Running head: Differential transport selectivities of HKTs in plant cells

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Title: Differential sodium and potassium transport selectivities of the rice OsHKT2;1 and OsHKT2;2 transporters in plant cells

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Footnotes

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ABSTRACT

Na⁺ and K⁺ homeostasis are crucial for plant growth and development. Two HKT transporter/channel classes have been characterized that mediate either Na⁺ transport or Na⁺ and K⁺ transport when expressed in *Xenopus* oocytes and yeast. However, the Na⁺/K⁺ selectivities of the K⁺ permeable HKT transporters have not yet been studied in plant cells. One study expressing 5’ UTR-modified HKT constructs in yeast has questioned the relevance of cation selectivities found in heterologous systems for selectivity predictions in plant cells (Haro et al., 2005). Here we therefore analyze two highly homologous HKT transporters in plant cells, OsHKT2;1 and OsHKT2;2, that show differential K⁺ permeabilities in heterologous systems. Upon stable expression in cultured *Nicotiana tabacum* Bright-Yellow 2 (BY2) cells, OsHKT2;1 mediated Na⁺ uptake, but little Rb⁺ uptake, consistent with earlier studies and new findings presented here in oocytes. In contrast, OsHKT2;2 mediated Na⁺-K⁺ co-transport in plant cells such that extracellular K⁺ stimulates OsHKT2;2-mediated Na⁺ influx and vice versa. Furthermore, at millimolar Na⁺ concentrations OsHKT2;2 mediated Na⁺ influx into plant cells without adding extracellular K⁺. In addition, the presence of external K⁺ and Ca²⁺ down-regulated OsHKT2;1-mediated Na⁺ influx in two plant systems, BY2 cells and intact rice roots, and also in *Xenopus* oocytes. The present study shows that the Na⁺/K⁺ selectivities of these HKT transporters in plant cells coincide closely with the selectivities in oocytes and yeast and furthermore that OsHKT transporter selectivities in plant cells depend on the imposed cationic conditions, supporting the model that HKT transporters are multi-ion pores.

Keywords: HKT channel; Na⁺ transporter; K⁺ transporter; Na⁺-K⁺ co-transport; salt stress
INTRODUCTION

Intracellular Na$^+$ and K$^+$ homeostasis play vital roles in growth and development of higher plants (Clarkson and Hanson, 1980). Low cytosolic Na$^+$ and high K$^+$ to Na$^+$ ratios aid in maintaining an osmotic and biochemical equilibrium in plant cells. Na$^+$ and K$^+$ influx and efflux across membranes require the function of transmembrane Na$^+$ and K$^+$ transporters/channels. Several Na$^+$ permeable transporters have been characterized in plants (Zhu, 2001; Horie and Schroeder, 2004; Apse and Blumwald, 2007). Na$^+$/H$^+$ antiporters mediate sequestration of Na$^+$ into vacuoles under salt stress conditions in plants (Blumwald and Poole, 1985, 1987; Sze et al., 1999). Na$^+$ (cation)/H$^+$ antiporters are encoded by 6 AtNHX genes in Arabidopsis (Apse et al., 1999; Gaxiola et al., 1999; Yokoi et al., 2002; Aharon et al., 2003). A distinct Na$^+$/H$^+$ antiporter, SOS1 (salt overly sensitive 1) mediates Na$^+$/H$^+$ exchange at the plasma membrane and mediates cellular Na$^+$ extrusion (Shi et al., 2000; Zhu, 2001; Shi et al., 2002; Ward et al., 2003). Electrophysiological analyses reveal that voltage-independent channels (VIC), also named non-selective cation channels (NSCC) mediate Na$^+$ influx into roots under high external Na$^+$ concentrations (Amtmann et al., 1997; Tyerman et al., 1997; Buschmann et al., 2000; Davenport and Tester, 2000), however, the underlying genes have remained unknown.

Potassium is the most abundant cation in plants and an essential nutrient for plant growth. The Arabidopsis genome includes 13 genes encoding KUP/HAK/KT transporters (Quintero and Blatt, 1997; Santa-María et al., 1997; Fu and Luan, 1998; Kim et al., 1998), and 17 genes have been identified encoding this family of transporters in rice (Oryza sativa cv. Nipponbare) (Bañuelos et al., 2002). Several KUP/HAK/KT transporters have been characterized as mediating K$^+$ uptake across the plasma membrane of plant cells (Rigas et al., 2001; Bañuelos et al., 2002; Gierth et al., 2005).
Ionic balance, especially the Na⁺/K⁺ ratio, is a key factor of salt tolerance in plants (Niu et al., 1995; Maathuis and Amtmann, 1999; Shabala, 2000; Mäser et al., 2002a; Tester and Davenport, 2003; Horie et al., 2006; Apse and Blumwald, 2007; Chen et al., 2007; Gierth and Mäser, 2007). Salinity stress is a major problem for agricultural productivity of crops worldwide (Greenway and Munns, 1980; Zhu, 2001). The Arabidopsis AtHKT1;1 transporter plays a key role in salt tolerance of plants by mediating Na⁺ exclusion from leaves (Mäser et al., 2002a; Berthomieu et al., 2003; Gong et al., 2004; Sunarpi et al., 2005; Rus et al., 2006; Davenport et al., 2007; Horie et al., 2009). athkt1;1 mutations cause leaf chlorosis and elevated Na⁺ accumulation in leaves under salt stress conditions in Arabidopsis (Mäser et al., 2002a; Berthomieu et al., 2003; Gong et al., 2004; Sunarpi et al., 2005). AtHKT1;1 and its homologue in rice, OsHKT1;5 (SKC1) mediate leaf Na⁺ exclusion by removing Na⁺ from the xylem sap to protect plants from salinity stress (Ren et al., 2005; Sunarpi et al., 2005; Horie et al., 2006; Davenport et al., 2007; Horie et al., 2009).

The land plant HKT gene family is divided into two classes based on their nucleic acid sequences and protein structures (Mäser et al., 2002b; Platten et al., 2006). Class 1 HKT transporters have a serine residue at a selectivity filter position in the first pore loop, which is replaced by a glycine in all but one known class 2 HKT transporter (Horie et al., 2001; Mäser et al., 2002b; Garciadeblás et al., 2003). While the Arabidopsis genome includes only one HKT gene, AtHKT1;1 (Uozumi et al., 2000), seven full-length OsHKT genes were found in the japonica rice cv. Nipponbare genome (Garciadeblás et al., 2003). Members of Class 1 HKT transporters, AtHKT1;1 and SKC1/OsHKT1;5 have a relatively higher Na⁺ to K⁺ selectivity in Xenopus oocytes and yeast than class 2 HKT transporters (Uozumi et al., 2000; Mäser et al., 2002b; Ren et al., 2005). The first identified plant HKT transporter, TaHKT2;1 from wheat is a Class 2 HKT transporter
(Schachtman and Schroeder, 1994). TaHKT2;1 was found to mediate Na\(^+\)-K\(^+\) co-transport and Na\(^+\) influx at high Na\(^+\) concentrations in heterologous expression systems (Rubio et al., 1995; Gassmann et al., 1996; Rubio et al., 1999; Mäser et al., 2002b). Thus, Class 1 HKT transporters have been characterized as Na\(^+\) preferring transporters with a smaller K\(^+\) permeability (Fairbairn et al., 2000; Uozumi et al., 2000; Su et al., 2003; Jabnoune et al., 2009), whereas Class 2 HKT transporters function as Na\(^+\)-K\(^+\) co-transporters or channels (Gassmann et al., 1996; Corratgé et al., 2007). In addition, at millimolar Na\(^+\) concentrations class 2 HKT transporters were found to mediate Na\(^+\) influx, without adding external K\(^+\) in *Xenopus* oocytes and yeast (Rubio et al., 1995; Gassmann et al., 1996; Rubio et al., 1999; Horie et al., 2001). However, the differential cation transport selectivities of the two types of HKT transporters have not yet been analyzed and compared in plant cells.

A study of the barley and wheat class 2 transporters has suggested that the transport properties of HvHKT2;1 and TaHKT2;1 expressed in yeast are variable, depending on the constructs from which the transporter is expressed and have led to questioning of the K\(^+\) transport activity of HKT transporters characterized in *Xenopus* oocytes and yeast (Haro et al., 2005). It was further proposed that the 5\(^\prime\) translation initiation of HKT proteins in yeast at non-conventional (non-ATG) sites affects the transporter selectivities of HKT transporters (Haro et al., 2005), although direct evidence for this has not yet been presented. However, recent research has shown a K\(^+\) permeability of OsHKT2;1 but not of OsHKT1;1 and OsHKT1;3 in *Xenopus* oocytes. These three OsHKT transporters show overlapping and also distinctive expression patterns in rice (Jabnoune et al., 2009).

