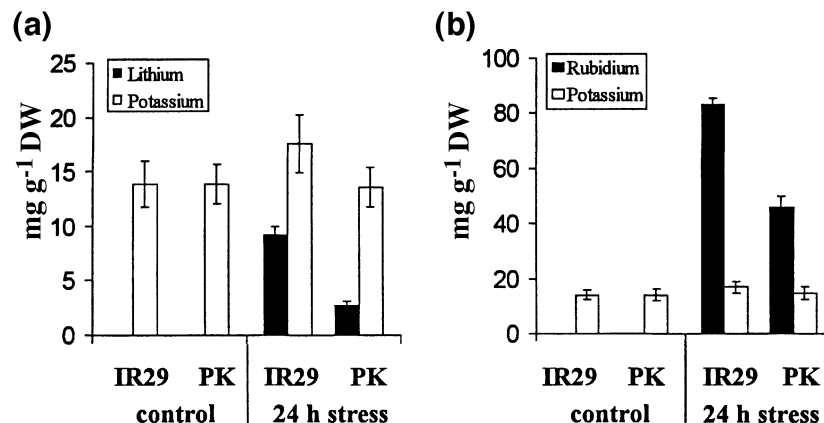


Figure 11. Accumulation of Li⁺ and Rb⁺ in rice.

The plants were grown in nutrition solution without K⁺ added. They were either grown as non-stressed control plants or treated with 150 mM LiCl (a) or with 150 mM RbCl (b) for 24 h.



OsHKT1 transcripts are expressed in specific cells and respond to alkali cations

OsHKT1 was expressed in leaves and roots of IR29 and Pokkali, but *in situ* hybridizations indicated differences in cell-specificity. The expression of *OsHKT1* in the rice root in the two lines is observed in different cell layers. In the tip region, some signal was localized to the epidermis, but strongest expression in both varieties was seen in a layer of cells subtending the epidermis. Cortex cells had fewer signals in contrast to what has been reported for wheat *HKT1* (Schachtman and Schroeder, 1994). A consistent difference between the rice varieties was seen for hybridization to cells of the vascular tissue. In IR29, strong signals to all cells of the developing vascular cylinder, strongest to xylem parenchyma cells, contrasted with less *OsHKT1*-signal in the vasculature in Pokkali. In addition, Pokkali signals generally appeared in patches while IR29 stained more uniformly. A possible interpretation of this cell specificity would be general transport of K⁺ towards the vasculature in the tip but lateral movement of K⁺ in the mature root, consistent with our physiological data on ion uptake into the roots (see also, Schachtman and Schroeder, 1994). In leaves, *OsHKT1* signals were found in cells adjacent to phloem vessels suggesting involvement of these transporters in K⁺-loading of the phloem. Based on kinetic studies in rye, White (1997) suggested

that, depending on the K⁺ concentration in shoot and root phloem, the K⁺ uptake rate into roots is regulated.

After NaCl stress, *OsHKT1* transcription was down-regulated in root-tips of both rice varieties as shown by *in situ* hybridizations. In mature roots the expression was reduced in the epidermis and vascular tissue with weaker transcript levels in Pokkali than in IR29. *OsHKT1* RT-PCR fragments were then used to probe for expression differences under salinity stress distinguishing the two lines. For the coding region of the gene, inhibition of the expression of *OsHKT1* in the roots of both rice lines by salt treatment was shown. Signals from the untranslated gene-specific region of *OsHKT1* indicated that in IR29 the specific expression of *OsHKT1* was not affected whereas in Pokkali the expression of the gene was inhibited. This comparison is valid because the 3' untranslated regions of HKT are sequence-identical in IR29, Pokkali and Nipponbare in those segments that are found in all three genes (Horie *et al.*, 2001).

