

Rice OshKT2;1 transporter mediates large Na⁺ influx component into K⁺-starved roots for growth

Tomoaki Horie¹, Alex Costa¹, Tae Houn Kim¹, Min Jung Han², Rie Horie¹, Ho-Yin Leung¹, Akio Miyao³, Hirohiko Hirochika³, Gynheung An² and Julian I Schroeder^{1,*}

¹Division of Biological Sciences, Cell and Developmental Biology Section, and Center for Molecular Genetics, University of California, San Diego, La Jolla, CA, USA, ²National Research Laboratory of Plant Functional Genomics, Department of Life Science, Pohang University of Science and Technology, Kyungbuk, Republic of Korea and ³Division of Genome and Biodiversity Research, National Institute of Agrobiological Sciences, Ibaraki, Japan

Excessive accumulation of sodium in plants causes toxicity. No mutation that greatly diminishes sodium (Na⁺) influx into plant roots has been isolated. The OshKT2;1 (previously named OshKT1) transporter from rice functions as a relatively Na⁺-selective transporter in heterologous expression systems, but the *in vivo* function of OshKT2;1 remains unknown. Here, we analyzed transposon-insertion rice lines disrupted in OshKT2;1. Interestingly, three independent *oshkt2;1*-null alleles exhibited significantly reduced growth compared with wild-type plants under low Na⁺ and K⁺ starvation conditions. The mutant alleles accumulated less Na⁺, but not less K⁺, in roots and shoots. OshKT2;1 was mainly expressed in the cortex and endodermis of roots. ²²Na⁺ tracer influx experiments revealed that Na⁺ influx into *oshkt2;1*-null roots was dramatically reduced compared with wild-type plants. A rapid repression of OshKT2;1-mediated Na⁺ influx and mRNA reduction were found when wild-type plants were exposed to 30 mM NaCl. These analyses demonstrate that Na⁺ can enhance growth of rice under K⁺ starvation conditions, and that OshKT2;1 is the central transporter for nutritional Na⁺ uptake into K⁺-starved rice roots.

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Introduction

Sodium (Na⁺) is an alkali cation, which is not accumulated at large concentrations in most plant cells, in contrast to the alkali cation potassium (K⁺), which is an essential macro-

nutrient for plant growth. High concentrations of external Na⁺ inhibit K⁺ absorption (Rains and Epstein, 1965, 1967b) and elevated Na⁺ concentrations in plant cells disturb functions of vital enzymes (Murguía *et al*, 1995) and photosynthesis (Tsugane *et al*, 1999), leading to Na⁺ toxicity and cell death. On the other hand, classical plant physiological studies have reported a positive effect of low Na⁺ concentrations on the growth of many plant species, but the underlying Na⁺ uptake mechanisms remain unknown (Flowers and Läuchli, 1983).

Several genes that encode Na⁺ permeable transporters/channels have been identified in plants. Biochemical analyses of vacuoles revealed a mechanism for the sequestration of Na⁺ into vacuoles under salt stress via Na⁺/H⁺ antiporters (Blumwald and Poole, 1985, 1987). Genome sequencing of *Arabidopsis thaliana* led to the identification of the corresponding plant Na⁺/H⁺ antiporter genes, *AtNHX1* to 6 (Apse *et al*, 1999; Gaxiola *et al*, 1999; Yokoi *et al*, 2002; Aharon *et al*, 2003). An essential salt tolerance gene of *Arabidopsis*, salt overly sensitive 1 (*SOS1*), also belongs to the same H⁺ exchanger family of transporters (Shi *et al*, 2000). The *SOS1* transporter has been reported to function in long-distance Na⁺ transport from roots to shoots in xylem parenchyma cells during salt stress (Shi *et al*, 2002).

Electrophysiological analyses using root cortex cells and suspension cultured cells of monocot plants suggest that toxic Na⁺ influx into roots under high external Na⁺ concentrations is mediated by voltage-independent channels (VIC) or non-selective cation channels (NSC) (Amtmann *et al*, 1997; Roberts and Tester, 1997; Tyerman *et al*, 1997; Buschmann *et al*, 2000; Davenport and Tester, 2000). VIC/NSC currents have also been reported in *Arabidopsis* roots and were shown to be downregulated by addition of cAMP and cGMP (Maathuis and Sanders, 2001). Recently, the cyclic nucleotide-gated channel 3 (*CNGC3*) gene was proposed to mediate non-selective cation uptake in the root of *Arabidopsis* plants (Gobert *et al*, 2006). The detailed molecular identities of VIC/NSC, however, remain unknown and no genetic mutations have been isolated in any plant species, to date, that show a strong reduction in Na⁺ influx into roots.

HKT-type transporters have been characterized in several plant and bacterial species (Schachtman and Schroeder, 1994; Rubio *et al*, 1995; Fairbairn *et al*, 2000; Uozumi *et al*, 2000; Horie *et al*, 2001; Gollmack *et al*, 2002; Garcíadeblás *et al*, 2003; Su *et al*, 2003; Ren *et al*, 2005; Tholema *et al*, 2005). TaHKT2;1 (previously named TaHKT1) (Platten *et al*, 2006), from wheat, has been shown to mediate at least two transport modes in heterologous expression systems, K⁺-Na⁺ co-uptake and Na⁺ influx at high Na⁺ concentrations (Rubio *et al*, 1995; Gassmann *et al*, 1996). Analyses of the ion transport specificity of AtHKT1;1, previously named AtHKT1, in *Arabidopsis* and OshKT2;1 (OshKT1) in rice revealed that these HKT1 transporters/channels showed a relatively Na⁺-

*Corresponding author. Division of Biological Sciences, Cell and Developmental Biology Section, and Center for Molecular Genetics, Room 5214, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0116, USA. Tel.: +1 858 534 7759; Fax: +1 858 534 7108; E-mail: julian@biomail.ucsd.edu

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selective transport activity relative to K⁺ transport activity in yeast and *Xenopus* oocytes (Uozumi *et al*, 2000; Horie *et al*, 2001; Garciadeblás *et al*, 2003). Null mutations in *AtHKT1;1* (Mäser *et al*, 2002a; Gong *et al*, 2004) and a reduced-function mutant allele of *AtHKT1;1* (Berthomieu *et al*, 2003) cause Na⁺ over-accumulation in shoots, resulting in leaf chlorosis. Detailed ²²Na⁺ influx studies showed that *AtHKT1;1* does not mediate Na⁺ influx into *Arabidopsis* roots (Berthomieu *et al*, 2003; Essah *et al*, 2003). Recent studies have shown that *AtHKT1;1* in *Arabidopsis* and its closest homologue, SKC1 or OshKT1;5 in rice, function by removing Na⁺ from the xylem sap, thus reducing Na⁺ accumulation in leaves (Ren *et al*, 2005; Sunarpi *et al*, 2005; Horie *et al*, 2006). Unlike *AtHKT1;1*, which is a single-copy gene in *Arabidopsis thaliana*, seven full-length *OshKT* genes were identified in the japonica rice genome based on the completed genome sequence (Garciadeblás *et al*, 2003).

Molecular genetic identification of transporters that mediate Na⁺ influx into plant roots is crucial for developing models of Na⁺ uptake mechanisms into plant roots. In this study, we have isolated and characterized *OshKT2;1*-disrupted rice plants from a *Tos17*-tagged population (Hirochika, 1997, 2001; Yamazaki *et al*, 2001), to determine the physiological role of the *OshKT2;1* transporter in rice. Here, we demonstrate that *oshkt2;1* loss-of-function mutant alleles cause a dramatic reduction in Na⁺ influx into K⁺-starved rice roots. We further demonstrate that *OshKT2;1* plays an exclusive role in nutritional Na⁺ uptake into K⁺-starved rice roots, with an apparent Na⁺ affinity (K_m) of ≈ 280 to 330 μ M. Interestingly, *OshKT2;1*-mediated Na⁺ influx does not cause Na⁺ toxicity, owing to a rapid down-regulation of the *OshKT2;1* transporter upon Na⁺ stress.