The report of Haro et al., (2005) has opened a central question addressed in the present study: Are the Na\(^+\)/K\(^+\) transport selectivities of plant HKT transporters
characterized in heterologous systems of physiological relevance in plant cells or do they exhibit strong differences in the cation transport selectivities in these non-plant vs. plant systems? To address this question, we analyzed the Na⁺/K⁺ transport selectivities of the OsHKT2;1 and OsHKT2;2 transporters expressed in cultured tobacco (*Nicotiana tabacum* L.) cv. Bright-Yellow 2 (BY2) cells. OsHKT2;1 and OsHKT2;2 are two highly homologous HKT transporters from *indica* rice cv. Pokkali, sharing 91% amino acid and 93% cDNA sequence identity (Horie et al., 2001). OsHKT2;1 mediates mainly Na⁺ uptake, which correlates with the presence of a serine residue in the first pore loop of OsHKT2;1 (Horie et al., 2001; Mäser et al., 2002b; Garciadeblás et al., 2003; Horie et al., 2007). In contrast, OsHKT2;2 mediates Na⁺-K⁺ co-transport in *Xenopus* oocytes and yeast (Horie et al., 2001). Furthermore, at millimolar Na⁺ concentrations OsHKT2;2 mediates Na⁺ influx in the absence of added K⁺ (Horie et al., 2001). Recent research on *oshkt2;1* loss-of-function mutant alleles has revealed that OsHKT2;1 from *japonica* rice mediates a large Na⁺ influx component into K⁺-starved roots, thus compensating for lack of K⁺ availability (Horie et al., 2007). But the detailed Na⁺/K⁺ selectivities of glycine containing, predicted K⁺ transporting class 2 HKT transporters have not yet been analyzed in plant cells.

Here, we have generated stable OsHKT2;1 and OsHKT2;2-expressing tobacco BY2 cell lines and characterized the cell lines by ion content measurements and tracer influx studies to directly analyze unidirectional fluxes (Epstein et al., 1963). These analyses showed that OsHKT2;1 exhibits Na⁺ uptake activity in plant BY2 cells in the absence of added K⁺, but little K⁺ (Rb⁺) influx activity. In contrast, OsHKT2;2 was found to function as a Na⁺-K⁺ co-transporter/channel in plant BY2 cells, showing K⁺-stimulated Na⁺ influx and Na⁺-stimulated K⁺ (Rb⁺) influx. The differential K⁺ selectivities of the two OsHKT2 transporters were consistently reproduced by voltage clamp experiments using
**RESULTS**

*OsHKT2;1* and *OsHKT2;2* expression in tobacco BY2 cell lines

Po-*OsHKT2;1* and Po-*OsHKT2;2* were identified in *indica* rice cv. Pokkali. These two highly homologous HKT transporters share 93% identical cDNA sequence and 91% identical amino acid sequence (Horie et al., 2001). The OsHKT2;1 and OsHKT2;2 transporters show differential Na⁺/K⁺ selectivities in heterologous systems. However their Na⁺/K⁺ transport selectivities have not yet been studied in plant cells. We cloned and transformed tobacco BY2 cells with the Po-*OsHKT2;1* and Po-*OsHKT2;2* cDNAs driven by the CaMV 35S promoter.
Stable transformants were selected on solid LS medium (Nagata et al., 1981) supplemented with 100 μg ml⁻¹ kanamycin and 250 μg ml⁻¹ carbenicilllin. Stable kanamycin-resistant subcultures of calli were selected by several sequential rounds of subcultures on solid LS plates (Fig. 1A). Each callus was transferred to liquid LS medium to initiate suspension culture BY2 cell lines (Fig. 1B). Real-time PCR was performed to determine the expression levels of OsHKT2;1 and OsHKT2;2 in the selected BY2 cell lines. OsHKT2;1 and OsHKT2;2 were stably expressed in transformed cell lines, but not expressed in vector control cells (Fig. 1C). Two lines, OsHKT2;1#37 and OsHKT2;2#5 were chosen for further analyses.

**OsHKT2;1, but not OsHKT2;2, exhibits Na⁺ uptake activity under low Na⁺ and without added K⁺.**

In order to study Na⁺/K⁺ transport mediated by OsHKT2;1 and OsHKT2;2 in plant cells, measurements of Na⁺ content and ²²NaCl tracer influx experiments were performed using transgenic BY2 cell lines. The Na⁺ content of four-day old BY2 cells expressing OsHKT2;1, OsHKT2;2 or empty vector, were determined by inductively coupled plasma - optical emission spectroscopy (ICP-OES) analyses after exposing each cell line to a 0.1 mM Na⁺-containing solution for 30 min and 60 min. Incubation in a 0.1 mM Na⁺-containing solution led to dramatic increases in the Na⁺ content only in OsHKT2;1-expressing BY2 cells (Fig. 2A). However, neither control nor OsHKT2;2-expressing BY2 cells showed a significant difference in Na⁺ accumulation compared to the initial Na⁺ content at time 0 (Fig. 2A). Time dependent tracer influx analyses at 0.01 mM external Na⁺ showed OsHKT2;1-mediated Na⁺ influx (Fig. 2B). Interestingly, however, no significant Na⁺ transport activity was found in OsHKT2;2-expressing BY2 cells compared to the control cell line (Fig. 2B). Results from time-dependent tracer influx
experiments are consistent with the Na\(^+\) accumulation phenotype of each BY2 cell line (Fig 2A and 2B).

Concentration-dependent short-term unidirectional \(^{22}\)Na\(^+\) influx experiments showed that OsHKT2;1 mediated Na\(^+\) influx in BY2 cells at 0-0.2 mM external Na\(^+\) with no added K\(^+\) (Fig. 2C). In contrast, OsHKT2;2 did not show Na\(^+\) influx activity compared to vector-transformed control cells (Fig. 2C). A kinetic analysis of OsHKT2;1-mediated Na\(^+\) influx in BY2 cells in the concentration range tested in this study showed an apparent Na\(^+\) affinity of \(\approx 0.014\) mM Na\(^+\), and a \(V_{\text{max}}\) of \(\approx 31\) nmol mg of protein\(^{-1}\) min\(^{-1}\) (Table Ia). Note that the apparent affinity of a transporter depends on multiple parameters, including membrane potential, electrical coupling to other cells and intracellular ion concentrations (Schroeder et al., 1994). Taken together, these results suggest that OsHKT2;1 functions as a Na\(^+\) transporter/channel, while OsHKT2;2 does not, in the presence of low external Na\(^+\) and no added extracellular K\(^+\) in plant cells.

**OsHKT2;2 mediates K\(^+\)-stimulated Na\(^+\) uptake into tobacco BY2 cells**

As OsHKT2;2 showed clear Na\(^+\) transport activity in the presence of extracellular K\(^+\) when expressed in *Xenopus* oocytes (Horie et al., 2001), we next characterized Na\(^+\) and K\(^+\) transport properties of each transgenic BY2 cell line in the presence of both Na\(^+\) and K\(^+\) (Rb\(^+\)). ICP-OES analyses were initially performed. Four-day old BY2 cells expressing OsHKT2;1, OsHKT2;2 or empty vector were treated with a 0.1 mM Rb\(^+\) and 0.01 mM Na\(^+\) solution for 30 min and 60 min. Interestingly, Rb\(^+\) stimulated significant Na\(^+\) accumulation in OsHKT2;2-expressing BY2 cells (Fig. 3A), which was not the case when Na\(^+\) was added as a sole alkali cation source in the buffer solution (Fig. 2A).

To more directly analyze K\(^+\) stimulated-Na\(^+\) influx activity of OsHKT2;2, concentration-dependent short-term unidirectional \(^{22}\)Na\(^+\) influx experiments were
performed in the presence or absence of 0.1 mM K⁺. OsHKT2;2-mediated Na⁺ influx into BY2 cells at 0-0.2 mM external Na⁺ and 0.1 mM external K⁺ conditions, but not in the absence of added external K⁺ (Fig. 3B). A kinetic analysis showed an apparent Na⁺ affinity for OsHKT2;2-mediated Na⁺ influx into BY2 cells of ≈0.077 mM, and a $V_{\text{max}}$ of ≈26 nmol mg of protein⁻¹ min⁻¹ when 0.1 mM K⁺ was added (Table Ib). Taken together, these results strongly suggest that OsHKT2;2 mediates K⁺-stimulated Na⁺ uptake into plant BY2 cells. Furthermore, at millimolar Na⁺ concentrations of 1 mM and 10 mM Na⁺, OsHKT2;2 showed Na⁺ accumulation in tobacco BY2 cells without adding extracellular K⁺ (Fig. 3C), consistent with previous findings on class 2 HKT transporters in *Xenopus* oocytes and yeast (Rubio et al., 1995; Horie et al., 2001).