OsHKT1 transcripts were detected in leaves and roots of IR29 and Pokkali after adaptation to both medium without K⁺ and medium containing millimolar concentrations of K⁺. Horie *et al.* (2001) reported *OsHKT1* transcripts in the varieties Nipponbare and Pokkali to be higher in low potassium. In *Arabidopsis*, transcription levels of *AtHKT1* were not affected by the addition of up to 100 mM K⁺ or 100 mM Na⁺ (Uozumi *et al.*, 2000) whereas in roots of

wheat and barley accumulation of *HKT1* mRNA was stimulated upon K^+ starvation (Wang *et al.*, 1998). In barley this effect was most pronounced in young seedlings whereas at the age of 8- to 9-days *HKT1* expression was only weakly stimulated by K^+ starvation (Wang *et al.*, 1998). It seems possible that reports on differences in the induction of *HKT1* by K^+ deprivation may have their basis in age- or development-specific regulation of these transporters (either amount, location in the membrane system or activity) and not in intrinsic qualities of the encoded proteins.

Effects of alkali cations on function and expression of *OshKT*, respectively, were investigated by functional studies in *Xenopus* oocytes and by monitoring the transcript level of *OshKT1* in rice plants treated with different cations. In *Xenopus* oocytes expressing *OshKT1* inward currents were induced not only by K^+ but all alkali cations tested with a permeability sequence $Rb^+ > Cs^+ > Na^+ > K^+ > Li^+$, suggesting that *OshKT1* functions as a general alkali cation transporter. Permeability of *HKT1* to all the alkali cations has been reported for wheat (Schachtman and Schroeder, 1994) and *Eucalyptus camaldulensis* (*HKT1-2*; Liu *et al.*, 2001), although with a different permeability sequence. We have obtained similar results with the *Mesembryanthemum crystallinum* and *Triticum aestivum* *HKT1* homologues (unpublished results). The permeability sequence we report here for rice *HKT1* contrasts with that of Horie *et al.* (2001) who, using the same clone, showed a greater permeability to Na^+ compared with the other alkali cations. This difference is difficult to reconcile, however, our results were consistently observed in more than five oocytes from each of three different frogs with low SE (Figure 5). Interestingly, Horie *et al.* (2001) proposed that the differences in selectivity between *OshKT1* (a Na^+ transporter) and *OshKT2* (a Na^+/K^+ co-transporter) were due to a single amino acid change (Gly-88 in *OshKT2*, Ser-88 in *OshKT1*). However, this observation is not supported by our results with rice *HKT* and those of Feirbain *et al.* (2000) and Liu *et al.* (2001) for the two *HKT* orthologues from *E. camaldulensis*, which function as Na^+/K^+ co-transporters where the equivalent amino acid corresponds to Ser-95 (*EcHKT1*) and Ser-94 (*EcHKT2*). It may be argued that the currents reported here are smaller than those reported by Rubio *et al.* (1995), Gassman *et al.* (1996) and, in part, Horie *et al.* (2001). However, as a control we have injected the mammalian brain K^+ channel $K_v1.1$ into *Xenopus* oocytes and have recorded currents larger than 10 μA at 30 mV (data not shown), similar to those reported by other researchers for the same channel (Rolf *et al.*, 2000). Also, current levels similar to those reported by us for the rice *HKT* homologue were recorded by Schachtman and Schroeder (1994) for wheat, Horie *et al.* (2001) for rice, and those by Feirbain *et al.* (2000) and Liu *et al.* (2001) for the two *HKT*

orthologues from *E. camaldulensis*. In root and leaf tissue of both rice lines *OshKT1* expression was inhibited by exposure of plants to Na^+ and Li^+ . In Pokkali, Rb^+ and Cs^+ induced down-regulation of the transcripts. In IR29, in the presence of millimolar concentrations of K^+ (representing excess), and in the presence of Cs^+ and Rb^+ , we observed a different RT-PCR fragment based on the presence of an intron. This suggests incomplete splicing as a possible mechanism by which transcripts might be retained in the nucleus or by which translatable transcripts are reduced under stress conditions. *OshKT1* clearly acts as a general alkali cation transporter not only when expressed in *Xenopus* oocytes but also after expression *in planta*.

