Running title:
Different Na⁺ sensitivities of athkl1 and sos3 mutants

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Calcium Regulation of Sodium Hypersensitivities of sos3 and athkt1 Mutants.

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Abbreviations: MS, Murashige-Skoog; HKT, high-affinity K⁺ transporter; SOS, salt overly sensitive; ICP-OES, inductively coupled plasma optical emission spectroscopy
Abstract

T-DNA disruption mutations in the AtHKT1 gene have been previously shown to suppress the salt sensitivity of the sos3 mutant. However, both sos3 and athkt1 single mutants show sodium (Na\(^+\)) hypersensitivity. In the present study we further analyzed the underlying mechanisms for these non-additive and counteracting Na\(^+\) sensitivities by characterizing athkt1-1 sos3 and athkt1-2 sos3 double mutant plants. Unexpectedly, mature double mutant plants grown in soil clearly showed an increased Na\(^+\) hypersensitivity compared to wild type plants when plants were subjected to salinity stress. The salt sensitive phenotype of athkt1 sos3 double mutant plants was similar to athkt1 plants, which showed chlorosis in leaves and stems. The Na\(^+\) content in xylem sap samples of soil-grown athkt1 sos3 double and athkt1 single mutant plants showed dramatic Na\(^+\) over-accumulation in response to salinity stress. Salinity stress analyses using basic minimal nutrient medium and MS medium revealed that athkt1 sos3 double mutant plants show a more athkt1 single mutant-like phenotype in the presence of 3 mM external Ca\(^{2+}\), but show a more sos3 single mutant-like phenotype in the presence of 1 mM external Ca\(^{2+}\). Taken together multiple analyses demonstrate that the external Ca\(^{2+}\) concentration strongly impacts the Na\(^+\) stress response of athkt1 sos3 double mutants. Furthermore, the presented findings show that SOS3 and AtHKT1 are physiologically distinct major determinants of salinity resistance such that sos3 more strongly causes Na\(^+\) over-accumulation in roots, whereas athkt1 causes an increase in Na\(^+\) levels in the xylem sap and shoots and a concomitant Na\(^+\) reduction in roots.

Keywords: Salinity, HKT/Trk/Ktr transporter, Na\(^+\) transport, Na\(^+\) sensitivity, Arabidopsis thaliana
Introduction

Glycophytes including most crop plants are sensitive to saline conditions. Excessive salt build up in soils is an increasingly detrimental problem that causes growth impairment and is toxic to shoot and root growth (Glenn et al. 1999, Munns 2002, Zhu 2002, Horie and Schroeder 2004). Increasing soil salinity in irrigated lands is a major threat to agricultural production. Elevated Na⁺ levels directly or indirectly perturb important cellular processes including the functions of vital enzymes (Murguía et al. 1995), K⁺ absorption (Rains and Epstein 1965, Epstein 1998) and photosynthesis (Tsugane et al. 1999).


The *Arabidopsis* genome encodes a single *HKT1* gene, *AtHKT1* (Uozumi et al. 2000). Expression of the *Arabidopsis* transporter *AtHKT1* in *Xenopus laevis* oocytes and *Saccharomyces cerevisiae* has shown a relatively Na\(^+\) selective transport activity of *AtHKT1* (Uozumi et al. 2000, Mäser et al. 2002b, Berthomieu et al. 2003). Loss-of-function mutations in *AtHKT1* render plants Na\(^+\) hypersensitive and disturb the distribution of Na\(^+\) between roots and shoots, causing Na\(^+\) over-accumulation in leaves and chlorosis under salinity stress (Mäser et al. 2002a). Furthermore, an EMS mutation that causes reduced *AtHKT1* activity has been shown to cause growth inhibition and Na\(^+\) over-accumulation in shoots under salt stress (Berthomieu et al. 2003). *AtHKT1* expression was found in vascular tissues (Mäser et al. 2002a) and in particular in phloem tissues, causing reduced Na\(^+\) levels in the phloem sap of the *sas2-1* allele in *AtHKT1* (Berthomieu et al. 2003). Furthermore, microarray-based mutant mapping identified an *athkt1* (FN1148) mutation that causes hyper-accumulation of Na\(^+\) in shoots (Gong et al. 2004). These findings reveal that *AtHKT1* is an important Na\(^+\) resistance determinant in *Arabidopsis* and that Na\(^+\) transport via *AtHKT1* is essential for protection of plant leaves against salinity stress in *Arabidopsis* (Mäser et al. 2002a, Berthomieu et al. 2003, Gong et al. 2004, Sunarpi et al. 2005). Recent studies demonstrated that *AtHKT1* and the close rice ortholog *OsHKT8*, function in reducing the Na\(^+\) concentration in the xylem sap (Ren et al. 2005, Sunarpi et al. 2005).

Salt overly sensitive *sos1* to *sos3* mutant loci were identified as essential factors for salt tolerance (Wu et al. 1996, Zhu et al. 1998). *SOS3* encodes an EF hand type Ca\(^{2+}\) binding protein sharing significant sequence similarity with the Ca\(^{2+}\) binding domain of the calcineurin B subunit of yeast (CnB) and neuronal calcium sensors from animals (Liu and Zhu 1998, Sánchez-Barrena et al. 2005). SOS3 has been shown to physically interact with the SOS2 protein kinase in yeast (Halfter et al. 2000), and the SOS2/SOS3 complex regulates the plasma membrane Na\(^+\)/H\(^+\) antiporter SOS1, which is essential for plant salt tolerance (Shi et al. 2000, Qiu et al. 2002, Quintero et al. 2002, Shi et al. 2002).

*AtHKT1* mutations were found to suppress the Na\(^+\) hypersensitivity of *sos3-1* mutant seedlings (Rus et al. 2001). Interestingly, both *sos3* and *athkt1* single mutant alleles show Na\(^+\) hypersensitivity (Liu and Zhu 1998, Mäser et al. 2002a), but *athkt1 sos3-1* plants showed
resistance to salinity stress independent of whether they were grown in agar medium or as mature plants in soil (Rus et al. 2001, Rus et al. 2004). In this study we characterize the complex relationship between these essential salt tolerance determinants to uncover the mechanism underlying the non-additive and counteracting Na\(^+\) sensitivities of atHKT1 sos3 double mutants compared to single sos3-1 and atHKT1 mutants. Detailed characterization of atHKT1-1 sos3-1 and atHKT1-2 sos3-1 double mutants under salt stress show that SOS3 and AtHKT1 function in distinct physiological aspects of salt tolerance and that atHKT1 mutations in sos3 plants do not strictly repress the Na\(^+\) hypersensitivity of sos3 plants to wild type levels, but that the external Ca\(^{2+}\) concentration strongly impacts Na\(^+\) stress responses of atHKT1 sos3 double mutants.

Results

atHKT1 sos3 double mutant plants show atHKT1-like Na\(^+\) hypersensitivity on soil

Previously, two independent T-DNA insertions in the AtHKT1 gene, atHKT1-1 and atHKT1-2, were shown to suppress the Na\(^+\) hypersensitivity of sos3-1 plants (Rus et al. 2001), including recent findings in mature soil-grown plants (Rus et al. 2004). To analyze the underlying mechanisms, mature soil-grown Columbia wild type, atHKT1-1, sos3, atHKT1-1 sos3 and atHKT1-2 sos3 plants were subjected to NaCl stress to determine whether salt sensitivity was suppressed in mature atHKT1 sos3 double mutant alleles here.

Three to four week old plants were watered with 100 mM NaCl twice during 1 week and subsequently watered with de-ionized water for the following week and subsequently analyzed as shown in figure 1. Interestingly, both double mutant alleles clearly showed strong Na\(^+\) hypersensitivity in all experiments with soil-grown mature plants (Fig. 1D, E, I and J) compared to wild type plants (Fig. 1A, F) (≥ 50 plants analyzed for each genotype). These findings differ from those of Rus et al. (2004) in which soil-grown atHKT1 sos3 double mutant plants showed similar NaCl sensitivity as wild type plants and repression of the sos3 Na\(^+\) sensitivity. In over half of the plants, early during the salt stress phase, atHKT1 sos3 mutant plants appeared less chlorotic
than athkt1. This early stress phenotype was more apparent when enriched nutrient media were used. But these plants eventually showed chlorosis after several more days, exhibiting consistent salinity stress of athkt1 sos3 double mutant plants compared to wild type controls, as illustrated in Figure 1D and E.

Mutants in the AtHKT1 gene have been previously characterized as showing increased salt sensitivity under agar-grown conditions (Mäser et al. 2002a, Gong et al. 2004) and under hydroponic conditions (Berthomieu et al. 2003). Soil-grown mature salt-stressed athkt1-1 plants showed chlorotic withering as well (Fig. 1B). Under salt stress, leaves of double mutant athkt1-1 sos3 and athkt1-2 sos3 plants also showed chlorotic withering (Fig. 1D and E) and stems were also damaged, similar to athkt1-1 plants (Fig. 1G, I and J) (n=50 plants per genotype). On the other hand, salt stressed sos3 plants showed a necrotic yellowing phenotype (Fig. 1C) and normal silique development more similar to wild type than athkt1 under the imposed conditions (Fig. 1F, H). We also performed additional NaCl sensitivity analyses using soil grown plants watered with 50 mM NaCl as used in Rus et al. (2004). Experiments under these conditions also showed that athkt1 sos3 double mutant plants show chlorosis at leaf borders similar to athkt1 single mutant plants, but in contrast to wild type plants (Supplemental Figure 1). sos3 single mutant plants showed a yellowing of leaves under these conditions.

**athkt1 sos3 double and athkt1 mutants but not sos3 cause strong Na\(^+\) over-accumulation in the xylem sap**

We next performed ICP analyses using soil-grown plants to compare levels of Na\(^+\) accumulation in leaves. Leaves of soil-grown salt-stressed athkt1-1 plants showed a dramatically increased Na\(^+\) content compared to wild type plants under both 100 mM NaCl (Fig. 2A) and 50 mM NaCl watering conditions (Supplemental Fig. 2). Furthermore, double mutant athkt1-1 sos3 and athkt1-2 sos3 plants also showed Na\(^+\) over-accumulation in leaves similar to athkt1-1 plants, but unlike wild type plants (Fig. 2A and Supplemental Fig. 2). Increases in the Na\(^+\) content of shoots compared to wild type were also found in soil-grown sos3 plants (Fig. 2A and Supplemental Fig. 2).
Expression of the *AtHKT1* gene in vascular tissues was shown using transgenic plants carrying *AtHKT1* promoter-*GUS* gene constructs (Mäser et al. 2002a, Berthomieu et al. 2003). The *sas2-1* mutant, which has a transport-reducing point mutation in the *athkt1* gene, shows lower Na\(^+\) content in the phloem sap compared to wild type (Berthomieu et al. 2003). A recent study has shown that *athkt1* single mutants caused an increase in the Na\(^+\) level of xylem sap and that the AtHKT1 protein is targeted to the plasma membrane of xylem parenchyma cells (Sunarpi et al. 2005). Here, we analyzed whether the Na\(^+\) content of the xylem sap of *athkt1 sos3* plants is affected during salt stress. Xylem sap was collected from soil-grown wild type (n=8) and *athkt1-1* plants (n=7) watered with 100 mM NaCl or 1/20 MS solution (non-stress control condition), and the Na\(^+\) content of the xylem sap was measured by ICP-OES.