Results

Isolation of *oshkt2;1* mutants and corresponding *TosWT* plants

Ion selectivity analyses of *OshKT2;1* expressed in yeast and *Xenopus* oocytes showed that *OshKT2;1* functions as a Na⁺ transporter in these heterologous expression systems (Horie *et al*, 2001; Mäser *et al*, 2002b; Garciadeblás *et al*, 2003). But the *in vivo* function of *OshKT2;1* in rice remains unknown. We searched the rice *Tos17* insertion mutant database (Hirochika, 1997, 2001; Miyao *et al*, 2003) for a putative *OshKT2;1* gene-disrupted line to be used for uncovering the physiological role of *OshKT2;1* in rice plants. The retro-transposon *Tos17* undergoes local transposition events only during tissue culture, but is stable and non-motile in rice plants. We identified five putative *OshKT2;1* insertion alleles. Among them, three *oshkt2;1* alleles, named *oshkt2;1-1*, *oshkt2;1-2* and *oshkt2;1-3* (Figure 1A), were chosen for further characterization, based on the fertility of plants and the germination rate of seeds of the next generation. In addition, we pursued isolation of related *OshKT2;1* wild-type (WT) control plants, named 'TosWT'. Each *Tos17* mutant line comprises an average of 8–10 insertions, which demands additional WT controls for characterization of mutants. *TosWT* control lines were isolated from the same seed populations as *oshkt2;1-1*, *oshkt2;1-2* and *oshkt2;1-3* by screening for individuals that show no insertion in the *OshKT2;1* gene. Southern hybridization showed polymorphisms in autoradiographs, confirming the insertion in *OshKT2;1* in each line

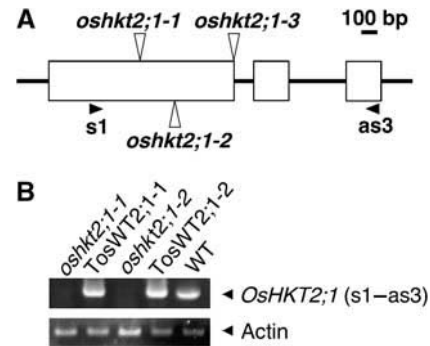


Figure 1 Isolation of homozygous *Tos17* insertion mutants in the *OshKT2;1* gene. (A) A schematic diagram of *oshkt2;1-1*, *oshkt2;1-2* and *oshkt2;1-3* alleles. White boxes represent exons. *oshkt2;1-3* has an insertion at the exon–intron boundary. s1 and as3 are primers used for RT-PCR. (B) RT-PCR analyses using cDNA derived from whole plant tissues of *oshkt2;1* mutant and WT plants (10-day old plants), which were grown under K⁺ starvation conditions.

(data not shown). RT-PCR analyses using the primer set shown in Figure 1A showed that mature *OshKT2;1* mRNA is missing in *oshkt2;1-1*, *oshkt2;1-2* plants (Figure 1B) and *oshkt2;1-3* (Supplementary Figure 1A). These data show the disruption of the *OshKT2;1* gene in the *oshkt2;1-1*, *oshkt2;1-2* and *oshkt2;1-3* mutant lines.

oshkt2;1 mutant plants exhibit reduced growth under low Na⁺ and K⁺-starved conditions

oshkt2;1 and *TosWT* plants showed normal growth and were indistinguishable from WT cv. Nipponbare plants, either on soil or on ordinary nutrient media (Figure 2A and data not shown). A low-affinity Na⁺ uptake mode of *OshKT2;1* has been hypothesized based on functional analyses, using heterologous expression systems (Horie *et al*, 2001). We thus first imposed NaCl stress (50–200 mM) on both soil grown and hydroponically grown plants. However, no notable difference in the visual phenotypes was observed between *oshkt2;1* and WT plants (data not shown).

The accumulation of *OshKT2;1* mRNA was reported to dramatically increase in response to K⁺ starvation (Horie *et al*, 2001; Garciadeblás *et al*, 2003). Ten-day old *oshkt2;1* plants grown in a 1 mM CaSO₄ (K⁺ deprived) solution showed no visible growth defects and no differences in the fresh weight compared with *TosWT* plants (Figure 2B, $P > 0.36$ for *TosWT2;1-1* versus *oshkt2;1-1*; $P > 0.08$ for *TosWT2;1-2* versus *oshkt2;1-2*; Supplementary Figure 2A, $P > 0.22$ for *TosWT2;1-3* versus *oshkt2;1-3*). The plants were subsequently grown for 15 additional days in hydroponic culture medium containing 0.5 mM NaNO₃ (see Materials and methods). Fifteen days later, *oshkt2;1-1*, *oshkt2;1-2* and *oshkt2;1-3* mutant plants showed remarkably reduced growth, accompanied with a withering of the oldest leaf compared with *TosWT2;1-1*, *TosWT2;1-2*, *TosWT2;1-3* and WT plants (Figure 2C and D and Supplementary Figure 2B and C). Fresh weights of *oshkt2;1* mutant plants were reduced approximately by 30–40% compared with *TosWT* plants (Figure 2E, $P < 1.6 \times 10^{-5}$ for *TosWT2;1-1* versus *oshkt2;1-1*; $P < 7.1 \times 10^{-7}$ for *TosWT2;1-2* versus *oshkt2;1-2*; Supplementary Figure 2D, $P < 5.7 \times 10^{-6}$ for *TosWT2;1-3* versus *oshkt2;1-3*). These data show that the *OshKT2;1* transporter

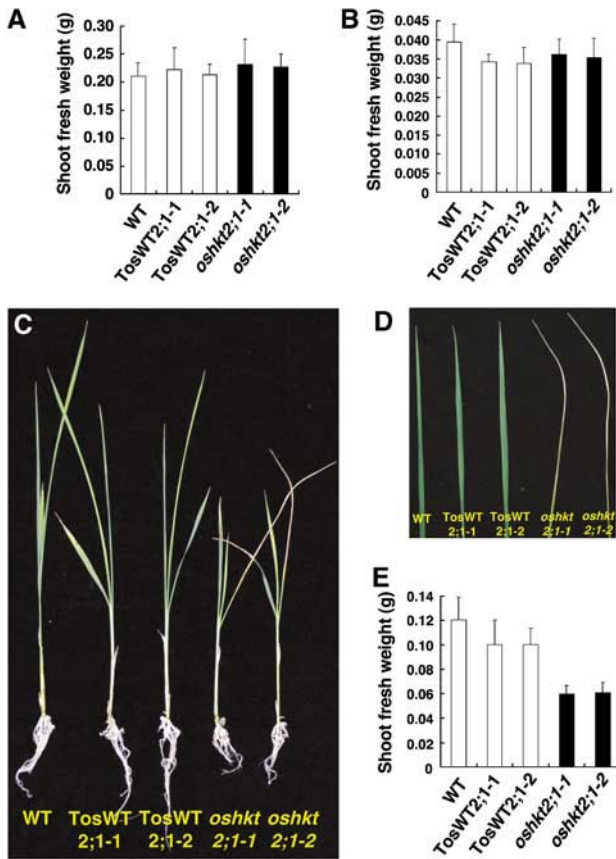


Figure 2 *oshkt2;1* mutant plants show reduced growth under low Na⁺ and K⁺-starved growth conditions. (A) Fresh weights of 25-day-old WT, TosWT2;1-1, TosWT2;1-2, *oshkt2;1-1* and *oshkt2;1-2* plants, which were grown under 1.25 mM K⁺ conditions for the last 15 days ($n = 12$; \pm s.d.). (B) Fresh weights of WT, TosWT2;1-1, TosWT2;1-2, *oshkt2;1-1* and *oshkt2;1-2* plants, which were germinated and grown in 1 mM CaSO₄ solution for 10 days ($n = 18$; \pm s.d.). (C, D) Twenty-five-day-old plants, which were grown under hydroponic conditions for the last 15 days in the presence of 0.5 mM Na⁺ in a K⁺-free medium. *oshkt2;1* mutant plants showed reduced growth (C) and chlorotic withering of the oldest leaves (D). Representative photographs of plants are shown and were observed in three independent experiments with over 18 total plants of each line. (E) Fresh weights of 25 day-old WT, TosWT2;1-1, TosWT2;1-2, *oshkt2;1-1* and *oshkt2;1-2* plants, which were grown under the same 0.5 mM Na⁺ condition for the last 15 days as in (C) and (D) ($n = 12$; \pm s.d.).

allows improved growth of WT plants under low nutrient K⁺-starved conditions when 0.5 mM NaNO₃ was added to the growth solution.

***oshkt2;1* mutant plants accumulate less Na⁺ in both shoots and roots**

We determined Na⁺ contents of *oshkt2;1* mutant, TosWT and WT plants. Ten-day-old plants grown in 1 mM CaSO₄ solution were transferred onto minimal medium containing 0.5 mM Na⁺ and grown for nine additional days. Interestingly, *oshkt2;1-1*, *oshkt2;1-2* and *oshkt2;1-3* mutant plants were found to accumulate considerably less Na⁺ in both roots and shoots compared with TosWT and WT plants (Figure 3 and Supplementary Figure 3). These results show that *OshKT2;1* functions in Na⁺ accumulation in roots and also in shoots.

