AtHKT1 Facilitates Na$^+$ Homeostasis and K$^+$ Nutrition in Planta[1]

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Genetic and physiological data establish that Arabidopsis AtHKT1 facilitates Na$^+$ homeostasis in planta and by this function modulates K$^+$ nutrient status. Mutations that disrupt *AtHKT1* function suppress NaCl sensitivity of *sos1-1* and *sos2-2*, as well as of *sos3-1* seedlings grown in vitro and plants grown in controlled environmental conditions. *hkt1* suppression of *sos3-1* NaCl sensitivity is linked to higher Na$^+$ content in the shoot and lower content of the ion in the root, reducing the Na$^+$ imbalance between these organs that is caused by *sos3-1*. *AtHKT1* transgene expression, driven by its innate promoter, increases NaCl but not LiCl or KCl sensitivity of wild-type (Col-0 *gl1*) or of *sos3-1* seedlings. NaCl sensitivity induced by AtHKT1 transgene expression is linked to a lower K$^+$ to Na$^+$ ratio in the root. However, *hkt1* mutations increase NaCl sensitivity of both seedlings in vitro and plants grown in controlled environmental conditions, which is correlated with a lower K$^+$ to Na$^+$ ratio in the shoot. These results establish that AtHKT1 is a focal determinant of Na$^+$ homeostasis in planta, as either positive or negative modulation of its function disturbs ion status that is manifested as salt sensitivity. K$^+$-deficient growth of *sos1-1*, *sos2-2*, and *sos3-1* seedlings is suppressed completely by *hkt1-1*. *AtHKT1* transgene expression exacerbates K$^+$ deficiency of *sos3-1* or wild-type seedlings. Together, these results indicate that AtHKT1 controls Na$^+$ homeostasis in planta and through this function regulates K$^+$ nutrient status.

Na$^+$ disequilibrium is a primary consequence of salt stress and leads to catastrophic pathologies affecting cell survival, division, and growth (Blumwald, 2000; Hasegawa et al., 2000; Tester and Davenport, 2003; Zhu, 2003). Some of the critical Na$^+$ homeostasis determinants are now identified because of recent research discoveries (Blumwald, 2000; Hasegawa et al., 2000; Tester and Davenport, 2003; Zhu, 2003). Mutations that render the plasma membrane Na$^+$/H$^+$ antiporter SOS1 dysfunctional cause NaCl hypersensitivity of Arabidopsis plants (Wu et al., 1996; Shi et al., 2002). Overexpression of the vacuolar NHX1 or the plasma membrane SOS1 Na$^+$/H$^+$ antiporter increases NaCl tolerance of plants by controlling Na$^+$ homeostasis under saline stress conditions (Apse et al., 1999; Zhang and Blumwald, 2001; Zhang et al., 2001; Shi et al., 2003). These results confirm that greater capacity to maintain Na$^+$ homeostasis enhances salt adaptation of plants.

Another consequence of Na$^+$ disequilibrium that is caused by salt stress is the detrimental effect of Na$^+$ on K$^+$ acquisition and nutrition. K$^+$ has essential functions in plant metabolism (e.g. charge balance, osmotic adjustment, and enzyme catalysis) and in growth and development (Maathuis and Sanders, 1996; Rigas et al., 2001; Elumalai et al., 2002). Na$^+$ in the soil solution disturbs K$^+$ homeostasis in plants presumably because the cytotoxic ion competes for binding sites in transport systems that mediate K$^+$ uptake (Epstein, 1961; Rains and Epstein, 1965; Niu et al., 1995; Hasegawa et al., 2000). Ca^{2+} is necessary to control K$^+$/Na$^+$ selective accumulation into plants, which effectively reduces Na$^+$ uptake and increases NaCl tolerance (Epstein, 1961; Cramer et al., 1987; Hasegawa et al., 2000; Demidchik et al., 2002; Zhu, 2003). Data from physiological research of more than 40 years ago identified high- and low-affinity transport systems that mediate K$^+$ uptake into roots and determined that both Ca^{2+} and Na$^+$ modulate the acquisition of this essential mineral nutrient (Epstein, 1961). K$^+$/Na$^+$ selective accumulation by both systems is Ca^{2+} dependent with the high-affinity system transporting predominantly K$^+$ over Na$^+$ when Ca^{2+} is present (Epstein, 1961; Rains and Epstein, 1965). In addition, plants have high- and low-affinity systems for Na$^+$ uptake (Epstein, 1961; Rains and Epstein, 1967). K$^+$ competes effectively with Na$^+$ for uptake by the low-affinity system in the presence of Ca^{2+}, while Na$^+$ uptake by the high-affinity system is predominant

even in the presence of K$^+$ and Ca^{2+} (Epstein, 1961; Rains and Epstein, 1967).

There is now more comprehensive understanding about the transport systems and regulatory mechanisms that mediate K$^+$ and Na$^+$ homeostasis in plants, including some Ca^{2+}-dependent processes that control K$^+$/Na$^+$ selective uptake (Epstein, 1998; Zhu, 2002, 2003). The Ca^{2+}-dependent salt overly sensitive (SOS) ion homeostasis pathway transduces salt stress signals to activate the plasma membrane SOS1 Na$^+$/H$^+$ antiporter, which mediates Na$^+$ efflux and homeostasis necessary for salt adaptation (Qiu et al., 2002; Zhu, 2002, 2003; Shi et al., 2003). *sos* mutant plants (*sos1*, *sos2*, and *sos3*) are NaCl sensitive and exhibit K$^+$ deficiency at μM external concentrations of the essential nutrient. The later phenotype implicates a positive regulatory effect on K$^+$ acquisition by the SOS signaling pathway, although neither the regulatory components nor the determinants that are regulated have been identified. Pretreatment of seedlings with 50 mM NaCl reduces K$^+$ permeability of plasma membranes from *sos1* root cells but not from those of wild-type seedlings (Qi and Spalding, 2001). Reduced K$^+$ uptake into and K$^+$ deficiency of *sos1* plants is attributed to elevated cytosolic Na$^+$ levels, which occur because the plasma membrane Na$^+$/H$^+$ antiporter is inoperative (Qi and Spalding, 2001; Qiu et al., 2002; Quintero et al., 2002).

Ca^{2+} affects K$^+$/Na$^+$ selective uptake also because it inhibits Na$^+$ influx across the plasma membrane, which is attributed to its negative regulation of non-selective cation channels (NSCCs; Roberts and Tester, 1997; Davenport and Tester, 2000; Demidchik and Tester, 2002; Tester and Davenport, 2003). NSCCs are characterized by the lack of monovalent cation selectivity and, for many, by Ca^{2+} inhibition of ion conductance (Demidchik et al., 2002; Tyerman, 2002; Tester and Davenport, 2003). The similar Ca$^{2+}_{ext}$ sensitivity of NSCC-mediated Na$^+$ currents and Na$^+$ influx into roots has implicated NSCCs as potential Na$^+$ uptake systems (Roberts and Tester, 1997; Tyerman et al., 1997; Amtmann and Sanders, 1999; Schachtman and Liu, 1999; Davenport and Tester, 2000; Tyerman, 2002). Ca^{2+}-inhibition of NSCC conductance is not absolute so it is possible these ion channels are a substantial leak for Na$^+$ influx, particularly in saline conditions (Schachtman and Liu, 1999; Davenport and Tester, 2000; Demidchik and Tester, 2002). However, the precise contribution of NSCCs to Na$^+$ uptake into plant roots awaits the identification of the molecular determinants and the necessary genetic resources for physiological dissection, although it seems evident that multiple molecular entities contribute to Na$^+$ influx at <mM Ca$^{2+}_{ext}$.

hkt1 alleles are genetic suppressors of *sos3-1* NaCl sensitivity (Rus et al., 2001). Physiological characterization of *hkt1* genotypes established that AtHKT1 controls plant Na$^+$ homeostasis and is not a K$^+$ transport system. Reduction of *TaHKT1* expression by RNAi or cosuppression decreased Na$^+$ uptake into wheat roots and increased plant salt tolerance (Laurie et al.,

2002), despite electrophysiological data from experimentation based on expression in heterologous systems that implicated a Na$^+$-dependent K$^+$ cotransport function for TaHKT1 (Rubio et al., 1995). High- and low-affinity Na$^+$ uptake into rice (*Oryza sativa*) roots is mediated by at least two independent OsHKT transport systems (Garciadeblás et al., 2003). Recently, it was determined that AtHKT1 controls Na$^+$ accumulation in shoots through a proposed mechanism in which the transport system loads Na$^+$ into the shoot phloem and facilitates recirculation of the ion from the shoot to the root (Mäser et al., 2002; Berthomieu et al., 2003). HKT function in the control of Na$^+$ acquisition (Rus et al., 2001; Laurie et al., 2002; Mäser et al., 2002; Berthomieu et al., 2003; Garciadeblás et al., 2003) and *hkt1* suppression of K$^+$ deficiency of *sos3-1* seedlings establishes that AtHKT1 is a focal determinant of K$^+$/Na$^+$ homeostasis and implicates a function as a downstream effector of the Ca^{2+}-dependent SOS signaling pathway (Rus et al., 2001).