Salt-dependent regulation of *OshKT1* expression in rice

Molecular mechanisms governing Na^+ uptake into plants are not well understood, but experimental data suggest non-specific influx through K^+ uptake systems (Amtmann and Sanders, 1999; Blumwald *et al.*, 2000; Lagarde *et al.*, 1996; Maathuis and Sanders, 1997; Maathuis *et al.*, 1997; Schroeder *et al.*, 1994). Several protein families could account for inadvertent Na^+ uptake-low-affinity channels, dual- or high-affinity transporters and voltage-independent cation channels (VIC). Another possible uptake vehicle are non-selective cation channels (Schachtman and Liu, 1999; White, 1997) and proteins like the wheat low-affinity cation transporter LCT1 (Schachtman *et al.*, 1997). Many low-affinity channels (AKT/KAT-type) are highly selective for K^+ , but other monovalent cations are not completely excluded (Blumwald *et al.*, 2000; Maathuis *et al.*, 1997; Schachtman *et al.*, 1992). The expression of the homologue of inward-rectifying K^+ channels *OsAKT1* was not modified in the root epidermis and endodermis of IR29 under salt stress conditions but was inhibited in Pokkali (D Gollmack, F Quigley, CB Michalowski, U Kamasani and HJ Bohnert; unpublished). Dual-affinity K^+ -transporters (HAK/AT/KUP-type) have recently been shown to be affected by Na^+ , or they may act as low-affinity Na^+ transporters (Fu and Luan, 1998; Quintero and Blatt, 1997; Santa-Maria *et al.*, 1997; Su, 2001). The K^+ uptake protein represented by *HKT1* from wheat has been shown to function as a K^+/Na^+ -co-transporter. Its action as a low-affinity uptake protein in the presence of 100 mM Na^+ (Rubio *et al.*, 1995) could explain Na^+ accumulation. Our results with rice showed a difference between two rice lines with respect to the expression of *OshKT* in the root vascular tissue whereas expression in the root tip was identical. The difference correlated with salt sensitivity in IR29 and tolerance in Pokkali, indicating a possible involvement of *OshKT1* in uptake and long-distance transport of cations in IR29 that is probably realised in Pokkali to a much lower degree. As a response to alkali-salt stress *OshKT1* transcription was down-regulated in roots

and leaves of Pokkali, whereas that in IR29 was either unchanged or down-regulated to a minor extent (Figures 6 and 8). If OsHKT1 functions as a Na⁺ transporter (Figures 4 and 5), our *in situ* and RT-PCR results (Figures 6,8,9 and 10) suggest that regulation of OsHKT1 by salt stress may be an important mechanism in salt tolerance. This view is supported by the consistent down-regulation of OsHKT1 caused by salt stress in the salt tolerant rice line Pokkali, leading to a lower level of Na⁺ (Li⁺ and Rb⁺) accumulation *in planta*, as compared to the amount taken up by the salt sensitive rice line IR29 (Figures 1 and 11). Further support comes from the recent detection of an *Arabidopsis* mutant in which salinity stress tolerance could be based on down-regulation (knockout) of the *AtHKT1* gene and protein (Rus et al., 2001). Our study suggests different cell-specificity for HKT transcripts and, also, intraspecies specific differences under high salt conditions that distinguish the Na⁺-tolerant line Pokkali from Na⁺-sensitive IR29. Accordingly, HKT-type transporters may contribute to the differences in Na⁺-uptake behaviour of these rice lines in high salinity. Possibly of high significance is that *OsHKT* seems to be involved in xylem loading and long distance transport of alkali cations from roots to shoots and in leaf phloem loading. The OsHKT transporter may thus determine how much Na⁺ is ultimately taken up and transported to leaves in a feedback regulation influencing other K⁺/Na⁺ uptake systems in high salinity. The differences in *OsHKT1* expression between IR29 and Pokkali observed under salt stress conditions may be due to variations in promoter sequence, transcription factors, or in, as yet unknown, signalling events that control expression. A comparative analysis of such a control system in Pokkali and IR29 will be necessary to gauge the relative importance of HKT1 in K⁺ homeostasis or Na⁺ exclusion and uptake in rice.

