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High-Affinity K^+ Transporters from a Halophyte, *Sporobolus virginicus*, Mediate Both K^+ and Na^+ Transport in Transgenic Arabidopsis, *X. laevis* Oocytes and Yeast

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Class II high-affinity potassium transporters (HKTs) have been proposed to mediate Na^+–K^+ co-transport in plants, as well as Na^+ and K^+ homeostasis under K^+-starved and saline environments. We identified class II HKTs, namely SvHKT2;1 and SvHKT2;2 (SvHKTs), from the halophytic turf grass, *Sporobolus virginicus*. *SvHKT2;2* expression in *S. virginicus* was up-regulated by NaCl treatment, while *SvHKT2;1* expression was assumed to be up-regulated by K^+ starvation and down-regulated by NaCl treatment. Localization analysis revealed SvHKTs predominantly targeted the plasma membrane. SvHKTs complemented K^+ uptake deficiency in mutant yeast, and showed both inward and outward K^+ and Na^+ transport activity in *Xenopus laevis* oocytes. When constitutively expressed in Arabidopsis, SvHKTs mediated K^+ and Na^+ accumulation in shoots under K^+-starved conditions, and the K^+ concentration in xylem saps of transformants was also higher than in those of wild-type plants. These results suggest transporter-enhanced K^+ and Na^+ uploading to the xylem from xylem parenchyma cells. Together, our data demonstrate that SvHKTs mediate both outward and inward K^+ and Na^+ transport in *X. laevis* oocytes, and possibly in plant and yeast cells, depending on the ionic conditions.

Keywords: Arabidopsis • High-affinity potassium transporter • K^+ and Na^+ transport • K^+ starvation • Salt tolerance • *Sporobolus virginicus*.

Introduction

Soil salinity is an environmental stress of major concern, causing a significant loss of agricultural productivity worldwide, particularly in irrigated soils (Boyer 1982, Zhu 2001, Flowers 2004, Horie and Schroeder 2004, Munns and Tester 2008). Therefore, improving salt tolerance in crops is essential for sustainable food production, and has prompted research into plant responses to salt stress. Potassium (K^+) and sodium (Na^+) homeostasis is important for salt tolerance in plants (Horie et al. 2001). Generally, Na^+ is excluded from shoots and K^+ accumulates in glycophytes under salt stress, thereby stabilizing the high cytosolic K^+/Na^+ ratio, particularly in leaves (Berthomieu et al. 2003, Ren et al. 2005, Davenport et al. 2007, Møller et al. 2009, Hauser and Horie 2010). On the other hand, halophytes are able to select K^+ from a mixture dominated by Na^+ and yet accumulate sufficient Na^+ for the purposes of osmotic adjustment (Flowers and Colmer 2008).

K^+ is the most abundant cation in the cells of non-halophytic higher plants (Maathuis et al. 1997), and it has a wide variety of indispensable functions in plant cells, including osmoregulation, cell expansion, enzyme activation, protein synthesis, membrane polarization and photosynthesis (Véry and Sentenac 2003, Gierth et al. 2005, Wang and Wu 2015). Plants absorb and transport K^+ through K^+ channels and transporters located in the cell membrane (Véry et al. 2014). K^+ transporters are categorized into three major families: (i) the KT/HAK/KUP transporters; (ii) the high-affinity K^+ transporters (HKTs); and (iii) the cation/proton antiporters (CPAs) (Gierth and Mäser 2007). Genes encoding the KT/HAK/KUP transporter family are present in all plant genomes, but not in the genomes of Protista or Animalia (Grabov 2007). Originally, CPA families were described as Na^+/H^+ exchangers involved in salt tolerance; however, it has been demonstrated that several CPAs also transport K^+ (Gierth and Mäser 2007). HKTs were generally believed to have evolved from bacterial KcsA K^+ channels (Doyle et al. 1998), and to have two functions: (i) to take up Na^+ from the soil to reduce K^+ requirements when K^+ is a limiting factor; and (ii) to reduce Na^+ accumulation in leaves by removing Na^+ from the xylem sap and loading it into the phloem sap (Rodríguez-Navarro and Rubio 2006).

Phylogenetic analyses of the identified HKT proteins demonstrated that HKTs can be divided into at least two subgroups: class I and II transporters. Class I is represented by

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Arabidopsis AtHKT1;1, which contains a serine in the first P-loop of the four-pore domain structure, and selectively transports Na^+. Class II is typified by wheat TaHKT2;1, which has a glycine in place of the serine, and acts as a symporter for both Na^+ and K^+ (Mäser et al. 2002, Horie et al. 2009, Hauser and Horie, 2010, Horie et al. 2011a). A recent phylogenetic analysis of extended HKTs suggested that class II HKTs can be further classified into two subgroups, class II and III (Su et al. 2015). Class III contains class II HKTs which have been found in the more primitive higher plants, such as *Selaginella moellendorffii* and *Physcomitrella patens*. Molecular genetic research demonstrated that Na^+ transport activity mediated by class I HKTs plays a key role in Na^+ tolerance and in the exclusion of Na^+ from leaves in Arabidopsis, rice and wheat (Mäser et al. 2002, Berthomieu et al. 2003, Ren et al. 2005, Sunarpi et al. 2005, Huang et al. 2006, Davenport et al. 2007, Horie et al. 2009, Møller et al. 2009). Class II HKTs, predominantly found in monocots, generally mediate Na^+–K^+ co-transport (Schachtman and Schroeder 1994, Rubio et al. 1995, Gassmann et al. 1996, Horie et al. 2001, Golldack et al. 2002, Takahashi et al. 2007, Ardie et al. 2009, Yao et al. 2010, Horie et al. 2011b, Mian et al. 2011, Oomen et al. 2012, Sassi et al. 2012, Suzuki et al. 2016). Although each class II HKT demonstrated distinct Na^+ and K^+ selectivity under different K^+/Na^+ concentrations in heterologous backgrounds, such as yeast and *Xenopus laevis* oocytes, high-affinity K^+ transport activity mediated by typical class II HKTs has not yet been robustly demonstrated in plants (Sassi et al. 2012, Suzuki et al. 2016). In the present study, we isolated class II HKTs (SvHKT2;1 and SvHKT2;2) from a halophytic monocot turf grass, *Sporobolus virginicus*, that demonstrates remarkable salinity tolerance up to 1.5 M NaCl, the mechanisms of which are gradually being elucidated via omics data (Tada et al. 2014, Yamamoto et al. 2015, Endo et al. 2017). Herein, we report that SvHKT2;1 and SvHKT2;2 mediate distinct outward and inward K^+ transport activity in plants, as well as in *X. laevis* oocytes and yeast.

Results

Comparison of amino acid sequences of SvHKT2;1, SvHKT2;2 and other class II HKTs

Among the unigenes of *S. virginicus* (Yamamoto et al. 2015) that are homologous to known HKTs, *SvHKT2;1* and *SvHKT2;2* belong to class II *HKT* genes because their deduced amino acid sequences contain a glycine in the first P-loop (Mäser et al. 2002, Platten et al. 2006) (Supplementary Fig. S1). The nucleotide sequence of *SvHKT2;1* comprises a deletion of 63 nucleotides from the 5′ end of the *SvHKT2;2* sequence, resulting in a 21 amino acid deletion, as well as six nucleotide substitutions that result in two amino acid substitutions (99.6% identity) (Supplementary Fig. S1). Phylogenetic analysis following alignment of the amino acid sequences of SvHKT2;1 and SvHKT2;2 with 11 other class II HKTs indicated an evolutionarily distant relationship between them (**Fig. 1**). Alignment of the amino acid sequences of SvHKT2;1, SvHKT2;2 and seven HKTs of the same clade showed their relatively low (42–62%) identities