To evaluate the relative purity of xylem sap samples, we analyzed sucrose levels of the xylem sap by HPLC analysis. Sucrose levels provide a measure of the level of contamination from phloem sap, as phloem sap in *Arabidopsis* contains high millimolar sucrose levels (Deeken et al. 2002). The average sucrose content of xylem sap samples was 8.02±1.75 µM (n=3). Average sucrose content concentrations have been reported in the phloem sap of *Arabidopsis* of 341±52 mM (Deeken et al. 2002). Thus our xylem sap samples contain negligibly low (< 0.01%) phloem sap contamination.

We measured the Na\(^+\) content in xylem sap samples of *sos3, athkt1, athkt1-1 sos3*, and *athkt1-2 sos3* soil-grown plants in the presence and absence of salinity stress. Interestingly, xylem sap samples from *athkt1 sos3* double mutant plants contained dramatically higher Na\(^+\) than those from *sos3* single mutant and wild type plants (Fig. 2B). The *athkt1 sos3* double mutant plants showed a similar phenotype to *athkt1-1* (Fig. 2B). In contrast single *sos3* mutant plants showed a moderate increase in xylem Na\(^+\) content similar to wild type plants (Fig. 2B), supporting an important role of AtHKT1 (Sunarpi et al. 2005) in maintaining low Na\(^+\) concentrations in xylem vessels during salinity stress. The purity of our xylem sap samples was also supported by the finding that *athkt1* mutants increase the Na\(^-\) content in xylem sap (Fig. 2B), but decrease the Na\(^+\) content in phloem sap (Berthomieu et al. 2003, Sunarpi et al. 2005). These results show that *athkt1-1 sos3* and *athkt1-2 sos3* double mutant plants display *athkt1-1*-like xylem sap Na\(^+\) levels,
which correlates with the Na⁺ hypersensitivity and leaf chlorosis in these mutants when grown on soil (Fig. 1 and Supplemental Fig. 1).

*athkt1 sos3* double mutant plants show *sos3*-like Na⁺ hypersensitivity on minimal medium

To further evaluate the Na⁺ sensitivity of *athkt1 sos3* double mutant plants in response to salt stress, we analyzed the Na⁺ sensitivity of mature plants grown on agar medium. The Na⁺ hypersensitive chlorotic phenotype of *athkt1* shoots was identified using minimal agar medium containing high concentrations of NaCl (Mäser et al. 2002a). Minimal medium was developed to more closely mimic physiological conditions compared to nutrient rich MS medium, while allowing plant growth (see Materials and Methods). Plants grown for 15-17 days on agar plates were transferred onto minimal agar medium supplemented with 75 mM NaCl and grown for 16 additional days. In minimal medium agar-grown plants, *sos3* showed necrotic lesions in the middle of the stress period and eventually showed chlorotic leaf death (Fig. 3C). Similarly, in soil-grown *sos3* plants, more severe salt stress (continuous 100 mM NaCl treatment for 2 weeks) caused *sos3* leaves to turn black, reflecting leaf necrosis (data not shown).

*athkt1-1 sos3* and *athkt1-2 sos3* plants grown in agar medium showed Na⁺ hypersensitivity under the imposed conditions as well and did not show a recovery from salt sensitivity approaching the wild type phenotype (Fig. 3 A, D and E; n=3 experiments, each for *athkt1-1 sos3* and *athkt1-2 sos3*). More surprisingly, double mutant plants showed *sos3*-like features under the imposed conditions, exhibiting necrotic leaf lesions in the middle of the 16-day stress-period followed by chlorosis. To quantitatively analyze this observed phenotype, leaf-chlorophyll content was determined by measuring absorbance at 646.8 nm and 663.8 nm (Porra et al. 1989) as shown in Figure 3F. The data demonstrate that *athkt1 sos3* double mutant plants show a similar time course of Na⁺ stress-induced chlorophyll content reduction to that of *sos3* plants, with *athkt1* plants showing an intermediate Na⁺ sensitivity to that of wild type plants and *athkt1 sos3* double mutants (Fig. 3F).

We next measured Na⁺ contents of shoots and roots of minimal medium grown plants to compare Na⁺ accumulation among the different genotypes. Plants were grown under hydroponic
conditions on minimal medium for 2-3 weeks and then subjected to 75 mM NaCl stress for 2 days. Na\(^+\) contents of shoots and roots were measured by ICP-OES. *athkt1-1* plants over-accumulated Na\(^+\) in shoots (Fig. 4A, n=8) and maintained lower Na\(^+\) levels in roots (Fig. 4B, n=8) compared to wild type plants. In the case of *sos3* plants, no significant difference was found in the Na\(^+\) content of shoots compared to wild type plants under these conditions (Fig. 4A; P>0.08). However, roots of *sos3* plants accumulated more Na\(^+\) compared to wild type (P<1.0x10\(^{-6}\)) and dramatically more Na\(^+\) compared to *athkt1* mutants (P<1.0x10\(^{-13}\)) (Fig. 4B). *athkt1-1 sos3* and *athkt1-2 sos3* double mutant plants, on the other hand, showed interesting Na\(^+\) accumulation profiles in which Na\(^+\) over-accumulation appeared in both shoots (P<1.3x10\(^{-5}\)) and roots (P<1.7x10\(^{-4}\)), indicating that double mutant plants potentially have detrimental features derived from both single mutant loci: shoot Na\(^+\) over-accumulation (*athkt1*-like) and root Na\(^+\) over-accumulation (*sos3*-like) under the imposed conditions (Fig. 4, A and B), which correlates with the strong Na\(^+\) sensitivity of *athkt1 sos3* double mutant plants (Fig. 3).

**sos3-like or athkt1-like Na\(^+\) hypersensitivities in athkt1 sos3 double mutants are primarily controlled by extracellular Ca\(^{2+}\)**

Results from soil-grown *athkt1-1 sos3* and *athkt1-2 sos3* plants showed that double mutant plants exhibit an *athkt1-1*-like Na\(^+\) sensitivity, shoot Na\(^+\) accumulation and elevated Na\(^+\) levels in the xylem sap (Figs. 1 and 2). Furthermore plants grown in minimal medium in agar showed that the Na\(^+\) sensitivity of double mutant plants exhibited a more *sos3*-like sensitivity to Na\(^+\) (Fig. 3); or in hydroponic medium the double mutants exhibited a Na\(^+\) sensitivity reflecting a combination of both *athkt1* and *sos3* impairments (Fig. 4). Previous research demonstrated suppression of the *sos3* phenotype in seedlings by *athkt1* in nutrient rich Murashige-Skoog (MS) agar medium (Rus et al. 2001). To analyze whether Na\(^+\) sensitivities depend on the nutrient status of agar-grown plants, we performed Na\(^+\) sensitivity analyses using MS medium.

Mature 15-17 day old MS medium agar-grown plants were transferred onto MS medium supplemented with 75 mM NaCl and grown for 18 days. A portion of leaves of *athkt1-1* plants showed chlorosis (Supplemental Figure 3B) and leaves of *sos3* plants showed necrotic
yellowing (Supplemental Figure 3C). *athkt1-1 sos3* and *athkt1-2 sos3* double mutant plants showed *athkt1*-like Na$^+$ sensitivity exhibiting chlorosis (white bleaching) in a portion of the leaves and a partial visual repression of the *sos3-1* Na$^+$ sensitivity (Supplemental Figure 3D and E). We subsequently performed additional experiments to determine whether specific components or the overall enrichment of nutrients in MS medium is responsible for showing *sos3*-like or *athkt1*-like Na$^+$ hypersensitivities of double mutants. We compared compositions and concentrations of all substances that differed between minimal medium and MS medium by testing each component individually using synthetic MS media in which the target substance was altered (n=2 experiments, 8 plants per condition). These agar growth analyses showed three ion species that affected the NaCl sensitivity of *Arabidopsis*: K$^+$, NH$_4^+$ and Ca$^{2+}$. These analyses revealed that the higher Ca$^{2+}$ concentration in normal MS medium (3 mM Ca$^{2+}$) compared to minimal medium (1 mM Ca$^{2+}$) affected the phenotypes of *athkt1 sos3* double mutant plants.

We prepared minimal agar medium containing 3 mM Ca$^{2+}$ and 75 mM Na$^+$ and performed further experiments. *athkt1 sos3* double mutant and *athkt1* plants showed chlorosis on minimal medium containing 3 mM Ca$^{2+}$ and 75 mM Na$^+$ (Fig. 5B, D and E). *athkt1 sos3* double mutant plants also showed a significant reduction in the chlorophyll content in response to 75 mM NaCl stress (Fig. 5F). The difference in chlorophyll reduction between *athkt1* and *sos3* plants in 3 mM Ca$^{2+}$ is much smaller compared to that in 1 mM Ca$^{2+}$ because *athkt1* plants become more sensitive and *sos3* plants become more insensitive to Na$^+$ in terms of the chlorophyll reduction rate at 3 mM Ca$^{2+}$ (compare Figs. 3F and 5F; see also Supplemental Figure 4).

We next performed ICP analyses using plants grown hydroponically on minimal medium containing 3 mM Ca$^{2+}$ and 75 mM Na$^+$. *athkt1 sos3* plants accumulated more Na$^+$ than WT in shoots similar to *athkt1* plants (Fig. 6A, P>0.20 for *athkt1-1* vs. *athkt1-1 sos3*, P>0.14 for *athkt1-1* vs. *athkt1-2 sos3*; n=8). Interestingly, *athkt1 sos3* double mutant plants accumulated less Na$^+$ in roots than wild type plants in the presence of 3 mM Ca$^{2+}$ (Fig. 6B; P<1 x 10$^{-4}$ for wild type vs. *athkt1-1 sos3*; P<3 x 10$^{-4}$ for wild type vs. *athkt1-2 sos3*; n=8). The reduced Na$^+$ accumulation in roots of *athkt1 sos3* plants with 3 mM Ca$^{2+}$ in minimal medium (Fig. 6B) lies in contrast to experiments with 1 mM Ca$^{2+}$ in minimal medium (Fig. 4B), and may explain the suppression of
sos3 by athkt1 in seedling growth assays with 3 mM Ca²⁺ in MS medium (Rus et al., 2001).

To test the above hypothesis and compare seedling phenotypes with those of Rus et al. (2001), we performed additional Na⁺ sensitivity analyses using immature agar-grown plants. Ten-day old seedlings germinated on MS plates were directly transferred onto minimal medium containing 75 mM NaCl and 1 mM or 3 mM Ca(NO₃)₂ and grown for 7 additional days (Fig. 7). In the presence of 1 mM Ca²⁺, athkt1-1 sos3 and athkt1-2 sos3 double mutant plants again showed a sos3-like phenotype (Fig. 7C; and left panels in Fig. 7D and E). In the presence of 3 mM Ca²⁺, sos3-like necrosis was suppressed in athkt1 sos3 double mutant plants as reported by Rus et al. (2001) (right panels in Fig. 7D and E). Furthermore, athkt1 sos3 plants showed curled leaves, as also did athkt1-1 under the imposed conditions (Fig. 7B; and right panels in Fig. 7D and E), but wild type plants did not show curled leaves (Fig. 7A). sos3 seedlings showed a severe root growth defect (Rus et al. 2004), however, athkt1 plants did not (Mäser et al. 2002a) under these conditions (Fig. 7B and C). athkt1 sos3 double plants showed a severe root growth defect similar to sos3 plants in 75 mM Na⁺ and 1 mM Ca²⁺ condition, but showed less inhibition of root growth in 75 mM Na⁺ and 3 mM Ca²⁺ condition (Fig. 7D and E, compare left and right panels).

