Loss of Halophytism by Interference with SOS1 Expression

Dong-Ha Oh, Eduardo Leidi, Quan Zhang, Sung-Min Hwang, Youzhi Li, Francisco J. Quintero, Xingyu Jiang, Matilde Paino D’Urzo, Sang Yeol Lee, Yanxiu Zhao, Jeong Dong Bahk, Ray A. Bressan, Dae-Jin Yun, José M. Pardo, Hans J. Bohnert

Division of Applied Life Science (BK21 program) and Environmental Biotechnology National Core Research Center, Graduate School of Gyeongsang National University, Jinju 660-701, Korea (D.-H.O., S.-H., S.Y.L., J.D.B., D.-J.Y.); Departments of Plant Biology and of Crop Sciences, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA (D.-H.O., Q.Z., Y.L., H.J.B.); Instituto de Recursos Naturales y Agrobiología, Consejo Superior de Investigaciones Científicas, Sevilla 41012, Spain (E.L., F.J.Q., X.J., J.M.P.), Key Laboratory of Plant Stress Research, College of Life Science, Shandong Normal University, Jinan 250014, PR China (Q.Z., Y.Z.); Key Laboratory of Ministry of Education for Microbial and Plant Genetic Engineering, Guangxi University, Nanning, Guangxi 530005, PR China (Y.L.); Department of Horticulture and Landscape Architecture, Purdue University, West-Lafayette, IN 47907, USA (M.P.D., R.A.B.)

1The work has been supported by the Environmental Biotechnology National Core Research Center Project (grant #, R15-2003-012-01002-00), the Biogreen 21 project of the Rural Development Administration (#20070301034030), NSF (DBI-0223905), UIUC and Purdue institutional funds, and by support from the Spanish Ministerio de Ciencia e Innovacion (Grant BFU2006-06968). S.-M. H. was supported by scholarships from the Brain Korea 21 program, Korea.

*Corresponding author ; e-mail hbohnert@illinois.edu; fax +1 217 333 5574
[Abstract]

The contribution of SOS1, encoding a sodium/proton antiporter, to plant salinity tolerance was analyzed in wild type and RNAi (RNA-interference) lines of the halophytic Arabidopsis-relative Thellungiella salsuginea. Under all conditions, SOS1 mRNA abundance was higher in Thellungiella than in Arabidopsis. Ectopic expression of the Thellungiella homolog ThSOS1 suppressed the salt-sensitive phenotype of a Saccharomyces cerevisiae strain lacking Na⁺ efflux transporters and increased salt tolerance of wild-type Arabidopsis. Thsos1-RNAi lines of Thellungiella were highly salt-sensitive. A representative line, thsos1-4, showed faster Na⁺ accumulation, more severe water loss in shoots under salt stress, and slower removal of Na⁺ ion from the root after removal of stress, compared to wild type. Thsos1-4 showed drastically higher sodium-specific fluorescence visualized by CoroNa-Green, a sodium-specific fluorophore, than wild type and inhibition of endocytosis in root tip cells, and cell death in the adjacent elongation zone. After prolonged stress, sodium ions accumulated inside the pericycle in thsos1-4, while sodium was confined in vacuoles of epidermis and cortex cells in wild type. RNAi-based interference of SOS1 caused cell death in the root elongation zone, accompanied by fragmentation of vacuoles, inhibition of endocytosis and apoplastic sodium influx into the stele and hence the shoot. Reduction in SOS1 expression changed Thellungiella salsuginea that normally can grow in sea water-strength NaCl solutions into a plant as sensitive to sodium ions as Arabidopsis thaliana.
[Introduction]

Accompanying production and accumulation of osmolytes and other protective molecules, an important aspect of plant responses leading to salt stress tolerance is the regulation of uptake, re-export and control over the distribution of sodium ions (Hasegawa et al., 2000; Tester and Davenport, 2003). Sodium (Na\(^+\)) ions appear to enter the root by several pathways (Essah et al., 2003; Pardo et al., 2006), although the nature of participating genes and their interaction in pathways requires further investigation. Once Na\(^+\) has entered the root endodermis, a tissue that represents a barrier to ions (Peng et al., 2004), it is generally assumed that the ion enters the xylem following the movement of water to aerial parts of the plant. Despite substantial efflux of Na\(^+\) across the plasma membrane of root cells, the net flux of Na\(^+\) is unidirectional from soil to roots and then to the shoot, except for possible re-circulation via the phloem (Tester and Davenport, 2003). In a range of species, the severity of damaging symptoms is positively correlated with the content of Na\(^+\) reaching photosynthetic tissues (Davenport et al., 2005; Ren et al., 2005; Munns et al., 2006). However, halophytic species can accumulate very high amounts of Na\(^+\) in vacuoles, such that Na\(^+\) may account for most of the total cellular osmotic potential (Tester and Davenport, 2003), and the presence of Na\(^+\) accelerates growth in eu-halophytes to some degree (Adams et al., 1998). Emerging as the major advantage of halophytes appears their exceptional control over Na\(^+\) influx combined with export mechanisms, the ability to coordinate its distribution to various tissues, and efficient sequestration of Na\(^+\) into vacuoles. These characters are of particular advantage when plants are subjected to a sudden increase of Na\(^+\) salts in their environment (Hasegawa et al., 2000), whereas
gradual increases in $\text{Na}^+$ may be tolerated even by plants that are not halophytic in nature.