Here, we present genetic (loss- and gain-of-function) and physiological evidence that AtHKT1 controls Na$^+$ homeostasis in planta, a function that apparently is not dependent on Ca^{2+} or the SOS signal pathway. Furthermore, AtHKT1 regulation of K$^+$ nutrient status is linked to its Na$^+$ homeostasis function and occurs both under conditions of normal or stress Na$^+$ levels.

RESULTS

hkt1 Mutations Are Genetic Suppressors of *sos1-1*, *sos2-2*, and *sos3-1* NaCl Hypersensitivity

Previously, we determined that AtHKT1 controls Na$^+$ homeostasis in Arabidopsis because *hkt1-1* or *hkt1-2* can suppress the NaCl sensitivity of *sos3-1* seedlings by regulating Na$^+$ accumulation in planta (Rus et al., 2001). *hkt1-1* is also a genetic suppressor of *sos1-1* and *sos2-2* since *sos1-1 hkt1-1* and *sos2-2 hkt1-1* double mutant seedlings exhibit greater root growth (Fig. 1, A–C) and shoot development with less leaf anthocyanin accumulation (data not shown) when grown in medium supplemented with NaCl. NaCl sensitivity of *sos1-1* and *sos2-2* seedlings is suppressed to a lesser extent than that of *sos3-1* seedlings by *hkt1-1*, presumably because intracellular Na$^+$ homeostasis is more impaired in *sos1-1* and *sos2-2* seedlings (Fig. 1, A–C; Qiu et al., 2002). NaCl sensitivity suppression of *sos* mutations by *hkt1-1* is less effective when seedlings are grown in medium without Ca^{2+} supplement (Fig. 1, A′–C′; Rus et al., 2001), presumably because the Ca^{2+}-sensitive Na$^+$ uptake system(s) is activated due to the reduced Ca$^{2+}_{ext}$ (Tyerman, 2002; Tester and Davenport, 2003). Interestingly, *hkt1-1* also suppresses the growth defect of *sos1-1* and *sos2-2* seedlings that is evident on medium without NaCl supplement (Fig. 1, A and B). AtHKT1 mRNA abundance is not altered in *sos1-1*, *sos2-2*, or *sos3-1* plants (data not shown), indicating that *AtHKT1* is not a transcriptional output of the SOS pathway.

Figure 1. *hkt1-1* is a genetic suppressor of *sos* mutations. In vitro grown seedlings (4 d on germination medium) were transferred to a basal medium (MS salts without CaCl$_2$, 30 g L^{-1} Suc, and 12 g L^{-1} Bacto Agar, DIFCO; Becton-Dickinson, Le Pont de Claix, France) supplemented with various amounts of NaCl, and either 3 mM (A, B, C, and D) or without CaCl$_2$ (A′, B′, C′, and D′). Root length increase was determined 5 d after transfer; values are the mean ± SE ($n \geq 8$).
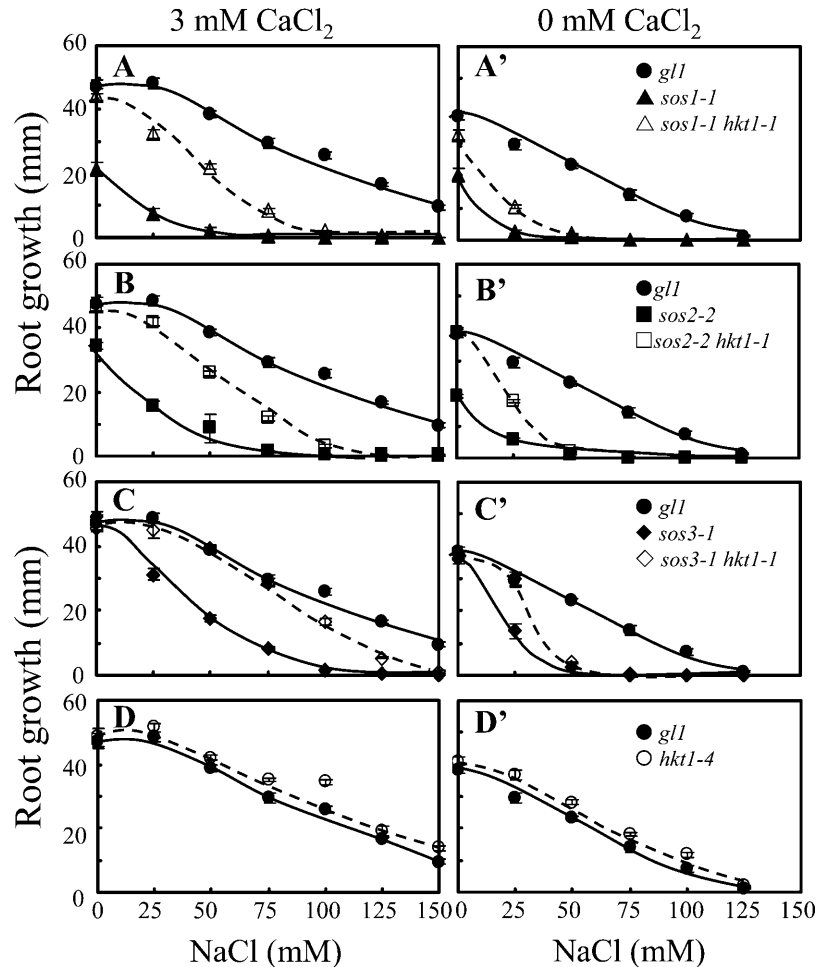
hkt1-1 suppression of *sos1-1*, *sos2-2*, and *sos3-1* and *hkt1-4* suppression of *sos3-1* NaCl sensitivity is evident also when plants are grown in soil (Fig. 2) or Turface (Fig. 3) under controlled environmental conditions. The *hkt1-4* allele has a 16-bp deletion in the third exon (position 3,496–3,511 of the open reading frame) that is predicted to result in a frame-shift causing a nonsense mutation. *hkt1-4* suppresses *sos3-1* NaCl sensitivity and K$^+$ deficiency of seedlings grown in vitro to the same extent as *hkt1-1* and *hkt1-2* (data not shown), indicating that the deletion results in a dysfunctional allele. These results establish that the capacity of *hkt1* to attenuate NaCl salt hypersensitivity of *sos* mutations is operative in plants that are actively transpiring. NaCl-treated *sos3-1 hkt1-4* plants exhibit a similar shoot and root Na$^+$ content as wild type (Fig. 4), indicating that *hkt1* restores Na$^+$ homeostasis of *sos3-1* plants to near that of wild type.

Salt Responses of *hkt1* Plants

hkt1-1 and *hkt1-4* alleles were segregated from *sos3-1* after backcrossing of plants to wild-type plants (*gl1* × *sos3-1 hkt1-1* and *gl1* × *sos3-1 hkt1-4*, respectively).

There was no difference in root (Fig. 1, D and D′) or shoot growth (data not shown) between *hkt1* and wild-type seedlings 6 d after transfer to medium without or supplemented with NaCl. After prolonged growth on medium supplemented with 75 mM NaCl, *hkt1* plants exhibited reduced shoot growth and tip senescence of mature leaves (data not shown). *hkt1-4* also caused NaCl sensitivity of plants grown in Turface that was manifested as leaf tip senescence and shoot growth inhibition (Fig. 3) and higher and lower Na$^+$ content in the shoot and root, respectively, relative to wild type (Fig. 4). Tip senescence was most pronounced for mature leaves (Fig. 3) 2 weeks after initiation of the salt treatment. The timing of symptom development is indicative of salt rather than hyperosmotic stress pathology (Munns, 2002).