**OsHKT2;2 mediates Na⁺-stimulated K⁺ uptake into tobacco BY2 cells**

Experiments were pursued to investigate whether OsHKT2;1 and OsHKT2;2 have the ability to mediate K⁺ influx. As K⁺ is fairly abundant in plant cells and endogenous K⁺ could mask the effect of the OsHKT2;2 protein-mediated K⁺ accumulation, external K⁺ was replaced with the K⁺ analog Rb⁺ (Epstein et al., 1963; Kochian and Lucas, 1988) in measurements of ion contents to monitor K⁺ transport properties of OsHKT2;1 and OsHKT2;2 in BY2 cells. Four-day old BY2 cells expressing *OsHKT2;1, OsHKT2;2* or empty vector were exposed to a 0.1 mM Rb⁺ and 0.01 mM Na⁺ buffer solution for 30 min and 60 min and the Rb⁺ content was determined by ICP-OES. Only *OsHKT2;2*-expressing BY2 cells exhibited significantly increased Rb⁺ accumulation compared to that of control BY2 cells (Fig. 4A).

More direct time-dependent tracer influx analyses using $^{86}\text{Rb}^+$ showed that OsHKT2;2 mediated Rb⁺ influx, whereas OsHKT2;1 did not compared to control BY2 cells (Fig. 4B), consistent with ICP data in Fig. 4A. Concentration-dependent
short-term unidirectional $^{86}\text{Rb}^+$ influx experiments at 0-0.2 mM external $\text{Rb}^+$ and 0.01 mM $\text{Na}^+$ revealed that only OsHKT2;2 exhibited significant $\text{Rb}^+$ ($K^+$) influx activity showing increases in the influx rate at increasing external $\text{Rb}^+$ concentrations (Fig. 4C). A kinetic analysis of OsHKT2;2-mediated $K^+$ ($\text{Rb}^+$) influx into BY2 cells showed an apparent affinity of $\approx 0.035$ mM $\text{Rb}^+$ and $V_{\text{max}} \approx 19$ nmol mg of protein$^{-1}$ min$^{-1}$ (Table Ic).

OsHKT2;2-mediated $\text{Na}^+$ uptake into BY2 cells was found to be external $K^+$ dependent (Figs. 2 and 3A to 3B). We therefore compared $\text{Rb}^+$ influx rates of OsHKT2;2-expressing BY2 cells at external 0-0.2 mM $\text{Rb}^+$ with or without 0.01 mM $\text{Na}^+$. OsHKT2;2 mediates $\text{Na}^+$-stimulated $\text{Rb}^+$ influx into BY2 cells (Fig. 4D). Taken together the findings presented in figures 3 and 4, showed that OsHKT2;2 mediates $\text{Na}^+$-stimulated $K^+$ uptake as well as $K^+$-stimulated $\text{Na}^+$ influx in BY2 cells, suggesting that OsHKT2;2 functions as a $\text{Na}^+$-$K^+$ co-transporter/channel in plant cells.

**OsHKT2;2 shows a large $K^+$ permeability in *X. laevis* oocytes, whereas OsHKT2;1 shows little $K^+$ permeability depending on conditions**

Results from other independent laboratories that have analyzed the $K^+$ selectivity of OsHKT2;1 expressed in yeast and *Xenopus* oocytes are controversial (Horie et al., 2001; Golldack et al., 2002; Garciadeblás et al., 2003; Jabnoune et al., 2009). Voltage clamp experiments using *Xenopus* oocytes showed a low $K^+$ permeability of OsHKT2;1 (Horie et al., 2001). However, similar electrophysiological analyses showed either relatively non-selective alkali cation selectivity (Golldack et al., 2002) or strong $K^+$ permeability of OsHKT2;1 (Jabnoune et al., 2009). We therefore recorded OsHKT2;1- and OsHKT2;2-mediated currents in *Xenopus* oocytes. Increasing external $K^+$ concentration from 1 mM to 10 mM led to small positive shifts in the reversal
potential of OsHKT2;1-expressing oocytes, consistent with the results reported by Horie et al. (2001) (Fig.5A). While, OsHKT2;2-expressing oocytes exhibited larger positive shifts in the reversal potential in response to an identical 10-fold increase in the K+ concentration (Fig.5B). These results were independently found in two laboratories (see Material and Methods) and were consistent with tobacco BY2 cell analyses.

Further experiments were pursued with modified experimental procedures in voltage clamp experiments. OsHKT2 cRNA-injected oocytes were incubated in a modified extracellular incubation solution including 0.5 mM Na+ (instead of 96 mM Na+; for details see the MATERIALS AND METHOD). Interestingly, OsHKT2;1-expressing oocytes incubated in the low Na+ buffer exhibited only very small reversal potential shifts in response to a 10-fold increase in the K+ concentration that were not statistically significant (Fig. 5C, E and G). In contrast, OsHKT2;2-expressing oocytes continued to exhibit significant positive shifts in the reversal potential when the external K+ concentration was increased (Fig. 5D, F and H). Average positive shifts in the reversal potential of OsHKT2;1--expressing oocytes incubated in the low Na+ buffer were of -0.2 ± 1.7 mV and in OsHKT2;2-expressing oocytes were 20.4 ± 1.3 mV, when the external K+ concentration was increased from 1 mM to 10 mM in the presence of 1 mM Na+ (Fig. 5C, D, E, F, G and H). 

A 10-fold increase in the Na+ concentration in the presence of 1 mM K+ led to significant positive shifts in the reversal potential of both OsHKT2;1- and OsHKT2;2-expressing oocytes (Fig. 5), consistent with tobacco BY2 cell transport analyses. When a 10-fold increase in the external Na+ concentration was imposed in the presence of 1 mM K+, both OsHKT2;1- and OsHKT2;2-expressing oocytes showed substantial positive shifts in the reversal potential, which were 19.8 ±1.96 mV and 23.0 ±1.10 mV, respectively (Fig. 5C, D, E, F, G and H). These
results are consistent with the results of ion accumulation and tracer influx analyses using transgenic BY2 cells (Figs. 2 to 4) and previous studies showing a low K⁺ permeability of OsHKT2;1 compare to OsHKT2;2 (Horie et al., 2001; García-de Blás et al., 2003), but do not reproduce studies suggesting the OsHKT2;1 is non-selective among cations (Golldack et al., 2002).

**External K⁺ and Ca²⁺ inhibit OsHKT2;1-mediated Na⁺ influx into plant cells and X. laevis oocytes**

To determine whether K⁺ inhibits OsHKT2;1-mediated Na⁺ influx into plant cells, we performed ²²Na⁺ tracer influx experiments using OsHKT2;1-expressing BY2 cells at 0.1 mM external Na⁺ with or without external K⁺. The presence of 0.1 mM external K⁺ had no significant impact on Na⁺ influx of OsHKT2;1-expressing BY2 cells. However, the addition of 1 mM extracellular K⁺ resulted in an approximately 51 to 55% reduction in OsHKT2;1-mediated Na⁺ influx into BY2 cells (Fig. 6A). We further tested the effect of extracellular K⁺ addition on OsHKT2;1-dependent Na⁺ influx into K⁺-starved intact rice roots. A previous study demonstrated that Na⁺ influx into K⁺-starved rice roots at low external Na⁺ concentrations is primarily dependent on the OsHKT2;1 transporter (Horie et al., 2007). We analyzed ²²Na⁺ influx analyses using the wild-type japonica rice cultivar Nipponbare, oshkt2;1 mutants that are disrupted in the OsHKT2;1 gene by an insertion of the endogenous retrotransposon Tos17 and their corresponding wild-type “TosWT” plants (Hirochika, 1997, 2001; Miyao et al., 2003; Horie et al., 2007). Experiments were performed at 0.1 mM external Na⁺ with or without 0.1 mM external K⁺. Interestingly, the presence 0.1 mM external K⁺ triggered strong reductions in the OsHKT2;1-dependent Na⁺ influx into roots of wild-type plants with approximately 80 to 88% reductions (Fig. 6B). Note, however, that a remarkable difference in the sensitivity of the OsHKT2;1-dependent Na⁺ influx to
external K\(^+\) was found between the Na\(^+\) influx profiles from BY2 cells and those from intact rice root cells (Fig. 6A and 6B).