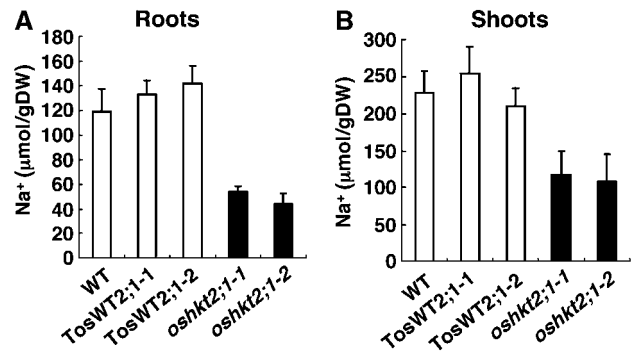


Figure 3 *oshkt2;1* mutant plants accumulate less Na⁺ in roots and shoots. Nineteen-day-old plants, which were hydroponically cultured under 0.5 mM Na⁺- and K⁺-free conditions for the last 9 days, were used. Na⁺ contents of roots (A) and shoots (B) were measured by ICP-OES. Error bars represent standard deviations ($n = 6$).

***OshKT2;1* expression in cortical and endodermal cells in roots and in vascular bundle regions in leaves**

Transgenic rice plants carrying the 1.6 kb *OshKT2;1* promoter- β -glucuronidase (GUS) or green fluorescence protein (GFP) gene constructs were produced in order to determine the expression pattern of *OshKT2;1*. Strong GUS signals were found in the main root but not in the root tip region (Figure 4A and B). Sections of GUS-stained roots further showed that cortex cells and endodermis cells were strongly stained (Figure 4C, see red stain). Sections of GUS-stained leaves showed the expression of *OshKT2;1* in vascular bundle regions (Figure 4D and E).

Tissue expression of *OshKT2;1* was further analyzed using transgenic plants expressing GFP driven by the 1.6 kb *OshKT2;1* promoter. Roots of hygromycin B-resistant plants were briefly stained with propidium iodide (Swarup *et al*, 2004). Fluorescence was analyzed by confocal microscopy. A strong GFP fluorescence was found in root cortex cells of K⁺-starved main roots (Figure 4 F, H and I). The tissue specificity of *OshKT2;1* expression in K⁺-starved roots of japonica rice presented in this study overlaps with, but also slightly differs from the expression pattern in indica rice varieties (Gollmack *et al*, 2002). The reason for this discrepancy could be the difference in the regulation of *OshKT2;1* expression between the two rice varieties and experimental conditions.

To analyze the transcriptional regulation of *OshKT2;1* in response to external K⁺ and Na⁺ concentrations, *OshKT2;1* promoter-GFP plants were grown in 1 mM CaSO₄ solution supplemented with either 30 mM K⁺ or 30 mM Na⁺. Addition of 30 mM K⁺ or 30 mM Na⁺ led to substantial reductions in GFP fluorescence of root cells (Figure 4J and K) compared with those of K⁺-starved roots (Figure 4H). GFP and propidium iodide fluorescence intensities were quantified based on 3D reconstruction images obtained by z-series stacking and GFP fluorescence intensities and normalized to propidium iodide fluorescence intensities. The average GFP fluorescence intensity of K⁺-starved roots was approximately five times higher than that of 30 mM K⁺- or 30 mM Na⁺-treated roots (Figure 4L). Taken together, these results demonstrate that the *OshKT2;1* gene is mainly expressed at the cortex cell layer and the endodermal cell layer of K⁺-starved japonica rice roots, and that *OshKT2;1* gene expres-

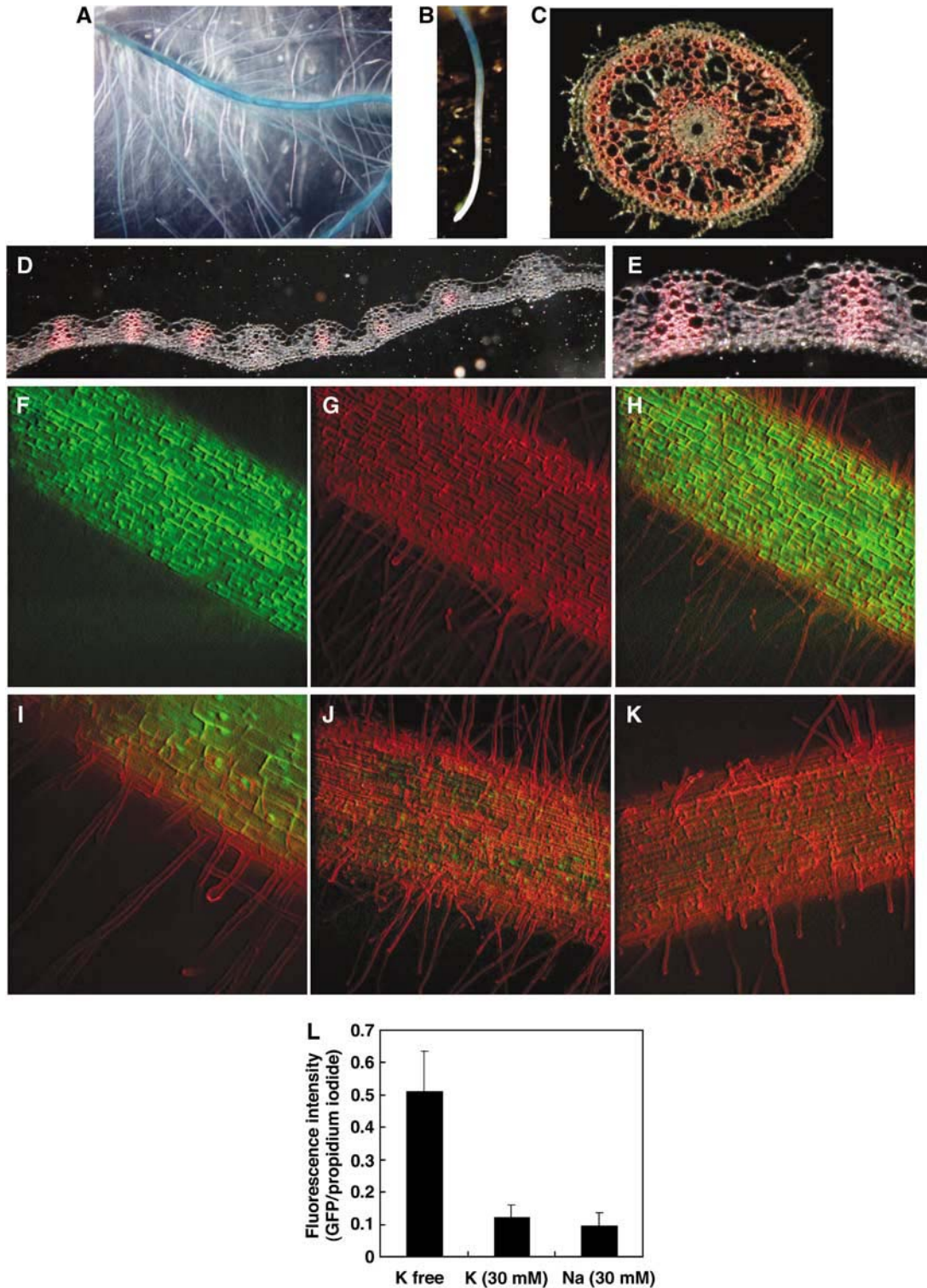


Figure 4 *OsHKT2;1* gene expression in the cortex and endodermis of K⁺-starved roots and in leaf vascular bundles. Transgenic rice plants expressing GUS or GFP reporter genes under the control of a 1.6 kb *OsHKT2;1* promoter were grown in 1 mM CaSO₄ solution in the presence of hygromycin. (A, B) Strong GUS staining was detected in main roots (A) but not in root tip regions (B). (C) A dark-field microscopic image of sections derived from GUS-stained main roots. Sections of GUS-stained (red staining) main roots showed strong signals in cortical and endodermal cells. (D, E) Dark-field microscopic images of sections derived from GUS-stained leaves. The sections showed strong GUS signals in vascular bundle regions. In (E) is an enlarged image of a section of the region shown in (D). Note that the reddish staining in (C–E) shows GUS signals, as these were obtained using dark-field microscopy. (F) A 3D reconstruction image of GFP fluorescence derived from K⁺-starved roots of *OsHKT2;1* promoter-GFP plants, showing strong GFP fluorescence in root cortex cells. (G) A 3D reconstruction image of propidium iodide fluorescence derived from K⁺-starved roots of the same plant shown in (F). (H) Combined images of GFP and propidium iodide fluorescence shown in (F) and (G). (I) An enlarged image of K⁺-starved roots shown in (H). (J, K) Combined images of GFP and propidium iodide fluorescence derived from 10-day-old *OsHKT2;1* promoter-GFP plants grown in 1 mM CaSO₄ solution supplemented with either 30 mM K⁺ (J) or 30 mM Na⁺ (K). (L) GFP fluorescence from three different conditions were quantified and normalized relative to propidium iodide fluorescence. Fluorescence intensities of GFP and propidium iodide in (J) were measured using three independent plants for each ionic condition (\pm s.d.).

sion is controlled by external K⁺ and Na⁺ concentrations at the transcriptional level.