We measured the Ca²⁺ content of the soil solution extracted from soil used for Na⁺ sensitivity analyses because soil grown athkt1 sos3 plants showed more athkt1-1-like Na⁺ hypersensitivity (Figs. 1 and 2; and Supplemental Figs. 1 and 2). Soil solution extracted from 3 independent soil samples contained 6.89±1.40 mM Ca²⁺. Together these results demonstrate that extracellular Ca²⁺ controls whether athkt1 sos3 double mutant plants display a more athkt1-like or a more sos3-like phenotype and that Ca²⁺ strongly impacts Na⁺ homeostasis in athkt1 sos3 double mutants.

Discussion

In the present study we demonstrate that athkt1 sos3 double mutant plants show differential Na⁺ hypersensitivities and that the concentration of extracellular Ca²⁺ controls
whether athkt1 sos3 double mutant plants display a more athkt1-like or a more sos3-like phenotype. Furthermore, the present findings also demonstrate distinct physiological roles of AtHKT1 and SOS3 in protecting plants from salinity stress. athkt1 sos3 double and athkt1 single mutants but not sos3-1, cause significantly increased Na⁺ levels in the xylem sap (Fig. 2B) (Sunarpi et al. 2005), resulting in reduction in root Na⁺ content and Na⁺ over-accumulation in leaves and eventual chlorosis. In contrast, the sos3 mutation more strongly caused Na⁺ over-accumulation in roots (Fig. 4 and 6).

_athkt1 sos3 double mutants show increased xylem sap Na⁺ content under salt stress_

_athkt1_ mutant alleles have been found to show Na⁺ hypersensitivity and Na⁺ over-accumulation in shoots under salt stress (Mäser et al. 2002a, Berthomieu et al. 2003, Gong et al. 2004, Rus et al. 2004). Decreased Na⁺ levels in the phloem sap were found in the reduced function point mutation _athkt1_ allele, _sas2-1_, compared to wild type, but no effect was found in the Na⁺ content of the xylem sap of the mutant (Berthomieu et al. 2003). Moreover, phloem tissue expression of _AtHKT1_ was found in promoter GUS analyses and _in situ_ hybridization (Berthomieu et al. 2003). A Na⁺ recirculation model for _AtHKT1_ has been proposed based on these observations, in which _AtHKT1_ functions in loading Na⁺ into the phloem cells in shoots and unloading Na⁺ from the phloem in roots (Berthomieu et al. 2003).

Xylem sap collected from _athkt1 sos3_ double and _athkt1_ single mutant alleles contained dramatically higher Na⁺ compared to _sos3-1_ and wild type controls (Fig. 2B). These findings support an expanded model for _AtHKT1_ functions in reducing Na⁺ in the xylem sap under salt stress, thus preventing plants from over-accumulating Na⁺ in shoots (Sunarpi et al. 2005). The rice gene _OsHKT8_ is a close orthologue of _AtHKT1_ and has recently been demonstrated to also control Na⁺ levels in the xylem sap (Ren et al. 2005). Direct immunoelectron microscopy of _AtHKT1_ demonstrated localization of the _AtHKT1_ protein in the plasma membrane of xylem parenchyma cells (Sunarpi et al. 2005). Note that these data do not exclude the complementary function of _AtHKT1_ in enhancing Na⁺ levels in the phloem (Berthomieu et al. 2003, Sunarpi et al. 2005). _AtHKT1_ may cooperate in both xylem and phloem Na⁺ content control thus mediating Na⁺
recirculation to roots. Recirculation of Na$^+$ from the xylem sap via the phloem to roots protects leaves from salinity stress and may allow plants to subsequently excrete Na$^+$ from roots. Na$^+$ recirculation may also enhance the overall vacuolar Na$^+$ sequestration in different types of root cells via tonoplast H$^+$/Na$^+$ exchangers (Blumwald and Poole 1985, Apse et al. 1999, Gaxiola et al. 1999). Important questions, including how AtHKT1 functions in both the xylem and the phloem and whether the activity and expression of AtHKT1 is differentially regulated in these tissues will be an important subject of investigation.

Salinity stress also caused an increase in the xylem sap of sos1-1 plants (Shi et al. 2002), but had a more limited effect in sos3-1 plants compared to athkt1 mutants, with sos3-1 xylem levels of Na$^+$ being closer to those in wild type plants (Fig. 2B; P>0.06 for sos3-1 compared to WT). The dramatically increased Na$^+$ accumulation in roots of sos3 plants (Fig. 4B and 6B) thus appears to result in a mild increase in the Na$^+$ content in the xylem sap.

_athkt1 sos3_ plants show an _athkt1_-like phenotype in 3 mM external Ca$^{2+}$, which can account for suppression of Na$^+$ hypersensitivity of sos3-1 seedlings

_athkt1-1_ and _athkt1-2_ alleles function as genetic suppressor alleles of sos3-1 seedlings and AtHKT1 was proposed to function as a Na$^+$ uptake pathway into roots (Rus et al. 2001). However, _athkt1_ mutant alleles were subsequently shown to cause Na$^+$ hypersensitivity in leaves (Mäser et al. 2002a, Berthomieu et al. 2003, Gong et al. 2004). No expression of _AtHKT1_ in the root epidermis or root cortex has been observed thus far (Mäser et al. 2002a, Berthomieu et al. 2003). Moreover, detailed tracer influx experiments using $^{22}$Na$^+$ showed no measurable disruption of Na$^+$ influx into Arabidopsis roots due to _athkt1_ mutations, despite analyzing several conditions (Berthomieu et al. 2003, Essah et al. 2003). Thus the mechanism by which _athkt1_ mutations suppress the Na$^+$ sensitivity of sos3-1 has remained unclear. The present study shows that _athkt1 sos3_ plants show an _athkt1_-like phenotype with 3 mM Ca$^{2+}$ in the nutrient medium in 2 to 4 weeks old plants (Figs. 5, 6 and 7). Under these conditions _athkt1_ alleles completely suppressed the characteristic Na$^+$ over-accumulation in roots of the sos3 mutant (Fig. 6B). Our analyses suggest that this “protective” effect of _athkt1_ on root Na$^+$ levels may explain why _athkt1_
mutations suppress the Na\(^+\) hypersensitivity of sos3-1 seedlings (Rus et al. 2001).

A more recent study showed however that athkt1 mutations also reverted sos3-1-dependent Na\(^+\)-induced damage of leaves of mature soil-grown plants (Rus et al. 2004). It was shown that mature soil-grown athkt1 sos3 plants are more salt tolerant than athkt1 single mutant plants, exhibiting similar growth and leaf health as wild type control plants under salinity stress conditions (Rus et al. 2004). However, our results obtained from mature soil-grown plants were inconsistent with these findings (Figs. 1 and 2; Supplemental Figs. 1 and 2). In the present study, mature soil-grown athkt1 sos3 plants showed Na\(^+\) hypersensitivity similar to athkt1 mutants (Figs. 1 and 2; Supplemental Figs. 1 and 2). The reason for this discrepancy is not clear and may depend on growth conditions. Nevertheless, the physiological effects of athkt1 in athkt1 sos3 double mutant plants on Na\(^+\) accumulation in leaves (Fig. 2A and Supplemental Fig. 2), xylem sap (Fig. 2B), and shoots (Figs. 4 and 6), and on Na\(^+\)-induced chlorophyll reduction (Figs. 3F and 5F), make it unlikely that athkt1 mutations can protect mature sos3-1 mutant plants from salinity stress and the repression of sos3-1 Na\(^+\) hypersensitivity in mature plants by athkt1 loss-of-function mutations appears unlikely under most physiological conditions.

**Ca\(^{2+}\)-dependence of SOS3 Ca\(^{2+}\) sensor and Na\(^+\) hypersensitivities of athkt1 sos3 double mutants**

Na\(^+\) influx channels and currents into root cells have been characterized in electrophysiological studies (Amtmann et al. 1997, Roberts and Tester 1997, Tyerman et al. 1997, Buschmann et al. 2000, Davenport and Tester 2000, Maathuis and Sanders 2001). However, transporter mutants that cause major reduction in root Na\(^+\) influx have not yet been found and the underlying genes remain to be identified. Several electrophysiological studies have shown partial block of Na\(^+\) influx by Ca\(^{2+}\) (Tyerman et al. 1997, Buschmann et al. 2000, Davenport and Tester 2000), suggesting that multiple transporters contribute to Na\(^+\) influx in roots. These findings correlate with classical studies showing that calcium enhances the relative absorption of K\(^+\) at high Na\(^+\) concentrations (Epstein 1961, LaHaye and Epstein 1969, Epstein 1998). SOS3 encodes a Ca\(^{2+}\) sensor that regulates the activity of the plasma membrane SOS1 Na\(^+\)/H\(^+\) exchanger, thus mediating cellular Na\(^+\) extrusion (Liu and Zhu 1998, Qiu et al. 2002, Shi et al. 2002). Thus
calcium appears to impact several mechanisms in mediating salt tolerance.

Findings presented here demonstrate that AtHKT1 mutations do not strictly repress the Na\(^+\) hypersensitivity of sos3 plants to wild type levels, but that athkt1 sos3 plants exhibit more athkt1-like or more sos3-like Na\(^+\) hypersensitivities, depending on the external Ca\(^{2+}\) concentration. sos3-like Na\(^+\) hypersensitivity became predominant in athkt1 sos3 mutants under salt stress in the presence of 1 mM Ca\(^{2+}\) (Figs. 3 and 4). On the other hand, AtHKT1 was the strongest salt tolerance determinant in athkt1 sos3 mutants in the presence of 3 mM Ca\(^{2+}\) as athkt1-like Na\(^+\) hypersensitivity becomes more prevalent (Figs. 5 and 6).

The partial Ca\(^{2+}\) block of Na\(^+\) influx channels contributes to reduction in salinity stress (Tyerman et al. 1997, Buschmann et al. 2000, Davenport and Tester 2000). Furthermore, the Ca\(^{2+}\)-dependent differential Na\(^+\) hypersensitivities of athkt1 sos3 double mutants can likely be explained by a model in which an increase in cytoplasmic Ca\(^{2+}\) can partially suppress the sos3-1 phenotype. This model is supported by results shown in Supplemental Figures S-3C, S-3F and S-4E. Note that young sos3 seedlings did not show this effect possibly because the effect is not clearly detected in young seedlings (Fig. 7C right panel). The SOS3 gene encodes a Ca\(^{2+}\) binding protein that is essential for plant salt tolerance (Liu and Zhu 1998). Recently, the three-dimensional structure of the SOS3 protein was reported (Sánchez-Barrena et al. 2005). The SOS3 protein includes four EF-hand Ca\(^{2+}\)-binding motifs and forms a dimer when Ca\(^{2+}\) concentrations are increased (Sánchez-Barrena et al. 2005). The sos3-1 mutation causes a three amino acid deletion in the third EF-hand (Liu and Zhu 1998, Sánchez-Barrena et al. 2005). This third EF-hand is hypothesized to stabilize the dimer structure of the SOS3 protein upon Ca\(^{2+}\) binding (Sánchez-Barrena et al. 2005). The SOS3-1 protein shows a reduced affinity for Ca\(^{2+}\) (Ishitani et al. 2000). Thus increasing extracellular Ca\(^{2+}\) may partially activate the SOS3-1 protein, thus reducing the sos3-1 phenotype resulting in the observed athkt1-like phenotypes of athkt1 sos3 plants at 3 mM external Ca\(^{2+}\) (Figs. 5, 6 and 7) (Rus et al. 2001). Furthermore, at high Ca\(^{2+}\) SOS3-independent mechanisms may be activated, as the Arabidopsis genome encodes 9 homologues of the SOS3 gene (Zhu 2002, Kolukisaoglu et al. 2004).

