AtHKT1 is a salt tolerance determinant that controls Na^+ entry into plant roots

Ana Rus*, Shuji Yokoi*, Altanbadralt Sharkhuu*, Muppala Reddy†, Byeong-ha Lee‡, Tracie K. Matsumoto*, Hisashi Koiwa*, Jian-Kang Zhu‡, Ray A. Bressan*, and Paul M. Hasegawa*§

*Center for Plant Environmental Stress Physiology, Purdue University, West Lafayette, IN 47907-1165; †Central Salt and Marine Chemicals Research Institute, Bhavanagar-364 002, India; and ‡Department of Plant Sciences, University of Arizona, Tucson, AZ 85721

Communicated by André T. Jagendorf, Cornell University, Ithaca, NY, September 21, 2001 (received for review June 19, 2001)

Two *Arabidopsis thaliana* extragenic mutations that suppress NaCl hypersensitivity of the *sos3–1* mutant were identified in a screen of a T-DNA insertion population in the genetic background of Col-0 *gl1 sos3–1*. Analysis of the genome sequence in the region flanking the T-DNA left border indicated that *sos3–1 hkt1–1* and *sos3–1 hkt1–2* plants have allelic mutations in *AtHKT1*. *AtHKT1* mRNA is more abundant in roots than shoots of wild-type plants but is not detected in plants of either mutant, indicating that this gene is inactivated by the mutations. *hkt1–1* and *hkt1–2* mutations can suppress to an equivalent extent the Na^+ sensitivity of *sos3–1* seedlings and reduce the intracellular accumulation of this cytotoxic ion. Moreover, *sos3–1 hkt1–1* and *sos3–1 hkt1–2* seedlings are able to maintain $[K^+]_{int}$ in medium supplemented with NaCl and exhibit a substantially higher intracellular ratio of K^+/Na^+ than the *sos3–1* mutant. Furthermore, the *hkt1* mutations abrogate the growth inhibition of the *sos3–1* mutant that is caused by K^+ deficiency on culture medium with low Ca^{2+} (0.15 mM) and <200 μM K^+. Interestingly, the capacity of *hkt1* mutations to suppress the Na^+ hypersensitivity of the *sos3–1* mutant is reduced substantially when seedlings are grown in medium with low Ca^{2+} (0.15 mM). These results indicate that AtHKT1 is a salt tolerance determinant that controls Na^+ entry and high affinity K^+ uptake. The *hkt1* mutations have revealed the existence of another Na^+ influx system(s) whose activity is reduced by high $[Ca^{2+}]_{ext}$.

High $[NaCl]_{ext}$ disturbs intracellular ion homeostasis of plants, which leads to membrane dysfunction, attenuation of metabolic activity, and secondary effects that cause growth inhibition and lead ultimately to cell death (1). Both glycophytes and halophytes use a similar strategy that involves regulation of net Na^+ flux across the plasma membrane and vacuolar compartmentalization of the internalized cation to mediate intracellular Na^+ homeostasis. This strategy requires the coordinated function of numerous ion transport determinants and effectively partitions the toxic ion away from critical cytosolic and organellar machinery. Under conditions of high $[Na^+]_{ext}$, the functioning of these determinants also facilitates the use of Na^+ as an osmolyte to mediate osmotic adjustment that is necessary for cell expansion (1–3). Because vacuolar expansion is the primary mechanism of plant cell enlargement, this strategy is likely to be an essential adaptation to saline environments.

Recently, putative plasma membrane and tonoplast localized Na^+/H^+ transporters were identified in plants that are presumed to mediate energized transport of Na^+ outward from the cytosol to the apoplast or into the vacuole (4–7). These transporters are apparently the molecular effectors of Na^+/H^+ antiporter activities associated with plasma membrane and tonoplast vesicles that were described more than a decade ago (1, 3, 8, 9). The plasma membrane Na^+/H^+ antiporter is the principal facilitator of cellular Na^+ efflux, whereas the tonoplast antiporter is the primary transport system for Na^+ compartmentalization into the vacuole.

Na^+ uptake across the plasma membrane has been attributed to low Na^+ permeability properties of systems that transport the essential nutrient K^+ (3, 10, 11). Transport systems that have high affinity for K^+ but also have low affinity for Na^+ include inward rectifying K^+ channels (KIRCs) like AKT1, outward rectifying K^+ channels (KORCs), and the KUP/HAK family of K^+–H^+ symporters (3, 11, 12). However, there have been some suggestions that Na^+ influx may be mediated also by low affinity cation or nonspecific cation transport systems (1, 3, 10, 11). The high affinity K^+ transporter (HKT1), low affinity cation transporter (LCT1), and nonselective cation channels are considered to be the most likely specific transport systems that mediate high Na^+ influx (10, 11, 13–16).

Support for the hypothesis that K^+ transport systems also mediate Na^+ entry derive from physiological studies conducted more than 30 years ago, which established that Na^+ adversely affects K^+ acquisition to the extent that plants growing in saline environments can be deficient for this essential nutrient (17, 18). Regulation of net K^+/Na^+ selective intracellular uptake is Ca^{2+}-dependent (19, 20), although regulatory mechanisms and focal control points have not been described in detail (21–23). Extensive screening of *Arabidopsis* mutants for increased salt sensitivity has identified the Ca^{2+}-dependent salt-overly sensitive (SOS) stress signal pathway. Three genetically linked loci (*SOS1*, *SOS2*, and *SOS3*) express determinants that control both Na^+ and K^+ homeostasis and salt tolerance (21–23). The *sos1*, *sos2*, and *sos3* mutants are Na^+ and Li^+ sensitive and exhibit K^+ deficiency when grown in solutions containing μM concentrations of this essential nutrient. High $[Ca^{2+}]_{ext}$ can suppress Na^+/Li^+ sensitivity and K^+ deficiency of *sos3* but not of *sos1* seedlings (24). SOS3 is an EF-hand type Ca^{2+} binding protein with sequence similarity to the calcineurin B subunit and neuronal Ca^{2+} sensors (25). Ca^{2+} binding is required for *in vivo* function (26) that includes the activation of the SOS2 kinase (27, 28), which in turn regulates *SOS1* expression (7). *SOS1* encodes a putative plasma membrane-localized Na^+/H^+ antiporter that is presumed to be involved in the control of net Na^+ flux across the plasma membrane. Present models based on these findings indicate that the SOS pathway mediates both Ca^{2+} activation of plasma membrane Na^+ efflux through SOS1 and K^+ acquisition at low external concentrations (μM) through an unidentified K^+ transport system (21–23, 29).