***oshkt2;1* mutant plants accumulate less Na⁺ in the xylem sap**

We next determined Na⁺ contents of the xylem sap. Ten-day-old *oshkt2;1* mutant and TosWT plants grown in 1 mM Ca²⁺ solution were transferred to hydroponic nutrient medium containing 0.5 mM Na⁺ and cultured for 2 days. Xylem sap was collected and Na⁺ concentrations were determined by inductively coupled plasma-optic emission spectroscopy (ICP-OES). The results clearly showed that *oshkt2;1-1* and *oshkt2;1-2* mutant plants contained much less Na⁺ compared with TosWT plants (Figure 5). Na⁺ levels in the xylem sap of *oshkt2;1-1* were reduced by approximately 81.0% compared with TosWT2;1-1 (Figure 5A, *P* < 0.0008 for *oshkt2;1-1* versus TosWT2;1-1) and those of *oshkt2;1-2* were reduced by approximately 89% compared with TosWT2;1-2 (Figure 5B, *P* < 0.001 for *oshkt2;1-2* versus TosWT2;1-2).

***OsHKT2;1* localization at the plasma membrane**

To gain insight into the subcellular localization of the OsHKT2;1 protein in plant cells, a chimeric EGFP-*OsHKT2;1* cDNA was constructed and placed downstream of the CaMV 35S promoter. Transient expression analyses were performed using tobacco leaf mesophyll cells. A bright fluorescence bordering the cell was found in mesophyll cells expressing EGFP-*OsHKT2;1* (Figure 6A). Cells were subsequently stained with the plasma membrane marker FM4-64 (Bolte *et al*, 2004). As shown in Figure 6B, FM4-64-derived red fluorescence was detected at the border of cells. Combined images showed an overlap of the two different fluorescence emissions at the border of cells (Figure 6C and E), indicating that the EGFP-*OsHKT2;1* protein localizes at the plasma membrane. Fluorescence images derived from EGFP and FM4-64 were analyzed in control experiments. In controls, green fluorescence was more broadly observed in the cell, including in the nucleus (Figure 6D), compared with EGFP-*OsHKT2;1*-derived fluorescence (Figure 6A). Native red

chloroplast fluorescence showed the localization of chloroplasts, in addition to the plasma membrane staining with FM4-64 (Figure 6D). Combined images showed that EGFP-derived fluorescence and FM4-64-derived fluorescence did not overlap at the plasma membrane (Figure 6D and F), indicating that the EGFP control protein does not localize at the plasma membrane.

Subcellular localization of EGFP-*OsHKT2;1* was also tested using *Arabidopsis* leaf epidermal cells. An EGFP-*OsHKT2;1* and a control construct were introduced into epidermal cells by particle bombardment. Epidermal cells expressing the EGFP-*OsHKT2;1* construct showed fluorescence at the border of cells (Figure 6G). In contrast, cells harboring a control

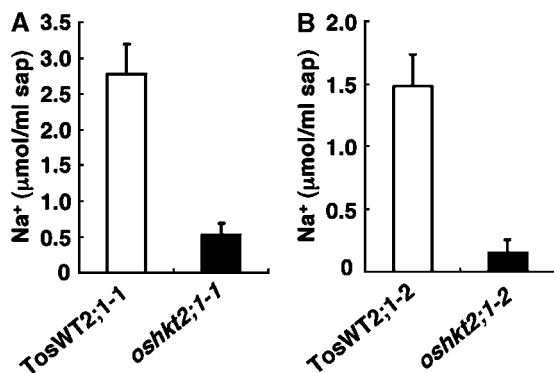


Figure 5 *oshkt2;1* mutant plants accumulate less Na⁺ in the xylem sap. Ten-day-old seedlings grown in 1 mM CaSO₄ solution were subsequently grown in 0.5 mM Na⁺- and K⁺-free conditions two more days. Xylem sap was collected from WT, TosWT2;1-1, TosWT2;1-2, *oshkt2;1-1* and *oshkt2;1-2*. Na⁺ contents were measured by ICP-OES. (A) TosWT2;1-1 versus *oshkt2;1-1*. (B) TosWT2;1-2 versus *oshkt2;1-2*. Xylem sap extractions for (A) were performed at a different time of year than (B). Error bars represent standard error (*n* = 8).

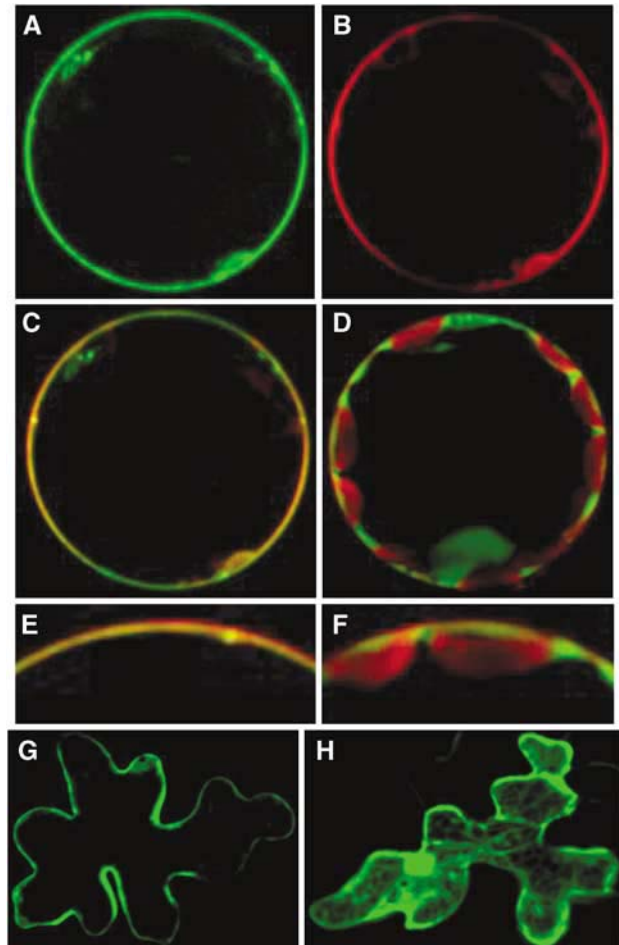


Figure 6 *OsHKT2;1* localizes at the plasma membrane of plant cells. EGFP-*OsHKT2;1* cDNA and EGFP were transiently expressed in protoplasts of tobacco mesophyll cells (A–F) and *Arabidopsis* epidermal cells (G, H) under the control of the 35S promoter. Fluorescence was analyzed by confocal microscopy. (A) GFP fluorescence of tobacco mesophyll cell protoplast expressing EGFP-*OsHKT2;1*. (B) FM4-64 plasma membrane marker fluorescence emitted from the same protoplast shown in (A). (C) Combined images of GFP and FM4-64 fluorescence shown in (A) and (B). (D) Combined images of GFP control and FM4-64 fluorescence, derived from cells harboring a control construct. Note that native red chloroplast autofluorescence was observed in addition to red FM4-64 fluorescence at the plasma membrane (D, F). (E) An enlarged image of (C). (F) An enlarged image of (D). (G) GFP fluorescence of *Arabidopsis* epidermal cells expressing EGFP-*OsHKT2;1*. (H) GFP fluorescence of *Arabidopsis* epidermal cells harboring a GFP control construct.

construct showed ubiquitous fluorescence in the cytoplasm and in the nucleus (Figure 6H). Taken together, these transient expression analyses provide evidence that the OshKT2;1 transporter localizes at the plasma membrane of plant cells.

Severe disruption of Na⁺ influx in *oshkt2;1* mutant roots

Less Na⁺ accumulation in shoots and roots of *oshkt2;1* mutants (Figure 3) and localization analyses of OshKT2;1 (Figures 4 and 6) indicated that OshKT2;1 may mediate Na⁺ influx into rice roots. In order to test this hypothesis, we performed short-term tracer influx experiments using ²²Na⁺. Time-dependent Na⁺ influx into roots of intact rice plants was monitored at 0.1 mM external Na⁺ using 10-day-old *oshkt2;1-1*, *oshkt2;1-2*, *oshkt2;1-3*, TosWT2;1-1, TosWT2;1-2, TosWT2;1-3 and WT plants, which were grown in 1 mM CaSO₄ solution. Interestingly, short-term Na⁺ influx into rice roots at 0.1 mM external Na⁺ was almost completely abolished in *oshkt2;1-1*, *oshkt2;1-2* and *oshkt2;1-3* plants, while TosWT2;1-1, TosWT2;1-2, TosWT2;1-3 and WT plants

exhibited similar Na⁺ influx time courses (Figure 7A and Supplementary Figure 4A).