Experimental procedures

Plant material

Seeds of rice (*Oryza sativa* L. indica) var. IR29 and Pokkali were obtained from IRRI (Los Banos, Philippines), germinated in sand, transferred to aerated hydroponic tanks and grown for 3 weeks in all experiments (12-h light/dark at 28°C/22°C) in controlled environment chambers (Conviron, Winnipeg, Canada). Plants were grown in half-strength Hoagland's nutrient solution with K⁺ concentrations as indicated for the experiments. The addition of chelated iron was doubled. In experiments with reduced K⁺ the NO₃⁻ counter ion was substituted by NH₄⁺. For salt stress experiments, the nutrient solution contained 150 mM NaCl (or LiCl, RbCl or CsCl) for 24 h and 72 h, respectively. Unstressed control plants were grown in parallel and harvested at the same time.

Cloning and sequencing

A partial cDNA clone with homology to wheat *HKT1* was obtained from the Cornell Collection (clone RZ405) and sequenced with an

automated ABI sequencer (model 377, version 2.1.1). Amplification of the 5'-end was done by 5'RACE-PCR (Gibco-BRL, Life Technologies, Inc., Rockville, MD, USA). The full-length cDNA was assembled and cloned in the *Xho*I and *Hind*III restriction sites of pBluescript (Stratagene, La Jolla, CA, USA) and the entire cDNA-fragment was re-sequenced. The sequence of *OsHKT1* was deposited at GenBank (AF313388).

Southern hybridizations

Genomic DNA was extracted from IR29 and Pokkali leaves according to Gustinich *et al.* (1991). DNA blotting and hybridization were performed according to standard procedures (Sambrook *et al.*, 1989). The probe, corresponding to nucleotides #283-1779 of the full-length clone, was labelled with [³²P]dCTP (Amersham-Pharmacia Biotech Inc., Piscataway, NJ, USA) by random priming. After hybridization in 2× SSC, filters were washed with 0.2× SSC at 42°C for 30 min. For an estimation of the copy number in the rice genome (560 Mb) plasmid DNA was loaded on the gels to produce signals equivalent to 0.5–4 copies.

In situ hybridizations

Root and leaf tissues were fixed with FAA, dehydrated and embedded. Root tips and segments approximately 5 cm from the tip were taken. Leaf sections were probed from the mid-section of the second youngest leaf of each plant. For tissue embedding Paraplast Plus (Fisher Scientific, Pittsburgh, PA, USA) was used. Sections 10 μm thick were mounted on poly-L-lysine coated slides. Sense and antisense RNA transcripts were synthesized by T3 and T7 RNA-polymerase from linearized pBluescript harbouring the cDNA (nucleotide #283-1779) using digoxigenin-UTP (Boehringer, Mannheim, Germany) as a label. Transcripts were hydrolyzed by alkaline treatment to an average length of 200 nucleotides. *In situ* hybridizations were done according to Golldack and Dietz (2001) and signals detected by antidigoxigenin alkaline phosphatase-conjugated Fab fragments (Boehringer, Mannheim, Germany) and 5-bromo-4-chloro-3-indolyl-phosphate and Nitroblue tetrazolium as a substrate. Microscopic analyses were carried out using an Axiophot fluorescence microscope (Zeiss, Oberkochen Germany). Photographs were processed through Nikon Scan 2.1 and Adobe Photoshop.