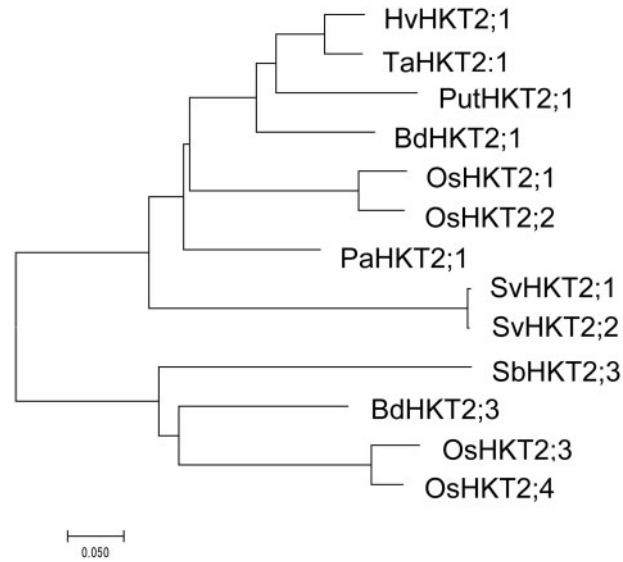

Fig. 1 Phylogenetic analysis of class II HKTs. A phylogenetic analysis of the HKT amino acid sequences was performed using the UPGMA method with the MEGA7 software package. Amino acid sequences of SvHKT2;1, SvHKT2;2, TaHKT2;1 (AAA52749), HvHKT2;1 (AEM55590.1), OsHKT2;1 (BAB61789), OsHKT2;2 (BAB61791), OsHKT2;3 (CAD37187), OsHKT2;4 (CAD37199), PaHKT2;1 (BAE44385.1), PutHKT2;1 (FJ716169.1), SbHKT2;3 (EER90327.1), BdHKT2;1 (KQK17402.1) and BdHKT2;3 (KQK17401.1) were used for the analysis. The branch length is proportional to the evolutionary distance between the HKTs, indicating the number of amino acid changes per site. The scale bar shows a length corresponding to 0.050 of the value.

(Supplementary Table S1), and revealed the existence of SvHKT2;1- and SvHKT2;2-specific short insertions in three regions (three, one and three amino acid insertions) (Supplementary Fig. S1).

Expression of *SvHKT2;1* and *SvHKT2;2* genes is differentially regulated by K^+ and Na^+ concentration

The expression profiles of *SvHKT2;1* and *SvHKT2;2* genes in *S. virginicus* under saline stress conditions were determined using quantitative reverse transcription–PCR (qRT–PCR) with primer pairs specific to *SvHKT2;2* and common to both *SvHKT2;1* and *SvHKT2;2* transcripts (**Fig. 2**). Expression levels relative to that in shoots at 0 h after treatment (1.0) are shown to compare the relative expression levels of shoots and roots. Both of the transcripts were found to accumulate preferentially in roots compared with shoots (**Fig. 2A–H**). In a time-course experiment, *SvHKT2;2* expression in roots increased approximately 30-fold 48 h after treatment with NaCl, whereas the combined expression of *SvHKT2;1* and *SvHKT2;2* in roots decreased following NaCl treatment (**Fig. 2B**, **F**). These results indicate that expression of *SvHKT2;1* and *SvHKT2;2* in roots is differentially regulated by salinity stress, and expression of *SvHKT2;1* is much higher than that of *SvHKT2;2* under conditions of high salinity. However, expression of *SvHKT2;2* in both roots and shoots was almost unchanged by K^+ starvation, whereas the combined

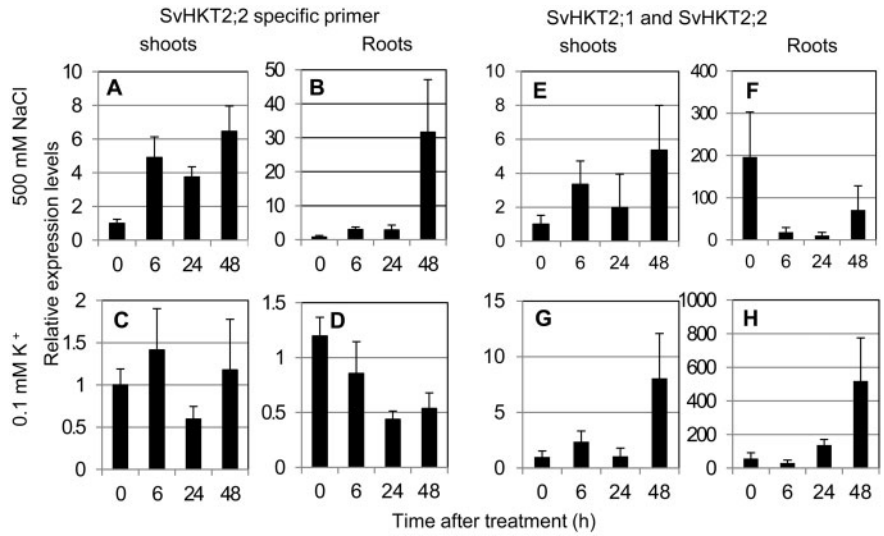

Fig. 2 Expression levels of *SvHKT2;1* and *SvHKT2;2* genes in *Sporobolus virginicus*. (A–D) Expression levels of SvHKT2;2; (E–H) combined expression levels of both *SvHKT2;1* and *SvHKT2;2*. Hydroponically grown *S. virginicus* plants grown in 1/2 MS salt medium were transplanted to 1/2 MS medium supplemented with 500 mM NaCl (A, B, E, F) or modified 1/2 MS medium in which the potassium concentration was reduced to 0.1 mM (C, D, G, H). The shoots (A, C, E, G) and roots (B, D, F, H) were harvested at the indicated time points and used for qRT–PCR. Expression levels relative to that in shoots at 0 h after treatment (1.0) are shown. Data are presented as the mean ± SE (n = 3, biological replicates).

expression of *SvHKT2;1* and *SvHKT2;2* increased by K^+ starvation at 48 h after treatment, especially in roots (**Fig. 2C**, **D**, **G**, **H**). These results indicate that *SvHKT2;1* expression is solely and significantly up-regulated by K^+ starvation in roots, whereas *SvHKT2;2* expression in roots is up-regulated by NaCl treatment.

Localization of SvHKT2;1 and SvHKT2;2 in *Nicotiana benthamiana* cells

To investigate the intracellular localization of enhanced green fluorescent protein (EGFP)–SvHKT2;1 and EGFP-SvHKT2;2 fusion proteins, *Agrobacterium* expressing *EGFP-SvHKT2;1*, *EGFP-SvHKT2;2* or a control *EGFP*-only construct were infiltrated into *N. benthamiana* leaf cells, and the fluorescent signals were observed using confocal laser scanning microscopy (**Fig. 3**). When EGFP alone was expressed, it localized to the nucleus and the cytoplasm (**Fig. 3C**, **F**, **I**). In contrast, EGFP–SvHKT2;1 and EGFP–SvHKT2;2 fusion proteins specifically localized to the plasma membrane (**Fig. 3A**, **B**, **D**, **E**, **G**, **H**). This pattern was verified by treatment with a hypertonic solution, 0.5 M mannitol, which induced plasmolysis (Supplementary Fig. S2), indicating that both SvHKT2;1 and SvHKT2;2 localized to the plasma membrane.

Functional analysis of SvHKT2;1 and SvHKT2;2 in *Xenopus laevis* oocytes

To examine K^+ and/or Na^+ channel/transporter activities in *X. laevis* oocytes, *SvHKT2;1* and *SvHKT2;2* cRNAs were injected into oocytes, and their electrophysiological profiles were analyzed (**Figs. 4**, **5**).

Using two-electrode voltage clamp (TEVC) experiments, positive shifts in the reversal potential (Erev) of both SvHKT2;1- and SvHKT2;2-expressing oocytes were observed when the concentration of external K- or Na-gluconates increased (**Fig. 4A**–D; Supplementary Table S2). These results indicate that both SvHKT2;1 and SvHKT2;2 transporters mediate K^+ and Na^+ transport. SvHKT2;1 and SvHKT2;2 have similar electrophysiological characteristics, such that both transporters produce outward rectification of K^+ currents, although weak inward rectification was also observed (**Fig. 4A**, **B**). Note that larger inward currents were detected in SvHKT2;1- and SvHKT2;2-expressing oocytes when compared with water-injected controls at −120 mV, not only with external Na^+ (**Fig. 4C**, **D**), but also with external 2 mM K^+ and 0.2 mM Na^+ (**Fig. 5D**). Furthermore, both SvHKT2s produce less rectification of Na^+ currents, with enhanced conductance in proportion to the concentration of Na^+ (**Fig. 4C**, **D**). Current–voltage relationships of controls (water-injected oocytes) are shown in the insets in **Fig. 4A**, **C** and D.