Detailed concentration-dependent Na⁺ influx studies were performed in the 'high-affinity' Na⁺ uptake range, at 1, 5, 10, 25, 50, 100 and 200 μM external Na⁺, using *oshkt2;1* mutant plants and TosWT plants. *oshkt2;1-1* and *oshkt2;1-2* plants showed severe Na⁺ influx reductions compared with TosWT2;1-1 and TosWT2;1-2 plants (Figure 7B; 92.7% reduction at 200 μM). We further characterized ²²Na⁺ influx kinetics of *oshkt2;1* mutants and TosWT plants at higher external Na⁺ concentrations of up to 5000 μM. Sodium influx into roots of *oshkt2;1-1* and *oshkt2;1-2* plants within this range was severely reduced compared with that of TosWT2;1-1 and TosWT2;1-2 (Figure 7C). Sodium influx in *oshkt2;1* mutant lines showed a reduction of ²²Na⁺ influx by approximately 88–90% of WT controls at 500 μM Na⁺, 86–88% at 1000 μM Na⁺ and 63–67% at 5000 μM Na⁺, respectively.

Michaelis–Menten analyses were performed using concentration-dependent Na⁺ influx data obtained at 1–5000 μM external Na⁺. A single-uptake phase formalism described the

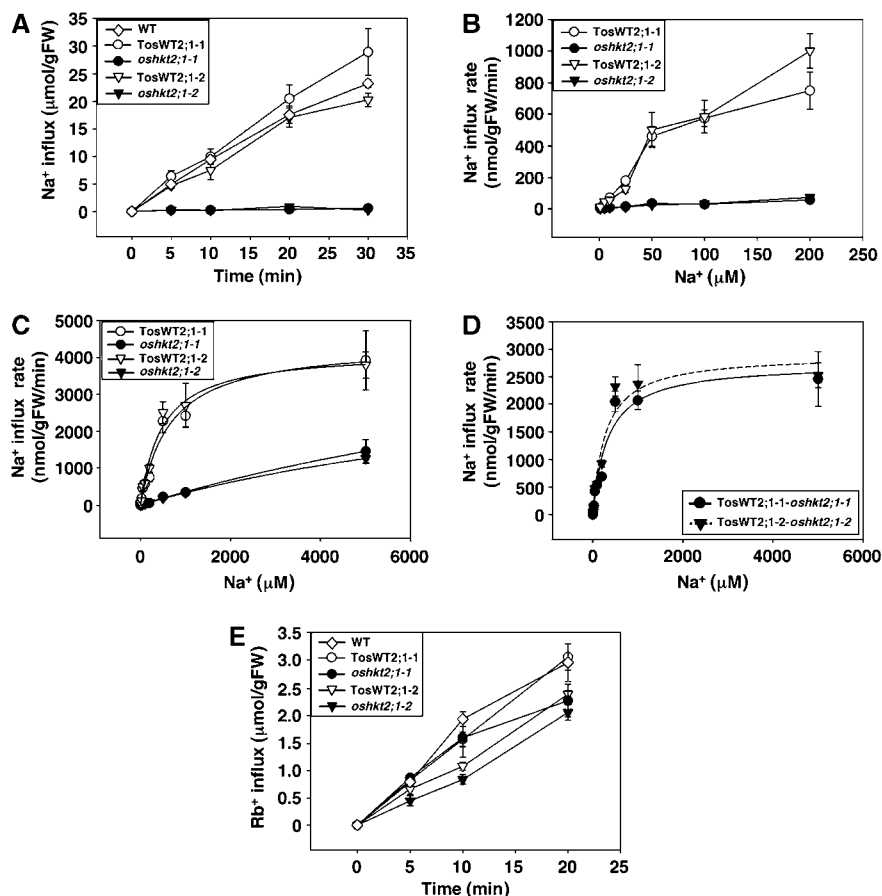


Figure 7 Short-term ²²Na⁺ influx analyses show that OshKT2;1 functions as a major Na⁺ uptake pathway in K⁺-starved rice roots. Plants were grown in 1 mM CaSO₄ solution for 10 days. Time-dependent and concentration-dependent ²²Na⁺ influx experiments were performed using ²²NaCl as a tracer. (A) Time-dependent Na⁺ influx into roots of WT, TosWT2;1-1, TosWT2;1-2, *oshkt2;1-1* and *oshkt2;1-2* at 0.1 mM external Na⁺ ($n = 3; \pm$ s.d.). (B) Concentration-dependent Na⁺ influx into roots of TosWT2;1-1, TosWT2;1-2, *oshkt2;1-1* and *oshkt2;1-2* at 1, 5, 10, 25, 50, 100 and 200 μM external Na⁺ ($n = 3; \pm$ s.d.). (C) Concentration-dependent Na⁺ influx into roots of TosWT2;1-1, TosWT2;1-2, *oshkt2;1-1* and *oshkt2;1-2* at 1–5000 μM external Na⁺ ($n = 3; \pm$ s.d.). Curves in (C) are plotted separately for each WT and *oshkt2;1* mutant allele derived from Michaelis–Menten analyses of the data (Table 1a). (D) OshKT2;1-dependent Na⁺ influx rates of rice roots. Differences in Na⁺ influx rates between TosWT and *oshkt2;1* mutant plants are shown (\pm s.d.). Curves in (D) were derived from Michaelis–Menten analyses of the data (Table 1b). (E) Time-dependent Rb⁺ (K⁺) influx into roots of WT, TosWT2;1-1, TosWT2;1-2, *oshkt2;1-1* and *oshkt2;1-2* at 0.1 mM external Rb⁺ ($n = 6; \pm$ s.d.).

Table I Michaelis–Menten curve-fitting results for ²²Na⁺ influx kinetics in intact rice roots of K⁺-starved *oshkt2;1* mutant and TosWT2;1 control plants (Figure 7C), and for OsHKT2;1-dependent Na⁺ uptake rates in rice roots (Figure 7D)

		R ²	K _M (μM)	V _{max} (nmol/gFW/min)
a	TosWT2;1-1	0.981	655.52 ± 114.17	4390.10 ± 272.26
	TosWT2;1-2	0.985	477.82 ± 74.45	4176.83 ± 219.04
	<i>oshkt2;1-1</i>	0.998	15 764.47 ± 3639.95	6051.83 ± 1082.91
	<i>oshkt2;1-2</i>	0.999	11 499.50 ± 579.23	4165.29 ± 149.14
b	TosWT2;1-1- <i>oshkt2;1-1</i>	0.958	280.4 ± 74.8	2901.1 ± 236.6
	TosWT2;1-2- <i>oshkt2;1-2</i>	0.964	330.9 ± 81.6	2744.5 ± 213.2

Curve fitting was performed with SigmaPlot 8.0 (SPSS, Chicago) software. Results from a one-term Michaelis–Menten equation. Fitting results to the data derived from TosWT and *oshkt1* mutant plants are shown in (a) (see results and Figure 7C). (b) Fitting results to the values, which represent the difference in Na⁺ influx rates between *oshkt2;1* mutant and TosWT2;1 plants.

kinetics of Na⁺ influx better in rice roots than a two-uptake phase analysis (Table Ia). The fitting results are plotted in Figure 7C for each WT and mutant allele. These results demonstrated that the disruption of the *OsHKT2;1* gene dramatically affected the affinity of rice roots for Na⁺ and caused a severe Na⁺ uptake reduction (average K_m shift from 550 μM to 13.6 mM) (Table Ia). To approximate OsHKT2;1-dependent Na⁺ influx into rice roots, Na⁺ influx rates of *oshkt2;1* mutants were subtracted from the corresponding rates of TosWT plants. Michaelis–Menten analysis predicted an apparent affinity for the OsHKT2;1-dependent Na⁺ influx into roots of ≈280 to 330 μM Na⁺ and a V_{max} of ≈2750 to 2900 nmol/gFW/min (Figure 7D; Table Ib). These flux analyses demonstrate that OsHKT2;1 mediates Na⁺ influx into roots and composes a major component for Na⁺ uptake into K⁺-starved rice roots in a broad concentration range.