A screen of a T-DNA-tagged *Arabidopsis* (Col-0 *gl1 sos3–1*) population was conducted to identify mutations that suppress the salt hypersensitivity of the *sos3–1* mutant. Potential suppressor mutations might result in activated alleles encoding signal components downstream of SOS3, components of a parallel regulatory pathway(s), or other salt tolerance effectors regulated by stress signal pathways, or might be intragenic mutations in the *sos3–1* allele. Alternatively, mutants with functional disruptions in genes encoding negative regulators of the SOS pathway that are downstream of SOS3 would exhibit enhanced salt tolerance

Abbreviations: SOS, salt-overly sensitive; MS, Murashige and Skoog.

§To whom reprint requests should be addressed. E-mail: paul.m.hasegawa.1@purdue.edu.

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relative to *sos3–1* plants. Herein is reported the identification of two allelic T-DNA insertion mutations in *AtHKT1* (*hkt1–1* and *hkt1–2*) that functionally disrupt its expression and suppress the Na^+ hypersensitive phenotype of the *sos3–1* mutant. Our results implicate AtHKT1 as an important Na^+ influx system in plant roots. Further phenotypic analyses of the *sos3–1 hkt1* mutants provide *in planta* evidence of another Na^+ entry system(s) that is inhibited by $[Ca^{2+}]_{ext}$ and indicate that AtHKT1 negatively regulates high affinity K^+ transport.

Materials and Methods

Plant Materials. The activation T-DNA vector pSKI015 (ref. 30, provided by D. Weigel, The Salk Institute, La Jolla, CA) was used to generate an insertion mutant population (T_1) in the genetic background of *Arabidopsis thaliana* Col-0 *gl1 sos3–1* based on glufosinate herbicide selection. Plants were grouped into 10-line pools, and T_2 progenies were screened for mutants that exhibit less NaCl hypersensitivity than the *sos3–1* mutant. Seeds were surface sterilized and sown onto cellophane membrane (Bio-Rad, no. 1650963) that was placed over germination medium [1× of the Murashige and Skoog (MS) salt formulation (31), B5 vitamins, 30 g·liter^{-1} sucrose, and 12 g·liter^{-1} agar, pH 5.7]. Four-day-old seedlings were inoculated onto NaCl-containing medium (basal constituents and 120 mM NaCl) by transferring the entire cellophane membrane onto the fresh medium. The confirmatory screen for suppressor mutations of *sos3–1* NaCl hypersensitivity was conducted by using between 35 and 40 T_3 progeny of each putative mutant and the root-bending assay procedure of Liu and Zhu (24).

PCR Analyses. *Identification of* SOS3 *and* sos3–1 *alleles.* DNA was isolated from T_3 mutant progeny by using a modified rapid DNA mini-preparation procedure (32) and used as template to confirm the *sos3–1* genetic background. The forward primer for *SOS3* was 5′-ATGTGCTTTCAAGTTGTACG-3′ and for *sos3–1* was 5′-GCTGTGCTTTCAAGTTACG-3′. The same reverse primer was used to detect either allele (5′-TTTATCTT-TCCTTGCATGGC-3′).

Thermal assymetric interlaced-PCR identification of T-DNA flanking region. The genomic sequence flanking the T-DNA left border was determined by thermal assymetric interlaced-PCR analysis (32). A nested set of three primers (oriented outward) corresponding to sequence in the T-DNA left border was used in successive PCRs: left border (LB) primer 1 = 5′-ATACGACGGATCGTAATTTGTC-3′, LB primer 2 = 5′-TAATAACGCTGCGGACATCTAC-3′, and LB primer 3 = 5′-TTGACCATCATACTCATTGCTG-3′. The degenerate (Deg) primers were Deg 1 = 5′-WGCNAGTNAGWANAAG-3′ and Deg 2 = 5′-AWGCANGNCWGANATA-3′; W = A/T and N = A/T/G/C. The nucleotide sequence of the PCR product was determined and subjected to BLASTN analysis.

Detection of AtHKT1 *and* hkt1 *alleles.* The *AtHKT1* wild-type allele was identified by PCR analysis by using forward (5′-AGTAGACCTCTCTACACTTTCC-3′) and reverse (5′-TTACTTCCTCAATCCATGGGAG-3′) primers corresponding to genome regions flanking both sides of the T-DNA insertion in the *sos3–1 hkt1–1* mutant. Detection of the mutant *hkt1–1* and *hkt1–2* alleles was based on reactions using the forward primer for amplification of *AtHKT1* sequence and, as a reverse primer, the T-DNA LB primer 3.

Reverse transcription–PCR analysis of AtHKT1 *expression.* Total RNA was isolated from shoots or roots, and 2 μg of total RNA was the template for first-strand cDNA synthesis. PCR was carried out by using forward-nested gene-specific primers for *AtHKT1*, as follows: first reaction, 5′-GCTTAAACCGACTC-GAGAAC-3′; second reaction, 5′-ATGGACAGAGTGGTG-GCAAA-3′. The same reverse primer was used for both PCRs (5′-TTAGGAAGACGAGGGGTAAA-3′). *AtNHX1* gene-specific primers were used to amplify a positive control PCR product (not shown) (S.Y., unpublished work).

Salt Stress and Nutrient Growth Responses. Experiments were conducted with plants of wild-type Col-0 (*SOS3 HKT1*), *sos3–1*, *sos3–1 hkt1–1* (T_4 homozygous progeny), and *sos3–1 hkt1–2* (T_3 homozygous progeny). Four-day-old seedlings were transferred to fresh medium that was supplemented with various salts, as indicated in the figure legends.