RT-PCR

RNA from root and leaf tissue of rice (IR29 and Pokkali) was isolated by guanidinium thiocyanate extraction according to Chomczynski and Sacchi (1987). cDNA was synthesized from total RNA using Superscript RT II (Gibco-BRL). Equal aliquots of cDNA were used as template for amplification in 50 μl standard PCR reactions. A sequence specific forward primer (5'-GCACTG-TCCAATGGAGAT-3'; position 1192-1209) and a reverse primer (5'-TTCCTTCATCACTCCACC-3', position 1493-1510) were used for the amplification of a fragment of the 3' translated region of the sequence. A partial fragment of the 3' non-translated region was amplified using the same forward primer and the reverse primer 5'-TGCACTTGATGCACTTGC-3' (position 1626-1643). The PCR amplifications were carried out as described before (Golldack and Dietz, 2001). A PCR-fragment with increased size from IR29 plants was cloned into PCR2.1 TOPO (Invitrogen, Carlsbad, CA, USA) and sequenced.

Analysis of ion contents

Root tissue and the second leaf of each plant were collected and ground in liquid nitrogen. After homogenization in ethanol : chloroform : water (12 : 5 : 3) and re-extraction with water, the aqueous phase was used for cation HPLC analysis (IonPac cation exchange; Dionex, Sunnyvale, CA, USA) (Adams *et al.*, 1992). Alternatively, analyses of ion concentrations were performed with an inductively coupled plasma atomic emission spectrometer (ICP-AES, Perkin-Elmer, Boston, MA, USA) according to Brune *et al.* (1995). The results shown are means from 5 plants.

Yeast complementation

The full-length cDNA of *OsHKT1* was cloned in the *Bam*HI and *Xho*I restriction sites of pYES2 (Invitrogen) under control of the inducible Gal1-promoter into *S. cerevisiae* CY162 (Gietz *et al.*, 1992; Ko and Garber, 1991). Initial selections of transformants were done on glucose containing SC-agar plates without uracil supplemented with 100 mM K⁺. Positive transformants were replica-plated on galactose containing arginine/phosphate medium (Rodriguez-Navarro and Ramos, 1984) without uracil containing 7 mM K⁺ and selected a second time on arginine-phosphate top-agar plates without uracil supplemented with galactose and 300 µM K⁺. For growth studies, positive transformants were transferred to arginine/phosphate-based liquid medium of K⁺ concentrations as indicated. Growth experiments, repeated at least three times with different transformants, were carried out in 20 ml aliquots and samples of 0.3 ml were taken to measure OD₆₀₀ (nm). The results shown are taken from one representative experiment. The plasmid was re-extracted from positive transformants and maintained in *E. coli* (d'Enfert *et al.*, 1995). As a control, *S. cerevisiae* CY162 was transformed with the pYES2 vector without any insert.

Preparation of template DNA, in vitro transcription and capping of mRNA

The coding region of the *OsHKT1* gene was cloned into the pGEM-HE vector which contains a T7 RNA polymerase promoter and 5'- and 3'-UTR of the *Xenopus* β-globin gene for enhanced expression (Liman *et al.*, 1992). The plasmid DNA was purified by CsCl gradient ultracentrifugation and linearized with *Nhe*I that cleaves the plasmid downstream of the inserted cDNA. *OsHKT1* RNA was synthesized *in vitro* using the mCAP(TM) mRNA Capping Kit (Stratagene) a procedure, which tends to enhance the translation efficiency of RNA transcripts in *Xenopus* oocytes.

Two-electrode voltage clamping of *Xenopus* oocytes

Oocytes injected with 25 ng of mRNA were used 2–5 days after injection for the recording of currents induced by the expression of HKT1 with the two-electrode voltage clamp technique. Initially, the voltage electrode was introduced into the oocyte. After a stable membrane potential had been reached for several minutes, the current electrode was inserted. 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⁻¹ with D-mannitol. K⁺ and Na⁺ were added as glutamate salts in experiments where only these two cations were studied. In the experiments where the selectivity of HKT1 was assayed, different alkali cations were added as

chloride solutions. Recordings were obtained with a Dagan Oocyte Clamp amplifier (Dagan, Minneapolis, MI, USA); data acquisition and analysis were done with program pCLAMP6 (Axon Instruments, Foster City, CA, USA).

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