In summary, the present findings show that increasing the external Ca\(^{2+}\) concentration
strongly impacts the response of athkt1 sos3 double mutant plants to Na$^+$ stress. Ca$^{2+}$-dependent sos3-like and athkt1-like Na$^+$ hypersensitivities of athkt1 sos3 plants suggest that both the SOS pathway and the AtHKT1 pathway are mechanistically independent strong salt tolerance determinants in Arabidopsis. Under physiological conditions both pathways mediate distinguishable Na$^+$ tolerance mechanisms and loss-of-function mutations in AtHKT1 are unlikely to cause physiological Na$^+$ resistance of mature Arabidopsis plants. Furthermore, comparative xylem sap analyses in sos3 and athkt1 single and double mutants show that athkt1 sos3 double mutants show enhanced Na$^+$ levels in xylem sap, whereas sos3 alone did not greatly affect xylem sap Na$^+$ levels. Studies into the question whether specific signaling pathways control the activity of AtHKT1 and whether cross talk with the SOS pathway exists will be interesting subjects of future inquiry.

**Materials and Methods**

*Cultivation of Arabidopsis*

Seeds were surface sterilized and sown on MS agar medium supplemented with 1% sucrose. All mutant alleles were confirmed to have the reported homozygous mutations by sequencing genomic DNA at the SOS3 and AtHKT1 loci. Ten to twelve day-old seedlings were transferred onto soil (Professional Blend; McConkey Co., Sumner, WA) for experiments using soil-grown plants, or onto a 10 µm pore-nylon mesh (Spectrum laboratories, Inc., Rancho Dominguez, CA) placed on fresh MS medium or minimal medium (Mäser et al. 2002a) for the agar-growth analysis, or directly onto minimal medium for the short-term agar-growth analysis, or onto a plug (Jaece Industries, Inc., North Tonawanda, NY) soaked in minimal medium for ion content measurements for plants grown on hydroponic medium. Minimal medium contained 0.5 mM KH$_2$PO$_4$, 1.25 mM KNO$_3$, 0.5 mM MgSO$_4$, 20 µM FeNa-EDTA, and the additional micronutrients 7 µM H$_3$BO$_3$, 1.4 µM MnSO$_4$, 1 µM ZnSO$_4$, 4.5 µM KI, 0.1 µM CuSO$_4$, 0.2 µM Na$_2$MoO$_4$, 10 nM CoCl$_2$ supplemented with the indicated amounts of NaCl and Ca(NO$_3$)$_2$. Plants were subsequently grown for 2-3 weeks on soil, or 5 days on MS medium or minimal medium in the case of agar growth analyses, or for 2-3 weeks with a solution change every 3 days in the case of hydroponic cultures, before salt stress was imposed. Surgical tape (3M, Santa Fe Springs, CA) was used to tape plates instead of a paraffin film in all agar growth analyses.
Imposition of salt stress

Pots of all soil-grown plants were placed on plastic trays and irrigated by adding irrigation solutions (de-ionized water or 1/20 MS basal salt solution). Mature soil-grown plants at the bolting stage were irrigated two times with 100 mM NaCl added to one-twentieth MS basal salts-solution (Shi et al. 2002) over a period of 6 days for Na⁺ content determination, for 2 days for xylem sap collection experiments, or for 7 days for NaCl sensitivity analyses. Plants were subsequently irrigated with de-ionized water for another 7 days after the stress treatment in the case of NaCl sensitivity analyses and the NaCl sensitivities were subsequently analyzed. Plants were watered with one-twentieth MS basal salts solution in parallel as a negative control in all experiments using soil-grown plants.

For the NaCl sensitivity analyses of agar-grown plants, meshes on which mature plants were laying were transferred onto the indicated media containing 75 mM NaCl. Plants were subjected to NaCl stress for 16-18 days. Hydroponically grown plants were subjected to minimal medium containing 75 mM NaCl (pH was adjusted to 5.5 with N-methyl-D-Glucamine) for 2 days.

Xylem sap collection and sucrose content determination

Xylem sap samples were collected as described previously (Gaymard et al. 1998, Shi et al. 2002). All rosette leaves were removed and the inflorescence stem was cut with a very sharp razor blade from soil-grown 4 to 5 week-old plants. The tray and plants were then covered with a transparent plastic dome to maintain high humidity. Xylem sap drops that accumulated at the cut surface of the inflorescence stem were then collected using a micropipette.

For sucrose content determinations xylem sap samples were first purified before HPLC analysis by passing through a Dionex H cartridge (Dionex corp., CA). The run-through and 3 x 0.2 ml water washes were collected in the same container and dried in a Speedvac and then re-dissolved in an appropriate amount of de-ionized water. Separation and detection of the sugars were achieved by injecting an aliquot of the sample into a Dionex DX 500 HPLC system (Dionex corp., CA) using the CarboPac MA1 column (Dionex corp., CA) in an isocratic mobile phase (480 mM NaOH at 0.4ml/min. for 50 min.) and electrochemical detector at the UCSD Glycobiology Center (UCSD School of Medicine).

Chlorophyll content determination

Chlorophyll content was determined by the method of Porra et al. (1989). Leaves of plants were harvested at the indicated time points and 4 leaf-disks were punched out of leaves. Punched disks were soaked in 3 ml of Dimethyl formamide (Fisher, Fair lawn, NJ) and kept at 4 °C for 12-16 hrs. Absorbances at 646.8 nm and 663.8 nm were then measured by photo
absorption spectrometry (Spectronic GENESYS5; Milton Roy USA, Ivyland, PA). Contents of chlorophyll a, b and a+b were calculated with formulas described by Porra et al. (1989).

**Ion content determination**

Ion content of the described samples was measured by inductively coupled plasma-optic emission spectroscopy (ICP-OES) at the Scripps Institution of Oceanography UCSD analytical facility. Shoot and root samples were isolated as described previously (Mäser et al. 2002a). Xylem sap samples were diluted in 5% HNO₃ and directly measured.

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**References**


Bot. 48: 459-480

Legends to figures

Figure 1. Increased salt sensitivity of athkt1-1 sos3 and athkt1-2 sos3 plants grown on soil. Plants were irrigated twice with one-twentieth MS basal salt-solution with 100 mM NaCl (right panels in A to E, and panels F to J) or without NaCl (left panels in A to E). Visual phenotypes are shown of leaves and shoots of (A, F) wild type/Columbia; (B, G) athkt1-1; (C, H) sos3; (D, I) athkt1-1 sos3; and (E, J) athkt1-2 sos3. Plants were watered twice during a 7-day period, and then all plants were irrigated with de-ionized water for the following 7 days prior to taking photographs.

Figure 2. athkt1-1 sos3 and athkt1-2 sos3 double mutant plants over-accumulate Na\(^+\) in the xylem sap similar to athkt1-1, but not sos3. Na\(^+\) contents of leaves and xylem sap, derived from soil-grown plants. (A) Na\(^+\) content measurements in leaves of plants watered with one-twentieth MS basal salt solution with 100 mM NaCl (black bars) or without NaCl (white bars) for 6 days. (B) Na\(^+\) content measurements in the xylem sap of soil-grown plants watered for 2 days with the same solutions used in (A). Na\(^+\) content was measured by ICP-OES. Error bars represent standard deviations (n=5 to 8).

Figure 3. Agar-grown athkt1-1 sos3 and athkt1-2 sos3 double mutant plants show sos3-like Na\(^+\) hypersensitivity under salt stress in the presence of 1 mM Ca\(^{2+}\). 15 to 17 day-old mature plants were transferred onto minimal medium containing 75 mM NaCl and 1 mM Ca\(^{2+}\). (A-E) Plants were grown for 16 additional days and photographs were taken at the end of this period. (F) Contents of chlorophyll a + b extracted from agar-grown plants, which were subjected to 75 mM NaCl stress for 0, 3, 6, 9, 12 or 15 days on minimal medium. Error bars represent standard deviations (n=3 to 4).
**Figure 4.** *athkt1-1 sos3* and *athkt1-2 sos3* double mutant plants over-accumulate Na⁺ in both roots and shoots in the presence of 1 mM Ca²⁺ showing cumulative detrimental phenotypes of the two single *athkt1* and *sos3* mutations. (A) Na⁺ contents in shoots. (B) Na⁺ contents in roots. Plants were hydroponically grown using minimal medium for 2-3 weeks. Mature, bolting plants were then subjected to 75 mM NaCl stress for 2 days. The Na⁺ content was measured by ICP-OES. Error bars represent standard deviations (n=7 to 8).

**Figure 5.** Agar-grown *athkt1-1 sos3* and *athkt1-2 sos3* double mutant plants show Na⁺ hypersensitivity under salt stress in the presence of 3 mM Ca²⁺. 15 to 17 day-old mature plants were transferred onto minimal medium containing 75 mM NaCl and 3 mM Ca²⁺. (A-E) Plants were grown for 16 additional days and photographs were then taken. (F) Contents of chlorophyll a + b extracted from agar-grown plants, which were subjected to 75 mM NaCl stress for 0, 3, 6, 9, 12 or 15 days on minimal medium containing 3 mM Ca²⁺. Error bars represent standard deviations (n=3 to 4).

**Figure 6.** *athkt1-1 sos3* and *athkt1-2 sos3* double mutant plants show ICP profiles similar to single *athkt1* mutants in 3 mM Ca²⁺ and 75 mM NaCl. (A) Na⁺ contents in shoots. (B) Na⁺ contents in roots. Plants were hydroponically grown using minimal medium containing 1 mM Ca²⁺ for 2-3 weeks. Mature, bolting plants were then subjected to 75 mM NaCl stress for 2 days in the presence of 3 mM Ca²⁺. Error bars represent standard deviations (n=7 to 8).

**Figure 7.** Young *athkt1-1 sos3* double mutant seedlings exhibit *sos3*-like or *athkt1*-like phenotypes on minimal agar medium, depending on the external Ca²⁺ concentration. The salt sensitivities of 17-day old seedlings are shown of (A) Wild type, (B) *athkt1-1*, (C) *sos3*, (D) *athkt1-1 sos3* and (E) *athkt1-2 sos3*. Seedlings were grown on MS agar medium for 10 days and then transferred to minimal agar medium for 7 days containing 75 mM NaCl and 1 mM Ca²⁺ (left in each panel) or 3 mM Ca²⁺ (right in each panel). Photographs of 2 representative plants from each condition are shown. Twenty seedlings per line were analyzed in 3 independent experiments and all plants showed the depicted phenotypes.
Running title:
Different Na⁺ sensitivities of athkt1 and sos3 mutants

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Calcium Regulation of Sodium Hypersensitivities of *sos3* and *athkt1* Mutants.