Intact K⁺ (Rb⁺) uptake in *oshkt2;1* mutant plants

To determine whether *OsHKT2;1* mutations have an influence on K⁺ uptake in intact rice roots, tracer influx experiments using ⁸⁶Rb⁺ were performed. *oshkt2;1* mutant, TosWT and WT plants, grown in 1 mM CaSO₄ solution, were used for time-dependent Rb⁺ influx assays at 100 μM external Rb⁺. Only very small differences were detected between *oshkt2;1* mutants and the WT lines, which showed a linear increase in Rb⁺ influx under the imposed condition (Figure 7E), in contrast to Na⁺ influx (Figure 7A). These results show that mutations in the *OsHKT2;1* gene do not have a strong impact on high-affinity K⁺ (Rb⁺) uptake into intact rice roots. Small differences may be due to indirect effects of the *oshkt2;1* mutations (e.g. on membrane potential).

Toxic sodium levels rapidly downregulate *OsHKT2;1* transporter function in rice roots

To determine whether OsHKT2;1-mediated Na⁺ influx is affected by the amount of applied K⁺ or Na⁺ concentrations, *oshkt2;1* mutant, TosWT and WT plants were grown in 1 mM CaSO₄ solution including either 5 mM KCl or 30 mM NaCl. ²²Na⁺ influx was monitored at 0.1 mM external Na⁺ (Figure 8A and Supplementary Figure 4B) and 1.0 mM external Na⁺ (Figure 8B and Supplementary Figure 4C). Interestingly, when the growth medium was supplemented with either 5 mM K⁺ or 30 mM Na⁺, Na⁺ influx into roots of TosWT and WT plants was dramatically repressed (Figure 8A and B). Comparisons with ²²Na⁺ influx rates of *oshkt2;1* knockout mutant plants demonstrated that the *OsHKT2;1*-mediated Na⁺ influx is tightly regulated by the level

of external K⁺ and Na⁺ in the growth medium (Figure 8A, and B).

The time course of Na⁺ influx repression was further analyzed. Sodium influx in WT plants at 0.1 mM Na⁺ was monitored at six time points after the addition of 30 mM NaCl into the 1 mM CaSO₄ growth solution. The ²²Na⁺ influx time course demonstrated that OsHKT2;1-dependent Na⁺ influx is rapidly repressed in response to addition of 30 mM NaCl (Figure 8C; half time = 1.45 ± 0.11 h).

Several inhibitors were applied to gain initial insight into the biological mechanism(s) that mediates the rapid repression of the OsHKT2;1 transporter upon Na⁺ stress. A solution composed of *N*-methyl-D-glucamine (NMDG) was first tested as a source of a relatively membrane impermeable cation. The results showed that 30 mM NMDG solution was not effective at evoking the repression of the OsHKT2;1 compared with 30 mM Na⁺ (Figure 8D, b). The transcriptional inhibitor actinomycin D (20 μg/ml), the translational inhibitor cycloheximide (100 μM), as well as the proteasome inhibitor MG132 (50 μM), all did not greatly affect the 30 mM NaCl-induced rapid repression (Figure 8D, d–f). On the other hand, the serine/threonine protein kinase inhibitor K252a caused a more severe repression of Na⁺ influx than the effect of 30 mM NaCl (Figure 8D, g).

Quantitative real-time RT-PCR analyses of *OsHKT2;1* mRNA upon the addition of 30 mM NaCl were subsequently performed. Interestingly, time-course analyses showed that the addition of NaCl caused a rapid reduction of *OsHKT2;1* mRNA (Figure 8E; *n* = 4 replicates). These data suggest that both posttranslational (Figure 8D) and transcript expression (Figure 8E) mechanisms control OsHKT2;1 activity in intact rice roots.

Discussion

Several *Arabidopsis* mutants such as *sos1* (Wu *et al*, 1996; Shi *et al*, 2002), *atnhx1* (Apse *et al*, 2003) and *athkt1;1* (Rus *et al*, 2001; Mäser *et al*, 2002a; Berthomieu *et al*, 2003), which are disrupted or mutated in genes encoding Na⁺ transporters, have been characterized. These reports have demonstrated that the Na⁺ transporters in *Arabidopsis*, SOS1, AtNHX1 and AtHKT1;1, function to protect *Arabidopsis* plants from salt stress by different mechanisms (Zhu, 2002; Horie and Schroeder, 2004). *HKT* cDNAs have been isolated and characterized from many plant species (Fairbairn *et al*, 2000; Uozumi *et al*, 2000; Horie *et al*, 2001; Gollmack *et al*, 2002; Garcíadeblás *et al*, 2003; Su *et al*, 2003; Ren *et al*, 2005).

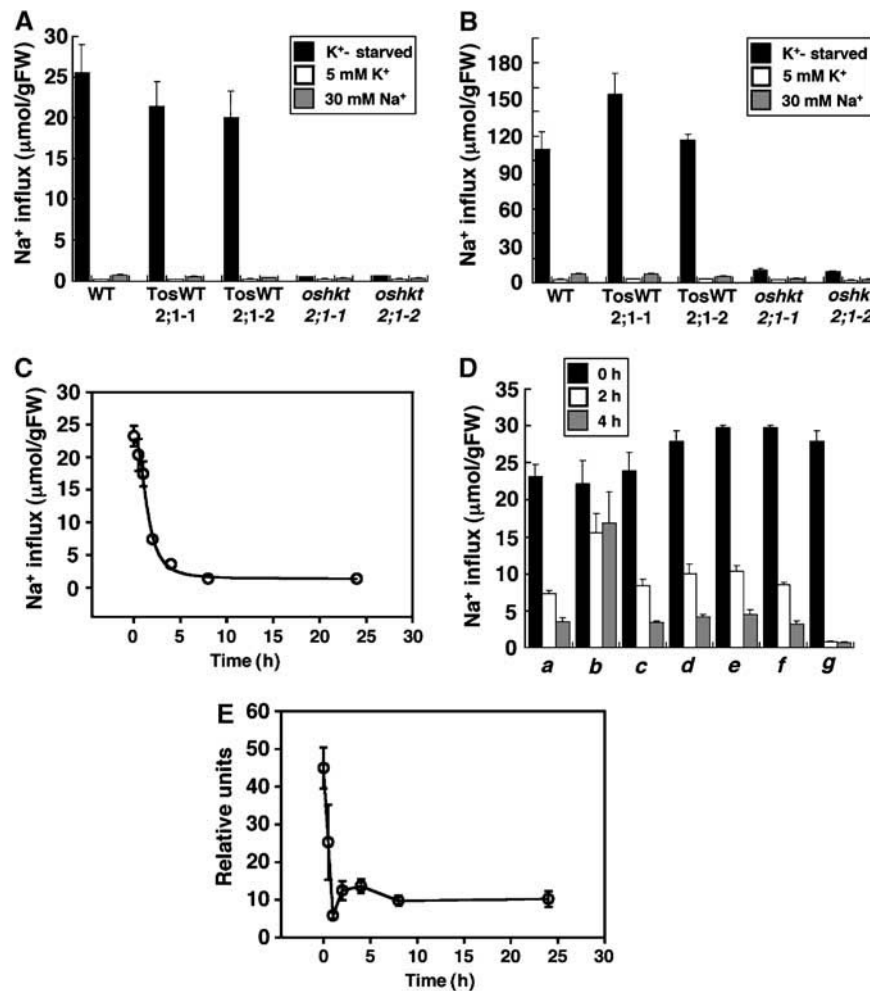


Figure 8 Sodium influx into K⁺-starved rice roots via *Oshkt2;1* is subjected to posttranslational inactivation in response to high external Na⁺. (A, B) Sodium influx into roots of 10-day-old WT, *TosWT2;1-1*, *TosWT2;1-2*, *oshkt2;1-1* and *oshkt2;1-2*, which were grown either in 1 mM CaSO₄ solution (black bars), or the same solution supplemented with 30 mM NaCl (gray bars). Sodium influx into roots was monitored at 0.1 mM external Na⁺ (A) and 1.0 mM external Na⁺ concentrations (B) ($n = 3$; \pm s.d.) 20 min after exposure of roots to the indicated solutions. (C) Time-dependent repression of Na⁺ influx into K⁺-starved rice roots via *Oshkt2;1* in response to addition of 30 mM NaCl ($n = 3$; \pm s.d.). (D) Repression of *Oshkt2;1*-mediated Na⁺ influx in response to inhibitors. Sodium influx into roots of 10-day-old WT plants grown in 1 mM CaSO₄ solution (0 h black bars) or plants incubated with the indicated test solutions for 2 h (white bars), or plants incubated with test solutions for 4 h (gray bars) are shown. Solutions contained (a) 30 mM NaCl, (b) 30 mM *N*-methyl-D-glucamine-Cl, (c) 30 mM NaCl and 0.1% DMSO, (d) 30 mM NaCl and the proteasome inhibitor MG132 (50 μ M), (e) 30 mM NaCl and actinomycin D (20 μ g/ml), (f) 30 mM NaCl and the translational inhibitor cycloheximide (100 μ M), and (g) 30 mM NaCl and 5 μ M K252a. Time-dependent sodium influx into roots shown in (C, D) was monitored at 0.1 mM external Na⁺ ($n = 3$; \pm s.d.). (E) Time-course expression analyses of the *Oshkt2;1* mRNA in roots in response to 30 mM NaCl by quantitative real-time RT-PCR. Expression levels of *Oshkt2;1* were monitored at 0, 0.5, 1, 2, 4, 8 and 24 h after the addition of 30 mM NaCl. The *Oshkt2;1* expression level of plants grown in 1 mM CaSO₄ solution supplemented with 30 mM KCl was also analyzed as a reference of basal *Oshkt2;1* expression. All data were normalized to the level of the constitutively expressed *OsSMT3* mRNA expression. *Oshkt2;1* mRNA levels are plotted relative to the basal *Oshkt2;1* expression level. Data at each time point show the average \pm s.e. of four independent experiments.