$\text{Na}^+$-ATPases, major $\text{Na}^+$ export systems in organisms such as fungi and the moss, *Physcomitrella patens*, have not been found in higher plants (Lunde et al., 2007). In *Arabidopsis*, transporters of monovalent (alkali) cations, such as HKT1 (Berthomieu et al., 2003; Rus et al., 2004), members of the NHX-family (Yamaguchi et al., 2005; Pardo et al., 2006), and SOS1 (Shi et al., 2000; Shi et al., 2002; Shi et al., 2003) have been shown to play roles in the movement and distribution of $\text{Na}^+$ ions. Studies have shown the involvement of non-selective ion channels with roles in the transport of $\text{Na}^+$ ions but the genes encoding such function(s) have not been identified (Demidchik and Maathuis, 2007). *SOS1*, whose deletion resulted in a strong salt sensitivity phenotype in *Arabidopsis*, encodes a plasma membrane $\text{Na}^+/\text{H}^+$-antiporter involved in removing $\text{Na}^+$ ions from cells (Shi et al., 2000). This efflux strategy, which may be sufficient for the survival of unicellular organisms, must be accompanied by other means of $\text{Na}^+$ confinement to avoid carryover of $\text{Na}^+$ between cells in futile cycles. Hence, the physiological role of a plasma membrane $\text{Na}^+/\text{H}^+$-antiporter must be embedded in the context of tissue, organ, and whole plant distribution of ions and their transporters. A recent discovery on cell-layer specific differential responses to the salt stress of root cells supported this notion (Dinneny et al., 2008).

In *Arabidopsis*, the *SOS1* gene is most strongly expressed in the epidermis of the root tip region and in cells adjacent to vascular tissues (Shi et al., 2002). Based on the salt concentration in shoot, root and xylem sap of *Arabidopsis* wild type and its *sos1* knockout mutants, the SOS1 antiporter is assumed to function in $\text{Na}^+$ export under severe salt stress condition (Shi et al., 2002). However, detailed knowledge about how
a Na⁺ excluder achieves salt tolerance in a multicellular eukaryote is still missing. 

Significantly also, even though SOS1 has been an intensely studied component of the ion homeostasis mechanism, its involvement in the exceptional salt tolerance of halophytes is not known.

*Thellungiella salsuginea* (salt cress), which had before been called *T. halophila* by us, is a close relative of *Arabidopsis*, which has become a model to study the genetic basis of this plant’s extreme tolerance to a variety of abiotic stress factors, including salinity (Inan *et al*., 2004; Gong *et al*., 2005; Vera-Estrella *et al*., 2005; Volkov and Amtmann, 2006; Amtmann, 2009). Salt cress lacks specialized morphological structures, such as salt glands or large sodium storage cells found in other halophytes, making it a useful model for studying stress tolerance mechanisms that could be applicable to further understanding or embark on engineering of conventional crops (Inan *et al*., 2004). Recently, it has been reported that *Thellungiella* had lower net Na⁺ uptake compared with *Arabidopsis*. The unidirectional influx of Na⁺ ions to roots appeared to be more restricted and/or tightly controlled in *Thellungiella* than in *Arabidopsis*. To compensate for greater influx, *Arabidopsis* roots showed higher Na⁺ efflux (Wang *et al*., 2006).

Here, we wished to explore the role(s) by which ThSOS1, the SOS1 homolog in *Thellungiella salsuginea*, could be involved in shaping the halophytic character of the species using ectopic expression of the gene in yeast and in *Arabidopsis* and *Thellungiella* SOS1-RNAi lines. The results identified ThSOS1 as a genetic element whose activity limits Na⁺ accumulation and affects the distribution of Na⁺ ions at high concentration thus acting as a major tolerance determinant.
RESULTS