Na^+ and K^+ Ion Content Determinations. Seeds were inoculated onto germination medium with 8 g·liter^{-1} agar. Three-week-old seedlings (n = 50) were transferred to 50 ml of liquid medium (1/2 × MS salts, 20 g·liter^{-1} sucrose at pH 5.7). After 2 days, the medium was supplemented with NaCl to a final concentration of 100 mM by the addition of a 5 M stock solution. The seedlings were harvested after 2 days, rinsed with deionized water, and dried at 65°C for 2 days. One-hundred milligrams of ground dry matter was then extracted with 10 ml of 0.1 M HNO_3 for 30 min and then filtered through Whatman no. 1 filter paper. K^+ and Na^+ contents in the solution were determined by using an atomic absorption spectrophotometer (Varian SpectrAA-10). Na^+ and K^+ content data were verified by inductively coupled plasma analysis (Perkin–Elmer Plasma 400 emission spectrometer).

Results

Extragenic Mutations That Suppress *sos3–1* NaCl Hypersensitivity Include Two *hkt1* Mutant Alleles. T_2 seedlings of 65,000 individual T-DNA insertion lines generated in the *sos3–1* genetic background were screened for salt tolerance gain-of-function mutants. Putative mutants were identified because they were more NaCl tolerant than *sos3–1* seedlings based primarily on criteria of shoot and root growth, root tip swelling, and shoot anthocyanin accumulation. T_3 progeny from 15 different putative mutants were obtained after self-fertilization, and the suppression of the salt hypersensitive phenotypes was confirmed by comparing growth responses to wild-type and *sos3–1* seedlings in medium supplemented with NaCl. PCR analysis confirmed that all mutants in the T_3 generation were homozygous for the *sos3–1* allele (not shown).

Two mutants were identified that are phenotypically identical, having better root and shoot growth and reduced anthocyanin accumulation relative to the *sos3–1* mutant on medium with NaCl (Fig. 1). Root growth comparison (Fig. 1*A*) as well as visual observation of shoot anthocyanin pigment accumulation (Fig. 1*B*) indicated that the suppression of *sos3–1* salt sensitivity by the mutations is complete at 75 mM NaCl. These genotypes have T-DNA insertions in *AtHKT1* (located in chromosome IV) at nucleotide positions 39,033 (first intron) and 40,894 (second intron) of bacterial artificial chromosome clone F24G24 (GenBank accession no. AL049488) for the *sos3–1 hkt1–1* and *sos3–1 hkt1–2* mutants, respectively. PCR analysis determined that *sos3–1 hkt1–1* and *sos3–1 hkt1–2* T_3 progenies were homozygous for the respective mutant allele. The *hkt1* mutants were crossed to Col-0 *sos3–1*, and the F_1 progeny (n = 122 for *sos3–1* × *sos3–1 hkt1–1* and n = 108 for *sos3–1* × *sos3–1 hkt1–2*) all exhibited the *sos3–1* NaCl-sensitive phenotype and were herbicide-resistant (not shown). F_2 analysis of *sos3–1 hkt1–1* (n > 1,000 seedlings) and *sos3–1 hkt1–2* (n = 375 seedlings) backcrosses revealed a segregation ratio of 3:1 for NaCl sensitivity/NaCl sensitivity suppression (χ^2 test, $P > 0.05$).

AtHKT1 transcript is expressed predominantly in roots of wild-type plants but is not detected either in roots or shoots of *sos3–1 hkt1–1* or *sos3–1 hkt1–2* plants, indicating that the *AtHKT1* gene is inactive in these mutants (Fig. 2). Increasing the PCR amplification from 20 (Fig. 2) to 30 (not shown) cycles per reaction resulted in detection of a prominent *AtHKT1* transcript in the shoot of wild type but did not amplify a product from RNA

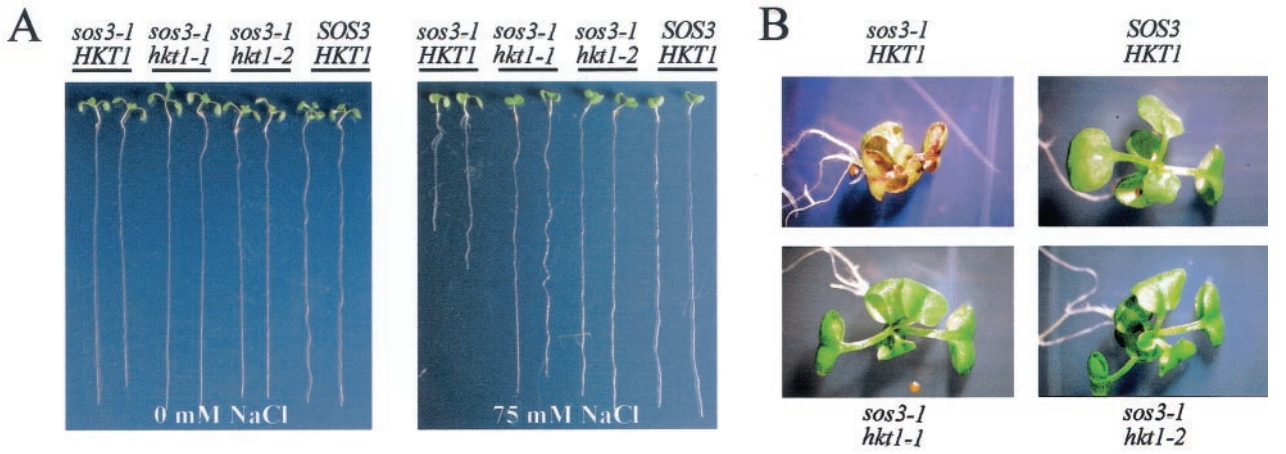

Fig. 1. The *hkt1–1* and *hkt1–2* mutations suppress the NaCl hypersensitive phenotypes of *sos3–1*. Photographs are of representative Col-0 wild-type (*SOS3 HKT1*), *sos3–1* (*sos3–1 HKT1*), and *sos3–1 hkt1–1* and *sos3–1 hkt1–2* T_3 seedlings. Four-day-old seedlings were transferred to fresh medium and illustrated is root growth on medium without (0) or supplemented with 75 mM NaCl after 6 days (*A*) and shoot growth and anthocyanin accumulation on medium with 75 mM NaCl after 15 days (*B*).

of the shoot or root of *sos3–1 hkt1* mutants. Together, these results indicate that the T-DNA insertions in *sos3–1 hkt1–1* and *sos3–1 hkt1–2* cause single gene recessive mutations that suppress the NaCl hypersensitivity of the *sos3–1* mutant by disrupting the expression of the *AtHKT1* gene.