AtHKT1-Mediated Salt Sensitivity Is Na$^+$ Specific

Expression of *AtHKT1* (*AtHKT1:AtHKT1*) in *sos3-1 hkt1-1* seedlings results in a phenocopy of *sos3-1* Na$^+$ sensitivity (data not shown) based on analysis of progeny from three independent T$_2$ lines that are homozygous for the transgene. This same binary
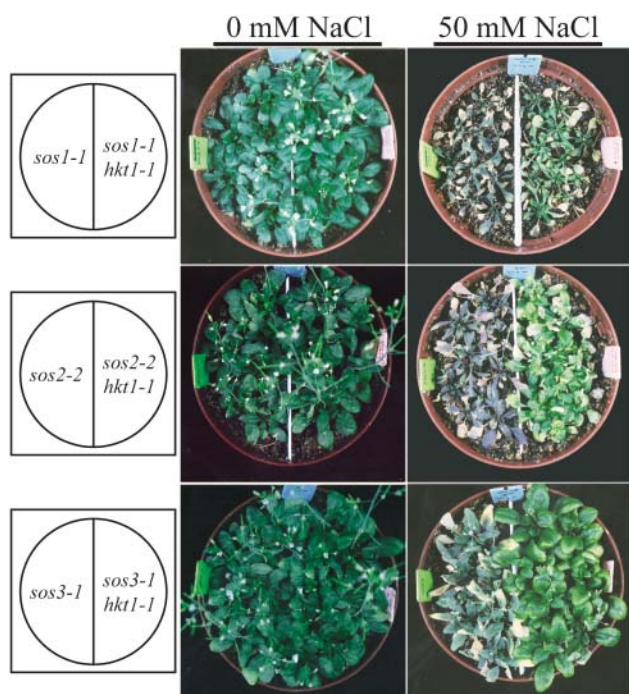
Figure 2. *hkt1-1* suppresses NaCl hypersensitivity of soil-grown *sos* plants. Three-week-old plants in soil were irrigated by placing the pots in a container filled with deionized water without or with 50 mM NaCl until the soil was saturated by capillarity. Plants were rewatered weekly, and pictures were taken 3 weeks later.

vector was used to express the *AtHKT1* transgene in *sos3-1* (*sos3-1-HKT1:HKT1*) and wild-type (*gl1-HKT1:HKT1*) plants, and five and six independent lines, respectively, were identified, based on segregation for kanamycin resistance of transgenic lines with a single functional insertion, i.e. 3:1 segregation ratio. Plants of all these lines were not preselected for altered NaCl growth response, and all have greater steady-state *AtHKT1* transcript abundance (data not shown). NaCl treatment (150 mM NaCl) does not affect *AtHKT1* transcript abundance either in plants of wild-type (Uozumi et al., 2000; Rus et al., 2001) or *AtHKT1* transgene expressing lines (data not shown). *AtHKT1* transgene expression increases NaCl sensitivity of *sos3-1* (*sos3-1-HKT1:HKT1*; Fig. 5) and wild-type *gl1* (*gl1-HKT1:HKT1*; Fig. 6) seedlings. Salt sensitivity is Na$^+$-specific as seedlings expressing the *AtHKT1* transgene are not more adversely affected by LiCl or KCl than the nonexpressing lines (Fig. 7).

AtHKT1 transgene expression exacerbates NaCl-induced stress symptoms of plants that are grown in soil (data not shown). *AtHKT1* transgene expressing plants have a lower K$^+$ to Na$^+$ ratio in the roots due primarily to decreased absolute K$^+$ accumulation (Fig. 8). Together, these results indicate that any modulation of in planta AtHKT1 function causes Na$^+$ disequilibrium and stress symptoms.

### *hkt1* Mutations Are Genetic Suppressors of *sos1-1*, *sos2-2*, and *sos3-1* K$^+$ Deficiency

hkt1-1, *hkt1-2*, and *hkt1-4* mutations suppress the growth defect of *sos3-1* seedlings that occurs on medium without or with μM levels of KCl supplement and 0.15 mM CaCl$_2$ (Rus et al., 2001; Fig. 9C; data not shown for *hkt1-4*). *hkt1-1* also suppresses the K$^+$ deficiency of *sos1-1* (Fig. 9A) and *sos2-2* (Fig. 9B) seedlings. No differences in shoot or root growth of wild-type or *hkt1* seedlings occurred on medium without or with KCl supplement (data not shown). Presumably, some growth is supported on medium that is devoid of KCl supplement because there is a trace level of the mineral element in the basal medium (approximately 0.12 mM) and the seedlings accumulate some K$^+$ while on germination medium. These results are genetic evidence that AtHKT1 is not a K$^+$ uptake system in planta but, either directly or indirectly, is a negative regulator of K$^+$ nutrition.

AtHKT1 Regulation of K$^+$ Nutrition Is Dependent on Na$^+$ Acquisition

AtHKT1 transgene expression that increases Na$^+$ sensitivity of *sos3-1* seedlings (Fig. 5) also causes greater K$^+$ deficiency that is manifested as reduced
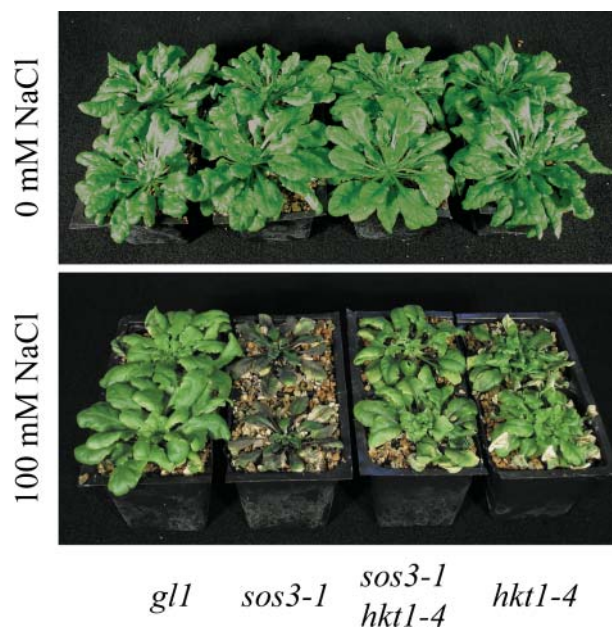
Figure 3. *hkt1-4* suppresses NaCl sensitivity of *sos3-1* plants but causes NaCl sensitivity of wild-type (*gl1*) plants. Stratified seeds in soil were transferred to a growth chamber for germination and plant growth. Plants were irrigated by placing the pots in a container of EXCEL solution until the soil was saturated. EXCEL includes 167 mg L^{-1} K$^+$, 67 mg L^{-1} Ca^{2+}, and no detectable Na$^+$, according to the manufacturer's specifications. One-month-old plants were transplanted into Turface. After 1 week of acclimation, plants were watered every 3 or 4 d with EXCEL solution (0 mM NaCl) or supplemented with NaCl (50 mM NaCl for four treatments followed by 100 mM NaCl for 7 treatments). Photographs were taken 5 weeks after beginning of treatment.
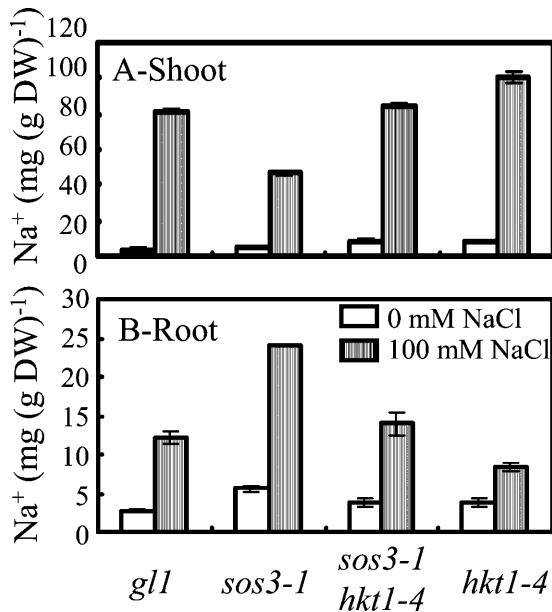
Figure 4. *hkt1-4* restores Na$^+$ content of *sos3-1* plants to a level similar to that of wild-type plants (*gl1*). Plants were grown as described in Figure 3. Plants were harvested 5 weeks after the start of NaCl treatment. Shoots and roots ($n \geq 7$ for each genotype per treatment) were separated, and materials were rinsed with deionized water and pooled into independent groups (minimum of approximately 50 mg after drying) for ion content analyses. A, Na$^+$ content in the shoot; values are the mean $\pm$ SE ($n = 3$). The fresh weight of the plants not treated with NaCl is *gl1*, 2.95 $\pm$ 0.2 g; *sos3-1*, 2.37 $\pm$ 0.25 g; *sos3-1 hkt1-4*, 2.32 $\pm$ 0.21 g; and *hkt1-4*, 2.63 $\pm$ 0.24 g, and of plants treated with NaCl (100 mM final concentration) is *gl1*, 2.34 $\pm$ 0.16 g; *sos3-1*, 0.44 $\pm$ 0.03 g; *sos3-1 hkt1-4*, 1.23 $\pm$ 0.09 g; and *hkt1-4*, 1.27 $\pm$ 0.04 g. B, Na$^+$ content in the root; values are the mean $\pm$ SE ($n \geq 2$), except for *sos3-1* treated with 100 mM NaCl. These data are representative of those obtained in replicate experiments.