*Thellungiella* expresses *SOS1* at higher levels than *Arabidopsis*. *ThSOS1* (EF207775) is most closely related to *Arabidopsis* *SOS1* (*AtSOS1*; At2g01980) in the deduced amino acid sequences (83% identity) among *SOS1* coding regions from other plants (Figure S1). *ThSOS1* exists as a single copy gene in the *Thellungiella* genome (data not shown). Transcript abundance of *SOS1* was compared between *Arabidopsis* and *Thellungiella* by RT-PCR. Reference genes were selected among genes more than 90% identical in their sequence identities between both species, while they showed unaltered expression level under stress conditions (Czechowski et al., 2005). All primers were designed to be identical in both species. In *Thellungiella*, *SOS1* mRNA was significantly more abundant than in *Arabidopsis* in both shoot and root, more prominently expressed in roots in the absence of salt stress, while the levels of reference gene expression were indistinguishable between the two species (Figure 1a). *SOS1* mRNA was quantified by real time RT-PCR (Figure 1b). Compared to *Arabidopsis* wild type, *ThSOS1* mRNA abundance was 2.9-fold and 7.6-fold higher under normal condition in shoot and root, respectively, and up to 5.7-fold higher compared to salt-stressed *Arabidopsis* roots under stressed condition. This difference in *SOS1* mRNAs in both species persisted in older plants of similar mass and growth stages, grown on artificial soil as described (Gong et al., 2005) under either non-salinized or highly saline conditions (Figure S2).

*ThSOS1* expression suppressed the salt-sensitive phenotype of a yeast strain lacking Na⁺ transporters and increased the salt tolerance in *Arabidopsis*. The *S. cerevisiae* strain AXT3K (Δena1-4 Δnha1 Δnhx1), lacking major Na⁺ transporters essential for tolerance of yeast, showed growth inhibition at Na⁺ concentrations higher
than 50mM, while the wild type was not affected (Quintero et al., 2002). Expression of ThSOS1 partially suppressed the salt sensitivity of AXT3K (Figure 2a). In Arabidopsis, AtSOS1 is activated by the SOS2/SOS3 protein kinase complex (Qiu et al., 2002; Quintero et al., 2002). Co-expression of Arabidopsis SOS2 and SOS3 in AXT3K together with ThSOS1 dramatically increased the salt tolerance of the transformed cells, leading to growth in media with 400 mM sodium (Figure 2b). Expression of ThSOS1 conferred salt tolerance to the yeast mutant at concentrations higher than those sustained by the expression of AtSOS1, both alone and after activation by SOS2/SOS3 (Figures 2a, b). To test whether the unequal Na⁺ tolerance was related to differential activity of AtSOS1 and ThSOS1, the Na⁺/H⁺ exchange activity was measured in plasma membrane vesicles purified by two-phase partitioning from yeast transformants (Figure 2c). The strong inhibition (ca. 84%) of ATP hydrolysis by vanadate, an inhibitor of plasma membrane H⁺-ATPases, demonstrated that vesicle preparations were enriched in plasma membranes (data not shown). Cells were grown on selective AP medium containing 1 mM KCl and transferred to the same medium supplemented with 100 mM NaCl for 1 hour to ensure activation of the SOS2/SOS3 kinase complex when present. Maximal Na⁺/H⁺ exchange activity was observed in cells co-expressing SOS1 proteins and the Arabidopsis SOS2/SOS3 kinase complex (Figure 2d). No significant differences were found in the Na⁺/H⁺ exchange activity of plasma membrane vesicles containing ThSOS1 or AtSOS1, with and without co-expression of the SOS2/SOS3 kinase complex (Figure 2d, and data not shown).

Transgenic Arabidopsis expressing ThSOS1 under control of the CaMV-35S promoter were analyzed together with AtSOS1-overexpressing lines (Shi et al., 2003) for SOS1 expression and salt tolerance (Figure 3). The transgenic lines showed
various levels of SOS1 expression, highest in a ThSOS1-overexpressing line (Figure 3a, line 30). The survival rate of Col0 and SOS1 over-expressing lines was quantified by rescuing the seedlings onto non-saline media after a brief stress. Seedlings that resumed growth were counted as survivors (Figure 3b). The survival rates of SOS1-overexpressing lines were proportional to the level of SOS1 under stress ($R^2 = 0.95$), regardless of SOS1 origin, indicating that expression strength/mRNA stability or protein amount/activity are determining factors in tolerance acquisition. However, even the highest tolerance observed in the line 30 (Figure 3b) did not approach that shown by wild type Thellungiella (Inan et al., 2004).

**RNAi-based reduction of ThSOS1 expression resulted in decreased salt tolerance with faster Na+ accumulation in shoots.** We have developed transgenic Thellungiella transformed with a ThSOS1 RNAi vector (Figure 4a). Two lines, designated thsos1-4 and thsos1-6 were used for further analyses. Both showed 3:1 segregation in BASTA resistance and salt sensitivity, indicating a single insertion locus of the RNAi construct (not shown).