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Abbreviations: MS, Murashige-Skoog; HKT, high-affinity K⁺ transporter; SOS, salt overly sensitive; ICP-OES, inductively coupled plasma optical emission spectroscopy
Abstract

T-DNA disruption mutations in the \textit{AtHKT1} gene have been previously shown to suppress the salt sensitivity of the sos3 mutant. However, both sos3 and \textit{athkt1} single mutants show sodium (Na\(^+\)) hypersensitivity. In the present study we further analyzed the underlying mechanisms for these non-additive and counteracting Na\(^+\) sensitivities by characterizing \textit{athkt1-1 sos3} and \textit{athkt1-2 sos3} double mutant plants. Unexpectedly, mature double mutant plants grown in soil clearly showed an increased Na\(^+\) hypersensitivity compared to wild type plants when plants were subjected to salinity stress. The salt sensitive phenotype of \textit{athkt1 sos3} double mutant plants was similar to \textit{athkt1} plants, which showed chlorosis in leaves and stems. The Na\(^+\) content in xylem sap samples of soil-grown \textit{athkt1 sos3} double and \textit{athkt1} single mutant plants showed dramatic Na\(^+\) over-accumulation in response to salinity stress, which was not observed to this degree in sos3-1 plants. We then performed salinity stress analyses using basic minimal nutrient medium and MS medium. These analyses revealed that \textit{athkt1 sos3} double mutant plants show a more \textit{athkt1} single mutant-like phenotype in the presence of 3 mM external Ca\(^{2+}\), but show a more sos3 single mutant-like phenotype in the presence of 1 mM external Ca\(^{2+}\). Taken together results from chlorophyll content, Na\(^+\) accumulation and Na\(^+\) sensitivity analyses in high and low Ca\(^{2+}\) demonstrate that the external Ca\(^{2+}\) concentration strongly impacts the Na\(^+\) stress response of \textit{athkt1 sos3} double mutants. Furthermore, the presented findings show that SOS3 and AtHKT1 are physiologically distinct major determinants of salinity resistance in \textit{Arabidopsis}.

Keywords: Salinity, HKT/Trk/Ktr transporter, Na\(^+\) transport, Na\(^+\) sensitivity, \textit{Arabidopsis thaliana}
Introduction

Glycophytes including most crop plants are sensitive to saline conditions. Excessive salt build up in soils is an increasingly detrimental problem that causes growth impairment and is toxic to shoot and root growth (Glenn et al. 1999, Munns 2002, Zhu 2002, Horie and Schroeder 2004). Increasing soil salinity in irrigated lands is a major threat to agricultural production. Elevated Na\(^+\) levels directly or indirectly perturb important cellular processes including the functions of vital enzymes (Murguía et al. 1995), K\(^+\) absorption (Rains and Epstein 1965, Epstein 1998) and photosynthesis (Tsugane et al. 1999).


The Arabidopsis genome encodes a single HKT1 gene, AtHKT1 (Uozumi et al. 2000). Expression of the Arabidopsis transporter AtHKT1 in Xenopus laevis oocytes and Saccharomyces cerevisiae has shown a relatively Na\(^+\) selective transport activity of AtHKT1 (Uozumi et al. 2000, Mäser et al. 2002b, Berthomieu et al. 2003). Loss-of-function mutations in AtHKT1 render plants Na\(^+\) hypersensitive and disturb the distribution of Na\(^+\) between roots and shoots, causing Na\(^+\) over-accumulation in leaves and chlorosis under salinity stress (Mäser et al. 2002a). Furthermore, an EMS mutation that causes reduced AtHKT1 activity has been shown to cause growth inhibition and Na\(^+\) over-accumulation in shoots under salt stress (Berthomieu et al. 2003). AtHKT1 expression was found in vascular tissues (Mäser et al. 2002a) and in particular in phloem tissues, causing reduced Na\(^+\) levels in the phloem sap of the sas2-1 allele in AtHKT1 (Berthomieu et al. 2003). Furthermore, microarray-based mutant mapping identified an athkt1 (FN1148) mutation that causes hyper-accumulation of Na\(^+\) in shoots (Gong et al. 2004). These findings reveal that AtHKT1 is an important Na\(^+\) resistance determinant in Arabidopsis and that Na\(^+\) transport via AtHKT1 is essential for protection of plant leaves against salinity stress in Arabidopsis (Mäser et al. 2002a, Berthomieu et al. 2003, Gong et al. 2004, Sunarpi et al. 2005). Recent studies demonstrated that AtHKT1 and the close rice ortholog OsHKT8, function in reducing the Na\(^+\) concentration in the xylem sap (Ren et al. 2005, Sunarpi et al. 2005).

Salt overly sensitive sos1 to sos3 mutant loci were identified as essential factors for salt tolerance (Wu et al. 1996, Zhu et al. 1998). SOS3 encodes an EF hand type Ca\(^{2+}\) binding protein sharing significant sequence similarity with the Ca\(^{2+}\) binding domain of the calcineurin B subunit of yeast (CnB) and neuronal calcium sensors from animals (Liu and Zhu 1998, Sánchez-Barrena et al. 2005). SOS3 has been shown to physically interact with the SOS2 protein kinase in yeast (Halfter et al. 2000), and the SOS2/SOS3 complex regulates the plasma membrane Na\(^+\)/H\(^+\) anti-porter SOS1, which is essential for plant salt tolerance (Shi et al. 2000, Qiu et al. 2002, Quintero et al. 2002, Shi et al. 2002).

AtHKT1 mutations were found to suppress the Na\(^+\) hypersensitivity of sos3-1 mutant seedlings (Rus et al. 2001). Interestingly, both sos3 and athkt1 single mutant alleles show Na\(^+\) hypersensitivity (Liu and Zhu 1998, Mäser et al. 2002a), but athkt1 sos3-1 plants showed
Resistance to salinity stress independent of whether they were grown in agar medium or as mature plants in soil (Rus et al. 2001, Rus et al. 2004). In this study we characterize the complex relationship between these essential salt tolerance determinants to uncover the mechanism underlying the non-additive and counteracting Na⁺ sensitivities of athkt1 sos3 double mutants compared to single sos3-1 and athkt1 mutants. Detailed characterization of athkt1-1 sos3-1 and athkt1-2 sos3-1 double mutants under salt stress show that SOS3 and AtHKT1 function in distinct physiological aspects of salt tolerance and that athkt1 mutations in sos3 plants do not strictly repress the Na⁺ hypersensitivity of sos3 plants to wild type levels, but that the external Ca²⁺ concentration strongly impacts Na⁺ stress responses of athkt1 sos3 double mutants.

**Results**

*athkt1 sos3 double mutant plants show athkt1-like Na⁺ hypersensitivity on soil*

Previously, two independent T-DNA insertions in the *AtHKT1* gene, *athkt1-1* and *athkt1-2*, were shown to suppress the Na⁺ hypersensitivity of sos3-1 plants (Rus et al. 2001), including recent findings in mature soil-grown plants (Rus et al. 2004). To analyze the underlying mechanisms, mature soil-grown Columbia wild type, *athkt1-1, sos3, athkt1-1 sos3* and *athkt1-2 sos3* plants were subjected to NaCl stress to determine whether salt sensitivity was suppressed in mature *athkt1 sos3* double mutant alleles here.

Three to four week old plants were watered with 100 mM NaCl twice during 1 week and subsequently watered with de-ionized water for the following week and subsequently analyzed as shown in figure 1. Interestingly, both double mutant alleles clearly showed strong Na⁺ hypersensitivity in all experiments with soil-grown mature plants (Fig. 1D, E, I and J) compared to wild type plants (Fig. 1A, F) (≥ 50 plants analyzed for each genotype). These findings differ from those of Rus et al. (2004) in which soil-grown *athkt1 sos3* double mutant plants showed similar NaCl sensitivity as wild type plants and repression of the sos3 Na⁺ sensitivity. In over half of the plants, early during the salt stress phase, *athkt1 sos3* mutant plants appeared less chlorotic than
This early stress phenotype was more apparent when enriched nutrient media were used. But these plants eventually showed chlorosis after several more days, exhibiting consistent salinity stress of athkt1 sos3 double mutant plants compared to wild type controls, as illustrated in Figure 1D and E.

Mutants in the AtHKT1 gene have been previously characterized as showing increased salt sensitivity under agar-grown conditions (Mäser et al. 2002a, Gong et al. 2004) and under hydroponic conditions (Berthomieu et al. 2003). Soil-grown mature salt-stressed athkt1-1 plants showed chlorotic withering as well (Fig. 1B). Under salt stress, leaves of double mutant athkt1-1 sos3 and athkt1-2 sos3 plants also showed chlorotic withering (Fig. 1D and E) and stems were also damaged, similar to athkt1-1 plants (Fig. 1G, I and J) (n=50 plants per genotype). On the other hand, salt stressed sos3 plants showed a necrotic yellowing phenotype (Fig. 1C) and normal silique development more similar to wild type than athkt1 under the imposed conditions (Fig. 1F, H). We also performed additional NaCl sensitivity analyses using soil grown plants watered with 50 mM NaCl as used in Rus et al. (2004). Experiments under these conditions also showed that athkt1 sos3 double mutant plants show chlorosis at leaf borders similar to athkt1 single mutant plants, but in contrast to wild type plants (Supplemental Figure 1). sos3 single mutant plants showed a yellowing of leaves under these conditions.

athkt1 sos3 double and athkt1 mutants but not sos3 cause strong Na⁺ over-accumulation in the xylem sap

We next performed ICP analyses using soil-grown plants to compare levels of Na⁺ accumulation in leaves. Leaves of soil-grown salt-stressed athkt1-1 plants showed a dramatically increased Na⁺ content compared to wild type plants under both 100 mM NaCl (Fig. 2A) and 50 mM NaCl watering conditions (Supplemental Fig. 2). Furthermore, double mutant athkt1-1 sos3 and athkt1-2 sos3 plants also showed Na⁺ over-accumulation in leaves similar to athkt1-1 plants, but unlike wild type plants (Fig. 2A and Supplemental Fig. 2). Increases in the Na⁺ content of shoots compared to wild type were also found in soil-grown sos3 plants (Fig. 2A and Supplemental Fig. 2).

Expression of the AtHKT1 gene in vascular tissues was shown using transgenic plants
carrying *AtHKT1* promoter-*GUS* gene constructs (Mäser et al. 2002a, Berthomieu et al. 2003). The *sas2-1* mutant, which has a transport-reducing point mutation in the *athkt1* gene, shows lower Na\(^+\) content in the phloem sap compared to wild type (Berthomieu et al. 2003). A recent study has shown that *athkt1* single mutants caused an increase in the Na\(^+\) level of xylem sap and that the AtHKT1 protein is targeted to the plasma membrane of xylem parenchyma cells (Sunarpi et al. 2005). Here, we analyzed whether the Na\(^+\) content of the xylem sap of *athkt1 sos3* plants is affected during salt stress. Xylem sap was collected from soil-grown wild type (n=8) and *athkt1-1* plants (n=7) watered with 100 mM NaCl or 1/20 MS solution (non-stress control condition), and the Na\(^+\) content of the xylem sap was measured by ICP-OES.

To evaluate the relative purity of xylem sap samples, we analyzed sucrose levels of the xylem sap by HPLC analysis. Sucrose levels provide a measure of the level of contamination from phloem sap, as phloem sap in *Arabidopsis* contains high millimolar sucrose levels (Deeken et al. 2002). The average sucrose content of xylem sap samples was 8.02±1.75 µM (n=3). Average sucrose content concentrations have been reported in the phloem sap of *Arabidopsis* of 341±52 mM (Deeken et al. 2002). Thus our xylem sap samples contain negligibly low (< 0.01%) phloem sap contamination.