Calcium (Ca\(^{2+}\)) is known to partially inhibit Na\(^+\) influx into plant roots (Amtmann et al., 1997; Tyerman et al., 1997; Davenport and Tester, 2000; Maathuis and Sanders, 2001). We next tested whether the presence of external Ca\(^{2+}\) has any impact on OsHKT2;1-mediated Na\(^+\) influx into plant cells. In the standard uptake buffer solution 1 mM CaCl\(_2\) is included as a component (See MATERIALS AND METHODS). One mM Ca\(^{2+}\) in the influx buffer caused a 30 to 32% decrease in OsHKT2;1-mediated Na\(^+\) influx into BY2 cells compared to 0.1 mM Ca\(^{2+}\) (Fig. 6C). Ten-day-old wild-type Nipponbare, TosWT, and oskkt2;1 rice plants were also analyzed for the effect of external Ca\(^{2+}\) concentrations on OsHKT2;1-dependent Na\(^+\) influx into rice roots. Approximately 46 to 48% reductions in OsHKT2;1-dependent Na\(^+\) influx into wild-type rice roots was found in response to an increase in the external Ca\(^{2+}\) concentration from 0.1 mM to 1 mM (Fig. 6D).

Voltage clamp experiments were performed to determine whether Ca\(^{2+}\) may more directly inhibit OsHKT2;1-mediated Na\(^+\) transport. OsHKT2;1-mediated currents were recorded while bathing oocytes in a 0.3 mM Na\(^+\)-solution containing 0.18 mM or 1.8 mM Ca\(^{2+}\). Average current-voltage curves revealed a moderate Ca\(^{2+}\) inhibition of OsHKT2;1-mediated Na\(^+\) currents in Xenopus oocytes (Fig. 7A). A comparison of current amplitudes at -120 mV from OsHKT2;1-expressing oocytes exhibited an approximately 46% reduction in the OsHKT2;1-mediated currents by a 10 fold increase in the external Ca\(^{2+}\) concentration (P < 0.01; Fig. 7B), which was comparable to results from transgenic BY2 cells and intact rice roots (Fig. 6C and D). Taken together, these influx analyses in tobacco BY2 cells, intact rice roots and Xenopus oocytes show that OsHKT2;1-mediated Na\(^+\) influx into plant roots is reduced by the presence of K\(^+\) and Ca\(^{2+}\) (Fig. 5, 6 and 7).
DISCUSSION

Several cDNAs encoding plant HKT transporters have been identified from different plant species and their ion selectivities have been primarily characterized in *Xenopus* oocytes and yeast (Schachtman and Schroeder, 1994; Rubio et al., 1995; Gassmann et al., 1996; Rubio et al., 1999; Fairbairn et al., 2000; Uozumi et al., 2000; Horie et al., 2001; Golldack et al., 2002; García-Deblás et al., 2003; Su et al., 2003; Haro et al., 2005; Ren et al., 2005; Takahashi et al., 2007; Jabnoune et al., 2009). These past analyses led to the findings that plant HKT transporters can be divided into two different classes based on their Na⁺/K⁺ selectivities, a more Na⁺ over K⁺ selective transport and Na⁺-K⁺ co-transport (Rubio et al., 1995; Horie et al., 2001; Mäser et al., 2002b), with some exceptions (Fairbairn et al., 2000; Golldack et al., 2002). A later phylogenetic analysis of HKT transporter proteins further supported the classification of HKT transporters into two groups, which correlates with the differential Na⁺/K⁺ selectivities of HKT transporters found in heterologous systems (Platten et al., 2006).

The differential cation selectivities of HKT transporters, however, have not yet been analyzed and compared in plant cells. The present findings reveal that OsHKT2;1 mediates Na⁺ influx, but little Rb⁺ uptake into plant BY2 cells. Under identical experimental conditions, the closely-related OsHKT2;2 transporter mediates Na⁺-K⁺ co-transport in plant cells, consistent with findings in heterologous systems (Horie et al., 2001; Mäser et al., 2002b) with analogous correlating findings for the wheat TaHKT2;1 transporter (Rubio et al., 1995; Gassmann et al., 1996). Furthermore, at millimolar Na⁺ concentrations OsHKT2;2 and TaHKT2;1 can transport Na⁺ into plant cells in the absence of external K⁺ as has been shown in *Xenopus* oocytes and yeast (Rubio et al., 1995; Gassmann et al., 1996; Horie et al., 2001). These findings show a close correlation to the cation transport selectivities of plant transporters in heterologous systems.
Are class 2 plant HKT transporters able to mediate K\(^+\) transport in plant cells?

HKT transporters identified to date can be divided into two subgroups, class 1 and class 2 HKT transporters (Mäser et al., 2002b; Platten et al., 2006). The class 1 HKT transporters such as AtHKT1;1, OsHKT1;5 (SKC1), TmHKT1;4, TmHKT1;5 and TaHKT1;5 were demonstrated or suggested to play crucial roles in salinity resistance mediating Na\(^+\) removal from the xylem during salinity stress in different plant species (Uozumi et al., 2000; Mäser et al., 2002a; Ren et al., 2005; Sunarpi et al., 2005; Horie et al., 2006; Huang et al., 2006; Byrt et al., 2007; Davenport et al., 2007; Horie et al., 2009; Møller et al., 2009). In contrast, however, physiological functions and the ion selectivities of the class 2 HKT transporters have rarely been studied in plants. TaHKT2;1-antisense wheat plants showed enhanced growth and reduced Na\(^+\)/K\(^+\) ratios under salinity stress and had lower sodium contents in roots, but no apparent K\(^+\) transport-related phenotypes were found in whole roots despite the findings that TaHKT2;1 showed Na\(^+\)/K\(^+\) permeability and at high Na\(^+\) concentrations Na\(^+\) selective influx in heterologous systems (Laurie et al., 2002). The OsHKT2;1 transporter is an unusual class 2 HKT transporter as it retains a serine residue in the first p-loop region where a glycine residue is conserved in typical class 2 HKT transporters, which correlates with Na\(^+\) selectivity and a lower K\(^+\) permeability of OsHKT2;1 (Horie et al., 2001; Mäser et al., 2002b; Garcia-deblás et al., 2003; Tholema et al., 2005). Analyses of *oshkt2;1* null mutant rice plants further revealed that OsHKT2;1 did not contribute measurably to whole-root K\(^+\) influx into K\(^+\)-starved rice roots in contrast to a large contribution of OsHKT2;1 to Na\(^+\) influx (Horie et al., 2007) (Fig. 6B and 6D).

In this study, we analyzed the Na\(^+\)/K\(^+\) transport selectivity in plant BY2 cells of
OsHKT2;2, a class 2 HKT transporter with the typical 4 glycine residues of this subfamily (Horie et al., 2001; Mäser et al., 2002b). OsHKT2;2-expressing BY2 cells mediate Na\(^+\)-stimulated Rb\(^+\) (K\(^+\)) uptake (Fig. 4). Kinetic analyses show an apparent affinity of OsHKT2;2-mediated Rb\(^+\) (K\(^+\)) influx into BY2 cells of ≈0.035 mM Rb\(^+\), and a \(V_{\text{max}}\) of ≈19 nmol mg of protein\(^{-1}\) min\(^{-1}\) (Table Ic). This is the first study that analyzes the Na\(^+\) and K\(^+\) (Rb\(^+\)) transport activities and kinetics of a “4 glycine”-containing class 2 HKT transporter in plant cells. These findings provide evidence that class 2 plant HKT transporters are able to mediate K\(^+\) transport in plant cells as suggested by ion selectivity analyses in eukaryotic heterologous expression systems including the reproduced K\(^+\) permeability of OsHKT2;2 expressed in Xenopus oocytes, presented in this study (Fig. 5B, D, F and H).