The *hkt1* Mutations Preferentially Suppress the Na^+ over the Li^+-Sensitive Phenotype of the *sos3–1* Mutant. The *sos3–1* mutant hypersensitivity to NaCl and LiCl but not to KCl, CsCl, or mannitol is manifested as a reduction in root and shoot growth and shoot anthocyanin accumulation (Fig. 1; ref. 24). Comparison of root growth on medium with NaCl indicated that *hkt1–1* and *hkt1–2* mutations suppress the NaCl-sensitive phenotype of the *sos3–1* mutant (Fig. 3*A*). Root growth of *sos3–1 hkt1–1* and *sos3–1 hkt1–2* seedlings was similar to that of wild-type seedlings on medium with 75 mM NaCl, less than wild-type seedlings on medium with 100 mM NaCl, and completely inhibited if the medium was supplemented with 150 mM NaCl. On medium with 100 mM NaCl, root growth of *sos3–1 hkt1* mutants was nearly equivalent to wild type after 3 days (not shown) and decreased to about 85% and 65% of wild type after 6 (Fig. 3*A*) and 9 (not shown) days, respectively. NaCl-induced root growth inhibition of *sos3–1 hkt1* mutants was accompanied by reduced shoot growth and an accumulation of anthocyanin pigments as occurs with the *sos3–1* mutant.

The *sos3–1 hkt1* seedlings also exhibited more growth on medium with LiCl than those of the *sos3–1* mutant (Fig. 3*B*). However, the suppression of the Li^+ hypersensitivity of the *sos3–1* mutant by *hkt1* mutations was much less than the suppression of Na^+ sensitivity. These results and those of Uozumi *et al.* (33) indicate that AtHKT1 is an influx system for Na^+ and, perhaps, for Li^+. The time- and concentration-

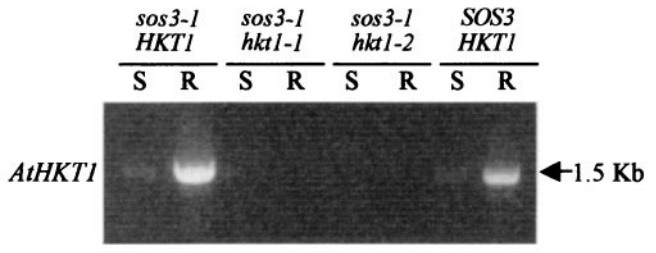

Fig. 2. AtHKT1 transcript abundance is greater in the root (R) than the shoot (S) of Col-0 wild-type plants, and expression is functionally disrupted in *sos3–1 hkt1–1* and *sos3–1 hkt1–2*. Two micrograms of total RNA was isolated from shoots or roots of Col-0 wild-type (*SOS3 HKT1*), *sos3–1* (*sos3–1 HKT1*), or *sos3–1 hkt1–1* or *sos3–1 hkt1–2* T_3 plants (3 weeks old) to produce first-strand cDNA. One microliter of this product was used as template for the first PCR amplification (20 cycles). One microliter of the reaction product was used for the second PCR (20 cycles).

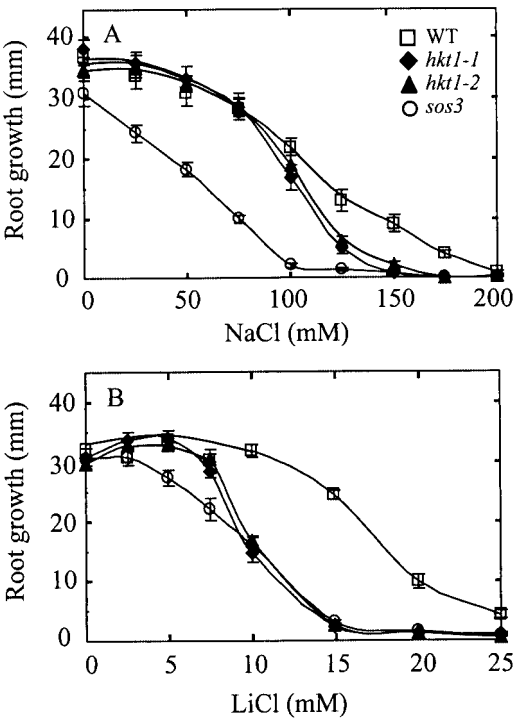

Fig. 3. The *hkt1–1* and *hkt1–2* mutations suppress *sos3–1* Na^+ hypersensitivity. Root growth of Col-0 wild-type (□), *sos3–1* (○), *sos3–1 hkt1–1* (◆), or *sos3–1 hkt1–2* (▲) seedlings was determined after 6 days on basal medium with MS salts and supplemented with NaCl (*A*) or LiCl (*B*). Values are the mean ± SE, $n = 13$.

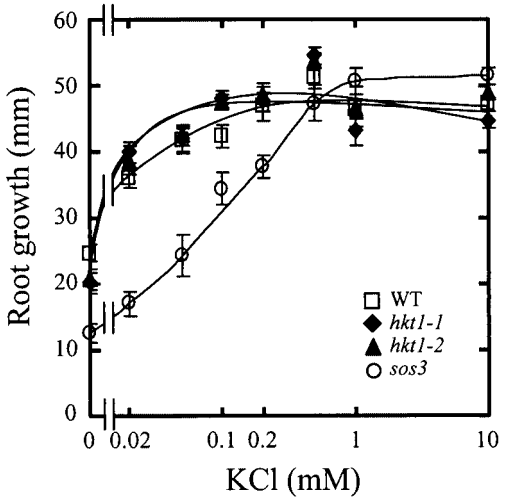

Fig. 4. The *hkt1–1* and *hkt1–2* mutations suppress the K^+ deficiency phenotype of *sos3–1*. Root growth of Col-0 wild-type (□), *sos3–1* (○), *sos3–1 hkt1–1* (◆), or *sos3–1 hkt1–2* (▲) seedlings was evaluated 9 days after their transfer directly onto basal medium with MS micronutrients (KI replaced by NaI), 1/20× macronutrients [KNO_3 eliminated and KH_2PO_4 replaced by $(NH_4)_2HPO_4$] and supplemented with various amounts of KCl. Illustrated are the mean values ± SE, $n = 30$.