In the presence of 2 mM external K-gluconate with 0, 0.2, 2 or 20 mM Na-gluconate, the Erev values in oocytes injected with *SvHKT2;1* cRNA were −74.5, −75.6, −70.1 and −46.3 mV, respectively (**Figs. 4A**, **5D**, **B**, **E**; Supplementary Table S2). This indicates that 2 mM K^+ is the greatest determinant of Erev in the presence of $\leq$2 mM Na^+, i.e. SvHKT2;1 has equal or lower permeability to Na^+ relative to K^+, although the presence of a >10-fold concentration of Na^+ leads to positive shifts in Erev, presumably due to an increase in the selectivity for Na^+ (**Figs. 4A**, **C**, **5E**).

In the presence of 2 mM external K-gluconate with 0, 0.2, 2 and 20 mM Na-gluconate, the Erev values in oocytes injected with the *SvHKT2;2* cRNA were −75.0, −53.4, −44.2 and −27.3 mV, respectively (**Figs. 4B**, 5D, B, E; Supplementary Table S2). This indicates that the Na^+ concentration has a greater influence on the Erev of SvHKT2;2 than SvHKT2;1, i.e. SvHKT2;2 has a higher affinity for Na^+ than SvHKT2;1. The Erevs of SvHKT2;1

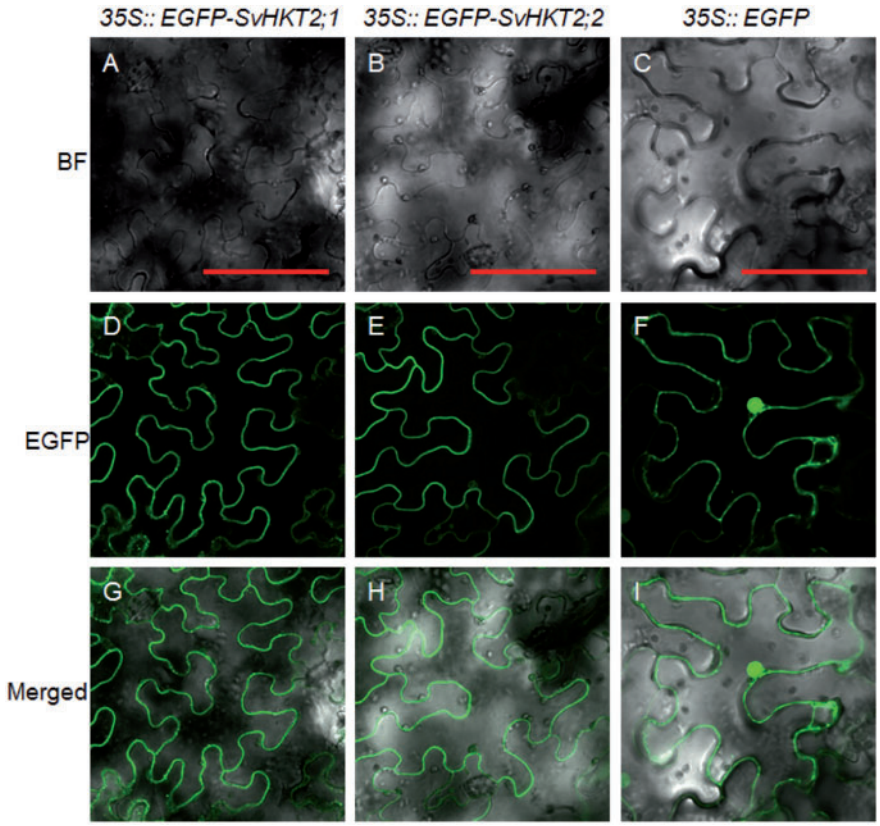

Fig. 3 Subcellular localization of EGFP-fused SvHKT2;1 and SvHKT2;2 proteins in *Nicotiana benthamiana* leaves. Confocal fluorescence images of EGFP (D–F) and differential interference contrast (A–C), as well as merged (G–I) images of *N. benthamiana* leaves expressing EGFP–SvHKT2;1 (A, D, G), EGFP–SvHKT2;2 (B, E, H) and EGFP control (C, F, I). The scale bar represents 100 μm. See also Supplementary Fig. S2 for data on plasmolyzed cells.

were nearer to the theoretical reversal potential than the Erevs of SvHKT2;2 in various external K^+ with constant external Na^+ (0 or 2 mM). This also indicates that permeability to K^+ is relatively higher in SvHKT2;1 than in SvHKT2;2.

SvHKT2;1- and SvHKT2;2-mediated K^+ (Rb^+) and Na^+ uptake was further confirmed by measuring uptake of radioactive Rb^+ (as a tracer for K^+) and Na^+. When oocytes injected with *SvHKT2;1*, *SvHKT2;2* or *OsHKT2;2/1* cRNAs, or a water control were incubated in radioactive Rb^+ or Na^+ solution including 88 mM Na^+ and 1 mM K^+, Rb^+ or Na^+ uptake was significantly higher in HKT2-expressing oocytes compared with those which were water injected (Supplementary Fig. S3A, B). Significantly enhanced Na^+ uptake in *SvHKT2*-expressing oocytes was also confirmed in solution including 4 mM Na^+ and 1 mM K^+ (data not shown).

Therefore, SvHKT2;1 and SvHKT2;2 mediate both K^+ and Na^+ transport in both directions.

Functional analysis of SvHKT2;1 and SvHKT2;2 in yeast

To examine K^+ and/or Na^+ channel/transporter activities in yeast, *SvHKT2;1* and *SvHKT2;2* were expressed in yeast strain 9.3 with defective K^+ transporters (**Fig. 6**). Yeast lines harboring *SvHKT2;1*, *SvHKT2;2* and positive control *HvHKT2;1* grew better on agar containing 0.2 mM K^+ than controls expressing an empty vector (**Fig. 6A**). These results suggest that both *SvHKT2;1* and *SvHKT2;2* complemented the K^+ uptake deficiency in the mutant yeast. Distinguishable effects were not observed between the growth of yeast expressing *SvHKT2;1*, *SvHKT2;2* and *HvHKT2;1* on medium including either higher K^+ or Na^+ concentrations (**Fig. 6B–F**).

Growth and K^+ and Na^+ concentrations of yeast expressing *SvHKT2;1*, *SvHKT2;2*, *HvHKT2;1* or an empty vector were examined further in a liquid medium containing 0.2 mM K^+ (Supplementary Fig. S4). The OD_{600} of yeasts expressing *SvHKT2;1* or *SvHKT2;2* increased to similar levels, comparable with those of the positive control expressing *HvHKT2;1*, which were significantly higher than that of yeasts expressing the empty vector (Supplementary Fig. S4A). K^+ and Na^+ concentrations in *SvHKT2;1*, *SvHKT2;2* and *HvHKT2;1* expressors were significantly higher than those expressing the empty vector in medium containing 0.2 mM K^+, though differences were not observed among them in medium containing 10 mM K^+ (Supplementary Fig. S4B, C). These results indicate these HKTs mediated both K^+ and Na^+ uptake in yeast under K^+-starved conditions.