root growth (Fig. 10) and increased root branching and leaf chlorosis (data not shown) in medium supplemented with 20 μM KCl but without added NaCl. The nutrient medium without added NaCl contains approximately 3 to 4 mM Na$^+$. No difference in growth was observed when *sos3-1* or *sos3-1-HKT1:HKT1* seedlings were grown on medium supplemented with 10 mM KCl (data not shown), indicating that AtHKT1 negatively affects K$^+$ acquisition by affecting uptake that occurs at μM K$^+_{ext}$. No difference in steady-state transcript abundance of *AKT1*, *TRH1*, or *SKOR* was detected in *sos3-1*, *sos3-1-pCAM* (vector control), and *sos3-1-HKT1:HKT1* plants (data not shown). These genes encode transport systems that are involved in K$^+$ uptake into roots or loading of the essential element into the xylem (Gaymard et al., 1998; Hirsch et al., 1998; Rigas et al., 2001). Consequently, AtHKT1 modulation of K$^+$ nutrient status in planta does not involve regulating the expression of the genes that encode these K$^+$ transport systems.

Interestingly, K$^+$ deficiency of *sos3-1* seedlings that is induced by *AtHKT1* transgene expression (65%–75%

reduction in root growth) is not affected by increasing the CaCl$_2$ level in the medium from 0.15 to 3 mM (Fig. 10), indicating that AtHKT1 activity is not Ca^{2+} dependent. An increase in Ca$^{2+}_{ext}$ enhances absolute root growth of all seedlings (Fig. 10) and reduces leaf chlorosis (data not shown), which may be attributable to partial Ca^{2+}-activation of the defective *sos3-1* protein (Ishitani et al., 2000).

AtHKT1 transgene expression affects K$^+$-dependent growth of wild-type seedlings only if the medium is supplemented with 20 mM NaCl (Fig. 11), unlike the situation of *sos3-1* seedlings (Fig. 10). The *AtHKT1*-induced root growth inhibition (Fig. 11B) apparently is not a Na$^+$ cytotoxicity effect since seedling root growth of all genotypes is similar at this concentration of NaCl if the medium is supplemented with 10 mM KCl (Fig. 11B). The impact of the Ca^{2+}-sensitive Na$^+$ entry system(s) was minimized in this experiment by in-
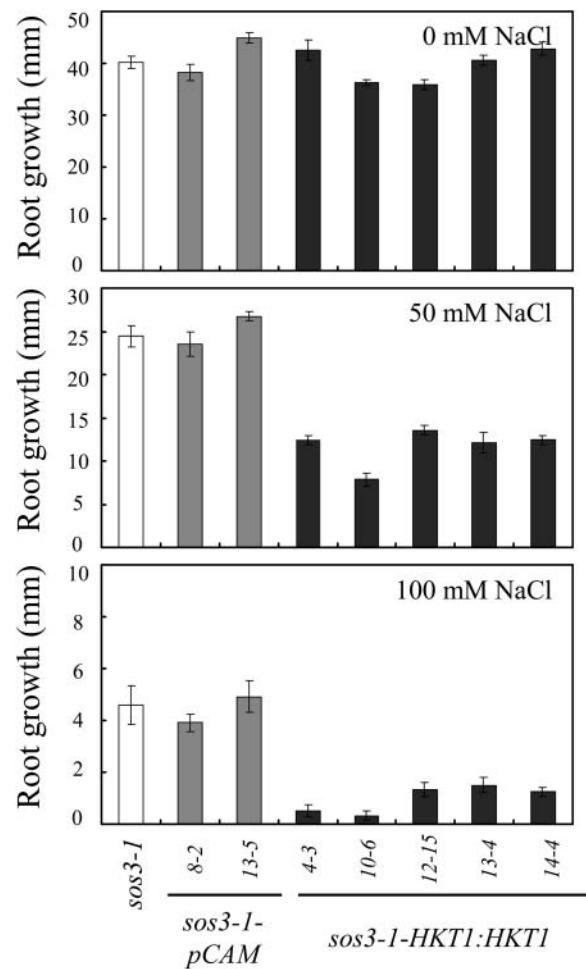
Figure 5. *AtHKT1:AtHKT1* transgene expression increases NaCl hypersensitivity of *sos3-1* seedlings. Four-day-old in vitro-grown seedlings were transferred to a basal medium (MS salts, 30 g L^{-1} Suc, and 12 g L^{-1} Bacto Agar) without or supplemented with NaCl; *sos3-1*, vector control (*sos3-1-pCAM*, two independent lines) and *AtHKT1:AtHKT1* transformed lines (*sos3-1-HKT1:HKT1*, five independent lines). Root growth was determined 6 d after transfer; values are the mean $\pm$ SE ($n \geq 11$).
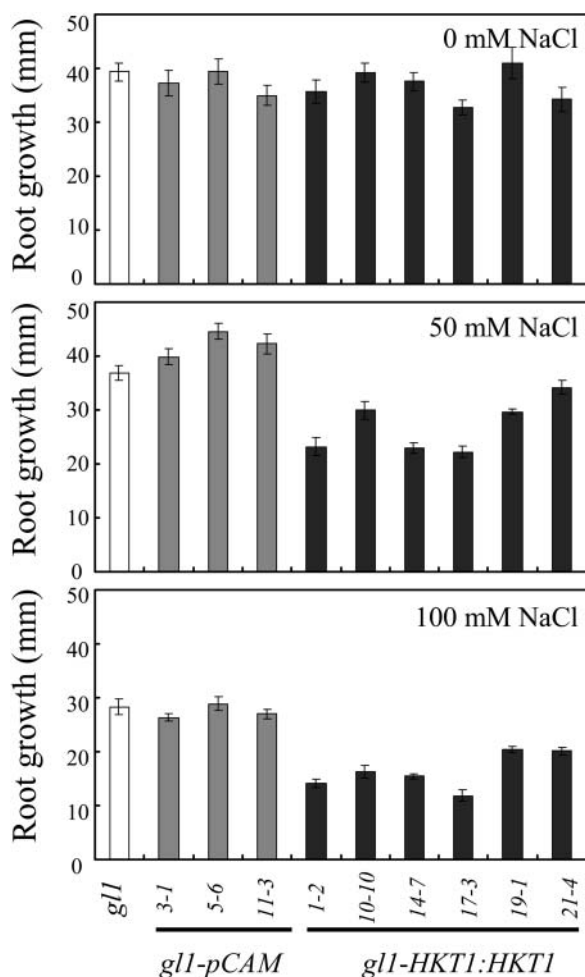
Figure 6. *AtHKT1:AtHKT1* transgene expression confers NaCl sensitivity to wild-type seedlings. Four-day-old seedlings were transferred to a basal medium (see Fig. 5 legend) without or supplemented with NaCl; wild-type (*gl1*), vector control (*gl1-pCAM*, three independent lines), and *AtHKT1:AtHKT1* transformed lines (*gl1-HKT1:HKT1*, six independent lines). Root growth was determined 6 d after transfer; values are the mean ± SE ($n \geq 10$).

clusion of 3 mM CaCl$_2$. Together, these results indicate that AtHKT1 negatively affects K$^+$ acquisition through its function as a Na$^+$ transport system. Presumably, addition of 20 mM NaCl is required to elicit the inhibitory effect of AtHKT1 on K$^+$ nutrition of wild-type seedlings, in contrast to that of *sos3-1* seedlings, because the SOS pathway is functional and cells are more capable of intracellular Na$^+$ homeostasis.