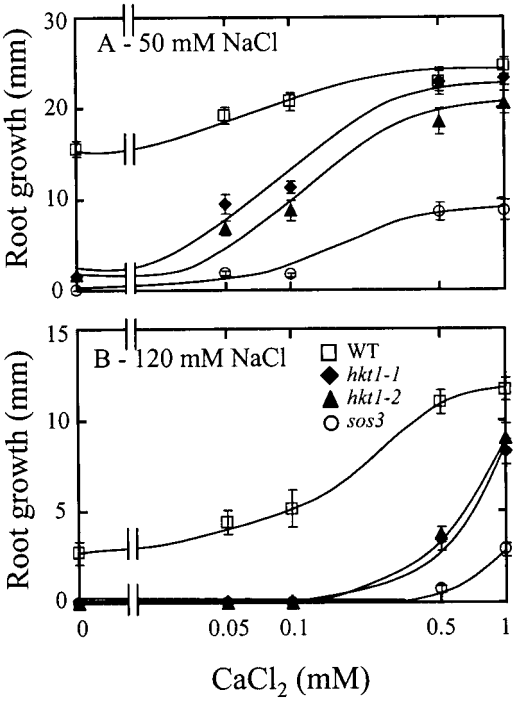

Fig. 5. Suppression of *sos3–1* Na^+ hypersensitivity by *hkt1* mutations is Ca^{2+}-dependent. Root growth of Col-0 wild-type (□), *sos3–1* (○), *sos3–1 hkt1–1* (◆), or *sos3–1 hkt1–2* (▲) seedlings was evaluated 9 days after their transfer directly onto basal medium with MS micronutrients and macronutrients with 0 or various amounts of $CaCl_2$. Data are for seedling root growth on medium supplemented with 50 mM (*A*) or 120 mM (*B*) NaCl; mean values ± SE, $n = 15$.

dependent suppression of the Na^+-sensitive phenotype of the *sos3–1* mutant indicates that AtHKT1 is not the only Na^+ entry system in *Arabidopsis*; otherwise, a tolerance phenotype greater than wild type would be expected.

K^+ Deficiency Phenotype of *sos3–1* Mutant Is Suppressed by *hkt1* Mutations. Root growth of *sos3–1* seedlings in medium with low (μM) levels of K^+ is substantially less than wild-type seedlings (24). Interestingly, root growth of both *sos3–1 hkt1* mutants was always similar to wild type over the $[K^+]_{ext}$ range that the *sos3–1* mutant exhibits a K^+ deficiency phenotype (Fig. 4). Similar relative root growth differences occurred if seeds were germinated on medium containing 20 μM or 20 mM KCl (not shown). The suppression of the K^+ deficiency phenotype of the *sos3–1* mutant by the *hkt1–1* and *hkt1–2* mutations was unaffected by $[Ca^{2+}]_{ext}$ (not shown). These results indicate that AtHKT1 is not a high affinity K^+ uptake system because its functional elimination results in alleviation and not exacerbation of the K^+-deficient phenotype of the *sos3–1* mutant. However, AtHKT1 may modulate activity of a high affinity K^+ transporter because the disruption mutations have a positive effect on K^+ acquisition by *sos3–1* mutant plants. Of known K^+ transporters, most likely this modulated system is AKT1 because the 1 mM $[NH_4^+]_{ext}$ included in the medium should inhibit the KUP family of high affinity K^+ transporters (34).

***hkt1* Mutation Suppression of the *sos3–1* Mutant Na^+ Hypersensitivity Is Ca^{2+}-Dependent.** Na^+ hypersensitivity of *sos3–1* mutant seedlings is increased at lower $[Ca^{2+}]_{ext}$ (Fig. 5), and virtually no shoot (not shown) or root growth (Fig. 5*A*) occurred through 9 days on medium with 50 mM NaCl and low $[Ca^{2+}]_{ext}$. The capacity of *hkt1* mutations to suppress Na^+ hypersensitivity of *sos3–1* mutant seedlings also is Ca^{2+}-dependent (Fig. 5). Similar to the *sos3–1* mutant, root and shoot growth of *sos3–1 hkt1* mutants were severely inhibited on medium containing 50 mM NaCl and not supplemented with $CaCl_2$. Wild-type seedlings exhibited greater Na^+ sensitivity on medium with low $[Ca^{2+}]_{ext}$, particularly on medium with 120 mM NaCl (Fig. 5*B*), which is consistent with many past reports (19). All of these observations indicate the occurrence of another Na^+ entry system that is Ca^{2+}-dependent. NaCl inhibition of *sos3–1 hkt1* mutant seedling root growth at low $[Ca^{2+}]_{ext}$ was not substantially affected when the medium KCl level was lowered from 20 mM KCl (Fig. 5) to 20 μM (not shown). Germination of seeds on medium without or with 20 mM KCl did not affect the subsequent relative root growth of either wild-type or mutant seedlings on medium supplemented with NaCl and low $CaCl_2$ (not shown).

***hkt1–1* Mutation Affects Na^+ and K^+ Accumulation.** It was shown by Zhu *et al.* (29) that *sos3–1* mutants accumulate marginally more Na^+ than wild-type seedlings after 2 days in medium supplemented with NaCl. The Na^+ content of *sos3–1 hkt1–1* seedlings was less than *sos3–1* and wild-type seedlings 2 days after transfer to medium with 100 mM NaCl (Fig. 6*A*). Suppression of *sos3–1* mutant Na^+ hypersensitivity by *hkt1* mutations visually is absolute at this concentration and time interval. Seedlings of *sos3–1 hkt1–1* have greater capacity for K^+/Na^+ selective accumulation in medium with 100 mM NaCl than either wild-type or *sos3–1* seedlings as evidenced by their increased ability to maintain $[K^+]_{int}$ at high $[Na^+]_{ext}$ (Fig. 6 *B* and *C*). Na^+ hypersensitivity of *sos3–1* seedlings correlates with reduced $[K^+]_{int}$ in medium supplemented with NaCl (29). Thus, maintenance of intracellular $[K^+]$ in the presence of $[Na^+]_{ext}$ in *sos3–1 hkt1–1* seedlings is consistent with the suppression of *sos3–1* K^+-deficient phenotype by the *hkt1–1* and *hkt1–2* mutations.