Ion concentrations of transgenic Arabidopsis plants

We produced Arabidopsis transformants harboring *35S-SvHKT2;1* and *35S-SvHKT2;2*, and examined the expression levels of the transgenes in each of six randomly selected

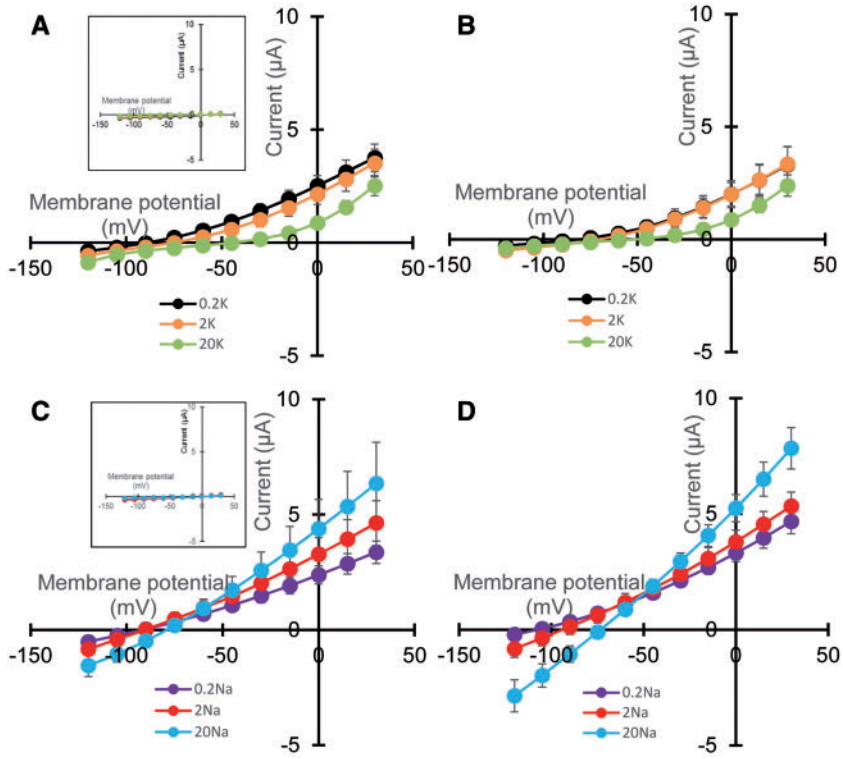

Fig. 4 Analyses of SvHKT2;1- or SvHKT2;2-mediated ion transport by two-electrode voltage clamp experiments using *Xenopus laevis* oocytes. Current–voltage relationship of oocytes injected with 12.5 ng of *SvHKT2;1* (A, C) or *SvHKT2;2* (B, D) cRNA bathed in solutions containing an indicated amount (mM) of K-gluconate only (A, B), and Na-gluconate only (C, D). Inserted graphs (A, C) show currents of water-injected oocytes (negative controls). Voltage steps ranged from −120 to +30 mV with 15 mV increments. Data are presented as the mean ± SD (n = 6–8 for samples and n = 3 for negative controls.

transgenic lines (T_2) grown on 1/2 Murashige and Skoog (MS) agar medium (containing 10 mM K^+ and 0.1 mM Na^+) (**Fig. 7A**). When their root growth on 0.1 mM K^+ medium (containing 0.725 mM Na^+) was examined (**Fig. 7B**, **C**), there was a weak correlation between the length of the elongated root on low-K^+ medium and expression levels of the transgene. Then, we determined ion concentrations in shoot and root from each of three selected lines grown on 0.1 mM K^+ agar medium (Supplementary Fig. S5). Shoot K^+, shoot Na^+ and root Na^+ concentrations in transgenic lines tended to be higher than those of the wild type (WT; Supplementary Fig. S5A–C) and root K^+ concentrations in SvHKT2;2 transgenic lines were significantly lower than that in the WT (Supplementary Fig. S5D). Based on these experiments, SvHKT2;1-1 and SvHKT2;2-15 transformants showing average root elongation and expression levels of the transgene and typical K^+ and Na^+ ion concentrations were selected as representatives for each line, and were used for further experiments.

The physiological conditions replicated in vitro are different from those in an open system; therefore, SvHKT transformants and WT plants were hydroponically cultured in 1/2 MS or 0.1 mM K^+ liquid medium, and their shoot and root ion concentrations were determined (**Fig. 8**). In 1/2 MS medium (0.2 mM Na^+ and 10 mM K^+), no significant differences were observed in Na^+ and K^+ concentrations in either shoots or roots between SvHKT transformants and WT plants (**Fig. 8A–D**). Shoot/root ratios of Na^+ and K^+ concentrations in the transgenic lines were also similar to those in the WT (**Fig. 8I**, **J**). However, in 0.1 mM K^+ medium, shoot K^+ and Na^+ concentrations in SvHKT transformants were significantly higher than those of WT plants, and root K^+ and Na^+ concentrations in transgenic lines were significantly or tended to be lower than those of WT plants (**Fig. 8E–H**). As a result, shoot/root ratios of Na^+ and K^+ concentrations in the transgenic lines were higher than those in the WT (**Fig. 8K**, **L**). These results suggested enhanced K^+ and Na^+ transportation from roots to shoots in SvHKT transformants under K^+-starved conditions.

K^+ and Na^+ concentrations in the xylem saps of Arabidopsis SvHKT transformants

To examine the mode of SvHKT-mediated K^+ and Na^+ transport in Arabidopsis, SvHKT transformants and WT plants were hydroponically cultured in 1/2 MS or 0.1 mM K^+ liquid medium, and K^+ and Na^+ concentrations in the xylem saps were determined at the bolting stage (**Fig. 9**). K^+ and Na^+ concentrations in the xylem saps were similar among transformants and WT plants in 1/2 MS medium (**Fig. 9A**, **B**), but K^+ concentrations were higher in SvHKT transformants than in WT plants in 0.1 mM K^+ medium (**Fig. 9D**), while Na^+ concentrations were higher than in WT plants only in SvHKT2;1 transformants (**Fig. 9C**). These results suggest that upload of K^+ and Na^+ to the root xylem was promoted in SvHKT transformants.

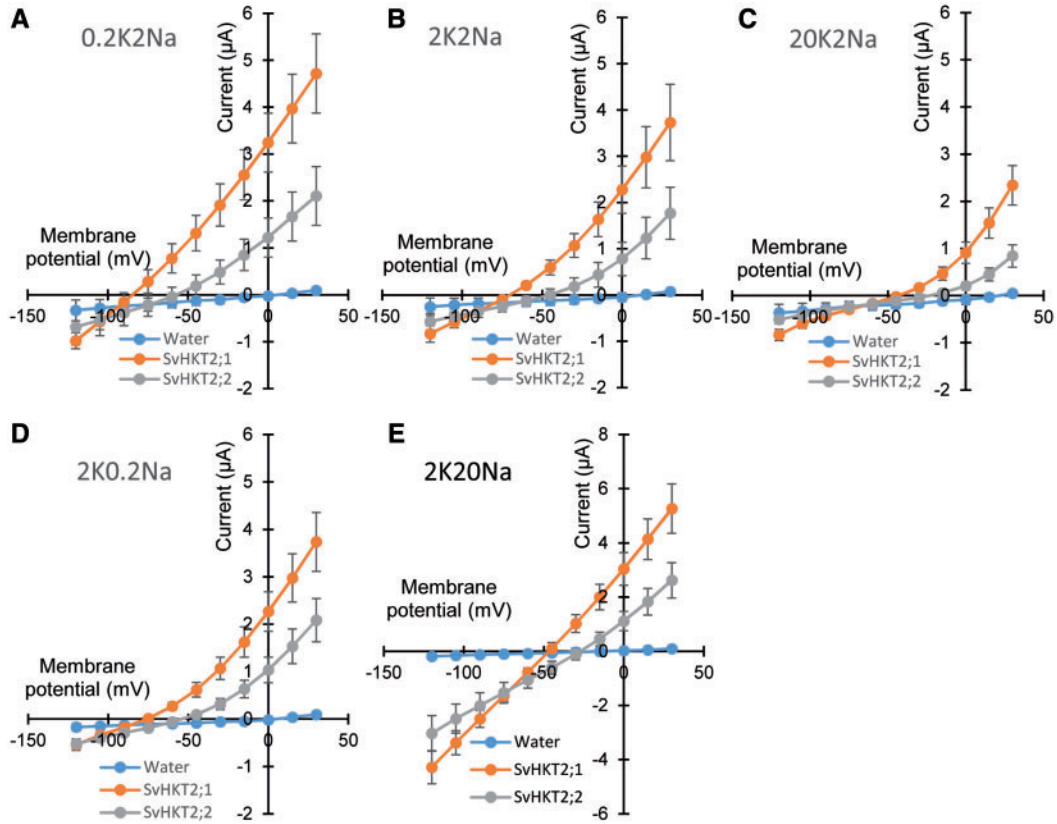

Fig. 5 Analyses of SvHKT2;1- or SvHKT2;2-mediated ion transport by two-electrode voltage clamp experiments using *Xenopus laevis* oocytes in the co-presence of external K^+ and Na^+. Current–voltage relationship of oocytes injected with 12.5 ng of *SvHKT2;1* or *SvHKT2;2* cRNA bathed in solutions containing 0.2 mM K-gluconate and 2 mM Na-gluconate (A), 2 mM K-gluconate and 2 mM Na-gluconate (B), 20 mM K-gluconate and 2 mM Na-gluconate (C), 2 mM K-gluconate and 0.2 mM Na-gluconate (D) or 2 mM K-gluconate and 20 mM Na-gluconate (E). Voltage steps ranged from −120 to +30 mV with 15 mV increments. Data are presented as the mean ± SD (n = 6–8 for samples and n = 3 for water-injected oocytes as negative controls).