DISCUSSION

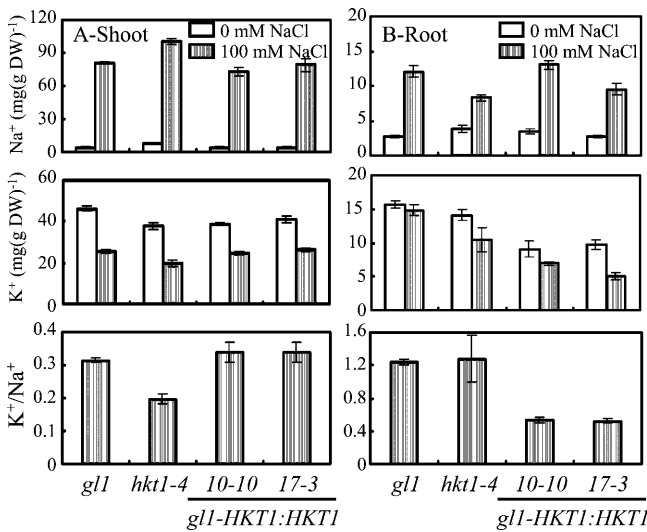### AtHKT1 Controls Na$^+$ Homeostasis in Planta

Herein comprehensive evidence from loss- and gain-of-function genetic experimentation establishes that AtHKT1 mediates Na$^+$ homeostasis in Arabidopsis. *hkt1* suppresses NaCl sensitivity of *sos1-1*, *sos2-2*, and

sos3-1 seedlings in vitro and plants that are grown in soil or Turface under controlled environmental conditions. Furthermore, *AtHKT1* transgene expression increases NaCl, but not LiCl or KCl, sensitivity of wild-type and *sos3-1* seedlings.

In vitro grown *hkt1-4* seedlings exhibit NaCl stress symptoms only after a prolonged period. However, actively transpiring *hkt1-4* plants (grown in Turface) are acutely more NaCl-sensitive and accumulate more Na$^+$ in the shoot and less in the root compared to wild-type plants (Figs. 3 and 4). Presumably, active transpi-
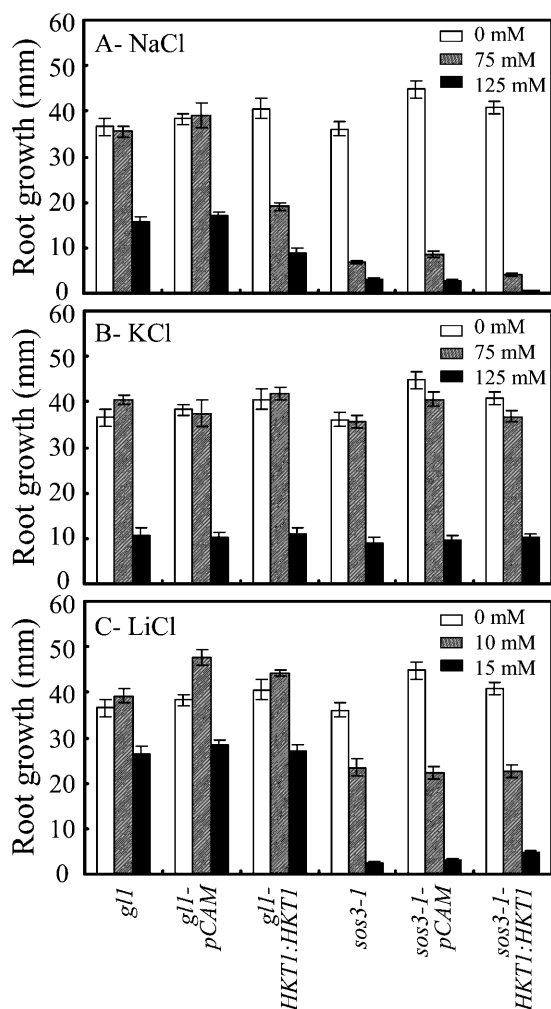
Figure 7. *AtHKT1:AtHKT1* transgene expression mediates Na$^+$-specific salt sensitivity of wild-type and *sos3-1* seedlings. Four-day-old seedlings were transferred to a basal medium (see Fig. 5 legend; white bars) or basal medium supplemented with 75 (hatched bars) or 125 (black bars) mM NaCl (A), 75 (hatched bars) or 125 (black bars) mM KCl (B), and 10 (hatched bars) or 15 (black bars) mM LiCl (C). Root growth of wild-type (*gl1*), wild-type vector control (*gl1-pCAM*, line no. 3-1 in Fig. 6), wild-type expressing *AtHKT1* transgene (*gl1-HKT1:HKT1*, line no. 10-10 in Fig. 6), *sos3-1*, *sos3-1* vector control (*sos3-1-pCAM*, line no. 13-5 in Fig. 5), and *sos3-1* expressing *AtHKT1* transgene (*sos3-1-HKT1:HKT1*, line no. 13-4 in Fig. 5) seedlings is illustrated. Root growth was determined 6 d after transfer; values are the mean ± SE ($n \geq 10$).

Figure 8. AtHKT1 loss- or gain-of-function affects K$^+$/Na$^+$ homeostasis in planta. Na$^+$ and K$^+$ content in the shoot (A) and root (B) of wild-type (*gl1*), *hkt1-4*, or *gl1* expressing the *AtHKT1* transgene (*gl1-HKT1:HKT1* line nos. 10-10 and 17-3 in Fig. 6) plants growing in Turface. Plants were grown as in Figure 3. Shoot content values are the mean ± SE (*n* = 3), root content values are the mean ± SE (*n* ≥ 2), and K$^+$/Na$^+$ was calculated from K$^+$ and Na$^+$ content.

rational flux is driving greater Na$^+$ transport to the shoot of plants grown in a growth chamber that exacerbates the Na$^+$ stress effects caused by *hkt1* mutations. It is hypothesized that AtHKT1 regulates Na$^+$ distribution between the root and the shoot, restricting the accumulation of the toxic cation in the aerial part of the plant (Mäser et al., 2002; Berthomieu et al., 2003). Greater Na$^+$ accumulation in the shoot and less in the root of *hkt1* plants indicates that AtHKT1 is not involved in the movement of this cation into the xylem and subsequent movement to the shoot via the transpirational stream (Fig. 4; Mäser et al., 2002; Berthomieu et al., 2003). Since *AtHKT1* is expressed predominantly in the phloem, it is hypothesized that, in the shoot, HKT1 loads Na$^+$ into the phloem, which is then translocated to the root, and removal of Na$^+$ from the root phloem occurs by efflux down the electrochemical potential (Berthomieu et al., 2003). Controlling Na$^+$ content of the shoot is a necessary salt adaptation strategy that restricts apoplast accumulation and net influx across the plasma membrane to coordinate effective compartmentalization by the NHX vacuolar Na$^+$/H$^+$ antiporter (Munns, 2002; Munns et al., 2002).