A study of the barley HvHKT2;1 and wheat TaHKT2;1 transporters expressed in yeast has suggested that the transport activities of these two class 2 HKT transporters in yeast are variable, showing Na\(^+\) or K\(^+\) uni-transport or Na\(^+\)-K\(^+\) co-transport depending on proposed differences in the 5′ translation start sites of HKT proteins at non-conventional (non-ATG) translation start sites (Haro et al., 2005). More importantly, the K\(^+\) transport function of HKT transporters characterized in Xenopus oocytes and yeast were further questioned as possible artifacts and concluded not to occur in plant cells (Haro et al., 2005). The basis for these observations may be complex. However, several important limitations indicate further analyses are needed, including that: (i) the proposed non-ATG translational start of HKT proteins was not analyzed or confirmed; (ii) the possibility of differential mRNA expression levels of the different 5′ UTR constructs in yeast appeared likely and was not tested at the mRNA level (Haro et al., 2005); (iii) bi-directional extracellular cation depletion was used as a method to deduce HKT transport selectivities (Haro et al., 2005), rather than the more robust unidirectional tracer flux analyses (Epstein et al., 1963). (iv) the non-ATG
translation start model has been now proposed to be unlikely by the same group (Bañuelos et al., 2008).

Not-with-standing the above limitations, the study of Haro et al (2005) called for analysis of HKT transporter selectivities in plant cells. The present study shows that a typical class 2 HKT transporter, OsHKT2;2, mediates inward K\(^+\) (Rb\(^+\)) transport showing a clear Na\(^+\)/K\(^+\) co-transport activity in plant BY2 cells (Figs. 3 and 4), correlating well with the results from voltage clamp experiments shown in Fig. 5B, D, F and H, which reproduced Na\(^+\)-K\(^+\) transport activity found in prior heterologous system studies (Horie et al., 2001; Mäser et al., 2002b), but not correlating with predictions of Haro et al. (2005). HKT transporters are likely to have several specialized physiological roles in plants. Further studies using gene knock-out plants will be required to elucidate the physiological functions of 4 glycine-containing class 2 HKT transporters. The present study provides evidence that the eukaryotic heterologous systems, *Xenopus* oocytes and yeast, provide facile systems for primary analyses of the ion selectivities of HKT transporters, and of other transporters/channels as well (Bichet et al., 2003; MacKinnon, 2004).

**Selectivity of OsHKT2;1**

Biophysical reversal potential analyses using *OsHKT2;1* cRNA-injected oocytes incubated in the normal ND96 solution (96 mM Na\(^+\)) show a small K\(^+\) permeability of OsHKT2;1 (Fig.5A, C, E and G), consistent with previous findings of small reversal potential shifts upon shifting the extracellular K\(^+\) concentration (Horie et al., 2001). These findings are also consistent with data suggesting a possible small K\(^+\) permeability of the *Arabidopsis* orthologue AtHKT1;1, based on *E. coli* complementation analyses (Uozumi et al., 2000). The present study did not reproduce the larger K\(^+\)-induced reversal potential shifts observed in a recent
study of OsHKT2;1 (Jabnoune et al., 2009). However, here we also show that the external K\(^+\) -induced shifts in reversal potentials depended on the pretreatment of oocytes in low (0.5 mM Na\(^+\)) or high (96 mM Na\(^+\)) buffer (Fig. 5C, E and G). These pre-incubation buffers are expected to shift the intracellular Na\(^+\) concentrations ([Na\(^+\)]\(_i\)) of oocytes, in particular, when oocytes express highly Na\(^+\) permeable channels/transporters like OsHKT2;1 compared to those incubated in a low Na\(^+\) buffer (Kellenberger et al., 1998).

The small K\(^+\) permeability of OsHKT2;1 found in oocytes in this study when oocytes were pre-incubated in 96 mM Na\(^+\) (Fig.5A) is comparable to the findings reported by Horie et al., (2001), but appears to differ from the lack of clear Rb\(^+\) permeability found in OsHKT2;1-expressing BY2 cells (Fig. 4 A to C) and also from the lack of K\(^+\) uptake complementation upon OsHKT2;1 expression in yeast (Horie et al., 2001; Garciadeblás et al., 2003) and the main \textit{in vivo} Na\(^+\) uptake activity of OsHKT2;1 in rice roots (Horie et al., 2007). Note however, that the K\(^+\) to Rb\(^+\) selectivity of HKT2 transporters is relatively high and therefore Rb\(^+\) accumulation and Rb\(^+\) influx analyses using transgenic BY2 cells may overlook the relatively small K\(^+\) permeability of OsHKT2;1 (Schachtman and Schroeder, 1994; Rubio et al., 1995; Gassmann et al., 1996; Horie et al., 2001). Furthermore, the present findings that the K\(^+\) permeability of OsHKT2;1 depends on pretreatment conditions of oocytes (Fig.5A, C, E and G) and can show no clear K\(^+\) permeability in OsHKT2;1-expressing oocytes incubated in a low Na\(^+\) buffer (Fig. 5C, E and G), suggest that the limited K\(^+\) permeability of OsHKT2;1 depends on ionic conditions. These data are also consistent with early findings that HKT transporters shift their selectivity depending on ion conditions (Rubio et al., 1995; Gassmann et al., 1996; Rubio et al., 1999). These findings correlate with data suggesting that HKT transporters are multi-ion occupancy pores (Corratgé et al., 2007). Multi-ion occupancy of transporters/channels is known to cause shifts in
the relative permeabilities of these proteins, as demonstrated in classical experiments (Hille, 2001).

**Inhibitory effects of external K⁺ and Ca²⁺ on OsHKT2;1-mediated Na⁺ influx into plant cells**

Potassium is an essential macro nutrient for plant growth and the most abundant cation in plant cells. In spite of the fact that K⁺ and Na⁺ are chemically similar, the majority of plants including glycophytes preferentially absorb and accumulate K⁺ to maintain growth (Flowers and Läuchli, 1983). The K⁺ selectivities of some plant K⁺ transporters are disturbed by the presence of mM concentrations of Na⁺ (Santa-María et al., 1997; Fu and Luan, 1998). OsHKT2;1-mediated Na⁺ transport is also inhibited by the presence of K⁺ in yeast cells (Garcia-deblás et al., 2003). These findings motivated us to analyze OsHKT2;1-mediated Na⁺ influx into plant BY2 cells and also into intact rice roots in the presence or absence of external K⁺. External K⁺ significantly inhibited Na⁺ influx in OsHKT2;1-expressing BY2 cells and K⁺-starved rice roots (Fig. 6A and 6B). Interestingly, however, the sensitivity of OsHKT2;1-mediated Na⁺ influx into rice roots to external K⁺ was remarkably higher than that found in BY2 cells (Fig. 6A and 6B). Tracer influx analyses using rice plants indicated that 0.1 mM external K⁺ was sufficient to trigger large approximately 80 to 88% reductions in Na⁺ influx via OsHKT2;1 in intact rice roots (Fig. 6B). In contrast, 1 mM K⁺ was needed to inhibit approximately 51 to 55% of OsHKT2;1-mediated Na⁺ influx into BY2 cells (Fig. 6A). These results suggest several possibly additive mechanisms controlling the activity of OsHKT2;1 in the presence of K⁺: (i) a simple competition of the two similar ions Na⁺ and K⁺ at the selectivity pore of OsHKT2;1 and (ii) post translational down-regulation of OsHKT2;1 by K⁺ in rice roots. A likely post-translational down-regulation of OsHKT2;1 was also observed in response
to elevated Na⁺ concentrations (Horie et al., 2007). (iii) In addition, the addition of 0.1 to 1.0 mM K⁺ would cause depolarization which could contribute to the reduction in OsHKT2;1-mediated Na⁺ influx.

Ca²⁺ is also an essential divalent cation, which acts as a second messenger in diverse signal transduction pathways. In plant stress physiology, Ca²⁺ is also known to partially inhibit or reduce toxic Na⁺-influx via voltage-independent channels (VIC) or non-selective cation channels (NSCC) that have been suggested to mediate Na⁺ influx leading to salinity stress (Amtmann et al., 1997; Roberts and Tester, 1997; Tyerman et al., 1997; Buschmann et al., 2000; Davenport and Tester, 2000; Maathuis and Sanders, 2001). Classical plant physiological studies have shown that increasing external concentrations of Ca²⁺ dramatically reduced Na⁺ influx into nutrients-starved (K⁺-starved) barely roots (Rains and Epstein, 1967). The transcript level of the barely HvHKT2;1 gene that is a close ortholog of the OsHKT2;1 gene has been found to be up-regulated in barely roots in response to K⁺-starvation (Wang et al., 1998). These findings led us to analyze the effect of external Ca²⁺ on OsHKT2;1-mediated Na⁺ influx into BY2 cells. Interestingly, an increase in the Ca²⁺ concentration of the uptake buffer from 0.1 mM to 1 mM led to significant partial reductions in the OsHKT2;1-mediated Na⁺ influx into both BY2 cells and intact rice roots (Figs. 6C and 6D). Current-voltage relationships from OsHKT2;1-expressing oocytes bathed in a solution containing 0.18 mM or 1.8 mM Ca²⁺ showed a moderate inhibitory effect of Ca²⁺ on the Na⁺ transport activity of OsHKT2;1 (Fig. 7A). Relatively little influence on the reversal potentials of OsHKT2;1-expressing oocytes in low and high Ca²⁺ solutions (Fig. 7A) and a significant 46% reduction in OsHKT2;1-mediated currents at -120 mV (p<0.01; Fig. 7B) upon a 10-fold increase in the external Ca²⁺ concentration suggest that high Ca²⁺ concentrations can inhibit OsHKT2;1-mediated Na⁺ currents in oocytes. Taken together, the
presented data sets reveal that not only K\(^+\) but also high concentrations of Ca\(^{2+}\) reduce Na\(^+\) transport by OsHKT2;1.