We measured the Na\(^+\) content in xylem sap samples of *sos3, athkt1, athkt1-1 sos3*, and *athkt1-2 sos3* soil-grown plants in the presence and absence of salinity stress. Interestingly, xylem sap samples from *athkt1 sos3* double mutant plants contained dramatically higher Na\(^+\) than those from *sos3* single mutant and wild type plants (Fig. 2B). The *athkt1 sos3* double mutant plants showed a similar phenotype to *athkt1-1* (Fig. 2B). In contrast single *sos3* mutant plants showed a moderate increase in xylem Na\(^+\) content similar to wild type plants (Fig. 2B), supporting an important role of AtHKT1 (Sunarpi et al. 2005) in maintaining low Na\(^+\) concentrations in xylem vessels during salinity stress. The purity of our xylem sap samples was also supported by the finding that *athkt1* mutants increase the Na\(^+\) content in xylem sap (Fig. 2B), but decrease the Na\(^+\) content in phloem sap (Berthomieu et al. 2003, Sunarpi et al. 2005). These results show that *athkt1-1 sos3* and *athkt1-2 sos3* double mutant plants display *athkt1-1*-like xylem sap Na\(^+\) levels, which correlates with the Na\(^+\) hypersensitivity and leaf chlorosis in these mutants when grown on
soil (Fig. 1 and Supplemental Fig. 1).

**athkt1 sos3 double mutant plants show sos3-like Na⁺ hypersensitivity on minimal medium**

To further evaluate the Na⁺ sensitivity of **athkt1 sos3** double mutant plants in response to salt stress, we analyzed the Na⁺ sensitivity of mature plants grown on agar medium. The Na⁺ hypersensitive chlorotic phenotype of **athkt1** shoots was identified using minimal agar medium containing high concentrations of NaCl (Mäser et al. 2002a). Minimal medium was developed to more closely mimic physiological conditions compared to nutrient rich MS medium, while allowing plant growth (see Materials and Methods). Plants grown for 15-17 days on agar plates were transferred onto minimal agar medium supplemented with 75 mM NaCl and grown for 16 additional days. In minimal medium agar-grown plants, **sos3** showed necrotic lesions in the middle of the stress period and eventually showed chlorotic leaf death (Fig. 3C). Similarly, in soil-grown **sos3** plants, more severe salt stress (continuous 100 mM NaCl treatment for 2 weeks) caused **sos3** leaves to turn black, reflecting leaf necrosis (data not shown).

**athkt1-1 sos3** and **athkt1-2 sos3** plants grown in agar medium showed Na⁺ hypersensitivity under the imposed conditions as well and did not show a recovery from salt sensitivity approaching the wild type phenotype (Fig. 3 A, D and E; n=3 experiments, each for **athkt1-1 sos3** and **athkt1-2 sos3**). More surprisingly, double mutant plants showed **sos3**-like features under the imposed conditions, exhibiting necrotic leaf lesions in the middle of the 16-day stress-period followed by chlorosis. To quantitatively analyze this observed phenotype, leaf-chlorophyll content was determined by measuring absorbance at 646.8 nm and 663.8 nm (Porra et al. 1989) as shown in Figure 3F. The data demonstrate that **athkt1 sos3** double mutant plants show a similar time course of Na⁺ stress-induced chlorophyll content reduction to that of **sos3** plants, with **athkt1** plants showing an intermediate Na⁺ sensitivity to that of wild type plants and **athkt1 sos3** double mutants (Fig. 3F).

We next measured Na⁺ contents of shoots and roots of minimal medium grown plants to compare Na⁺ accumulation among the different genotypes. Plants were grown under hydroponic conditions on minimal medium for 2-3 weeks and then subjected to 75 mM NaCl stress for 2 days.
Na⁺ contents of shoots and roots were measured by ICP-OES. *athkt1-1* plants over-accumulated Na⁺ in shoots (Fig. 4A, n=8) and maintained lower Na⁺ levels in roots (Fig. 4B, n=8) compared to wild type plants. In the case of *sos3* plants, no significant difference was found in the Na⁺ content of shoots compared to wild type plants under these conditions (Fig. 4A; P>0.08). However, roots of *sos3* plants accumulated more Na⁺ compared to wild type (P<1.0x10⁻⁶) and dramatically more Na⁺ compared to *athkt1* mutants (P<1.0x10⁻¹³) (Fig. 4B). *athkt1-1 sos3* and *athkt1-2 sos3* double mutant plants, on the other hand, showed interesting Na⁺ accumulation profiles in which Na⁺ over-accumulation appeared in both shoots (P<1.3x10⁻⁵) and roots (P<1.7x10⁻⁴), indicating that double mutant plants potentially have detrimental features derived from both single mutant loci: shoot Na⁺ over-accumulation (*athkt1*-like) and root Na⁺ over-accumulation (*sos3*-like) under the imposed conditions (Fig. 4, A and B), which correlates with the strong Na⁺ sensitivity of *athkt1 sos3* double mutant plants (Fig. 3).

*sos3*-like or *athkt1*-like Na⁺ hypersensitivities in *athkt1 sos3* double mutants are primarily controlled by extracellular Ca²⁺

Results from soil-grown *athkt1-1 sos3* and *athkt1-2 sos3* plants showed that double mutant plants exhibit an *athkt1*-like Na⁺ sensitivity, shoot Na⁺ accumulation and elevated Na⁺ levels in the xylem sap (Figs. 1 and 2). Furthermore plants grown in minimal medium in agar showed that the Na⁺ sensitivity of double mutant plants exhibited a more *sos3*-like sensitivity to Na⁺ (Fig. 3); or in hydroponic medium the double mutants exhibited a Na⁺ sensitivity reflecting a combination of both *athkt1* and *sos3* impairments (Fig. 4). Previous research demonstrated suppression of the *sos3* phenotype in seedlings by *athkt1* in nutrient rich Murashige-Skoog (MS) agar medium (Rus et al. 2001). To analyze whether Na⁺ sensitivities depend on the nutrient status of agar-grown plants, we performed Na⁺ sensitivity analyses using MS medium.

Mature 15-17 day old MS medium agar-grown plants were transferred onto MS medium supplemented with 75 mM NaCl and grown for 18 days. A portion of leaves of *athkt1-1* plants showed chlorosis (Supplemental Figure 3B) and leaves of *sos3* plants showed necrotic yellowing (Supplemental Figure 3C). *athkt1-1 sos3* and *athkt1-2 sos3* double mutant plants showed
**athkt1**-like Na\(^+\) sensitivity exhibiting chlorosis (white bleaching) in a portion of the leaves and a partial visual repression of the **sos3**-1 Na\(^+\) sensitivity (Supplemental Figure 3D and E). We subsequently performed additional experiments to determine whether specific components or the overall enrichment of nutrients in MS medium is responsible for showing **sos3**-like or **athkt1**-like Na\(^+\) hypersensitivities of double mutants. We compared compositions and concentrations of all substances that differed between minimal medium and MS medium by testing each component individually using synthetic MS media in which the target substance was altered (n=2 experiments, 8 plants per condition). These agar growth analyses showed three ion species that affected the NaCl sensitivity of *Arabidopsis*: K\(^+\), NH\(_4\)\(^+\) and Ca\(^{2+}\). These analyses revealed that the higher Ca\(^{2+}\) concentration in normal MS medium (3 mM Ca\(^{2+}\)) compared to minimal medium (1 mM Ca\(^{2+}\)) affected the phenotypes of **athkt1** sos3 double mutant plants.

We prepared minimal agar medium containing 3 mM Ca\(^{2+}\) and 75 mM Na\(^+\) and performed further experiments. **athkt1** sos3 double mutant and **athkt1** plants showed chlorosis on minimal medium containing 3 mM Ca\(^{2+}\) and 75 mM Na\(^+\) (Fig. 5B, D and E). **athkt1** sos3 double mutant plants also showed a significant reduction in the chlorophyll content in response to 75 mM NaCl stress (Fig. 5F). The difference in chlorophyll reduction between **athkt1** and **sos3** plants in 3 mM Ca\(^{2+}\) is much smaller compared to that in 1 mM Ca\(^{2+}\) because **athkt1** plants become more sensitive and **sos3** plants become more insensitive to Na\(^+\) in terms of the chlorophyll reduction rate at 3 mM Ca\(^{2+}\) (compare Figs. 3F and 5F; see also Supplemental Figure 4).

We next performed ICP analyses using plants grown hydroponically on minimal medium containing 3 mM Ca\(^{2+}\) and 75 mM Na\(^+\). **athkt1** sos3 plants accumulated more Na\(^+\) than WT in shoots similar to **athkt1** plants (Fig. 6A, P>0.20 for **athkt1**-1 vs. **athkt1**-1 sos3, P>0.14 for **athkt1**-1 vs. **athkt1**-2 sos3; n=8). Interestingly, **athkt1** sos3 double mutant plants accumulated less Na\(^+\) in roots than wild type plants in the presence of 3 mM Ca\(^{2+}\) (Fig. 6B; P<1 x 10\(^{-4}\) for wild type vs. **athkt1**-1 sos3; P<3 x 10\(^{-4}\) for wild type vs. **athkt1**-2 sos3; n=8). The reduced Na\(^+\) accumulation in roots of **athkt1** sos3 plants with 3 mM Ca\(^{2+}\) in minimal medium (Fig. 6B) lies in contrast to experiments with 1 mM Ca\(^{2+}\) in minimal medium (Fig. 4B), and may explain the suppression of **sos3** by **athkt1** in seedling growth assays with 3 mM Ca\(^{2+}\) in MS medium (Rus et al., 2001).
To test the above hypothesis and compare seedling phenotypes with those of Rus et al. (2001), we performed additional Na\(^+\) sensitivity analyses using immature agar-grown plants. Ten-day old seedlings germinated on MS plates were directly transferred onto minimal medium containing 75 mM NaCl and 1 mM or 3 mM Ca(NO\(_3\))\(_2\) and grown for 7 additional days (Fig. 7). In the presence of 1 mM Ca\(^{2+}\), \textit{athkt1-1 sos3} and \textit{athkt1-2 sos3} double mutant plants again showed a \textit{sos3}-like phenotype (Fig. 7C; and left panels in Fig. 7D and E). In the presence of 3 mM Ca\(^{2+}\), \textit{sos3}-like necrosis was suppressed in \textit{athkt1 sos3} double mutant plants as reported by Rus et al. (2001) (right panels in Fig. 7D and E). Furthermore, \textit{athkt1 sos3} plants showed curled leaves, as also did \textit{athkt1-1} under the imposed conditions (Fig. 7B; and right panels in Fig. 7D and E), but wild type plants did not show curled leaves (Fig. 7A). \textit{sos3} seedlings showed a severe root growth defect (Rus et al. 2004), however, \textit{athkt1} plants did not (Mäser et al. 2002a) under these conditions (Fig. 7B and C). \textit{athkt1 sos3} double plants showed a severe root growth defect similar to \textit{sos3} plants in 75 mM Na\(^+\) and 1 mM Ca\(^{2+}\) condition, but showed less inhibition of root growth in 75 mM Na\(^+\) and 3 mM Ca\(^{2+}\) condition (Fig. 7D and E, compare left and right panels).

We measured the Ca\(^{2+}\) content of the soil solution extracted from soil used for Na\(^+\) sensitivity analyses because soil grown \textit{athkt1 sos3} plants showed more \textit{athkt1-1}-like Na\(^+\) hypersensitivity (Figs. 1 and 2; and Supplemental Figs. 1 and 2). Soil solution extracted from 3 independent soil samples contained 6.89±1.40 mM Ca\(^{2+}\). Together these results demonstrate that extracellular Ca\(^{2+}\) controls whether \textit{athkt1 sos3} double mutant plants display a more \textit{athkt1}-like or a more \textit{sos3}-like phenotype and that Ca\(^{2+}\) strongly impacts Na\(^+\) homeostasis in \textit{athkt1 sos3} double mutants.