The model of phloem recirculation may not explain all relevant in planta functions of AtHKT1 as revealed by the results of this study that *hkt1* mutations suppress Na$^+$ sensitivity of *sos* mutants and *AtHKT1* transgene expression increases NaCl sensitivity of seedlings and plants. The plasma membrane Na$^+$/H$^+$ antiporter SOS1 is proposed to reduce net Na$^+$ flux into cells of the root meristem, which contain vacuoles of insufficient size to compartmentalize Na$^+$ effec-

tively, and to restrict Na$^+$ accumulation in the shoot by controlling xylem loading in the root (Shi et al., 2002). SOS1 overexpression increases NaCl tolerance that is linked to a reduction in Na$^+$ accumulation presumably because the Na$^+$/H$^+$ antiporter is retrieving the toxic cation from the xylem into the surrounding parenchyma cells reducing the salt load transported to the shoot (Shi et al., 2003). It is suggested also that when *SOS1* is driven by the 35S promoter, ectopic expression
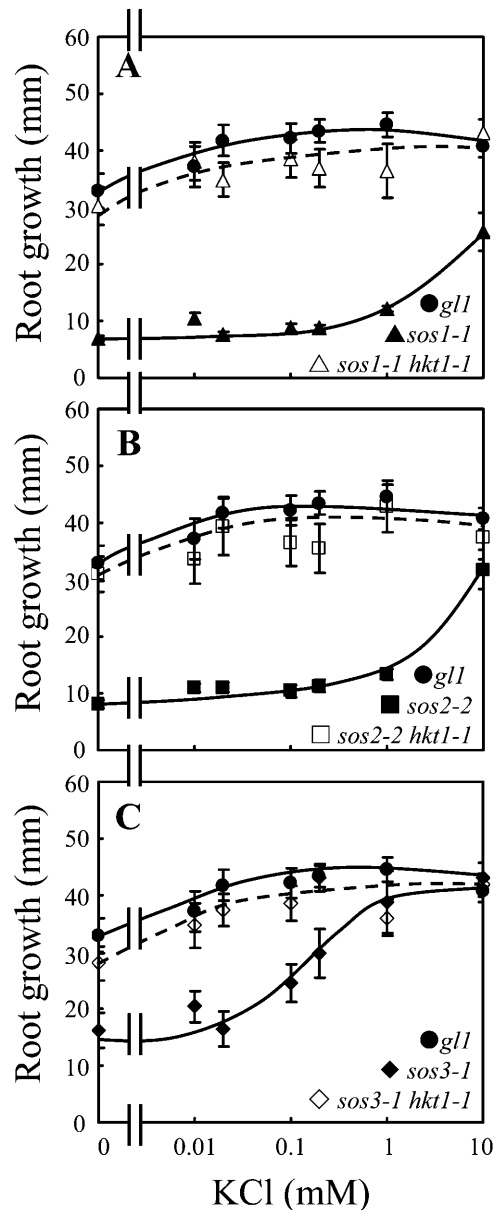
Figure 9. *hkt1-1* suppresses the K$^+$ deficiency of *sos* seedlings. Four-day-old seedlings were transferred to a basal medium containing 1/20× MS macronutrients [without KNO$_3$, and KH$_2$PO$_4$ replaced with (NH$_4$)$_2$HPO$_4$], MS micronutrients (KI replaced with NaI), 30 g L^{-1} Suc, and 12 g L^{-1} Bacto Agar, without or supplemented with various amounts of KCl. The basal medium contains 0.15 mM CaCl$_2$. Illustrated is *hk1-1* suppression of *sos1-1* (A), *sos2-2* (B), and *sos3-1* (C). Root growth was determined 9 d after transfer; values are the mean ± SE (*n* ≥ 8).
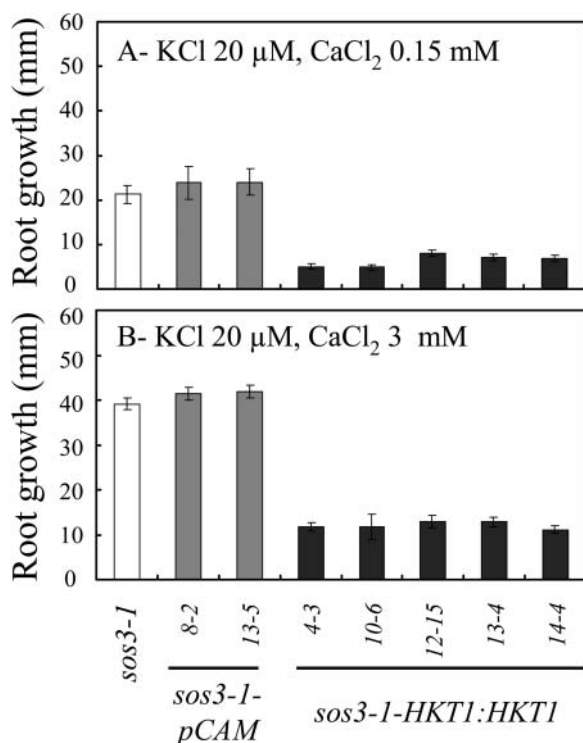
Figure 10. *AtHKT1:AtHKT1* transgene expression increases the K$^+$ deficiency of *sos3-1* seedlings independent of Ca$^{2+}_{ext}$. Four-day-old seedlings were transferred to a basal medium (1/20× MS macronutrients [without KNO$_3$, without CaCl$_2$, KH$_2$PO$_4$ replaced with (NH$_4$)$_2$HPO$_4$, with 20 µM KCl], MS micronutrients [KI replaced with NaI], 30 g L^{-1} Suc, and 12 g L^{-1} Bacto Agar) and 0.15 (A) or 3 mM CaCl$_2$ (B); genotypes are as in Figure 5. Root growth was determined 6 d after transfer; values are the mean ± SE ($n \geq 10$).

occurs in the epidermis thereby restricting transverse flux of Na$^+$ to the stele (Shi et al., 2003). In planta, Na$^+$ homeostasis apparently is linked tightly to the function of SOS1 and HKT1, and a disruption of either causes ion disequilibrium resulting in salt sensitivity. Combining mutations that reduce the function of these transporters partially restores ion homeostasis that is manifested as suppression of salt sensitivity. Interestingly, suppression of NaCl sensitivity of *sos3-1* plants by *hkt1* is associated with Na$^+$ profiles in the shoot and root similar to wild-type plants (Fig. 4). Thus *hkt1* in the *sos* background reestablishes Na$^+$ homeostasis of wild-type plants. However, suppression is not equivalent to the salt adaptive capacity of wild-type plants, which have functional SOS1 and HKT1 transport systems.

Plants expressing two additional copies of *AtHKT1* under the control of the innate promoter exhibit greater NaCl stress symptoms than parental plants whether wild type or *sos3-1*. In view of the proposed phloem recirculation mechanism whereby AtHKT1 transports Na$^+$ from the shoot to root (Berthomieu et al., 2003), increased *AtHKT1* expression should enhance salt tolerance rather than cause stress symp-

toms. It is predicted that enhanced Na$^+$ recirculation should reduce accumulation of the toxic cation in the shoot. These results together with *hkt1* suppression of Na$^+$ sensitivity of *sos* mutants implicate other, yet unidentified, function(s) for AtHKT1. It is likely that the Na$^+$ transport function of AtHKT1 is coordinated tightly, both intracellularly and intercellularly, with other transport systems and together these mediate Na$^+$ homeostasis. Either a dysfunctional allele or overexpression of *AtHKT1* would modulate Na$^+$ distribution within the plant altering the K$^+$/Na$^+$ homeostasis.

Evidence That AtHKT1 Is Not Regulated by the SOS Pathway

Genetic suppression of *sos3-1* and *sos2-2* by *hkt1* mutations implicate the SOS pathway in the regulation
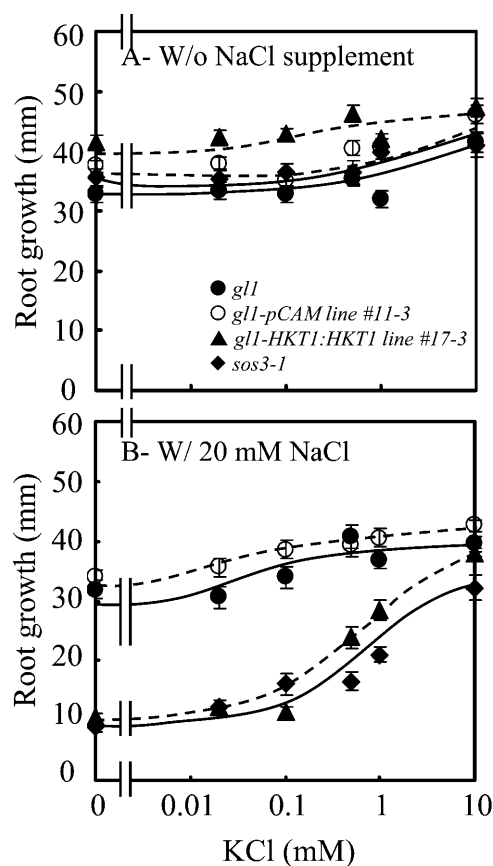
Figure 11. *AtHKT1:AtHKT1* transgene expression-mediated K$^+$ deficiency of wild-type (*gl1*) seedlings requires Na$^+_{ext}$. Four-day-old seedlings were transferred to a basal medium (1/20× MS macronutrients [without KNO$_3$, with 3 mM CaCl$_2$, KH$_2$PO$_4$ replaced with (NH$_4$)$_2$HPO$_4$], MS micronutrients [KI replaced with NaI], 30 g L^{-1} Suc, and 12 g L^{-1} Bacto Agar) without or supplemented with various amounts of KCl and without NaCl supplement (A) or with 20 mM NaCl (B); plants of wild-type (*gl1*), vector control (*gl1-pCAM*, line no. 11-3 in Fig. 6), *AtHKT1* transgene expressing line (*gl1-HKT1:HKT1*, line no. 17-3 in Fig. 6), and *sos3-1*. Root growth was determined 6 d after transfer; values are the mean ± SE ($n \geq 19$).