**CONCLUSIONS**

In conclusion, although OsHKT2;1 and OsHKT2;2, two highly homologous HKT transporters, share 93% identical cDNA sequence and 91% identical amino acid sequence (Horie et al., 2001), they exhibit differential Na\(^+\)/K\(^+\) transport selectivities when expressed in tobacco BY2 cells, which correlate remarkably well with ion selectivity studies of these transporters in *Xenopus* oocytes and yeast. Moreover, ionic conditions affect the K\(^+\)/Na\(^+\) selectivities of HKT transporters, as was found for the wheat TaHKT2;1 transporter (Rubio et al., 1995), supporting the model that HKT transporters are multi-ion pores (Gassmann et al., 1996; Corratgé et al., 2007). Furthermore, interesting inhibition and possible down-regulation of OsHKT2;1-mediated Na\(^+\) influx in rice roots is shown here. The genome of a *japonica* rice cultivar Nipponbare includes 7 functional OsHKT genes encoding five class 1 and two class 2 HKT transporters, respectively (García-de Blas et al., 2003). Other than OsHKT1;5 (Ren et al., 2005) and OsHKT2;1 (Horie et al., 2007), little in planta genetic mutant information exists on the physiological functions of the remaining HKT transporters in rice plants. Elucidation of the physiological roles of other HKT members in rice will be important for understanding Na\(^+\)/K\(^+\) homeostasis mechanisms mediated by both class 1 and class 2 HKT transporter proteins in plants. Further studies of loss-of-function mutant plants will be required to draw a complete picture of plant HKT transporter functions.

**MATERIALS AND METHODS**

Transformation of BY2 cells
Transformation of tobacco (*Nicotiana tabacum*) cv. Bright-Yellow 2 (BY2) cells was carried out as described previously (Nakayama et al., 2000). Briefly, four-day-old cells were co-incubated with 100 μl solution containing *Agrobacterium tumefaciens* strain C58 carrying the indicated DNA constructs at 25°C in the dark for 2 days. The cells were washed and then plated on modified LS medium (Nagata et al., 1981) containing 100 μg ml⁻¹ kanamycin and 250 μg ml⁻¹ carbenicillin. The plates were placed at 25°C in the dark for 3-4 weeks. Kanamycin-resistant calli were transferred onto solid LS medium and placed at 25°C in the dark for 2 weeks. A part of each callus was then transferred into liquid LS medium containing 100 μg ml⁻¹ kanamycin and 250 μg ml⁻¹ carbenicillin for 12 to 14 days. When the growth of each cell line became stable in liquid culture, the cells were maintained as described below.

**Culture conditions and maintenance of tobacco Bright Yellow 2 cells**

Tobacco BY2 cells were maintained in modified LS liquid medium (Nagata et al., 1981) at 25°C in the dark in an incubator shaker (Innova 4430, New Brunswick Scientific, NJ, USA) at 130 rpm. The liquid culture medium contained (mM): 19 KNO₃, 20 NH₄NO₃, 2.7 KH₂PO₄, 0.005 KI, 0.10 H₃BO₃, 0.10 MnSO₄·5H₂O, 0.03 ZnSO₄·7H₂O, 0.0010 Na₂MoO₄·2H₂O, 0.00010 CuSO₄·5H₂O, 0.00010 CoCl₂·6H₂O, 3.0 CaCl₂·2H₂O, 1.5 MgSO₄·7H₂O, 0.10 Fe(III)-EDTA, 88 Sucrose, 0.56 myo-inositol, 0.003 thiamine-HCl, 0.00090 2,4D, pH5.8 with KOH. One to two ml of suspension cell culture solution was transferred into 50 ml of LS liquid medium once a week. To maintain transformed BY2 cells, 100 μg ml⁻¹ kanamycin was added in liquid medium.

**Plant materials and growth**

*Japonica* rice (*Oryza sativa*) cv. Nipponbare was used in this study. Rice
plants were prepared as described previously (Horie et al., 2007). The line numbers of Tos17-insertion rice mutants used in this study are NF1009 for oshkt2;1-1 and TosWT2;1-1 and ND3042 for oshkt2;1-2 and TosWT2;1-2, respectively (Horie et al., 2007).

**Real-time PCR assays**

To analyze the expression levels of OsHKT2;1 and OsHKT2;2 in tobacco BY2 cell lines and vector control lines, quantitative real-time PCR analyses were performed. Total RNA samples were isolated from seven-day-old suspension cells using RNeasy plant mini kit (Qiagen) followed by DNase digestion and RNA purification, and then the first-strand cDNA was reverse transcribed with First-Strand cDNA synthesis kit (GE healthcare) at 37˚C for 1 hr. Specific primers were designed for OsHKT2;1 and OsHKT2;2 transcripts: for OsHKT2;1, forward 5' - GCATATTCACCCATTCTGGATTCAGT-3' and reverse 5' - CGATGGTGATGAGGCTGGAAAGT-3'; and for OsHKT2;2, forward 5' - GCATGTTCCACCATTCTGGATCCAAC-3' and reverse 5' - GGTGCTGAGGCCGGAAACG-3'. Amplification of the NtEF1α (forward 5' - GCTGTGAGGGACATGCGTCAAA-3' and reverse 5' - GTAGTAGATATCGCGAGTACCACCA-3') mRNA was used as an internal quantitative control. Real-time PCR was performed by using a LightCycler Carousel-Based System (Roche) and LightCycler FastStart DNA MasterPLUS SYBR Green I Master Mix (Roche). PCR amplification was carried out with an initial step at 95˚C for 10 min followed by 45 cycles of 10 sec at 95˚C and 1 min at 60˚C. An amplification of target genes was monitored every cycle by SYBR green fluorescence. Three independent replicates were performed for each line.

**Ion content determinations**
Four-day old OsHKT2;1, OsHKT2;2 and vector control BY2 cells were pre-treated with 19 mM NH$_4^+$ and 0 mM K$^+$ for 8 hours. NH$_4^+$ was added to inhibit residual K$^+$ uptake from LS growth medium. After washing 5 times with uptake buffer (2 mM MES-1,3 bis[tris(hydroxymethyl)9-methylamino]propane (BTP) pH 5.8, 1 mM CaCl$_2$ and 0.17 M mannitol), cells were treated with uptake buffer containing 0.1 mM Na$^+$ and 0 K$^+$ and 0.1 mM Rb$^+$ and 0.01 mM Na$^+$ for 0 min, 30 min and 60 min. And then the cells were washed with washing/uptake buffer again 3 times. Total protein contents were determined by the Bradford method and used to normalize ion concentration data from ICP-OES analyses (Bradford, 1976; Mendoza-Cózatl et al., 2002; Mendoza-Cózatl and Moreno-Sanchez, 2005). The rest of the cells were dried at 80 °C in an oven for 2 days. Dry samples were digested in nitric acid (trace metal grade, Fisher) (Gong et al., 2003) and analyzed by ICP-OES (Perkin-Elmer Optima 3000XL, Applied Biosystems) at the Scripps Institution of Oceanography (UCSD, La Jolla, CA, USA) (Gong et al., 2004).