Discussion

AtHKT1 Mediates Na^+ Acquisition. The genetic suppression of *sos3–1* NaCl hypersensitivity by *hkt1* mutations is consistent with AtHKT1 functioning as an important Na^+ influx system, as implicated by the electrophysiological determinations of Uozumi

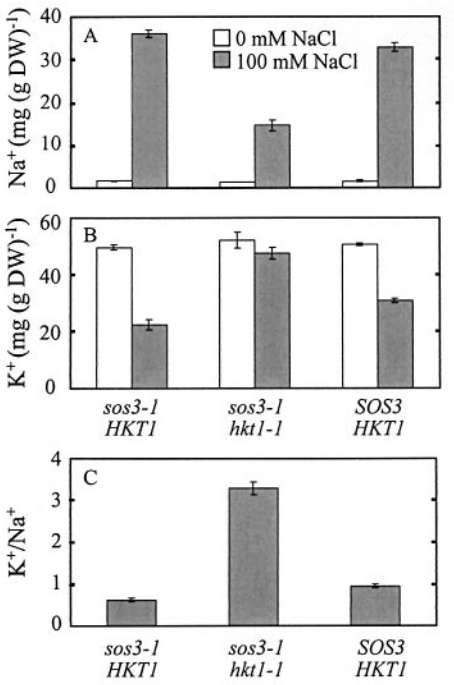

Fig. 6. *sos3–1 hkt1–1* accumulates less Na^+ and maintains intracellular K^+ relative to *sos3–1* in medium supplemented with NaCl. Col-0 wild-type (*SOS3 HKT1*) or *sos3–1* or *sos3–1 hkt1–1* seedlings (3 weeks old) were transferred to liquid medium and grown for 2 days. An aliquot of 5 M NaCl stock solution was then added into the flask to supplement the medium with 100 mM NaCl. An equivalent volume of H_2O was added to cultures that were not supplemented with NaCl. Illustrated are the mean values ± SE ($n = 3$) for Na^+ (*A*) or K^+ (*B*) content as determined by atomic absorption spectrophotometry or for intracellular K^+/Na^+ ratio calculated from the data in *A* and *B* (*C*). Data from one of two experiments with similar results are shown.

et al. (33). However, a regulatory function for AtHKT1 in Na^+ acquisition into plant roots is possible. This conclusion is supported by the fact that *hkt1* seedlings accumulate substantially less Na^+ than *sos3–1* seedlings under salt conditions, where the mutations suppress the NaCl hypersensitive phenotype of the *sos3–1* mutant.

In heterologous systems, *Arabidopsis* AtHKT1 mediates highly selective Na^+ and low affinity K^+ uptake (33). Electrophysiological data indicate that Na^+ does not energize K^+ transport through AtHKT1, and Na^+ flux is not coupled to K^+ or H^+ transport. This finding contrasts with the function of wheat HKT1 that mediates high affinity K^+ uptake energized by either H^+ or Na^+ (12). Residues presumed to affect K^+ binding in HKT1 are variant in AtHKT1, and these differences may contribute to the unique cation specificities of the wheat and the *Arabidopsis* proteins (33). HKT1 and AtHKT1 have sequence similarity to the yeast Trk1–2 and bacterial KtrAB transporters (33, 35, 36) that are hypothesized to have evolved from bacterial KcsA-like K^+ channels (35). The defining structure is composed of two transmembrane helices (M1 and M2) and an intervening hairpin segment (MPM) that forms the outer portion of the pore and mediates ion selectivity. AtHKT1 has four MPM domains that are interspersed between eight transmembrane-spanning segments and are presumed to form the channel pore (35, 36). Perhaps AtHKT1 has both a channel and a transporter function that facilitates downhill transport of Na^+, as well as a H^+-energized K^+ transport (33).

It is difficult initially to rationalize why the Na^+ transport function of AtHKT1 has been retained in glycophytes because, in nonsaline environments, Na^+ accumulation is not required for vacuolar expansion and it is not considered generally to be an essential element by nutritional criteria. However, recent evidence that vacuolar pH may be controlled by the Na^+/H^+ exchange activity of a putative tonoplast antiporter (37, 38) may offer insight about one evolutionary need for intracellular Na^+ uptake in the absence of high $[Na^+]_{ext}$ (37, 38).

Presumably, AtHKT1 imparts to plants the capacity to control the uptake of Na^+ during periods of salt exposure. Strict control of net intracellular Na^+ influx is likely essential in environments of high $[Na^+]_{ext}$, as this cation is an osmolyte required for cellular osmotic adjustment but which must be compartmentalized into the vacuole to prevent cytotoxicity. Although Na^+ can accumulate in the vacuole to several hundred mM, cytosolic concentrations $\geq$100 mM are metabolically poisonous (3, 39). Perhaps Na^+ is required for the fitness of plants by functioning in another yet undefined process such as mentioned previously in pH control (37, 38). Conditional and nonessential functions of AtHKT1 may be the reason mutations that disrupt the function of the single-copy *AtHKT1* gene are not lethal and why it has been retained in glycophytes.