Discussion

SvHKT2;1 and SvHKT2;2 facilitate K^+ and Na^+ transport in *X. laevis* oocytes, yeast and Arabidopsis cells

We isolated genes for class II HKTs, *SvHKT2;1* and *SvHKT2;2*, from the halophytic monocot turf grass, *S. virginicus*. SvHKT2;1 has high sequence similarity to SvHKT2;2, but with a 21 amino acid deletion at its 5′ end; however, both EGFP fusion proteins localized specifically to the plasma membrane (**Fig. 3**; Supplementary Fig. S2). Both SvHKT2;1 and SvHKT2;2 complemented K^+ uptake deficiency in mutant yeast (**Fig. 6**; Supplementary Fig. S4), and mediated Rb^+ (K^+) and Na^+ uptake in oocytes (Supplementary Fig. S3), indicating inward K^+ transport activity. Furthermore, TEVC experiments using *X. laevis* oocytes revealed that both SvHKT2;1 and SvHKT2;2 showed similar electrophysiological characteristics, producing outward rectification of currents in the presence of a low external K^+ concentration, and reduced rectification of currents in the presence of Na^+ at low external K^+ concentration, though *SvHKT2;1* showed higher K^+ selectivity than *SvHKT2;2* (**Figs. 4**, **5**). Although it is assumed that permeability to Na^+ ($P_{Na}{}^+$) and permeability to K^+ ($P_K{}^+$) is equal ($P_{Na}{}^+ = P_K{}^+$), and permeability to Cl^- is zero ($P_{Cl}{}^- = 0$) for theoretical reversal potential, actual $P_{Na}{}^+$ should be similar but not identical to $P_K{}^+$ in SvHKT2s. Also the oocyte membrane endogenously shows some permeability to Cl^- These factors may induce some discrepancy between values of theoretical and observed reversal potentials (Supplementary Table S2).

Those results indicated that SvHKT2s are bi-directional Na^+–K^+ co-transporters.

We investigated the function of SvHKT2;1 and SvHKT2;2 in transgenic Arabidopsis because our attempt to transform the genotype of *S. virginicus* was unsuccessful. When grown in media containing 0.1 mM K^+, Na^+ and K^+ concentrations, and shoot/root ratios of Na^+ and K^+ concentration in transformants were significantly higher than those of the WT plants, and root Na^+ and K^+ concentrations in transformants tended to be lower than those in the WT (**Fig. 8G–L**). These results suggested enhanced ion transportation from roots to shoots in transformants.

Furthermore, K^+ concentrations in the xylem sap of transformants were also higher than those in WT plants under these conditions, although Na^+ concentrations in SvHKT2;1 transformants, but not in SvHKT2;2 transformants, were higher than those in WT plants (**Fig. 9**). Together with the outward K^+ transport observed in oocytes, it is possible that K^+ (and

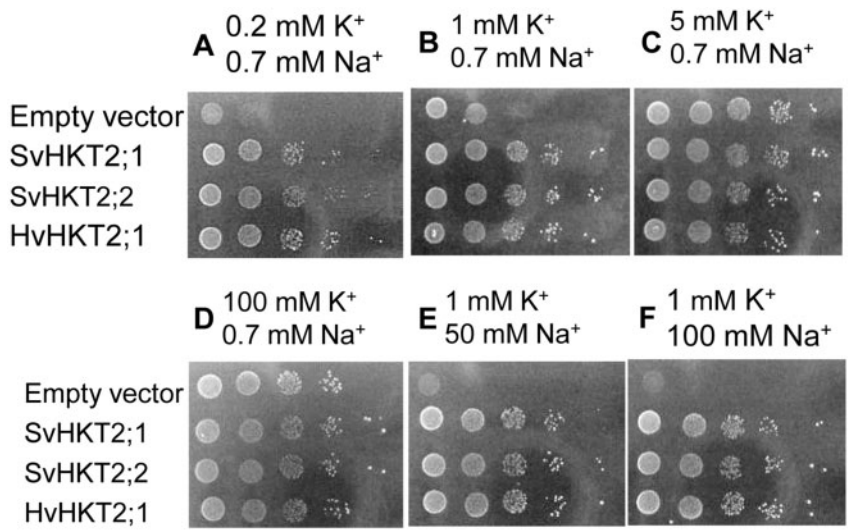

Fig. 6 Growth of yeast strain 9.3 transformed with the empty vector or the plasmid containing a plant HKT. SC/-His medium supplemented with different K^+ and Na^+ concentrations was inoculated with serially diluted yeast cell suspensions and incubated for 3 d. (A) 0.2 mM K^+ and 0.7 mM Na^+, (B) 1.0 mM K^+ and 0.7 mM Na^+, (C) 5.0 mM K^+ and 0.7 mM Na^+, (D) 100 mM K^+ and 0.7 mM Na^+, (E) 1.0 mM K^+ and 50 mM Na^+, and (F) 1.0 mM K^+ and 100 mM Na^+.

possibly Na^+ also) is uploaded to the xylem from xylem parenchyma and the surrounding cells via outward K^+ transport by SvHKT2;1 and SvHKT2;2 in transformants under K^+-starved conditions, which would promote translocation of K^+ and Na^+ from the roots to the shoots. However, in such cases, xylem parenchyma cells must be depolarized for K^+ and Na^+ efflux into the xylem. In plant roots, K^+ is absorbed by the epidermis and root hairs, and is then transported into the xylem through several layers of root cells, mediated by many K^+ channels and transporters (Han *et al.*, 2016). K^+ is released from xylem parenchyma cells into the xylem by SKOR in Arabidopsis, and an increase in the outward current was observed when the external concentration of K^+ was increased in the 0–10 mM concentration range in oocytes (Gaymard et al. 1998). SvHKT2;1 and SvHKT2;2 may have enhanced the K^+ loading when the SKOR channel is active in transgenic Arabidopsis plants grown under K^+-starved conditions. It is also possible that constitutive expression of SvHKT2s could mediate both increased Na^+ and K^+ uptake in root epidermal cells, followed by increased accumulation of Na^+ and K^+ in xylem parenchyma cells, which could lead to uploading of Na^+ and K^+ into the xylem. In such a case, root Na^+ and K^+ concentrations in transformants must be higher than those in the WT; however, this was not the case with SvHKT2 transformants (**Fig.8G**, **H**). Further investigation will be necessary to measure membrane potential in xylem parenchyma and the surrounding cells to examine this hypothesis. The tissue expression pattern of *SvHKT2;1* and *SvHKT2;2* should be determined to elucidate the roles of SvHKT2s in *S. virginicus*.

Comparison of the properties of SvHKT2;1 and SvHKT2;2 with those of other HKTs

Expression of *SvHKT2;1* and *SvHKT2;2* in *S. virginicus* was abundant in the roots compared with the shoots, and was differentially regulated by K^+ starvation and NaCl treatment. Expression levels of *SvHKT2;1* and *SvHKT2;2* were down-regulated and up-regulated in salt-treated roots, respectively, and expression levels of *SvHKT2;1* and *SvHKT2;2* were up-regulated and almost unchanged in K^+-starved roots, respectively (**Fig. 2**). Thus, it was suggested that SvHKT2;1 and SvHKT2;2 play major roles in *S. virginicus* roots under K^+-starved and high-saline conditions, respectively. The transcript levels of other class II *HKT* genes, including *TaHKT2;1*, *OsHKT2;2*, *HvHKT2;1*, *PutHKT2;1* and *PhaHKT1*, were also shown to increase in roots following K^+ starvation (Wang et al. 1998, Horie et al. 2001, Takahashi et al. 2007, Ardie et al. 2009, Mian et al. 2011). However, other HKTs respond differently to high Na^+ concentrations; *OsHKT2;2* (Suzuki et al. *2016)* was down-regulated in roots, but *PutHKT2;1*, *PaHKT2;1* (Takahashi et al. 2007, Ardie et al. 2009) and *HvHKT2;1* (Mian et al. 2011) were up-regulated in roots and shoots, respectively, by salt stress. Therefore, the transcriptional response of *SvHKT2;1* to ionic stress is similar to that of *OsHKT2;2*, while that of *SvHKT2;2* is similar to that of *PutHKT2;1*, *PaHKT2;1* and *HvHKT2;1*. Overexpression of *HvHKT2;1* in barley led to enhanced Na^+ uptake and higher Na^+ concentrations in the xylem sap (Mian et al. 2011). In these transgenic barley plants, increased K^+ accumulation in the leaf blade was observed under 0 mM K^+ plus 50 mM Na^+ conditions. These results suggested functional similarity between HvHKT2;1 and SvHKT2;1/SvHKT2;2.