**Tracer influx analyses**

Four-day old OsHKT2;1, OsHKT2;2 and vector control BY2 cells were treated and washed as described in “Ion content determinations”. The basic Influx buffer was composed of 2 mM MES-BTP pH 5.8, 1 mM CaCl$_2$ and 0.17 M mannitol supplemented with either non-radioactive NaCl and $^{22}$NaCl (Perkin-Elmer, Boston, MA, USA) or non-radioactive RbCl and $^{86}$RbCl (Perkin-Elmer, Boston, MA, USA) for Na$^+$ and Rb$^+$ influx assays, respectively (Gierth et al., 2005; Horie et al., 2007). Cells were incubated for 15 min except for time-dependent influx assays. After treatments, the cells were washed with a non-radioactive washing buffer, and collected with 0.45 μm HVHP membrane filter (Millipore). The washed cells were transferred to a scintillation vial and radioactivity was measured with a scintillation counter (LS6500 Beckman, UCSD, La Jolla, CA, USA). In K$^+$ inhibition assays,
OsHKT2;1-mediated $^{22}$Na$^+$ influx rates were analyzed and compared after the OsHKT2;1-expressing BY2 cells and vector controls were exposed to either a influx buffer with no added K$^+$ or with added K$^+$ buffer, 0.1 mM external K$^+$ and 1mM external K$^+$. The Ca$^{2+}$ concentration was lowered to 0.1 mM from the standard (1 mM Ca$^{2+}$) basic influx buffer when indicated. OsHKT2;1-mediated $^{22}$Na$^+$ influx rates were analyzed and compared after OsHKT2;1-expressing BY2 cells and vector controls were exposed to either a buffer with decreased Ca$^{2+}$ concentration, 0.1 mM Ca$^{2+}$ or a 1 mM Ca$^{2+}$ buffer.

$^{22}$Na$^+$ tracer Influx experiments using rice plants were performed as described previously (Horie et al., 2007) with minor modifications. Briefly, ten-day-old rice plants grown in 1 mM CaSO$_4$ solution (K$^+$-starvation) were used for influx analyses. The basic influx buffer was composed of 2 mM MES-BTP, pH5.5, 1 mM CaCl$_2$ supplemented with cold NaCl and $^{22}$NaCl (Amersham Bioscience, Pittsburgh, PA, USA). In K$^+$ inhibition analyses either 0.1 mM or 1 mM KCl was added to the basic influx buffer. In Ca$^{2+}$ inhibition analyses, a modified influx buffer that contains 0.1 mM CaCl$_2$ but maintains the same other components as the basic influx buffer was prepared and used. Plants were incubated for 20 min in each influx experiment and radioactivity from excised roots was measured as described above.

**OsHKT2 gene expression and electrophysiology in Xenopus laevis oocytes**

OsHKT2;1 and OsHKT2;2 cRNAs were transcribed from linearized plasmid constructs pXβG-ev1::OsHKT2;1 and OsHKT2;2 (Horie et al., 2001) using mMESSAGE mMACHINE in vitro transcription kit (Ambion, Austin, TX, USA) and 12.5 ng of each OsHKT2 cRNA was injected into X. laevis oocytes. Oocytes were kept for 1 ± 2 days at 18°C in either a normal ND96 solution including 96 mM NaCl or a modified ND96 solution composed of 92 mM Tris-HCl, 0.5 mM NaCl, 2
mM KCl, 1 mM CaCl₂, 2 mM MgCl₂, and 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)-Tris, pH 7.4. K⁺/Na⁺ selectivity experiments shown in Fig. 5 were independently reproduced at UCSD by S.X. and D.C.B. and at Okayama University by T.H. and M.K. Two-electrode voltage clamp experiments were performed using a Dagan TEV-200 amplifier (Dagan Corporation, Minneapolis, MN, USA) or a dual electrode voltage clamp amplifier (Nihon Kohden, Tokyo, Japan). Detailed descriptions of electrophysiological experiments were as reported by Rubio et al.(1995) and Horie et al. (2001). Oocytes were perfused with a solution containing 6 mM MgCl₂, 1.8 mM CaCl₂, 10 mM 2-(N-morpholino)-ethanesulfonic acid (MES)-1,3-bis (Tris[hydroxymethyl]methylamino) propane (BTP), pH 5.5, 180 mM D-mannitol and the indicated concentrations of Na- and K-glutamate. The ionic strength of the solutions for different Na⁺ and K⁺ concentrations was kept constant by adding Tris-glutamate. PCLAMP10 (Axon Instrument, CA, USA) or Lab-Trax-4/16 (World Precision Instruments, Inc. Sarasota, FL, USA) was used for electrophysiological measurements. A voltage ramp was generated from 0 mV to -150 mV at a rate of 0.15 mV ms⁻¹. For Ca²⁺ inhibition analysis, OsHKT2;1 cRNA-injected oocytes were incubated in a high Na⁺ solution for more than 24 hrs. Upon recording of OsHKT2;1-mediated currents, the perfusion solution described above was modified by replacing 1.62 mM CaCl₂ with additional MgCl₂, leading the final concentrations of CaCl₂ and MgCl₂ to 0.18 mM and 7.62 mM, respectively. Voltage steps were applied from 0 mV to -150 mV in -15 mV decrements. Microelectrodes were filled with 3 M KCl. 3M KCl agar bridge was used as bath electrode. All experiments were performed at room temperature (23°C).

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Figure legends:

Figure 1. A, Tobacco (Nicotiana tabacum L.) cv. BY2 calli expressing OsHK2;1 on selective LS medium as a representative example. B, Tobacco BY2 suspension cells expressing OsHK2;1 as a representative example. C, Real-time PCR analyses of OsHK2;1 and OsHK2;2 expression in tobacco BY2 cell lines expressing either OsHK2;1, OsHK2;2 or empty vector DNA constructs (n=3; ±S.E.M.).

Figure 2. OsHK2;1 mediates Na⁺ influx, whereas OsHK2;2 does not in the absence of external K⁺. A, OsHK2;1 increases Na⁺ accumulation in cultured tobacco BY2 cells. Na⁺ contents of transgenic BY2 cell lines exposed to a buffer containing 0.1 mM Na⁺ and no added K⁺ for either 0, 30, or 60 min. Na⁺ contents were determined by ICP-OES (n=6; ±S.D.; * p<0.001, compared to vector controls). B, OsHK2;1 mediates Na⁺ influx from a 0.01 mM Na⁺ and 0 K⁺ buffer, whereas OsHK2;2 does not compared to vector controls. Na⁺ influx profiles from time-dependent short-term ²²Na⁺ influx experiments using transgenic BY2 cell lines in influx buffer containing 0.01 mM Na⁺ and no added K⁺ (n=3; ±S.D.). C, OsHK2;1 mediates enhanced Na⁺ influx as a function of the external Na⁺ concentration with no added K⁺, whereas OsHK2;2 does not compared to vector controls. Concentration-dependent short-term ²²Na⁺ influx rates of transgenic BY2 cell lines, analyzed at 0-0.2 mM external Na⁺ without K⁺ (n=3; ±S.D.; t=15 min).

Figure 3. Extracellular K⁺ (Rb⁺) stimulated OsHK2;2-mediated Na⁺ influx.
A, OsHKT2;2-expressing tobacco BY2 cells exhibited increased Na⁺ accumulation in the presence of 0.1 mM Rb⁺. Na⁺ contents of transgenic BY2 cell lines exposed to a buffer containing 0.1 mM Rb⁺ and 0.01 mM Na⁺ for either 0, 30, or 60 min, determined by ICP-OES (n=6; ±S.D.; * p<0.001 compared to vector controls). B, OsHKT2;2 mediated K⁺-dependent Na⁺ influx. Na⁺ influx kinetics are shown from concentration-dependent short-term ²²Na⁺ influx analyses using transgenic BY2 cell lines in influx buffer containing 0-0.2 mM external Na⁺ with or without 0.1 mM K⁺ (n=3; ±S.D.; t=15 min). C, OsHKT2;2-expressing tobacco BY2 cells show enhanced Na⁺ accumulation at millimolar external Na⁺ concentrations without adding extracellular K⁺. Na⁺ contents of OsHKT2;2-expressing BY2 cell lines exposed to a buffer containing either 1 mM Na⁺ or 10 mM Na⁺ for 60 min, determined by ICP-OES (n=6; ±S.D.; * p<0.001 compared to vector controls).