Another Na^+ Entry System That Is Inhibited by $[Ca^{2+}]_{ext}$. Abrogation of *sos3–1* Na^+ hypersensitive suppression by *hkt1* mutations in medium with low $[Ca^{2+}]_{ext}$ is indicative that another Na^+ entry system(s) is operative in plants. Na^+ influx seems to be substantially higher at low $[Ca^{2+}]_{ext}$. However, this or another influx system must function to some extent at high $[Ca^{2+}]_{ext}$ because mutations that inactivate *AtHKT1* do not fully suppress the Na^+-sensitive phenotype of the *sos3–1* mutant, even when the divalent cation is present at mM levels. Perhaps the SOS pathway regulates Na^+ permeability of an alternative system(s) and the defective product of the *sos3–1* allele does not function adequately at μM $[Ca^{2+}]_{ext}$ but is partially operative at mM $[Ca^{2+}]_{ext}$. The *sos3–1* protein exhibits reduced capacity for binding of Ca^{2+} and activation of the SOS2 kinase (26). Alternatively, Ca^{2+} may block directly Na^+ influx through an alternative system (10, 11, 14). Evidence from physiological experimentation implicates nonselective cation channels as Ca^{2+}-sensitive Na^+ influx systems. Data indicate that about 50% of Na^+ permeability is Ca^{2+}-inhibited in maize and wheat protoplasts (10). Furthermore, unidirectional $^{22}Na^+$ flux measurements have identified two independent Na^+ influx systems, one Ca^{2+}-sensitive and one Ca^{2+}-insensitive (P. Essah, R. J. Davenport, and M. Tester, personal communication). Together, these observations seem to implicate AtHKT1 as a Ca^{2+}-insensitive and nonselective cation channels as Ca^{2+}-sensitive Na^+ uptake systems. However, it is problematic to conclude from present evidence whether AtHKT1 activity is Ca^{2+}-independent. Experimentally, there is ambiguity in assessing Ca^{2+} effects on AtHKT1 activity in the presence of a functional Ca^{2+}-inhibited Na^+ uptake system. Definitive conclusion will likely arise from experiments in which the Ca^{2+}-dependent uptake is attenuated, i.e., by mutations. It would not be surprising that AtHKT1 activity is controlled in a Ca^{2+}-dependent manner, perhaps through the SOS signal pathway.

***hkt1* Mutations Suppress K^+ Deficiency of the *sos3–1* Mutant.** *sos3–1* plants exhibit substantially less growth than wild type on medium with μM levels of K^+, indicating that the *sos3–1* mutant has a decreased capacity for K^+ accumulation at low $[K^+]_{ext}$ (Fig. 4, ref. 24). Suppression of the K^+-deficient phenotype of the *sos3–1* mutant by *hkt1* mutations and the fact that *sos3–1 hkt1* mutants are characterized by a capacity to maintain intracellular $[K^+]$ even in medium with NaCl indicates that AtHKT1 does not mediate high affinity K^+ uptake. It is unclear exactly how *hkt1* mutations suppress the K^+ deficiency of the *sos3–1* mutant. However, lower $[Na^+]_{int}$ caused by *hkt1* mutations is linked to

alleviation of K^+ deficiency at low $[K^+]_{ext}$ resulting from the *sos3–1* mutation. This could be a result of $[Na^+]_{int}$ directly inhibiting a high affinity K^+ transport system. Alternatively, elimination of a cation influx system such as AtHKT1 could result in maintenance of membrane potential that favors higher K^+/Na^+ selective accumulation through the activity of high affinity K^+ transport systems, particularly at lower $[Na^+]_{ext}$ (10). The membrane potential not only provides the driving force for cation influx but the highly K^+-selective KIRCs have greater conductance when the membrane potential is more polarized. Inclusion of 1 or 20 mM NH_4^+ in the medium, as in these experiments, should inhibit the activity of the high affinity K^+ carrier of the KUP family (34), implicating the involvement of AKT1 or some unidentified K^+ transport system. Because another cation influx system is functioning at low $[Ca^{2+}]_{ext}$ and suppression of K^+ deficiency by *hkt1* mutations is Ca^{2+}-independent, hypotheses that link membrane potential maintenance to K^+ uptake may be tenuous unless the alternative influx system has very low affinity for Na^+ and the membrane potential is maintained in medium not supplemented with NaCl. Regardless of the biochemical mechanism, by genetic criteria, suppression of *sos3–1* K^+ deficiency by *hkt1* mutations can be interpreted to mean that AtHKT1 negatively regulates high affinity K^+ uptake.

It has been long known that $[Ca^{2+}]_{ext}$ can minimize inhibition of K^+ acquisition by Na^+, presumably through the activation of a high affinity K^+ uptake system (17, 18). SOS3 has been implicated as a signaling intermediate between Ca^{2+} and high affinity K^+ uptake through the transduction of a stress-mediated Ca^{2+} signal that activates increased selectivity of K^+ transport (29). SOS3 also regulates, in a Ca^{2+}-dependent manner, the transcriptional activation of the putative plasma membrane Na^+/H^+ antiporter, SOS1. Thus, Ca^{2+} facilitation of increased selective accumulation of K^+ over Na^+ can be linked through the SOS signal pathway to altered K^+ uptake and to Na^+ efflux. The results herein identify AtHKT1 as an *in planta* regulator of Na^+ influx based on the capacity of *hkt1* mutations to suppress Na^+ accumulation and hypersensitivity of the *sos3–1* mutant. *In planta* evidence indicates the existence of another Na^+ entry system(s) that is inhibited by $[Ca^{2+}]_{ext}$. Na^+ toxicity caused by its entry through this alternative system seems to be unrelated to K^+ deficiency because Na^+-induced growth reduction of *sos3–1 hkt1* mutants at low $[Ca^{2+}]_{ext}$ is only marginally exacerbated by inclusion of 20 μM rather than 20 mM KCl in the medium. Mutations that affect the other Na^+ entry system(s) could be identified by screening for improved tolerance to Na^+ in the genetic background of *sos3–1 hkt1* mutants in medium with low $[Ca^{2+}]_{ext}$.

We thank Drs. Irina Sokolchik and Barbara Damsz, Ms. Wanda Hunter, and Mr. Adam Barb for assistance in this research. We also thank Ms. Terry Kirk for assistance with the atomic absorption spectrophotometer. This work was supported by a Spanish Government Fellowship (to A.R.), Japan Society for the Promotion of Science Japanese Junior Scientist Research Fellowship (to S.Y.), National Institutes of Health Grant R01GM59138 (to J.K.Z.), and National Science Foundation Plant Genome Award DBI-98–13360. This is Purdue University Agricultural Research Program Paper No. 16,611.