The transport selectivity of Na^+ and K^+ by class II HKTs depends on the ionic conditions, mediating selective Na^+ influx at high Na^+ concentrations (Rubio et al. 1995, Gassmann et al. 1996, Horie et al. 2001, Laurie et al. 2002, Jabnoune et al. 2009). Wheat TaHKT2;1 functions as an Na^+–K^+ symporter at micromolar ion concentrations, whereas in yeast it acts in the millimolar ion concentration range as an Na^+ uniporter (Rubio et al. 1995). Fungal TRKs also accomplish

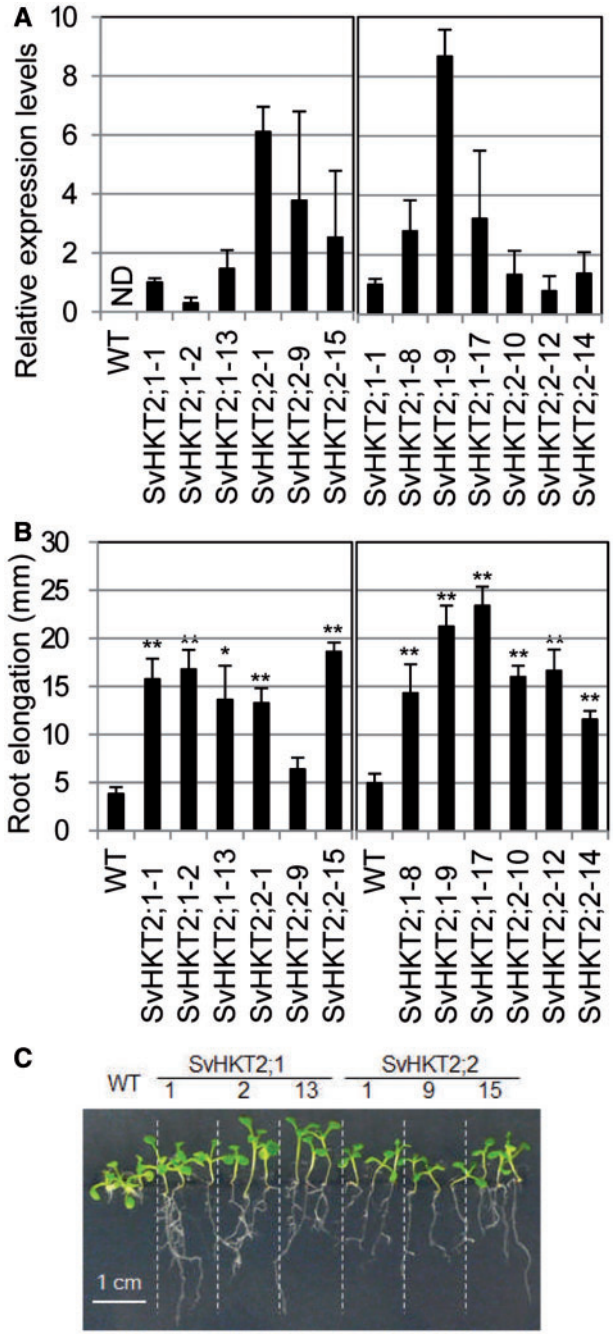

Fig. 7 Expression of transgenes and root growth of *SvHKT2;1* and *SvHKT2;2* transformants. (A) Expression levels of *SvHKT2;1* and *SvHKT2;2* genes in 12 transgenic Arabidopsis lines and WT plants. Ten-day-old whole plants were used for RNA extraction and qRT–PCR. Data are presented as the mean ± SE (n = 3, biological replicates). ND, not detected. (B) Root elongation of transgenic and WT seedlings on 0.1 mM K^+ medium. Transgenic and WT seedlings grown for 5 d on 0.1 mM K^+ medium were transplanted onto vertically positioned agar plates containing 0.1 mM K^+ medium and incubated for an additional 5 d, and the root length elongation over 5 d was then determined. Data are presented as the mean ± SE (n = 3–5, biological replicates). Single and double asterisks denote significant differences compared with the values of WT plants at $P < 0.05$ and $P < 0.01$, respectively, determined using Student's t-test. (C) The appearance of six transgenic lines and WT seedlings on 0.1 mM K^+ medium examined in (B).

conditional modulation of Na^+/K^+ selectivity according to the ionic environment and the K^+ status of the cell (Rodríguez-Navarro 2000). SvHKT2;1 and SvHKT2;2 were also greatly affected by ionic conditions, mediating Na^+ influx at high Na^+ (20 mM) concentrations in the presence of 2 mM K^+ in oocytes (**Fig. 5**). In the presence of 2 mM Na^+, increases in the external K^+ concentration lead to positive shifts in the Erev with decreases of the conductance (**Fig. 5A–C**) as observed in the case of Po-OsHKT2;2 in rice (Horie et al. 2001). Open probability should be down-regulated at the single-channel level at high K^+ in these HKTs, but future study is required to reveal such molecular mechanisms.

To date, the K^+ transport functions of plant class II HKTs, TaHKT2;1, HvHKT2;1, OsHKT2;2, OsHKT2;4, PutHKT2;1 and PhaHKT1 have been demonstrated in yeast and *X. laevis* oocytes (Gassmann et al. 1996, Wang et al. 1998, Horie et al. 2001, Takahashi et al. 2007, Ardie *et al.*, 2009, Horie et al. 2011a, Mian et al. 2011), but (interestingly) not in plants. Overexpression of plant *HKT* genes in plants through genetic engineering has thus far not identified a role in K^+ transport for these transporters (Sassi et al. 2012, Suzuki et al. 2016), except one study demonstrating an increasing K^+ accumulation in the leaf blades of *HvHKT2;1*-overexpressing barley under 0 mM K^+ plus 50 mM Na^+ conditions (Mian et al. 2011). In the present study, we demonstrate that class II HKTs, SvHKT2;1 and SvHKT2;2, can mediate K^+ and Na^+ transport activity under K^+-starved conditions when constitutively expressed in Arabidopsis, which possibly includes uploading of these ions into xylem. These characteristic ion transport properties in planta have not been reported in other class II HKTs. These transport properties and expression profiles of *SvHKT2;1* and *SvHKT2;2* may be partially responsible for the ability of *S. virginicus* to maintain K^+ homeostasis under salt stress (Tada et al. 2014). To examine this hypothesis, further experiments including localization analysis of *SvHKT2* expression in *S. virginicus* are needed.

Materials and Methods

Isolation of *SvHKT* genes

We searched for HKT and HAK gene homologs in previously constructed unigenes assembled from *S. virginicus* RNA-Seq data (Yamamoto et al. 2015), and found seven HKT-like and three HAK-like unigenes. Among them, two unigene sequences encoding putative class II-type HKTs, SvHKT2;1 and SvHKT2;2 (DDBJ accession Nos. LC271218 and LC271219), were PCR-amplified using specific primers, SvHKT1A1F and SvHKT1A2R, and SvHKT2A1F and SvHKT2A2R, respectively (Supplementary Table S3). Amplified sequences were cloned into pENTER vectors (Thermo Fisher Scientific) to form the entry vectors, pENTER-SvHKT2;1 and pENTER-SvHKT2;2.