Figure 4. OsHKT2;2 but not OsHKT2;1 mediates Rb⁺ (K⁺) accumulation and influx.
A, OsHKT2;2-expressing tobacco BY2 cells exhibit increased Rb⁺ accumulation. Rb⁺ contents of transgenic BY2 cell lines exposed to a buffer containing 0.1 mM Rb⁺ and 0.01 mM Na⁺ for either 0, 30, or 60 min, determined by ICP-OES (n=6; ±S.D.; * p<0.001 compared to vector controls). B, OsHKT2;2 mediates Rb⁺ (K⁺) influx, whereas OsHKT2;1 does not compared to vector controls. Rb⁺ influx kinetics are shown from time-dependent short-term ⁸⁶Rb⁺ influx assays using transgenic BY2 cell lines in influx buffer solution containing 0.1 mM Rb⁺ and 0.01 mM Na⁺ (n=3; ±S.D.). C, OsHKT2;2 mediates enhanced Rb⁺ (K⁺) influx as a function of the external Rb⁺ concentration, whereas OsHKT2;1 does not compared to vector controls. Concentration-dependent short-term ⁸⁶Rb⁺ influx rates of transgenic BY2 cell lines, analyzed at 0-0.2 mM external Rb⁺ with 0.01 mM Na⁺ (n=3; ±S.D.; t=15 min). D, OsHKT2;2 mediates Na⁺-stimulated K⁺ (Rb⁺) influx. Concentration-dependent short-term ⁸⁶Rb⁺ influx rates of transgenic BY2 cell lines, analyzed at 0-0.2 mM external Rb⁺ with or without 0.01 mM Na⁺ (n=3; ±S.D.; t=15 min). Samples in panels C and D were measured in parallel.

Figure 5. OsHKT2;1 and OsHKT2;2 display different Na⁺ and K⁺ selectivities in Xenopus laevis oocytes.
A and B, Average currents-voltage recordings in oocytes expressing OsHKT2;1 (A) and OsHKT2;2 (B), pre-incubated in a 96 mM Na⁺-containing solution. C and D Representative electrical recordings of OsHKT2;1- (C) and OsHKT2;2- (D) mediated currents in response to voltage ramps from 0 mV to –150 mV in oocytes pre-incubated in 0.5 mM Na⁺-containing solution. E and F Average current-voltage relationships of OsHKT2;1- (E) and OsHKT2;2- (F) expressing oocytes as recorded in C and D. G and H, Average reversal potentials of OsHKT2;1- (G) and OsHKT2;2- (H) mediated currents at the indicated bath Na⁺
and K⁺ solutions pre-incubated as in C and D. In C, D, G and H, number 1=1 Na⁺/10 K⁺, number 2=1 Na⁺/1 K⁺, number 3=10 Na⁺/1 K⁺ (in mM). In C, D, E, F, G and H, oocytes were pre-incubated in a modified medium with 0.5 mM Na⁺ after cRNA injection. Data are means ±S.D. (n=8 in A and B, n=5 in E, G and H, n=4 in F).

**Figure 6.** External K⁺ and Ca²⁺ inhibit OsHKT2;1-mediated Na⁺ influx into plant cells.

A, OsHKT2;1-mediated Na⁺ influx into BY2 cells at 0.1 mM external Na⁺ showed significant decreases in the presence of 1 mM K⁺. Short-term ²²Na⁺ influx analyses using transgenic BY2 cell lines (n=6; ±S.D.; t=15 min; * p<0.001 compared to OsHKT2;1-mediated Na⁺ influx rate from a 0.1 mM Na⁺ buffer without added K⁺). 0.1 mM K⁺ and 1 mM K⁺ were added to the influx buffer. B, The presence of 0.1 mM external K⁺ in influx buffer inhibited OsHKT2;1-mediated Na⁺ influx into K⁺-starved rice roots of wild-type plants. Short-term ²²Na⁺ influx assays at 0.1 mM external Na⁺ using wild-type, oshkt2;1 mutants and TosWT rice plants in the presence or absence of 0.1 mM external K⁺ (n=3; ±S.D.; t=20 min; * p<0.02; 0.1 mM Na⁺ vs. 0.1 mM Na⁺ + 0.1 mM K⁺). C, The presence of 1 mM Ca²⁺ in influx buffer inhibited OsHKT2;1-mediated Na⁺ influx into BY2 cells. Short-term ²²Na⁺ influx analyses using transgenic BY2 cell lines (n=6; ±S.D.; t=15 min; * p<0.001; 0.1 mM Na⁺ + 0.1 mM Ca²⁺ vs. 0.1 mM Na⁺ + 1 mM Ca²⁺). D, An increase in the external Ca²⁺ concentration of the influx buffer led to significant decreases in OsHKT2;1-dependent Na⁺ influx into K⁺-starved wild-type rice roots. Short-term ²²Na⁺ influx assays at 0.1 mM external Na⁺ using wild-type, oshkt2;1 mutants and TosWT rice plants in the presence of either 0.1 mM or 1 mM external Ca²⁺ (n=3; ±S.D.; t=20 min; * p<0.02; 0.1 mM Na⁺ + 0.1 mM Ca²⁺ vs. 0.1 mM Na⁺ + 1 mM Ca²⁺).

**Figure 7.** Increasing the external Ca²⁺ concentration reduces OsHKT2;1-mediated Na⁺ currents in *Xenopus laevis* oocytes.

A, Average current-voltage curves from OsHKT2;1-expressing oocytes bathed in a 0.3 mM Na⁺-solution containing either 0.18 mM Ca²⁺ (solid line, numbered 1) or 1.8 mM Ca²⁺ (dashed line, numbered 2). Currents were measured using a step command with 15 mV decrements (see MATERIALS AND METHODS). Error bars represent ± S.E. (n=13~15) from 2 independent experiments. B, Current amplitudes recorded at -120 mV, derived from OsHKT2;1-expressing oocytes in the presence of 0.18 mM (numbered 1; n=15) or 1.8 mM Ca²⁺ (numbered 2; n=13), which were extracted from the data sets presented in A. Error bars represent ± S.E. (* p<0.01; 0.18 mM Ca²⁺ vs. 1.8 mM Ca²⁺).
Table I.
Michaelis-Menten curve fitting results for $^{22}\text{Na}^+$ and $^{86}\text{Rb}^+$ influx kinetics of *OsHKT2;1*- and *OsHKT2;2*-expressing tobacco BY2 cells (n=3). Curve fitting was performed with Microcal origin 6.0 software (http://microcal-origin.software.informer.com/6.0/).

<table>
<thead>
<tr>
<th></th>
<th>$R^2$</th>
<th>$K_M$ (mM)</th>
<th>$V_{max}$ (nmol mg of protein$^{-1}$ min$^{-1}$)</th>
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<tbody>
<tr>
<td>OsHKT2;1$^a$ (Na$^+$)</td>
<td>0.996</td>
<td>0.014±0.002</td>
<td>31±0.9</td>
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<td>OsHKT2;2$^b$ (Na$^+$)</td>
<td>0.980</td>
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<tr>
<td>OsHKT2;2$^c$ (Rb$^+$)</td>
<td>0.986</td>
<td>0.035±0.01</td>
<td>19±1.6</td>
</tr>
</tbody>
</table>

(a) Results of fits to data from Fig. 2C, showing $^{22}\text{Na}^+$ influx kinetics of OsHKT2;1 transporter with no added K$^+$. (b) Results of fits to data from Fig. 3B, showing $^{22}\text{Na}^+$ influx kinetics of OsHKT2;2 transporter with 0.1 mM extracellular K$^+$. (c) Results of fits to data from Fig. 4C, showing $^{86}\text{Rb}^+$ influx kinetics of OsHKT2;2 transporter with 0.01 mM extracellular Na$^+$. 


Panel A: Graph showing the comparison of Na$^+$ (nmol/mg of protein) levels between Vector, OsHKT2;1, and OsHKT2;2 over time (0, 30, 60 min) under 0.1 mM Rb$^+$ and 0.01 mM Na$^+$ conditions.

Panel B: Graph showing the influx rate of Na$^+$ (nmol/mg of protein/min) for OsHKT2;2 under different Na$^+$ concentrations (0.05, 0.10, 0.15, 0.20 mM) with 0.1 mM K$^+$ and 0 K$^+$ conditions.

Panel C: Graph showing the comparison of Na$^+$ levels between Vector and OsHKT2;2 under 0 K$^+$ conditions with Na$^+$ concentrations of 0, 1, and 10 mM.
A. For 0.1 mM Na⁺, the Na⁺ influx rate is higher in OsHKT2;1 compared to the vector control. The rate decreases significantly with 1 mM K⁺.

B. The Na⁺ influx rate in WT is higher than that in mutants TosWT2;1-1, TosWT2;1-2, oshkt2;1-1, and oshkt2;1-2. The addition of 0.1 mM Na⁺ and 0.1 mM K⁺ further increases the influx rate.

C. For 0.1 mM Na⁺, the Na⁺ influx rate is significantly increased by OsHKT2;1.

D. The Na⁺ influx rate in WT is higher than that in mutants. The addition of 0.1 mM Na⁺ and 1 mM Ca²⁺ increases the influx rate.