**Discussion**

In the present study we demonstrate that \textit{athkt1 sos3} double mutant plants show differential Na\(^+\) hypersensitivities and that the concentration of extracellular Ca\(^{2+}\) controls whether \textit{athkt1 sos3} double mutant plants display a more \textit{athkt1}-like or a more \textit{sos3}-like phenotype.
Furthermore, the present findings also demonstrate distinct physiological roles of AtHKT1 and SOS3 in protecting plants from salinity stress. *athkt1 sos3* double and *athkt1* single mutants but not *sos3-1*, cause significantly increased Na\(^+\) levels in the xylem sap (Fig. 2B) (Sunarpi et al. 2005), resulting in reduction in root Na\(^+\) content and Na\(^+\) over-accumulation in leaves and eventual chlorosis. In contrast, the *sos3* mutation more strongly caused Na\(^+\) over-accumulation in roots (Fig. 4 and 6).

*ahtkt1 sos3* double mutants show increased xylem sap Na\(^+\) content under salt stress

*ahtkt1* mutant alleles have been found to show Na\(^+\) hypersensitivity and Na\(^+\) over-accumulation in shoots under salt stress (Mäser et al. 2002a, Berthomieu et al. 2003, Gong et al. 2004, Rus et al. 2004). Decreased Na\(^+\) levels in the phloem sap were found in the reduced function point mutation *athkt1* allele, *sas2-1*, compared to wild type, but no effect was found in the Na\(^+\) content of the xylem sap of the mutant (Berthomieu et al. 2003). Moreover, phloem tissue expression of *AtHKT1* was found in promoter GUS analyses and *in situ* hybridization (Berthomieu et al. 2003). A Na\(^+\) recirculation model for AtHKT1 has been proposed based on these observations, in which AtHKT1 functions in loading Na\(^+\) into the phloem cells in shoots and unloading Na\(^+\) from the phloem in roots (Berthomieu et al. 2003).

Xylem sap collected from *athkt1 sos3* double and *athkt1* single mutant alleles contained dramatically higher Na\(^+\) compared to *sos3-1* and wild type controls (Fig. 2B). These findings support an expanded model for AtHKT1 functions in reducing Na\(^+\) in the xylem sap under salt stress, thus preventing plants from over-accumulating Na\(^+\) in shoots (Sunarpi et al. 2005). The rice gene *OsHKT8* is a close orthologue of *AtHKT1* and has recently been demonstrated to also control Na\(^+\) levels in the xylem sap (Ren et al. 2005). Direct immuno-electron microscopy of AtHKT1 demonstrated localization of the AtHKT1 protein in the plasma membrane of xylem parenchyma cells (Sunarpi et al. 2005). Note that these data do not exclude the complementary function of AtHKT1 in enhancing Na\(^+\) levels in the phloem (Berthomieu et al. 2003, Sunarpi et al. 2005). AtHKT1 may cooperate in both xylem and phloem Na\(^+\) content control thus mediating Na\(^+\) recirculation to roots. Recirculation of Na\(^+\) from the xylem sap via the phloem to roots protects
leaves from salinity stress and may allow plants to subsequently excrete Na\textsuperscript{+} from roots. Na\textsuperscript{+} recirculation may also enhance the overall vacuolar Na\textsuperscript{+} sequestration in different types of root cells via tonoplast H\textsuperscript{+}/Na\textsuperscript{+} exchangers (Blumwald and Poole 1985, Apse et al. 1999, Gaxiola et al. 1999). Important questions, including how AtHKT1 functions in both the xylem and the phloem and whether the activity and expression of AtHKT1 is differentially regulated in these tissues will be an important subject of investigation.

Salinity stress also caused an increase in the xylem sap of sos1-1 plants (Shi et al. 2002), but had a more limited effect in sos3-1 plants compared to athkt1 mutants, with sos3-1 xylem levels of Na\textsuperscript{+} being closer to those in wild type plants (Fig. 2B; P>0.06 for sos3-1 compared to WT). The dramatically increased Na\textsuperscript{+} accumulation in roots of sos3 plants (Fig. 4B and 6B) thus appears to result in a mild increase in the Na\textsuperscript{+} content in the xylem sap.

\textit{athkt1 sos3} plants show an \textit{athkt1}-like phenotype in 3 mM external Ca\textsuperscript{2+}, which can account for suppression of Na\textsuperscript{+} hypersensitivity of sos3-1 seedlings

\textit{athkt1-1} and \textit{athkt1-2} alleles function as genetic suppressor alleles of sos3-1 seedlings and AtHKT1 was proposed to function as a Na\textsuperscript{+} uptake pathway into roots (Rus et al. 2001). However, \textit{athkt1} mutant alleles were subsequently shown to cause Na\textsuperscript{+} hypersensitivity in leaves (Mäser et al. 2002a, Berthomieu et al. 2003, Gong et al. 2004). No expression of \textit{AtHKT1} in the root epidermis or root cortex has been observed thus far (Mäser et al. 2002a, Berthomieu et al. 2003). Moreover, detailed tracer influx experiments using \textsuperscript{22}Na\textsuperscript{+} showed no measurable disruption of Na\textsuperscript{+} influx into \textit{Arabidopsis} roots due to \textit{athkt1} mutations, despite analyzing several conditions (Berthomieu et al. 2003, Essah et al. 2003). Thus the mechanism by which \textit{athkt1} mutations suppress the Na\textsuperscript{+} sensitivity of sos3-1 has remained unclear. The present study shows that \textit{athkt1 sos3} plants show an \textit{athkt1}-like phenotype with 3 mM Ca\textsuperscript{2+} in the nutrient medium in 2 to 4 weeks old plants (Figs. 5, 6 and 7). Under these conditions \textit{athkt1} alleles completely suppressed the characteristic Na\textsuperscript{+} over-accumulation in roots of the sos3 mutant (Fig. 6B). Our analyses suggest that this “protective” effect of \textit{athkt1} on root Na\textsuperscript{+} levels may explain why \textit{athkt1} mutations suppress the Na\textsuperscript{+} hypersensitivity of sos3-1 seedlings (Rus et al. 2001).
A more recent study showed however that *athkt1* mutations also reverted *sos3-1*-dependent Na\(^+\)-induced damage of leaves of mature soil-grown plants (Rus et al. 2004). It was shown that mature soil-grown *athkt1 sos3* plants are more salt tolerant than *athkt1* single mutant plants, exhibiting similar growth and leaf health as wild type control plants under salinity stress conditions (Rus et al. 2004). However, our results obtained from mature soil-grown plants were inconsistent with these findings (Figs. 1 and 2; Supplemental Figs. 1 and 2). In the present study, mature soil-grown *athkt1 sos3* plants showed Na\(^+\) hypersensitivity similar to *athkt1* mutants (Figs. 1 and 2; Supplemental Figs. 1 and 2). The reason for this discrepancy is not clear and may depend on growth conditions. Nevertheless, the physiological effects of *athkt1* in *athkt1 sos3* double mutant plants on Na\(^+\) accumulation in leaves (Fig. 2A and Supplemental Fig. 2), xylem sap (Fig. 2B), and shoots (Figs. 4 and 6), and on Na\(^+\)-induced chlorophyll reduction (Figs. 3F and 5F), make it unlikely that *athkt1* mutations can protect mature *sos3-1* mutant plants from salinity stress and the repression of *sos3-1* Na\(^+\) hypersensitivity in mature plants by *athkt1* loss-of-function mutations appears unlikely under most physiological conditions.

**Ca\(^{2+}\)**-dependence of SOS3 Ca\(^{2+}\) sensor and Na\(^+\) hypersensitivities of *athkt1 sos3* double mutants

Na\(^+\) influx channels and currents into root cells have been characterized in electrophysiological studies (Amtmann et al. 1997, Roberts and Tester 1997, Tyerman et al. 1997, Buschmann et al. 2000, Davenport and Tester 2000, Maathuis and Sanders 2001). However, transporter mutants that cause major reduction in root Na\(^+\) influx have not yet been found and the underlying genes remain to be identified. Several electrophysiological studies have shown partial block of Na\(^+\) influx by Ca\(^{2+}\) (Tyerman et al. 1997, Buschmann et al. 2000, Davenport and Tester 2000), suggesting that multiple transporters contribute to Na\(^+\) influx in roots. These findings correlate with classical studies showing that calcium enhances the relative absorption of K\(^+\) at high Na\(^+\) concentrations (Epstein 1961, LaHaye and Epstein 1969, Epstein 1998). SOS3 encodes a Ca\(^{2+}\) sensor that regulates the activity of the plasma membrane SOS1 Na\(^+\)/H\(^+\) exchanger, thus mediating cellular Na\(^+\) extrusion (Liu and Zhu 1998, Qiu et al. 2002, Shi et al. 2002). Thus calcium appears to impact several mechanisms in mediating salt tolerance.
Findings presented here demonstrate that \textit{AtHKT1} mutations do not strictly repress the Na\textsuperscript{+} hypersensitivity of \textit{sos3} plants to wild type levels, but that \textit{athkt1 sos3} plants exhibit more \textit{athkt1}-like or more \textit{sos3}-like Na\textsuperscript{+} hypersensitivities, depending on the external Ca\textsuperscript{2+} concentration. \textit{sos3}-like Na\textsuperscript{+} hypersensitivity became predominant in \textit{athkt1 sos3} mutants under salt stress in the presence of 1 mM Ca\textsuperscript{2+} (Figs. 3 and 4). On the other hand, AtHKT1 was the strongest salt tolerance determinant in \textit{athkt1 sos3} mutants in the presence of 3 mM Ca\textsuperscript{2+} as \textit{athkt1}-like Na\textsuperscript{+} hypersensitivity becomes more prevalent (Figs. 5 and 6).

The partial Ca\textsuperscript{2+} block of Na\textsuperscript{+} influx channels contributes to reduction in salinity stress (Tyerman et al. 1997, Buschmann et al. 2000, Davenport and Tester 2000). Furthermore, the Ca\textsuperscript{2+}-dependent differential Na\textsuperscript{+} hypersensitivities of \textit{athkt1 sos3} double mutants can likely be explained by a model in which an increase in cytoplasmic Ca\textsuperscript{2+} can partially suppress the \textit{sos3-1} phenotype. This model is supported by results shown in Supplemental Figures S-3C, S-3F and S-4E. Note that young \textit{sos3} seedlings did not show this effect possibly because the effect is not clearly detected in young seedlings (Fig. 7C right panel). The \textit{SOS3} gene encodes a Ca\textsuperscript{2+} binding protein that is essential for plant salt tolerance (Liu and Zhu 1998). Recently, the three-dimensional structure of the SOS3 protein was reported (Sánchez-Barrena et al. 2005). The SOS3 protein includes four EF-hand Ca\textsuperscript{2+}-binding motifs and forms a dimer when Ca\textsuperscript{2+} concentrations are increased (Sánchez-Barrena et al. 2005). The \textit{sos3-1} mutation causes a three amino acid deletion in the third EF-hand (Liu and Zhu 1998, Sánchez-Barrena et al. 2005). This third EF-hand is hypothesized to stabilize the dimer structure of the SOS3 protein upon Ca\textsuperscript{2+} binding (Sánchez-Barrena et al. 2005). The SOS3-1 protein shows a reduced affinity for Ca\textsuperscript{2+} (Ishitani et al. 2000). Thus increasing extracellular Ca\textsuperscript{2+} may partially activate the SOS3-1 protein, thus reducing the \textit{sos3-1} phenotype resulting in the observed \textit{athkt1}–like phenotypes of \textit{athkt1 sos3} plants at 3 mM external Ca\textsuperscript{2+} (Figs. 5, 6 and 7) (Rus et al. 2001). Furthermore, at high Ca\textsuperscript{2+} SOS3-independent mechanisms may be activated, as the \textit{Arabidopsis} genome encodes 9 homologues of the \textit{SOS3} gene (Zhu 2002, Kolukisaoglu et al. 2004).