Seedlings on plates (Figure 4b) and mature plants grown in artificial soil (Figure S3a) showed a salt-sensitive phenotype in the RNAi lines. Shoots of RNAi line plants showed partial bleaching and root growth stopped at 200mM NaCl, while wild type and vector control (thsos1-11) continued to grow without symptoms in NaCl solutions up to 300mM (Figures 4b, c). The abundance of ThSOS1 mRNA was determined by quantitative real-time PCR in roots and shoots with or without salt stress. The decline of SOS1 mRNA by RNAi was more prominent under salt stress, where thsos1-4 showed 30% and 84% and thsos1-6 17% and 40% of wild type expression in the shoot and root, respectively (Figure 4d). A similar reduction in SOS1 transcript amount
persisted throughout development and at different strength of NaCl (Figures S3b, S5). The vector control (thsos1-11) showed no significant difference in ThSOS1 expression from wild type (data not shown).

To further characterize the role of ThSOS1 in the stress response, 2-week-old plants of wild type and thsos1-4 were transferred to 200mM NaCl, and fresh weight, water and ion content of shoots were measured, using inductively coupled plasma-optical emission spectrometry (ICP-OES). Wild type shoots continued to gain weight, but thsos1-4 shoots showed no growth after day 4 (Figure 5a). Water content was deduced from a comparison of the fresh and dry weights of seedling shoots. Salt-stressed wild type maintained water content slightly lower than the unstressed control throughout the experiments. However, a more significant decrease in water content in thsos1-4 shoots became apparent after 7 days (Figure 5b). In contrast to wild type, which showed a linear, gradual increase of Na⁺ in the leaves, sodium content in thsos1-4 leaves peaked by day four and later declined, most probably by leakage due to the loss of water (Figure 5c). Loss of potassium was observed in both wild type and thsos1-4, with a seemingly insignificant higher decrease in thsos1-4 during later times of the experiment (Figure 5d). These results already pointed to ThSOS1 as important and required for the ability to tolerate extremely high levels of NaCl.

The changes in ion content of stressed plants after removal of the stress were compared between wild type and thsos1-4 (Figure 6). Hydroponically-grown plants were treated by increasing NaCl stepwise to the non-lethal concentration of 150mM within 2 weeks, transferred to media without NaCl, and harvested to determine Na⁺ and K⁺ concentrations. Both shoots and roots of thsos1-4 showed higher Na⁺ (Figures 6a, c) and lower K⁺ (Figures 6b, d) contents compared to wild type. In wild type, shoot
ion contents did not change over a 72h period after removal of the plants from NaCl solutions, while *thsos1-4* showed gradual decreases in Na\(^{+}\) and increases in K\(^{+}\), eventually converging on the Na\(^{+}\) contents observed in wild type (Figures 6a, b). The roots of wild type exhibited a sharp drop in Na\(^{+}\) ion content within 1 h after removal of NaCl, while the efflux of Na\(^{+}\) ions was much slower in *thsos1-4* roots, which failed to reach the level of Na\(^{+}\) content in the wild type at 72 h (Figure 6c). To assess the contribution of ThSOS1 to the reduction of net sodium uptake in the short-term, wild type and *thsos1-4* plants were transferred to hydroponic media supplemented with 0.1, 1 and 10 mM and the Na\(^{+}\) content was determined after minutes (15 and 30 min) and hours (2 and 48h). Roots of line *thsos1-4* started to show significantly greater Na\(^{+}\) contents than wild type after 2 hours of salt imposition (Figure S4).

**Down-regulation of ThSOS1 resulted in increased sodium accumulation in the root tip and cell death in the elongation zone.** To determine the role of ThSOS1 in specific cell types in the roots, the distribution of sodium was imaged using the fluorescent, sodium-specific dye CoroNa-Green AM (Invitrogen, Carlsbad, CA), as outlined in the methods section, comparing wild type and *thsos1-4* seedling roots by confocal microscopy. Incubations in 150mM NaCl did not result in rapid differences between wild type and *thsos1-4*, but within 24h *thsos1-4* roots showed higher fluorescence in the meristematic region than wild type (Figures 7a, b). When the vacuolar fluorescence intensities of root cortex cells from 5 individual plants from each line were quantified, the wild type showed a mean relative value of fluorescence intensity 29.68 (S.D. = 9.43; arbitrary units), while the mean was 68.80 (S.D. = 16.89) for *thsos1-4* (Figure 7c).

Cellular events in the root tip region at this time point were observed by CoroNa
Green AM and FM4-64 (Invitrogen). In wild type, CoroNa Green specifically stained the pre-vacuolar compartment of irregular shape in the meristematic and expanding cells (Figure 7d). Cells of *thsos1-4* showed significantly stronger CoroNa-Green fluorescence in small round intracellular vacuoles, which converged into one or two large bodies within 24h in medium with 150mM NaCl (Figure 7e). Interestingly, the endocytotic inclusion of the FM4-64 dye, which was apparent in wild type (Figure 7d, arrows in insert), was absent in *thsos1-4* root cells (Figure 7e, insert). Neither the shape of vacuoles nor endocytosis was affected under normal conditions in wild type or *thsos1-4* (data not shown).