1. Hasegawa, P. M., Bressan, R. A., Zhu, J.-K. & Bohnert, H. J. (2000) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **51,** 463–499.
2. Niu, X., Bressan, R. A., Hasegawa, P. M. & Pardo, J.-M. (1995) *Plant Physiol.* **109,** 735–742.
3. Blumwald, E., Aharon, G. S. & Apse, M. P. (2000) *Biochim. Biophys. Acta* **1465,** 140–151.
4. Apse, M. P., Akaron, G. S., Snedden, W. A. & Blumwald, E. (1999) *Science* **285,** 1256–1258.
5. Gaxiola, R. A., Rao, R., Sherman, A., Grisafi, P., Alper, S. L. & Fink, G. R. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 1480–1485.
6. Quintero, F. J., Blatt, M. R. & Pardo, J.-M. (2000) *FEBS Lett.* **471,** 224–228.
7. Shi, H., Ishitani, M., Kim, C.-S. & Zhu, J.-K. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 6896–6901. (First Published May 23, 2000; 10.1073/pnas.120170197)
8. Blumwald, E. & Poole, R. J. (1985) *Plant Physiol.* **78,** 163–167.
9. Hassidim, M., Braun, Y., Lerner, H. R. & Reinhold, L. (1990) *Plant Physiol.* **94,** 1795–1801.
10. Amtmann, A. & Sanders, D. (1999) *Adv. Bot. Res.* **29,** 75–112.
11. Maathuis, F. J. M. & Amtmann, A. (1999) *Ann. Bot. (London)* **84,** 123–133.
12. Schachtman, D. P. (2000) *Biochim. Biophys. Acta* **1465,** 127–139.
13. Amtmann, A., Fischer, M., Marsh, E. L., Stefanovic, A., Sanders, D. & Schachtman, D. P. (2001) *Plant Physiol.* **126,** 1061–1071.
14. Davenport, R. J. & Tester, M. (2000) *Plant Physiol.* **122,** 823–834.
15. Schachtman, D. P. & Schroeder, J. I. (1994) *Nature (London)* **370,** 655–658.
16. Schachtman, D. P., Kumar, R., Schroeder, J. I. & Marsh, E. L. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 11079–11084.
17. Rains, D. W. & Epstein, E. (1967) *Plant Physiol.* **42,** 314–318.
18. Maathuis, F. J. M., Verlin, D., Smith, F. A., Sanders, D., Fernandez, J. A. & Walker, N. A. (1996) *Plant Physiol.* **112,** 1609–1616.
19. Läuchli, A. (1990) in *Calcium in Plant Growth and Development*, eds. Leonard, R. T. & Hepler, P. K. (Am. Soc. Plant Physiol., Rockville, MD), Vol. 4, pp. 26–35.
20. Epstein, E. (1998) *Science* **280,** 1906–1907.
21. Hasegawa, P. M., Bressan, R. A. & Pardo, J.-M. (2000) *Trends Plant Sci.* **5,** 317–319.
22. Sanders, D. (2000) *Curr. Biol.* **10,** 486–488.
23. Zhu, J.-K. (2001) *Trends Plant Sci.* **6,** 66–71.
24. Liu, J. & Zhu, J.-K. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 14960–14964.
25. Liu, J. & Zhu, J.-K. (1998) *Science* **280,** 1943–1945.
26. Ishitani, M., Liu, J., Halfter, U., Kim, C.-S., Shi, W. & Zhu, J.-K. (2000) *Plant Cell* **12,** 1667–1677.
27. Halfter, U., Ishitani, M. & Zhu, J.-K. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 3735–3740. (First Published March 21, 2000; 10.1073/pnas.040577697)
28. Liu, J., Ishitani, M., Halfter, U., Kim, C.-S. & Zhu, J.-K. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 3730–3734. (First Published March 21, 2000; 10.1073/pnas.060034197)
29. Zhu, J.-K., Liu, J. & Xiong, L. (1998) *Plant Cell* **10,** 1181–1191.
30. Weigel, D., Ahn, J. H., Blázquez, M. A., Borevitz, J. O., Christensen, S. K., Fankhauser, C., Ferrándiz, C., Kardailsky, I., Malancharuvil, E. J., Neff, M. M., *et al.* (2000) *Plant Physiol.* **122,** 1003–1013.
31. Murashige, T. & Skoog, F. (1962) *Physiol. Plant* **15,** 473–497.
32. Liu, Y.-G., Mitsukawa, N., Oosumi, T. & Whittier, R. F. (1995) *Plant J.* **8,** 457–463.
33. Uozumi, N., Kim, E. J., Rubio, F., Yamaguchi, T., Muto, S., Tsuboi, A. Bakker, E. P. Nakamura, T. & Schroeder, J. I. (2000) *Plant Physiol.* **122,** 1249–1259.
34. Spalding, E. P., Hirsch, R. E., Lewis, D. R., Qi, Z., Sussman, M. R. & Lewis, B. D. (1999) *J. Gen. Physiol.* **113,** 909–918.
35. Durell, S. R., Hao, Y., Nakamura, T., Bakker, E. P. & Guy, H. R. (1999) *Biophys. J.* **77,** 775–788.
36. Kato, Y., Sakaguchi, M., Mori, Y., Saito, K., Nakamura, T., Bakker, E. P., Sato, Y., Goshima, S. & Uozumi, N. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 6488–6493. (First Published May 8, 2001; 10.1073/pnas.101556598)
37. Fukada-Tanaka, S., Inagaki, Y., Yamaguchi, T., Saito, N. & Iida, S. (2000) *Nature (London)* **407,** 581.
38. Yamaguchi, T., Fukada-Tanaka, S., Inagaki, Y., Saito, N., Yonekura-Sakakibara, K., Tanaka, Y., Kusumi, T. & Iida, S. (2001) *Plant Cell Physiol.* **42,** 451–461.
39. Binzel, M. L., Dana Hess, F., Bressan, R. A. & Hasegawa, P. M. (1988) *Plant Physiol.* **86,** 607–614.