In summary, the present findings show that increasing the external Ca\textsuperscript{2+} concentration strongly impacts the response of \textit{athkt1 sos3} double mutant plants to Na\textsuperscript{+} stress. Ca\textsuperscript{2+}-dependent
sos3-like and athkt1-like Na⁺ hypersensitivities of athkt1 sos3 plants suggest that both the SOS pathway and the AtHKT1 pathway are mechanistically independent strong salt tolerance determinants in Arabidopsis. Under physiological conditions both pathways mediate distinguishable Na⁺ tolerance mechanisms and loss-of-function mutations in AtHKT1 are unlikely to cause physiological Na⁺ resistance of mature Arabidopsis plants. Furthermore, comparative xylem sap analyses in sos3 and athkt1 single and double mutants show that athkt1 sos3 double mutants show enhanced Na⁺ levels in xylem sap, whereas sos3 alone did not greatly affect xylem sap Na⁺ levels. Studies into the question whether specific signaling pathways control the activity of AtHKT1 and whether cross talk with the SOS pathway exists will be interesting subjects of future inquiry.

Materials and Methods

Cultivation of Arabidopsis

Seeds were surface sterilized and sown on MS agar medium supplemented with 1% sucrose. All mutant alleles were confirmed to have the reported homozygous mutations by sequencing genomic DNA at the SOS3 and AtHKT1 loci. Ten to twelve day-old seedlings were transferred onto soil (Professional Blend; McConkey Co., Sumner, WA) for experiments using soil-grown plants, or onto a 10 μm pore-nylon mesh (Spectrum laboratories, Inc., Rancho Dominguez, CA) placed on fresh MS medium or minimal medium (Mäser et al. 2002a) for the agar-growth analysis, or directly onto minimal medium for the short-term agar-growth analysis, or onto a plug (Jaece Industries, Inc., North Tonawanda, NY) soaked in minimal medium for ion content measurements for plants grown on hydroponic medium. Minimal medium contained 0.5 mM KH₂PO₄, 1.25 mM KNO₃, 0.5 mM MgSO₄, 20 μM FeNa-EDTA, and the additional micronutrients 7 μM H₃BO₃, 1.4 μM MnSO₄, 1 μM ZnSO₄, 4.5 μM KI, 0.1 μM CuSO₄, 0.2 μM Na₂MoO₄, 10 nM CoCl₂ supplemented with the indicated amounts of NaCl and Ca(NO₃)₂. Plants were subsequently grown for 2-3 weeks on soil, or 5 days on MS medium or minimal medium in the case of agar growth analyses, or for 2-3 weeks with a solution change every 3 days in the case of hydroponic cultures, before salt stress was imposed. Surgical tape (3M, Santa Fe Springs, CA) was used to tape plates instead of a paraffin film in all agar growth analyses.
Imposition of salt stress

Pots of all soil-grown plants were placed on plastic trays and irrigated by adding irrigation solutions (de-ionized water or 1/20 MS basal salt solution). Mature soil-grown plants at the bolting stage were irrigated two times with 100 mM NaCl added to one-twentieth MS basal salts-solution (Shi et al. 2002) over a period of 6 days for Na\(^+\) content determination, for 2 days for xylem sap collection experiments, or for 7 days for NaCl sensitivity analyses. Plants were subsequently irrigated with de-ionized water for another 7 days after the stress treatment in the case of NaCl sensitivity analyses and the NaCl sensitivities were subsequently analyzed. Plants were watered with one-twentieth MS basal salts solution in parallel as a negative control in all experiments using soil-grown plants.

For the NaCl sensitivity analyses of agar-grown plants, meshes on which mature plants were laying were transferred onto the indicated media containing 75 mM NaCl. Plants were subjected to NaCl stress for 16-18 days. Hydroponically grown plants were subjected to minimal medium containing 75 mM NaCl (pH was adjusted to 5.5 with N-methyl-D-Glucamine) for 2 days.

Xylem sap collection and sucrose content determination

Xylem sap samples were collected as described previously (Gaymard et al. 1998, Shi et al. 2002). All rosette leaves were removed and the inflorescence stem was cut with a very sharp razor blade from soil-grown 4 to 5 week-old plants. The tray and plants were then covered with a transparent plastic dome to maintain high humidity. Xylem sap drops that accumulated at the cut surface of the inflorescence stem were then collected using a micropipette.

For sucrose content determinations xylem sap samples were first purified before HPLC analysis by passing through a Dionex H cartridge (Dionex corp., CA). The run-through and 3 x 0.2 ml water washes were collected in the same container and dried in a Speedvac and then re-dissolved in an appropriate amount of de-ionized water. Separation and detection of the sugars were achieved by injecting an aliquot of the sample into a Dionex DX 500 HPLC system (Dionex corp., CA) using the CarboPac MA1 column (Dionex corp., CA) in an isocratic mobile phase (480 mM NaOH at 0.4ml/min. for 50 min.) and electrochemical detector at the UCSD Glycobiology Center (UCSD School of Medicine).

Chlorophyll content determination

Chlorophyll content was determined by the method of Porra et al. (1989). Leaves of plants were harvested at the indicated time points and 4 leaf-disk were punched out of leaves. Punched disks were soaked in 3 ml of Dimethyl formamide (Fisher, Fair lawn, NJ) and kept at 4 °C for 12-16 hrs. Absorbances at 646.8 nm and 663.8 nm were then measured by photo absorption spectrometry (Spectronic GENESYS5; Milton Roy USA, Ivyland, PA). Contents of chlorophyll a,
b and a+b were calculated with formulas described by Porra et al. (1989).

**Ion content determination**

Ion content of the described samples was measured by inductively coupled plasma-optic emission spectroscopy (ICP-OES) at the Scripps Institution of Oceanography UCSD analytical facility. Shoot and root samples were isolated as described previously (Mäser et al. 2002a). Xylem sap samples were diluted in 5% HNO₃ and directly measured.

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**References**


Legends to figures

Figure 1. Increased salt sensitivity of athkt1-1 sos3 and athkt1-2 sos3 plants grown on soil. Plants were irrigated twice with one-twentieth MS basal salt-solution with 100 mM NaCl (right panels in A to E, and panels F to J) or without NaCl (left panels in A to E). Visual phenotypes are shown of leaves and shoots of (A, F) wild type/Columbia; (B, G) athkt1-1; (C, H) sos3; (D, I) athkt1-1 sos3; and (E, J) athkt1-2 sos3. Plants were watered twice during a 7-day period, and then all plants were irrigated with de-ionized water for the following 7 days prior to taking photographs.

Figure 2. athkt1-1 sos3 and athkt1-2 sos3 double mutant plants over-accumulate Na\(^+\) in the xylem sap similar to athkt1-1, but not sos3. Na\(^+\) contents of leaves and xylem sap, derived from soil-grown plants. (A) Na\(^+\) content measurements in leaves of plants watered with one-twentieth MS basal salt solution with 100 mM NaCl (black bars) or without NaCl (white bars) for 6 days. (B) Na\(^+\) content measurements in the xylem sap of soil-grown plants watered for 2 days with the same solutions used in (A). Na\(^+\) content was measured by ICP-OES. Error bars represent standard deviations (n=5 to 8).

Figure 3. Agar-grown athkt1-1 sos3 and athkt1-2 sos3 double mutant plants show sos3-like Na\(^+\) hypersensitivity under salt stress in the presence of 1 mM Ca\(^{2+}\). 15 to 17 day-old mature plants were transferred onto minimal medium containing 75 mM NaCl and 1 mM Ca\(^{2+}\). (A-E) Plants were grown for 16 additional days and photographs were taken at the end of this period. (F) Contents of chlorophyll a + b extracted from agar-grown plants, which were subjected to 75 mM NaCl stress for 0, 3, 6, 9, 12 or 15 days on minimal medium. Error bars represent standard deviations (n=3 to 4).

Figure 4. athkt1-1 sos3 and athkt1-2 sos3 double mutant plants over-accumulate Na\(^+\) in both roots and shoots in the presence of 1 mM Ca\(^{2+}\) showing cumulative detrimental phenotypes of the two single athkt1 and sos3 mutations. (A) Na\(^+\) contents in shoots. (B) Na\(^+\) contents in roots. Plants were hydroponically grown using minimal medium for 2-3 weeks. Mature, bolting plants were then
subjected to 75 mM NaCl stress for 2 days. The Na\(^+\) content was measured by ICP-OES. Error bars represent standard deviations (n=7 to 8).

Figure 5. Agar-grown *athkt1-1 sos3* and *athkt1-2 sos3* double mutant plants show Na\(^+\) hypersensitivity under salt stress in the presence of 3 mM Ca\(^{2+}\). 15 to 17 day-old mature plants were transferred onto minimal medium containing 75 mM NaCl and 3 mM Ca\(^{2+}\). (A-E) Plants were grown for 16 additional days and photographs were then taken. (F) Contents of chlorophyll a + b extracted from agar-grown plants, which were subjected to 75 mM NaCl stress for 0, 3, 6, 9, 12 or 15 days on minimal medium containing 3 mM Ca\(^{2+}\). Error bars represent standard deviations (n=3 to 4).

Figure 6. *athkt1-1 sos3* and *athkt1-2 sos3* double mutant plants show ICP profiles similar to single *athkt1* mutants in 3 mM Ca\(^{2+}\) and 75 mM NaCl. (A) Na\(^+\) contents in shoots. (B) Na\(^+\) contents in roots. Plants were hydroponically grown using minimal medium containing 1 mM Ca\(^{2+}\) for 2-3 weeks. Mature, bolting plants were then subjected to 75 mM NaCl stress for 2 days in the presence of 3 mM Ca\(^{2+}\). Error bars represent standard deviations (n=7 to 8).

Figure 7. Young *athkt1-1 sos3* double mutant seedlings exhibit sos3-like or athkt1-like phenotypes on minimal agar medium, depending on the external Ca\(^{2+}\) concentration. The salt sensitivities of 17-day old seedlings are shown of (A) Wild type, (B) *athkt1-1*, (C) sos3, (D) *athkt1-1 sos3* and (E) *athkt1-2 sos3*. Seedlings were grown on MS agar medium for 10 days and then transferred to minimal agar medium for 7 days containing 75 mM NaCl and 1 mM Ca\(^{2+}\) (left in each panel) or 3 mM Ca\(^{2+}\) (right in each panel). Photographs of 2 representative plants from each condition are shown. Twenty seedlings per line were analyzed in 3 independent experiments and all plants showed the depicted phenotypes.
Figure 1
Figure 2
**Figure 3**

- **A** WT/Col
- **B** *athkt1-1*
- **C** *sos3*
- **D** *athkt1-1 sos3*
- **E** *athkt1-2 sos3*
- **F**

Chlorophyll (a+b) [µM/cm²]

- **WTcol**
- **athkt1-1**
- **athkt1-1 sos3**
- **athkt1-2 sos3**
- **sos3**

Days

0 3 6 9 12 15 18

Chlorophyll (a+b) [µM/cm²]

0 1 2 3 4 5 6 7 8 9
Figure 4
Figure 5

Chlorophyll (a+b) [µM/cm²]

Days

WT/Col  athkt1-1  sos3  athkt1-1 sos3  athkt1-2 sos3

A  B  C  D  E
Figure 6
Figure 7