(presumably negative control) of AtHKT1 (Rus et al., 2001; herein). However, *AtHKT1* is not a transcriptional output of the SOS signal pathway (data not shown). Furthermore, genetic suppression of *sos1-1* by *hkt1-1* indicates that AtHKT1 activity is not regulated by the SOS pathway. Both SOS3 and SOS2 are operative in *sos1-1* plants, so *hkt1-1* should have little, if any, effect on the NaCl phenotype in these plants if AtHKT1 is negatively controlled by the pathway and SOS1 does not have regulatory function. Moreover, there is no indication that Ca^{2+} modulates the Na^+ transport function of AtHKT1 (Fig. 1). Furthermore, *AtHKT1* transgene expression increases K^+ deficiency of *sos3-1* seedlings, and this is Ca_{ext}^{2+}-independent (Fig. 10) even though the K^+ deficiency of *sos3-1* seedlings is suppressed by Ca_{ext}^{2+} (Liu and Zhu, 1997; Fig. 10). Despite evidence that AtHKT1 may not be regulated by the SOS pathway, genetic data indicate that AtHKT1 and SOS1 are linked in function to the control of Na^+ homeostasis in planta.

K^+ Nutrient Status Is Controlled by the Na^+ Homeostasis Function of AtHKT1

Herein, evidence establishes that the K^+ nutrient status of plants is linked to or is a consequence of the Na^+ homeostasis function of AtHKT1. *hkt1* mutations suppress the K^+ deficient phenotype of *sos* seedlings, and *AtHKT1* transgene expression exacerbates K^+ deficiency of wild-type and *sos3-1* seedlings. Several lines of evidence indicate that Na^+ transport by AtHKT1 controls Na^+ homeostasis in planta, which in turn affects K^+ acquisition and nutrient status. First, *hkt1*-induced NaCl sensitivity affects the shoot K^+ to Na^+ ratio in plants grown under controlled environmental conditions (Figs. 3 and 8). Second, *AtHKT1* transgene expression increases Na^+ sensitivity of wild-type plants that is linked to a lower K^+ to Na^+ ratio in the root (Fig. 8). Third, *hkt1* mutations suppress K^+ deficiency of *sos* mutants (Fig. 9). Fourth, *AtHKT1* transgene expression exacerbates K^+ deficiency of *sos3-1* seedlings, which are defective in Na^+ efflux

capacity (Fig. 10; Qiu et al., 2002). Fifth, *AtHKT1* transgene expression causes K^+ deficiency of wild-type seedlings only if the medium is supplemented with 20 mm NaCl, a level that does not constitute Na^+, Cl^-, or osmotic stress (Fig. 11). These results are substantial genetic evidence that Na_{int}^+ is a controller of K^+ acquisition or nutrient status, adding to the knowledge base of more than 40 years that Na_{ext}^+ competes with K^+ for uptake through high and low-affinity transport systems (Epstein, 1961).

Potential K^+ Uptake System Targets of AtHKT1 Regulation

The results herein indicate that *AtHKT1* negatively controls K^+ acquisition or nutrient status (Figs. 9–11). *hkt1* mutations suppress the K^+ deficiency of *sos* seedlings, which exhibit a growth defect that is mitigated by mm K_{ext}^+. *sos1* seedlings exhibit reduced K^+ uptake rate in medium supplemented with μm but not mm levels of K^+ (Wu et al., 1996). K^+ permeability of *sos1* root cells is inhibited in the presence of Na^+ (Qi and Spalding, 2001). *AtHKT1* transgene expression causes K^+ deficiency at μm K_{ext}^+ but not at mm K_{ext}^+ (Fig. 11; data not shown for *AtHKT1* transgene expression in *sos3-1* seedlings). Together, these results implicate an AtHKT1 function in the control of high-affinity K^+ acquisition. However, complete suppression of the growth defect of *sos1-1* and *sos2-2* seedlings that is evident on medium supplemented with mm K^+ may be indicative of a more global function of AtHKT1 to control K^+ nutrient status of plants.

AKT1 might be a target for negative regulation of AtHKT1 because the NH_4^+ concentration in the medium used in our experimentation (approximately 1.2 mm) should inhibit the activity of the high-affinity K^+ transporters (Figs. 9–11; Spalding et al., 1999; Rus et al., 2001). NH_4^+ inhibits the activity of HAK1/KUP1 high-affinity K^+ transporters in barley (*Hordeum vulgare*) and rice root cells (Santa-Maria et al., 2000; Bañuelos et al., 2002). Arabidopsis KT/HAK/KUP/TRH transporters are suggested to be components of the NH_4^+-

Table I. *Genotypes and genetic resources used in this study*

Abbreviation Used	Background Genotype	Binary Vector	Reference
Wild type, *gl1*	Col-0 *gl1*		
sos1-1	Col-0 *gl1 sos1-1*		Shi et al. (2000)
sos2-2	Col-0 *gl1 sos2-2*		Liu et al. (2000)
sos3-1	Col-0 *gl1 sos3-1*		Liu and Zhu (1998)
hkt1-1	Col-0 *gl1 hkt1-1*		This study
hkt1-4	Col-0 *gl1 hkt1-4*		This study
sos1-1 hkt1-1	Col-0 *gl1 sos1-1 hkt1-1*		This study
sos2-2 hkt1-1	Col-0 *gl1 sos2-2 hkt1-1*		This study
sos3-1 hkt1-1	Col-0 *gl1 sos3-1 hkt1-1*	pSKI015	Rus et al. (2001)
gl1-pCAM	Col-0 *gl1*	pCAMBIA 2300	This study
gl1-HKT1:HKT1	Col-0 *gl1*	pCAMBIA 2300 [*AtHKT1:AtHKT1*]	This study
sos3-1-pCAM	Col-0 *gl1 sos3-1*	pCAMBIA 2300	This study
sos3-1-HKT1:HKT1	Col-0 *gl1 sos3-1*	pCAMBIA 2300 [*AtHKT1:AtHKT1*]	This study

sensitive pathway for K$^+$ uptake (Spalding et al., 1999). Furthermore, AKT1-mediated K$^+$ currents are inhibited by intracellular Na$^+$ (E. Spalding, personal communication). K$^+$ transport through AKT1 occurs at μM concentrations (Hirsch et al., 1998; Spalding et al., 1999; Dennison et al., 2001), which is consistent with our results that AtHKT1 is a negative regulator of K$^+$ nutrient status in the presence of μM K$^+_{ext}$ (Figs. 9–11). Our analysis indicates that *AKT1*, *TRH1*, or *SKOR* expression is not controlled by the function of AtHKT1.

Evidence is mounting that HKT1 systems are conserved in plant species and that these function in Na$^+$ transport (Rus et al., 2001; Golldack et al., 2002; Laurie et al., 2002; Garciadeblás et al., 2003). It is evident why plants need to control Na$^+$ homeostasis during an episode of salt exposure, as this ion is a necessary osmolyte for vacuolar osmotic adjustment, yet is a cytotoxin (Blumwald, 2000; Hasegawa et al., 2000; Rus et al., 2001; Zhu, 2003). However, the necessity of a specific Na$^+$ transport system in nonsaline environments and the retention of this system in glycophytes are more enigmatic. It has been suggested that Na$^+$ may substitute for K$^+$ physiologically (pH control, volume regulation) under conditions where K$^+$ is limiting (Rodriguez-Navarro, 2000). Na$^+$-mediated pH control may be essential for biotic stress signaling and ontological development of plants, and available data implicate the tonoplast Na$^+$/H$^+$ antiporter as a pH-stat (Fukada-Tanaka et al., 2000; Yamaguchi et al., 2001; Viehweger et al., 2002). Clearly, the existence and function of AtHKT1 together with other transport systems that transport Na$^+$ with high specificity, e.g. SOS1 and perhaps NHX, are indicative that Na$^+$ has a necessary function in nonsaline environments. Also, it is conceivable that Na$^+_{int}$ may be a controller of K$^+$ nutrient status, and recent evidence establishes a direct connection between K$^+$ acquisition and regulation of growth and development (Rigas et al., 2001; Elumalai et al., 2002).