Whereas wild type did not show any symptoms even during longer term exposure to salt (Figure 7f), increasing Na⁺-specific fluorescence in *thsos1-4* was followed by cell death within and adjacent to the elongation zone, visualized by intracellular staining of propidium iodide (Figure 7g, arrows). The number of *thsos1-4* showing higher fluorescence and/or cell death at the root tip and elongation zone increased to two third of the tested seedlings (10 out of 15) within 36 h of stress, and the primary root of all *thsos1-4* seedlings (10 out of 10) had died within 48 h.

*Thsos1-4* accumulated sodium in the root stele after long-term stress. To visualize Na⁺ distribution in the stele, wild type and *thsos1-4* seedlings were incubated for longer time at low concentrations of CoroNa Green (see Materials and Methods). Fluorescein diacetate (FDA) was used as a control in separate experiments, to assure that dyes penetrated to the vasculature (not shown). In wild type, Na⁺ was confined in the vacuoles of epidermis, cortex and, less pronounced, endodermis cells and fluorescence was absent from pericycle cells and cells within the vasculature after treatment with 150mM NaCl for up to 4d (Figures 8a, b, e, g, i). In contrast, cell
damage in the elongation zone in *thsos1-4* extended to the older sections of the root as the stress continued (Figures 8c, d), resulting in compromised cells with intensive intracellular staining of propidium iodide (Figure 8f, arrow 1). Strongest CoroNa Green fluorescence was observed in cells adjacent to structurally compromised cells (Figure 8f, arrow 2). While epidermis and cortex cells were either terminally damaged or failed to confine sodium ions to vacuoles, the Na⁺-specific fluorescence was also found in cells inside the pericycle (Figure 8h, arrows). Within 4 d of incubation, pericycle cells revealed damage and CoroNa Green stained xylem vessel (Figure 8j) in *thsos1-4* at the low concentration of NaCl (150mM), which was not recognized in wild type plants as a stressful conditions, as shown by Figure 8i and also suggested by microarray experiments (Gong *et al.*, 2005).

The higher accumulation of Na⁺ ions inside the endodermis of *thsos1-4* was confirmed by scanning electron microscopy and energy dispersive X-ray microanalysis (SEM-EDX) in the roots of mature plants (Figure 9). After treatment with 250mM NaCl for 2d, *thsos1-4* root accumulated more than twice the amount of Na⁺ than wild type, concurrent with a dramatic decrease of K⁺ in the vacuole of xylem parenchyma that resulted in a more than twelve-times higher Na⁺/K⁺ ratio. In contrast, cortex cells did not show a significant difference between wild type and *thsos1-4*.

**DISCUSSION**

In *Arabidopsis*, the SOS pathway has been documented as an essential component of the ion homeostasis system. The known signal transduction components of the pathway, a complex of SOS2 and SOS3, control the activity of SOS1, a plasma membrane-localized Na⁺/H⁺-antiporter (Qiu *et al.*, 2002; Quintero *et al.*, 2002;
Chinnusamy et al., 2006). The importance of this mechanism in the glycophyte *Arabidopsis* notwithstanding (Shi et al., 2002; Shi et al., 2003), questions remained about the relative impact of this pathway in a naturally salt-tolerant species. We used the halophytic *Arabidopsis*-relative *Thellungiella salsuginea* [halophila] (Bressan et al., 2001) to address this question.

**SOS1 abundance determines the extent of shoot Na⁺ accumulation.**

While the sequence of SOS1 is highly conserved between *Thellungiella* and *Arabidopsis* (Oh et al., 2007), a conspicuous difference emerged from comparisons of expression strength: transcript levels in *Thellungiella* were 8 to 10-times higher in both non-salinized and stressed states, and the salt-dependent induction of expression or stabilization *SOS1* mRNA (Chung et al., 2008) known for *Arabidopsis* was significantly higher in *Thellungiella*. Consistent with earlier reports (Kawasaki et al., 2001; Taji et al., 2004), pre-stress elevated or constitutive high expression of stress-relevant genes could be the basis that determines relative abiotic stress tolerance differences between plants.