TaHKT2;1-antisense wheat plants have been shown to exhibit an approximately 42% decrease in ²²Na⁺ uptake at a high 100 mM external NaCl concentration, but no substantial difference in ²²Na⁺ influx at 20 and 50 mM NaCl concentrations (Laurie *et al*, 2002). Despite these advances, no strong Na⁺ influx genetic mutant line has been identified in plants, indicating overlapping redundancies in Na⁺ transporters that mediate Na⁺ influx into plants (Schroeder *et al*, 1994). The present findings reveal that *Oshkt2;1* mediates a major contribution to Na⁺ influx into K⁺-starved roots. An approximately 10–30% Na⁺ influx activity at millimolar concentrations remains in *oshkt2;1-1* and *oshkt2;1-2* mutant plants (Figures 7 and 8). Studies investigating whether the remaining Na⁺ influx in intact rice roots depends on other

Oshkt transporters and/or different Na⁺ permeable channels/transporters will be an interesting subject for future inquiry. Genome analysis of rice cv. Nipponbare led to the identification of seven expressed *Oshkt* genes (Garcia-deblás *et al*, 2003). *Oshkt1;5* (*Oshkt8* or *SKC1*) and its closest homologue in *Arabidopsis*, *AtHKT1;1* (*AtHKT1*), have been shown to function in salt tolerance by reducing Na⁺ levels in the xylem sap (Ren *et al*, 2005; Sunarpi *et al*, 2005; Horie *et al*, 2006). More recently from initial mapping experiments, the *TaHKT7* (*TaHKT1;4*) and *TaHKT8* (*TaHKT1;5*) genes in durum wheat, close homologues of *AtHKT1;1*, have also been hypothesized to play a protective role in maintaining viability of wheat leaves under saline conditions (Huang *et al*, 2006; Byrt *et al*, 2007).

Genetic evidence that Na⁺ enhances growth of rice plants and a major role for OsHKT2;1 in nutritional Na⁺ uptake

Unlike K⁺, which is an essential macronutrient and the most abundant cation in plant cells, Na⁺ is not required as an essential nutrient by most higher plants, including glyco-phytes, despite the fact that the availability of Na⁺ and K⁺ is in general similar. On the other hand, however, classic plant physiological studies have shown that moderate amounts of Na⁺ enhance the growth of many plant species (Mengel and Kirkby, 1982; Flowers and Läuchli, 1983). Nutritional effects of Na⁺ have been found on the growth of beet and tomato plants (Woolley, 1957; Marschner, 1971; Besford, 1978). Despite these physiological observations, no genetic evidence for growth enhancement by Na⁺ has been obtained and genes mediating this process have not been identified.

oshkt2;1-1 and *oshkt2;1-2* mutant plants were indistinguishable from WT plants under K⁺-rich nutrient conditions (Figure 2A and data not shown). Interestingly, however, *oshkt2;1-1*, *oshkt2;1-2* and *oshkt2;1-3* plants exhibited reduced growth, accompanied by leaf chlorosis under low Na⁺ and K⁺-limiting conditions (Figure 2C–E and Supplementary Figure 2B–D). These results, together with Na⁺ transport, accumulation and OsHKT2;1 localization analyses, provide first genetic evidence that Na⁺ enhances growth of rice plants under K⁺-starved condition, and that the plasma membrane transporter/channel OsHKT2;1 plays a central role in acquiring and distributing Na⁺ as a ‘nutrient’. Potassium depletion strongly induces *OsHKT2;1* gene expression (Figure 4H) (Horie *et al*, 2001; Gollmack *et al*, 2002; Garcíadeblás *et al*, 2003) and OsHKT2;1-dependent Na⁺ influx in roots (Figure 8A and B). However, Na⁺ does not completely rescue rice plants from K⁺ deficiency (Figure 2A and E). Presumably, Na⁺ replaces only some of the roles of K⁺, which functions in many processes, including osmotic adjustment, enzyme activation, cell expansion, maintenance of membrane potential, protein synthesis and photosynthetic activity. Possible main functions of Na⁺ for cellular viability in K⁺-starved plants may be maintenance of turgor pressure and cell expansion.

K⁺ (Rb⁺) uptake and the OsHKT2;1 transporter in K⁺-starved rice roots

A Na⁺-selective has been detected in ion selectivity analyses of OsHKT2;1 expressed in heterologous expression systems (Horie *et al*, 2001; Mäser *et al*, 2002b; Garcíadeblás *et al*, 2003), with an exception in which similar K⁺ and Na⁺ transport were observed (Gollmack *et al*, 2002). To test whether dysfunctional mutations in the *OsHKT2;1* gene affect net K⁺ influx *in planta*, we performed short-term ⁸⁶Rb⁺ tracer influx analyses and compared the results between *oshkt2;1* mutants and WT plants. Small reductions in ⁸⁶Rb⁺ influx were detected in ⁸⁶Rb⁺ influx assays performed at 0.1 mM external RbCl (Figure 7E). Two not mutually exclusive explanations may account for this difference. OsHKT2;1 may have a small permeability to K⁺. Alternatively, disruption of the *OsHKT2;1* gene may affect other K⁺ influx transporters, for instance, by causing less hyperpolarized membrane potential in the Na⁺-free Rb⁺ influx buffers used for Rb⁺ influx assays. A slightly less hyperpolarized membrane potential in *oshkt2;1* plants would

indirectly result in decreasing net Rb⁺ (K⁺) influx in *oshkt2;1* mutant roots compared to WT plants. The relatively small effect of *oshkt2;1* disruption on Rb⁺ influx compared with Na⁺ influx shows that OsHKT2;1 mainly transports Na⁺ *in planta* under the imposed conditions.

Downregulation of OsHKT2;1 in response to high levels of sodium

Sodium over-accumulation in plant cells triggers diverse toxicities by perturbing vital cellular processes and reduces plant viability (Glenn *et al*, 1999; Zhu, 2002; Ward *et al*, 2003). Sodium influx into barley roots has been found to show multiple kinetic components (Rains and Epstein, 1967a), and an increase in the concentration of either K⁺ or Ca²⁺ in 50 mM Na⁺ solution led to reductions of low-affinity Na⁺ influx into K⁺-starved barley roots (Rains and Epstein, 1967b), and in a reduction in external Na⁺ depletion at 100 μM external Na⁺ (Haro *et al*, 2005). *OsHKT2;1*-expressing yeast cells showed Na⁺ hypersensitivity even under relatively mild stress conditions and OsHKT2;1 expressed in *Xenopus* oocytes yielded large Na⁺-selective inward currents at 100 mM external Na⁺ concentration (Horie *et al*, 2001). Furthermore, we found in this study that OsHKT2;1 mediates Na⁺ influx into rice roots, with substantial V_{max} values of ≈2700 to 2900 nmol/gFW/min (Figures 7C and D, 8A; Table Ib). It cannot be excluded that a portion of the OsHKT2;1-dependent Na⁺ influx into intact roots (Figures 7, 8A and B) may be mediated by other transporters that are influenced by OsHKT2;1 activity (Schroeder *et al*, 1994). On the other hand, *oshkt2;1* knockout would cause more hyperpolarized potentials in the presence of Na⁺, which can drive more Na⁺ influx via other transporters, and thus ²²Na⁺ influx analyses of *oshkt2;1* mutants may underestimate Na⁺ influx transported by OsHKT2;1.

OsHKT2;1 has been suggested to mediate high-affinity Na⁺ uptake in roots of rice plants (Garcíadeblás *et al*, 2003). The determined K_m values in this study differ to a degree from the K_m (20 μM) values for OsHKT2;1 expressed in yeast (Garcíadeblás *et al*, 2003). The reason for this difference may be several factors, including homologous versus heterologous expression of OsHKT2;1, different membrane potentials and possibly measurements of external Na⁺ depletion in yeast (Garcíadeblás *et al*, 2003) compared to ²²Na⁺ influx here.