Phylogenetic analysis

A phylogenetic analysis of the HKT amino acid sequences using the Neighbor-Joining method, following their alignment using ClustalW, was performed using the MEGA7 software package (Kumar et al. 2016).

Real-time qRT–PCR

Hydroponic culture was carried out as described previously to test the response of *S. virginicus* to NaCl treatment (Yamamoto et al. 2015). To test the response

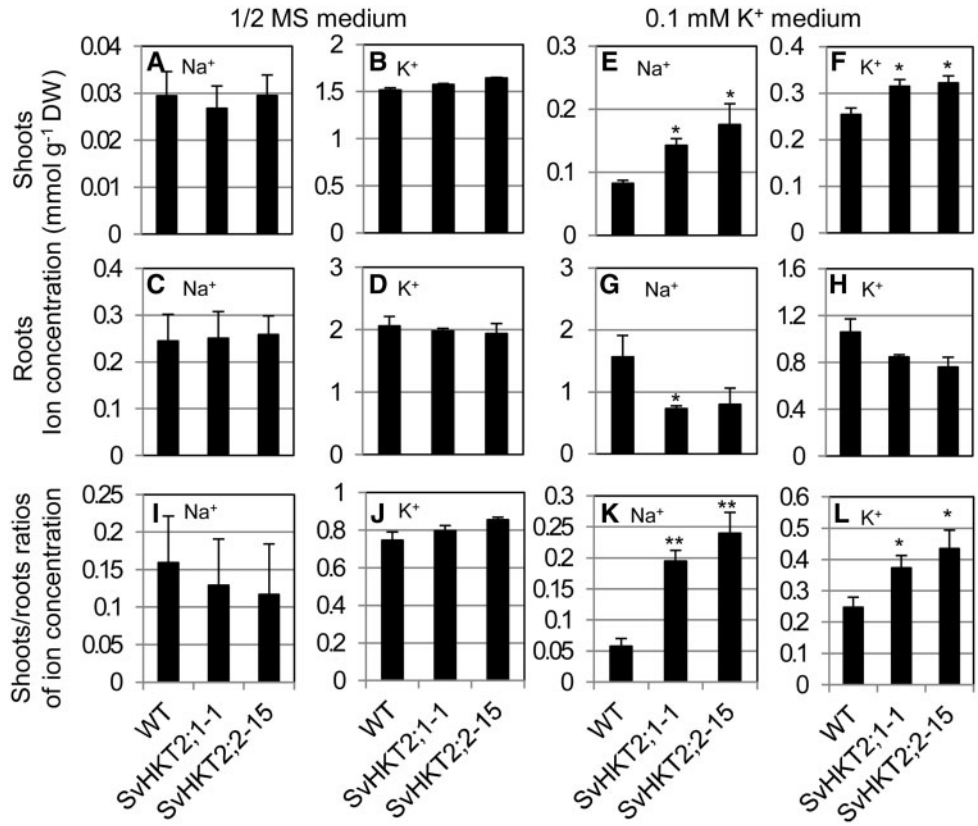

Fig. 8 Na^+ and K^+ concentrations and shoot/root ratios of Na^+ and K^+ concentrations in SvHKT transformants and WT plants grown in 1/2 MS or 0.1 mM K^+ medium. Two-week-old seedlings germinated on 1/2 MS medium were hydroponically cultured in 1/2 MS (A–D, I, J) or 0.1 mM K^+ (E–H, K, L) medium for an additional 2 weeks, and the Na^+ and K^+ concentrations in their shoots (A, B, E, F) and roots (C, D, G, H) and shoot/root ratios of Na^+ (I, K) and K^+ (J, L) concentrations were determined. Data are presented as the mean ± SE (n = 3–4, biological replicates). Single and double asterisks denote significant differences compared with the values of WT plants at $P < 0.05$ and $P < 0.01$, respectively, determined using Student's t-test.

to K^+ deficiency, *S. virginicus* plants were hydroponically cultivated in 1/2 MS salt solution, then transplanted to 0.1 mM K^+ medium (Supplementary Table S4), and harvested for RNA isolation at the time indicated.

Whole plants (n = 3, biological replicates) of WT and transgenic seedlings were harvested for RNA isolation 10 d after sowing to determine transcription levels of *SvHKT2;1* and *SvHKT2;2* in transgenic Arabidopsis lines. The RNeasy plant mini kit was used to extract the total RNA (Qiagen), and real-time qRT–PCR was performed as previously reported (Yamamoto et al. 2015). Two pairs of primer sets were used: one pair, qSvHKT2;2-F and qSvHKT2;2-R, is specific to SvHKT2;2 and the other, qSvHKT2;x-F and qSvHKT2;x-R, is common to both *SvHKT2;1* and *SvHKT2;2* (Supplementary Table S3). Specific primers for *SvHKT2;1* could not be designed because of its high sequence similarity to *SvHKT2;2*. The relative expression levels of the target to reference gene, actin for *S. virginicus* or ubiquitin extension protein (*UBQ5*, AT3G62250.1) for Arabidopsis, were calculated using the delta-delta Ct method.

Subcellular localization of SvHKT2;1 and SvHKT2;2 in *N. benthamiana* leaves

To examine the subcellular localization of SvHKT2;1 and SvHKT2;2, the entry vectors pENTER-SvHKT2;1 and pENTER-SvHKT2;2 were reacted with a destination vector pH7WGF2.0 encoding an N-terminal EGFP fusion (Karimi et al. 2002) using LR clonase reactions (Thermo Fisher Scientific). As a control, a non-fused EGFP construct was used. After confirmation by DNA sequencing, the recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101 then infiltrated into *N. benthamiana* leaves. Two days post-infiltration, GFP fluorescence images were observed using an LSM 5 EXCITER confocal fluorescence microscope with a C ApoChromat ×40/1.2 W Corr objective lens (water; Carl Zeiss). A Zeiss LSM Image Browser (Carl Zeiss) was used to process the images.

Functional analysis of SvHKT2;1 and SvHKT2;2 in *X. laevis* oocytes

The SvHKT2;1 and SvHKT2;2 cDNAs were excised from the entry vectors pENTER-SvHKT2;1 and pENTER-SvHKT2;2 using the restriction enzymes *Not*I and *Asc*I, then, inserted into the *Not*I and *Asc*I sites of pXBG-NA under the control of the T_3 promoter, which was constructed by inserting a *Bgl*II–*Not*I–*Asc*I–*Bgl*II linker–adaptor into the *Bgl*II site of pXBG-ev1 (Preston et al. 1992, Katsuhara et al. 2002). A mMESSAGE mMACHINE in vitro transcription kit (Thermo Fisher Scientific) was used to synthesize the capped RNA mimics. Oocytes and TEVC experiments were prepared and performed as described previously (Yao et al. 2010), with a minor modification. In brief, 12.5 ng of cRNA (*SvHKT2;1* or *SvHKT2;2*) was injected into *X. laevis* oocytes, and incubated at 18°C for 2 d. Water-injected oocytes were also prepared as controls in each experiment. The data recordings and analysis were performed using an Axoclamp 900 A amplifier and an Axon Instruments Digidata 1440 A with Clampex 10.3 and Clampfit 10.3 software (Molecular Devices). The analyses of ion selectivity using alkali cation salts utilized oocytes bathed in a solution containing 1.8 mM $CaCl_2(2H_2O)$, 1 mM $MgSO_4(7H_2O)$, 10 mM 2-MES and the indicated concentrations of Na or K glutamate salts, adjusted to pH 5.5 with 1,3-bis[tris(hydroxymethyl)methylamino] propane. The osmolality of each solution was measured using a vapor pressure Wescor 5200 osmometer (Wescor Inc.), and adjusted to 199 ± 6 (max/min) mOsmol kg^{-1} with D-mannitol. Voltage steps were applied from −120 to +30 mV in 15 mV increments, with a holding potential as a resting potential before voltage clamping. All the experiments were performed at 18°C.