Complementation by *SOS1* cDNAs of a yeast mutant lacking Na⁺ transporters suggested that *ThSOS1* can function in Na⁺ exclusion more efficiently than *AtSOS1*, especially at higher level of NaCl (Figures 2a, b). Co-expression of *AtSOS2/3* indicated that *ThSOS1* was activated by *Arabidopsis* SOS signaling components (Figure 2b), indicating conservation of the pathway in *Thellungiella*. However, no significant differences in Na⁺/H⁺ exchange rates were found in plasma membrane vesicles from cells expressing *AtSOS1* or *ThSOS1* (Figure 2d). These results indicate that the two highly conserved SOS1 proteins are substantially equivalent, and the long term effect of differential specific activity or protein abundance, too subtle to be discriminated in
transport ion assays, may become amplified over time to render cells with improved salt tolerance. This is in agreement with the correlation between the salt tolerance of *Arabidopsis* transgenic lines and the expression level of SOS1, regardless of the origin of the protein (Figure 3).

Sodium fluxes into and out of *Thellungiella* roots have been studied in comparison with *Arabidopsis* (Wang *et al*., 2006). A higher Na⁺ efflux in the roots of *Arabidopsis* was reported, which partly compensated for Na⁺ influx. Still, *Arabidopsis* plants accumulated more Na⁺ than *Thellungiella* (Wang *et al*., 2006). Consequently, it was proposed that limitation of Na⁺ influx, not higher efflux, should be the main mechanism by which *Thellungiella* could achieve lower net Na⁺ accumulation under salinized conditions in comparison to *Arabidopsis*. The staining by CoroNa-Green indicates Na⁺ exclusion by the activity of SOS1 in specific regions of the root, rather than in all regions (Figures 7 and 8). Thus, SOS1 may not be directly involved in plant level Na⁺ efflux, but rather may function in protecting the particularly vulnerable cells of the root elongation zone (Figures 7f, g). Indeed, older regions of the root are not characterized by higher Na⁺ content in *thsos1-4* roots, apart from the CoroNa-Green signal that indicated higher content in the xylem at later stages of salt stress (Figure 8). The protection of the young root cells by a more abundant SOS1 in the halophyte may contribute to counteract Na⁺ influx and, in turn, net Na⁺ accumulation.

The RNAi-induced reduction of SOS1 led to faster leaf senescence accompanied by severe shoot water loss during salt stress (Figures 4b, 5b, S3a). This water loss in RNAi lines was not based on impaired stomatal conductance as the RNAi plants showed severe stress symptoms even at 100% humidity (not shown). The phenotype was strictly Na⁺-specific; the RNAi lines did not show differences compared to wild type
at K\(^+\) concentrations of up to 300mM (Figure 4b). The phenotype rather appeared related to the rate of Na\(^+\)-accumulation during the initial stages of exposure to high Na\(^+\), and not to the absolute amount of Na\(^+\) in the shoots after long term exposure. Generally, tolerant species that have been categorized as Na\(^+\)-excluders accumulate large amounts of the ion over time but this accumulation proceeds more slowly than in sensitive “sodium-including” species (Tester and Davenport, 2003). Indeed, Na\(^+\) accumulation in \textit{thsos1-4} was faster and appeared less controlled than in wild type, which eventually contained more Na\(^+\) than the RNAi line without adverse effects on growth at the moderate concentrations of NaCl used (Figure 4c). Considering the positive correlation between SOS1 transcript abundance, the long-term overall high accumulation of Na\(^+\) and the control over the rate of accumulation during early stages of salt stress, \textit{Thellungiella} behaves like a true halophyte and \textit{SOS1} expression appears to constitute an essential trait at the basis of halophytic growth. Observations of Na\(^+\) efflux from plant tissues after removal from non-lethal concentrations of Na\(^+\) confirmed this halophytic nature (Figure 6). Thellungiella wild type maintained near 300mM of Na\(^+\) in the shoot tissue even after removal of Na\(^+\) from the media, indicating that this halophyte might utilize ions as an osmoticum (Figure 6a). In contrast, the higher Na\(^+\) contents in the shoot of \textit{thsos1-4} converged over time to the levels in wild type shoots (Figure 6a), indicating accumulation of Na\(^+\) ions in \textit{thsos1-4} shoots as an uncontrolled process requiring redistribution after the removal of external Na\(^+\). Under very low external Na\(^+\) (0.1, 1 and 10mM NaCl; Figure S4), \textit{thsos1-4} roots took up more Na\(^+\) than wild type after 2 h of treatment. This, together with the slower Na\(^+\) exclusion from roots in the RNAi lines (Figure 6c) emphasized the conserved function of \textit{ThSOS1} in Na\(^+\) exclusion/export under non-lethal stress conditions.
ThSOS1 activity leads to the exclusion of sodium from the root meristematic region and protects cells of the elongation zone. A main objective was to observe accumulation and distribution of Na⁺ ions in cell lineages of the root, considering that we lack information of the genetic makeup that allows halophytic multicellular plants to achieve control over the rate of Na⁺ accumulation. Sodium uptake was followed using the membrane-permeable fluorescent dye CoroNa-Green that binds Na⁺ ions only after it has been confined within cells (Meier et al., 2006), while propidium iodide staining permitted observations of membrane integrity.