MATERIALS AND METHODS

Plant Materials

Arabidopsis Col-0 *gl1* (wild type) and the other genetic resources reported in this paper are listed in Table I. Plant materials are available upon request.

sos1-1 hkt1-1 and sos2-2 hkt1-1

sos1-1 hkt1-1 and *sos2-2 hkt1-1* were obtained after crossing *sos1-1* (Shi et al., 2000) and *sos2-2* (Liu et al., 2000), respectively, to *sos3-1 hkt1-1* (Rus et al., 2001); *sos3-1 hkt1-1* × *sos1-1* and *sos3-1 hkt1-1* × *sos2-2*. Genotypic analysis of F$_2$ plants was accomplished by using PCR for detection of mutant and wild-type alleles. Primers used to identify *sos3-1* and *SOS3* alleles were dub23-3F, 5′-TCTCATGAATTTGCAGTTGC-3′ and dub23-3R, 5′-AAACTGTT-TAATCTGGAGGG-3′ (Halfter et al., 2000). The products are 112 (Δ9 bp in *sos3-1*) and 121 bp, respectively. Specific forward primers were: for *SOS2*, dub23-2FWT, 5′-AATTTGGATGATATTCGTGCAGTTTTTG-3′ and for *sos2-2*, dub23-2F, 5′-GGATGATATTCGTGCAGTTTGA-3′ together with the same reverse primer, dub23-2RUH, 5′-TTAACATTTAAATGGAATTGA-CC-3′. Primers to genotype the *SOS1* and *sos1-1* alleles were ssr1test-F, 5′-GGCATTGCATCAGTTATTTGG-3′ and ssr1test-R, 5′-GGTCTACAGA-

AAGACGTACAG-3′; 101 bp for *sos1-1* (Δ14 bp) and 115 bp for *SOS1*. PCR procedures for genotyping the *AtHKT1* locus were as described in Rus et al. (2001).

hkt1-1 and hkt1-4

sos3-1 hkt1-1 and *sos3-1 hkt1-4* were crossed to Col-0 *gl1*, and F$_2$ plants were genotyped to identify *hkt1-1* and *hkt1-4* homozygotes. Primers for analysis of the *SOS3* locus were as described above. PCR analysis to genotype *hkt1-1* and *HKT1* is as described by Rus et al. (2001). *hkt1-4* allele was distinguished from *HKT1* by using the primer pairs 265F, 5′-TGGTTTCACTACCGGGTACA-3′ and 265R, 5′-GCCAGATTTGGCTGTGAACT-3′ that produced 150- (Δ16 bp in *hkt1-4*) and 166-bp amplification products, respectively.

AtHKT1 Binary Vector and Generation of Transgenic Plants

An *AtHKT1* promoter fragment (−2,000 to −12 bp upstream of the translational initiation site) was amplified by PCR using the bacterial artificial chromosome (BAC) clone F24G24 (GenBank accession no. AL049488) and cloned into the *EcoRV-XhoI* sites of pBluescript SK($\pm$; Stratagene, La Jolla, CA). The nucleotide sequence of the cloned *AtHKT1* promoter fragment was the same as in F24G24. An *AtHKT1* open reading frame fragment (spanning from 12 bp upstream of the translational initiation site to 523 bp downstream of the stop codon, including the polyadenylation cis-element) was obtained by digestion of the BAC clone F24G24 (positions 37,557–41,752) with *XhoI* and *HindIII*, and this fragment was cloned into pBluescript SK($\pm$). After nucleotide sequence analysis, this fragment containing the *AtHKT1* coding sequence was fused, in frame, to the *AtHKT1*-promoter fragment. The *AtHKT1:AtHKT1* genomic fragment was cloned into *SacI* and *HindIII* sites of the binary vector pCAMBIA 2300 (CAMBIA, Canberra, Australia). The pCAMBIA 2300 (without insert) and pCAMBIA 2300/*AtHKT1:AtHKT1* vectors were introduced into *Agrobacterium tumefaciens* (strain GV3101) by electroporation.

Wild-type or *sos3-1* plants were transformed by spraying flower buds with an Agrobacterium suspension (50 g L^{-1} Suc and 2 μL mL^{-1} Silwet L-77 [OSI Specialties, Danbury, CT]; Chung et al., 2000). T$_1$ seeds were collected and then germinated in vitro on medium containing Murashige and Skoog (MS) salts, 30 g L^{-1} Suc, 8 g L^{-1} agar, and 50 mg L^{-1} kanamycin. T$_2$ seeds were collected from individual lines, and only those for which progeny segregated at a 3:1 ratio for kanamycin resistance (i.e. single transgene insertion) were used to obtain T$_3$ progeny. T$_3$ seeds of lines homozygous for kanamycin resistance, and not selected by any other criteria, were used for experimentation. The higher *AtHKT1* mRNA abundance in the *AtHKT1* transgene expressing lines was confirmed by reverse transcription PCR analysis. The transgenic lines are referred to as *gl1-pCAM* or *gl1-HKT1:HKT1* (expressing *AtHKT1* transgene) for wild-type plants transformed with vector without insert or containing *AtHKT1:AtHKT1* (as described above), respectively, and *sos3-1-pCAM* or *sos3-1-HKT1:HKT1* for *sos3-1* plants transformed with vector without insert or containing *AtHKT1:AtHKT1*, respectively.

Na$^+$, K$^+$, and Ca^{2+} Growth Responses of Seedlings in Vitro

After stratification, seeds were germinated in vitro on medium containing MS salts (JRH Biosciences, Lenexa, KS), 30 g L^{-1} Suc, and 10 g L^{-1} agar as described by Rus et al. (2001). Four-day-old seedlings were transferred from germination medium to test media and grown in vitro as described (Rus et al., 2001). Test media were prepared as described previously (Rus et al., 2001), and the constituents are listed in the figure legends. The pH of all media was adjusted to 5.7 prior to autoclave sterilization. Data are illustrated as root length increase after transfer to a test medium.

NaCl Growth Response of Plants

Seeds were sown onto moist soil (Scotts Potting Medium; Scotts-Sierra Horticultural Products, Marysville, OH) and stratified at 4°C for 3 d. NaCl growth response of plants in soil or Turface (Profile Products, Buffalo Grove, IL) was evaluated under controlled environmental conditions (8 h light:16 h dark, 150 μmol m^{-2} s^{-1} light intensity, 75% relative humidity).

The comparison of the NaCl response of wild-type, *sos3-1*, *sos3-1 hkt1-4*, *hkt1-4*, and two independent transgenic lines expressing *AtHKT1* (*gl1-HKT1:HKT1* no. 10-10 and no. 17-3) plants was conducted with plants grown on Turface (Figs. 3, 4, and 8). After stratification, pots with seeds were transferred to the growth chamber for germination. One-month-old seedlings were transplanted into Turface and watered with EXCEL solution (Scotts) by capillarity (167 mg L^{-1} K^{+}, 67 mg L^{-1} Ca^{2+}, and no detectable Na^{+} according to specification of the manufacturer; Scotts). NaCl treatment was initiated 1 week after transfer to Turface. Plants were watered every 3 or 4 d with EXCEL solution without or with NaCl using the following procedure: EXCEL solution only; or 50 mM NaCl (four treatments) followed by 7 treatments with 100 mM NaCl.

Na^{+} and K^{+} Content Determinations

For determination of shoot and root ion content data, plant material was dried in an oven at 65°C for 4 d. The dried material was ground with a mortar and pestle. Ground dry-matter (around 50 mg/sample) was extracted with HNO$_3$ 0.1 N. Na^{+} or K^{+} content in the extract was determined by using an atomic absorption spectrophotometer (Varian SpectrAA-10; Victoria, Australia).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AL049488.

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