At high Na⁺ concentrations of more than 25 mM, an apoplastic Na⁺ influx pathway contributes to Na⁺ uptake into rice roots (Yeo *et al*, 1987). The present study shows a major contribution of OsHKT2;1 to Na⁺ influx at a wide concentration range. The maximum Na⁺ influx rates of low-affinity Na⁺ uptake components in K⁺-starved barley roots, determined in the presence of 0.5–50 mM NaCl, can be estimated to approximately 4–5 times smaller than the V_{max} values of *OsHKT2;1*-dependent Na⁺ influx (Rains and Epstein, 1967a) (Table Ib). Maximum *OsHKT2;1*-dependent Na⁺ influx rates with 1 mM Ca²⁺ in the influx buffer (Figure 7D) were approximately twice as high as the Na⁺ influx rate of *Arabidopsis* roots exposed to 50 mM NaCl and mM Ca²⁺ (Essah *et al*, 2003). These findings and comparisons show that OsHKT2;1 can mediate substantial Na⁺ influx rates in intact plants and suggest that OsHKT2;1-mediated Na⁺ influx would potentially cause Na⁺ toxicity in K⁺-starved rice plants. In contrast, however, *oshkt2;1-1*

and *oshkt2;1-2* plants did not show any difference in salt sensitivity when treated with several high concentrations of NaCl, even under K⁺-starved conditions (data not shown).

To investigate whether OsHKT2;1 contributes to toxic Na⁺ influx, we performed tracer influx experiments, adding high levels of Na⁺ to K⁺-starved roots. Interestingly, OsHKT2;1-dependent Na⁺ influx was rapidly repressed upon the addition of 30 mM NaCl to K⁺-starved roots (Figure 8C). *OsHKT2;1* mRNA was also found to undergo rapid reduction in response to the addition of NaCl (Figure 8E). Use of proteasome, transcriptional, translational and protein kinase inhibitors suggests that rapid downregulation of OsHKT2;1-dependent Na⁺ influx might not be directly and solely mediated by ubiquitination-related protein degradation, (Figure 8D, d–g). The rapid downregulation process correlates with *OsHKT2;1* mRNA degradation (Figure 8C, D (g), E). However, it should be noted that a relatively large and measurable level of *OsHKT2;1* mRNA remains even after the OsHKT2;1-dependent Na⁺ influx is almost completely shut down (Figure 8C and E), suggesting that mechanisms such as phosphorylation-dependent posttranslational modifications could be contributing to the downregulation of OsHKT2;1-dependent Na⁺ influx (Figure 8D, g).

In summary, the present study reports the identification of genetic mutants, *oshkt2;1-1* and *oshkt2;1-2*, that strongly reduce Na⁺ influx into plant roots. Moreover, our findings provide robust genetic evidence that a moderate amount of Na⁺ enhances the growth of rice plants as a nutrient and the *OsHKT2;1* gene is responsible for a major portion of nutritional Na⁺ uptake and distribution in K⁺-starved rice plants. OsHKT2;1 functions in Na⁺-selective influx relative to K⁺ *in planta*. Our findings also reveal that OsHKT2;1-induced Na⁺ influx is rapidly downregulated by posttranslational and transcriptional mechanisms, which monitor and restrict the amount of Na⁺ influx via OsHKT2;1 to prevent Na⁺ over-accumulation and thus Na⁺ toxicity. A complete elucidation of the molecular mechanisms that mediate rapid inactivation of OsHKT2;1 under high Na⁺ concentrations will be an important subject for understanding mechanisms controlling nutritional Na⁺ uptake and salinity resistance.

Materials and methods

Plant materials and growth conditions

Rice seeds were sterilized as described previously (Horie *et al*, 2001). Sterilized seeds were then planted on a plastic screen (Caisson labs, Sugar City, ID, USA) that was subsequently placed in a sterilized vessel (Caisson labs, Sugar City, ID, USA) filled with 1 mM CaSO₄ solution or the same solution supplemented with KCl or NaCl. Seedlings were grown under light/dark cycles of 16/8 h and a temperature regime of 28/26°C. Hydroponic cultures were conducted using a modified basic nutrient medium (Mäser *et al*, 2002a) composed of 1.25 mM KNO₃, 0.5 mM NH₄H₂PO₄, 0.5 mM MgSO₄, 0.5 mM Ca(NO₃)₂, 50 μM Fe₂(SO₄)₃, 26 μM H₃BO₃, 5 μM ZnSO₄ and 40 μM MnSO₄. For 0.5 mM Na⁺-containing medium, KNO₃ was substituted with NaNO₃ and NMDG neutralized with HNO₃. pH was brought to 5.5 by NMDG. Seedlings grown in 1 mM CaSO₄ solution were transferred onto each modified medium using plugs (Jaecce Industries Inc., North Tonawanda, NY, USA). Solutions were changed every 3 days.

Tobacco (*Nicotiana tabacum* cv. SR1) seeds were surface sterilized and sown on one-half Murashige and Skoog agar medium (Murashige and Skoog, 1962) containing 15 g/l sucrose. Tobacco plants were grown at 25°C and maintained by *in vitro* sterile shoot culture under light/dark cycles of 16/8 h in a growth chamber.

Arabidopsis (*A. thaliana* ecotype Columbia) plants were grown on sterile soil for 20 days at 22°C under light/dark cycles of 16/8 h in a growth chamber.

Nucleic acid analyses and transgenic plants

Standard methods for nucleic acid isolations and analyses, for reporter genes analyses and for plant transformations are given in Supplementary data.

Ion content determinations

Ten-day-old plants grown in 1 mM CaSO₄ solution were transferred onto 0.5 mM Na⁺-containing basic nutrient medium and hydroponically grown 9 further days. Ion contents of shoots and roots were measured by ICP-OES.

Ten-day-old plants grown in 1 mM CaSO₄ solution were exposed to 0.5 mM Na⁺-containing basic nutrient medium for 2 days. Xylem sap samples were collected as described previously (Ma *et al*, 2004). Xylem sap samples were diluted in 5% HNO₃ and Na⁺ contents of the samples were measured by ICP-OES.

Transient gene expression analyses

The EGFP DNA fragment was amplified by PCR and subcloned into the pBI221 vector (Clontech, Mountain View, CA, USA). The coding region of the *OsHKT2;1* cDNA was also amplified and fused in frame to the 3' end of the EGFP DNA. EGFP-OsHKT2;1 and control constructs were introduced into protoplasts of leaf mesophyll cells of tobacco (*Nicotiana tabacum*) cv. SR1 by polyethylene-glycol-mediated transformation, as described previously (Sparla *et al*, 2006). Before confocal microscopy, FM4-64 (Invitrogen Molecular probes, Eugene, OR, USA) was added to protoplast incubation solutions at a final concentration of 17 μM and incubated for 2 min at room temperature. Fluorescence was analyzed by confocal microscopy (Eclipse TE2000-U; Nikon, Tokyo, Japan), using 488 nm excitation and either 510 nm emission filters for EGFP or 600 nm emission filters for FM4-64.

EGFP-OsHKT2;1 and control constructs were also introduced into leaf epidermal cells of *Arabidopsis* (Columbia). Abaxial sides of WT plants grown on soil for 20 days were transfected by bombardment (PDS-000/He; Bio-Rad, Hercules, CA, USA) (Arnim *et al*, 1998). After 1 day, GFP expression was examined by confocal microscopy (Eclipse TE2000-U, Nikon, Tokyo, Japan).

Tracer influx analyses

Rice plants were grown in 1 mM CaSO₄ solution or the same solution supplemented with the indicated concentrations of KCl or NaCl. Influx assays were performed as described previously (Gierth *et al*, 2005), with minor modifications described below. The influx buffer was composed of 2 mM MES-1,3 bis[tris(hydroxymethyl)9-methylamino]propane (BTP) pH5.5, 1 mM CaCl₂ supplemented with either cold NaCl and ²²NaCl (Amersham Bioscience, Pittsburgh, PA, USA) in Na⁺ influx assays or RbCl and ⁸⁶RbCl (Amersham Bioscience, Pittsburgh, PA, USA) in Rb⁺ uptake assays. The same solution, excluding radioisotope, was used as a washing buffer. K⁺-starved intact plants were pretreated with the indicated concentrations of each inhibitor in the presence of 1 mM CaSO₄ for 1 h. Plants were incubated for 20 min except for time-dependent influx assays. Excised roots were placed into a liquid scintillation vial and radioactivity was measured with a scintillation counter (LS6500 Beckman, Fullerton, CA, USA).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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