Early during salt stress, thsos1-4 roots showed little differences when compared to wild type in intensity or distribution of the Na⁺-specific fluorescence (not shown). Observable changes eventually originated at the root tip region, which began to show stronger fluorescence signals in thsos1-4, indicating SOS1’s function in Na⁺ exclusion (Figures 7a-c). Propidium iodide, which stained cell walls and dead cells, revealed that increased Na⁺ fluorescence was accompanied by a gradual loss of membrane integrity, initially confined to cells of the elongation zone (Figure 7g), spreading to cells of the root hair zone over time (Figures 8c, f). It appears that Na⁺ exclusion by SOS1 is most critical in cells that expand and consequently take up water, whereas cells closer to the quiescent center seem to be protected by the absence of large vacuoles. These observations correspond with results from a recent study that identified the beginning of the elongation zone as the most responsive to salt stress along the longitudinal axis of the primary root (Dinneny et al., 2008). Compromised membranes may result in increased apoplastic Na⁺ flux, deposition of excess (compared to wild type) Na⁺ into vacuoles, which then appeared to initiate cell death in adjacent cells, where the strongest Na⁺ specific signals were typically observed (Figure 7g). A chain reaction of
cell death events accompanied the influx of Na⁺ into the older part of the root as the stele began to accumulate more Na⁺. This behavior of the *thsos1-4* was in sharp contrast to wild type plants, which confined Na⁺ ions nearly exclusively into epidermis (and root cap) cells (Figures 7f, 8a and 8b). Only at concentrations higher than 350 mM NaCl in the medium did we observe the beginning deterioration of the root elongation zone in *Thellungiella* wild type, while *Arabidopsis* wild type showed this phenotype at >180 mM NaCl (not shown).

In roots incubated for an extended time (18 h) with the dye, wild type plants showed significantly lower Na⁺ -specific fluorescence in cells of the pericycle and the stele (Figures 8e, g, i). This visual observation matched studies using X-ray microanalysis for the localization of Na⁺ in roots and confirmed the root endodermis as a major barrier controlling ion influx into the stele (Peng *et al.*, 2004; Ottow *et al.*, 2005). This barrier function may be important under severe stress conditions as it has recently been reported that under moderate stress (50 mM NaCl) this role appears to be satisfied by cells of the epidermis and cortex of wheat (Läuchli *et al.*, 2008). In strong contrast, the localization of Na⁺ and cell viability staining in *thsos1-4* roots clearly revealed higher uptake into the root vasculature (Figures 8f, h, j) and greater movement most likely to the shoot under transpiring conditions. This identifies the crucial barrier at the pericycle/endodermis boundary, where indeed *Arabidopsis* showed strong expression of *SOS1* in a construct expressing *GUS* under control of the *AtSOS1* promoter (Shi *et al.*, 2002). Interestingly, the appearance of Na⁺-ion specific fluorescence inside the pericycle of *thsos1-4* occurred at the same time as root tip damage, suggesting that the protection of root tips by SOS1 may also contribute to the protection of the root stele from the intrusion of Na⁺ ions. SEM-EDX analysis on roots...
of mature plants confirmed higher accumulation of Na\(^+\) in the root stele and cells of the photosynthetic tissues of *thsos1-4* (Figure 9) as observed by measuring ion contents (Figures 5c and 6a) and monitoring Na\(^+\)-specific fluorescence (Figure 8) in younger plants.

**ThSOS1, endocytosis protection and halophytic adaptation.** New evidence has revealed SOS1 and the SOS pathway with a function not only as a Na\(^+\) exporter but as a mediator of intracellular Ca\(^{2+}\) and pH homeostasis (Cheng *et al.*, 2004; Shabala *et al.*, 2005). Involvement of SOS1 in regulating ROS metabolism through an interaction with RCD1, involved in radical-based signaling, through its long C-terminal cytoplasmic tail has been reported (Katiyar-Agarwal *et al.*, 2006). Mutations in SOS1 and other SOS pathway components are known to affect aspects of root development, such as cortical microtubule organization and gravitropism (Sun *et al.*, 2008) under salt stress. Considering the negative effect on endocytosis by a suppression of ThSOS1 expression, indicated by the abolishment of FM4-64 import in *thsos1-4* root cells (Figure 7e), protection of endocytosis may be suggested as an additional role of *SOS1*. In *Arabidopsis*, SOS1 affects cortical microtubule organization and endocytic vesicles are known to be transported along actin filaments and microtubules (Shoji *et al.*, 2006; Soldati and Schliwa, 2006). In affecting endocytosis under salinity conditions, lack of *SOS1* could also interfere with other functions of endocytosis, including polar auxin transport (Dhonukshe *et al.*, 2007), brassinosteroid signaling (Geldner *et al.*, 2007), recycling of plasma membrane receptors and ion channels (Murphy *et al.*, 2005; Sutter *et al.*, 2007) and cytokinesis (Dhonukshe *et al.*, 2006; Reichardt *et al.*, 2007), and therefore result in the termination of root growth and cell death (Figure 7f). SOS1 may be involved in endocytosis via the yet unknown interaction with a component or
regulator of intracellular trafficking, or via an indirect pathway including the regulation of cytosolic or vacuolar pH under salt stress (Shabala et al., 2005), which may then affect endomembrane and vesicle trafficking (Li et al., 2005; Shoji et al., 2006).