To measure Rb^+ (K^+) and Na^+ uptake of HKT-expressing *Xenopus* oocytes, oocytes were injected with 25 ng of SvHKT cRNAs, water as a negative control or 6.125 ng of OsHKT2;2/1 cRNA (Suzuki et al. 2016) as a positive control. Rb^+

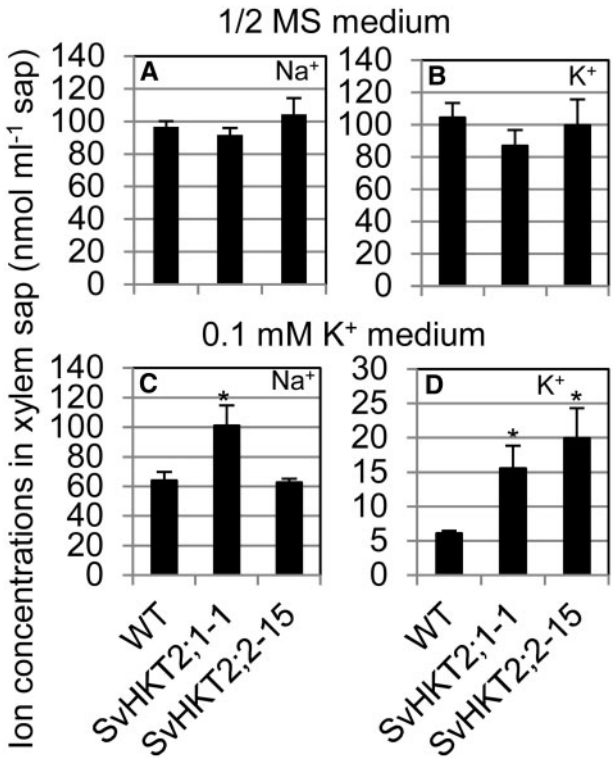

Fig. 9 Na^+ and K^+ concentrations in xylem and phloem sap from SvHKTs transformants and WT plants grown in 1/2 MS or 0.1 mM K^+ medium. Two-week-old seedlings germinated on 1/2 MS medium were hydroponically cultured in 1/2 MS (A, B) or 0.1 mM K^+ (C, D) medium until the bolting stage; their xylem saps were collected, and their Na^+ (A, C) and K^+ (B, D) concentrations were determined. Data are presented as the mean ± SE (n = 3–4, biological replicates). Single and double asterisks denote significant differences compared with the values of WT plants at $P < 0.05$ and $P < 0.01$, respectively, determined using Student's t-test.

was a tracer of K^+. Injected oocytes were incubated in modified Barth's solution (MBS) including 88 mM NaCl and 1 mM KCl (Katsuhara et al. 2002) for 1 d at 18°C. Five oocytes were grouped in a well with 1 ml of MBS, and 140 kBq of ^{86}RbCl (Perkin Elmer) per well or 92.5kBq of ^{22}NaCl (Perkin Elmer) per well was added to start Rb^+ (K^+) or Na^+ uptake, respectively. After 60 min, oocytes were washed four times with non-labeled MBS. Washed oocytes were transferred to scintillation vials, and radioactivity of ^{86}Rb was measured with a scintillation counter (Tri-Carb 2800TR, Perkin Elmer). For radioactivity of ^{22}Na, washed oocytes were transferred to plastic tubes, and measured with a γ-ray counter (AccuFLEX g ARC-7001, Aloka).

Functional analysis of SvHKT2;1 and SvHKT2;2 in yeast

The protein expression vector pAUT1 was produced by inserting the *Kpn*I–*Not*I–*Asc*I–*Sal*I linker–adaptor into *Kpn*I and *Sal*I sites of pAUR123 (TAKARA BIO INC.), in which the transgene was driven by the constitutive alcohol dehydrogenase 1 promoter from *Saccharomyces cerevisiae*. The SvHKT2;1 and SvHKT2;2 cDNAs were excised from the entry vectors using the restriction enzymes *Not*I and *Asc*I, and inserted into the expression vector, pAUT1. Resultant expression vectors were transformed into the yeast *S. cerevisiae* strain 9.3 (ATCC, No. 201409), in which the original potassium transporters (TRK1 and TRK2) and the P-type ATPases involved in Na^+ extrusion (ENA1–ENA4) were deleted. The yeast cells were transformed by electroporation, grown in YPD medium and amplified until the OD_{600} value reached approximately 1.0. Then, the cells were washed with SC/-His medium (Supplementary Table S5) supplemented with 0.2 mM K^+, and resuspended in the same medium at an OD_{600} value of 1.0. Basal SC/-His medium contains 0.125 mM K^+ and 0.678 mM Na^+, which originate form DO-His medium (TAKARA BIO INC.). For the growth tests on agar medium, resuspended yeast cells were diluted, and spotted onto SC/-His agar medium that contained different concentrations of KCl and/or NaCl. For the growth test in liquid medium, 1 ml of resuspended yeast cells were transferred to 100 ml of SC/-His medium (Supplementary Table S5) supplemented with 0.2 mM K^+, incubated for 24 h, then 10 mM K^+ was added and the culture was incubated for an additional 20 h. To measure ion concentrations in yeast, a portion of the yeast cells were harvested from each culture by centrifugation at 10,000 r.p.m. for 10 min at 4°C, resuspended in 1.5 mM $CaCl_2$ solution, collected again by centrifugation, vacuum-dried for 2 min and dried in an oven at 60°C for 5 h. Dried yeast was suspended in 0.5% HNO_3, and ions were extracted using an ultrasonicator 'ULTRA S Homogenizer VP-55' (TAITEC). K^+ and Na^+ concentrations in the extracts were determined using an Ion analyzer IA-300 (TOA DKK). All the yeast cell incubations were carried out at 30°C.

Production of transgenic Arabidopsis

To produce transgenic Arabidopsis, expression vectors of SvHKT2;1 and SvHKT2;2 were constructed by Gateway technology. The entry vectors were reacted by LR enzyme with a destination vector, pGH1, in which the NOS terminator of pGWB2 (GenBank AB289765.1, a gift from Dr. Nakagawa) was excised by restriction enzymes *Sac*I and *Stu*I and replaced by the Arabidopsis heat shock protein terminator gene from pRI201-AN (TAKARA BIO INC.) excised by restriction enzymes *Sac*I and *Sma*I, followed by deletion of the neomycin phosphotransferase gene with its promoter and terminator by digestion with restriction enzymes *Hin*dIII and *Eco*81I, and self-ligation. Arabidopsis WT plants (ecotype Columbia) were transformed with expression vectors by floral dipping (Clough and Bent 1998). *Agrobacterium* strain GV3101 was used for transformation.

Plant growth conditions

To examine growth and ion concentrations in transgenic and WT Arabidopsis plants, seeds were sown on 1/2 MS medium (containing 10 mM K^+ and 0.1 mM Na^+) or 0.1 mM K^+ medium (containing 0.725 mM Na^+) (Supplementary Table S4) supplemented with 1% sucrose and 30 μg ml^{-1} hygromycin, and the germinated seedlings were transplanted onto agar medium supplemented with different concentrations of K^+ and/or Na^+. The plants were grown at 23°C under a 16 h/8 h light/dark cycle with approximately 60 μmol m^{-2} s^{-1} light intensity. To measure root elongation, agar plates were positioned vertically, and the root length was measured on the day of transplantation and at the end of incubation.

Hydroponic culture of Arabidopsis was performed using the Home Hyponica Karen (Kyowa Co., LTD.) system with 1/2 MS or 0.1 mM K^+ medium as the hydroponic culture solution. Fourteen-day-old plants grown on 1/2 MS agar medium were transplanted to the hydroponic system. Roots and shoots were sampled and their ion content was determined after a further 14 d of cultivation.

Measurement of ion content in plants

To measure ion content, dried plant materials were powdered using a mortar and pestle or a SK mill (Tokken) manual crusher, then suspended in 0.5% HNO_3, and the mixture was incubated at 60°C overnight. The ion content in the extracts was determined using an Ion Analyzer IA-300, and expressed as micromoles per gram of dry weight (μmol g DW^{-1}).

Collection of xylem sap

Transgenic and WT Arabidopsis plants were grown on 1/2 MS plate medium for 2 weeks, then were transplanted to liquid 1/2 MS or 0.1 mM K^+ (Supplementary Table S4) medium until they reached the bolting stage. Collection of xylem sap was carried out according to the methods of Sunarpi et al. (2005). The collected samples were diluted in 0.5% HNO_3 solution and were used for ion measurement using an Ion Analyzer IA-300.

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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