SOS1 activity could protect endocytosis of cells in the root tip and elongation zone and ultimately sustain membrane integrity, thus providing an essential stopgap measure or temporary protective solution allowing for other defensive measures to become established in the plants. Supporting this view is the fact that cells in Thellungiella wild type roots that developed after the plants had adapted to increased salinity showed a stronger fluorescence than cells that developed during the stress imposition period (Figure 8b, comparing 1 and 2). Apparently, the halophyte achieved adaptation in newly developed root cells within less than 2d, which was absent from thsos1-4 roots that suffered extensive damage, cell death and uncontrolled apoplastic Na⁺ influx into the root stele and shoot (Figure 8d). Stress “preparedness” by Thellungiella seems to extend to other functions because the plant contains, compared to Arabidopsis, higher amounts of metabolites, including proline, trehalose, inositols and several organic acids and substantially unique yet unknown compounds (Taji et al., 2004; Gong et al., 2005; Oh et al., 2007) that are additionally salt stress-inducible. Although Arabidopsis shows induction, often stronger than Thellungiella, for a number of putatively protective pathways, the ultimate accumulation of metabolites achieved by Arabidopsis is less than that seen in Thellungiella (Gong et al., 2005). Similarly, thsos1-4 shared with Arabidopsis the absence of drastically increased levels of metabolites (Oh et al., 2007). By providing a temporal barrier to a sudden exposure to high Na⁺ in the root cell elongation zone, ThSOS1 prohibited the onset of a chain reaction leading to cell death and apoplastic Na⁺ influx into the shoots. ThSOS1 activity
then resulted in adaptation of the entire plant allowing higher levels of sodium accumulation in the shoot to be non-toxic.

**MATERIALS AND METHODS**

**Transformation of yeast and plants.** Transformation and salt treatment of yeast AXT3K mutant and *Arabidopsis* Columbia wild type were performed as described (Shi *et al.*, 2003; Martinez-Atienza *et al.*, 2007) using the full-length ThSOS1 cDNA (accession: EF207775). Transgenic *Arabidopsis* lines expressing *AtSOS1* (lines 1-1 and 7-1) were kind gifts from Dr. Huazhong Shi (Shi *et al.*, 2003). *Thellungiella* expressing ThSOS1 RNAi were developed as described in (Oh *et al.*, 2007).

**Plant growth and stress treatment.** All plants were grown under 14-hour day and 10-hour night condition. For the assessment of salt tolerance of *Arabidopsis* expressing ThSOS1 and AtSOS1, seedlings were transferred to media containing 200mM NaCl as described in (Shi *et al.*, 2003). Except when indicated otherwise, seedlings were grown on ¼ strength of MS (Murashige-Skoog) media supplemented with 2% sucrose and 0.8% Select-Agar (Invitrogen), with the petridish sealed using porous Micropore tape. For root growth assay, 10-day-old seedlings were transferred to media containing various concentrations of NaCl with the plates oriented vertically. Salt treatment on mature plants was performed as described in (Oh *et al.*, 2007).

**Transcript level.** All analyses used pools of ten plants for each sample. For the interspecific comparison, two-week (for *Arabidopsis*) or 3-week old (for *Thellungiella*) plants, thus accounting for identical growth stage (Gong *et al.*, 2005), were incubated on vertical plates containing salt for 12h and their tissues were pooled and harvested for RNA extraction and RT-PCR analyses. Quantitative real time PCR results were
normalized to ACT2 gene expression. For the list of primers used see Table S1.

**Ion measurements.** For measurement of ion contents in the seedling shoot, media containing 200mM NaCl were made in single compartments of half-divided Petri dish. Two-week old seedlings were grown vertically with their shoot placed in the empty compartment not contacting the medium. Harvested shoot samples were dried, dissolved in HNO₃, and analyzed by an ICP-OES (Optima 2000; Perkin Elmer, Waltham, Ma). For measurement of ion contents in hydroponically grown plants, atomic absorption spectrophotometry (Perkin-Elmer 1100B, Norwalk, CT, USA) was applied to saps extracted from leaf and root frozen samples as described (Gorham et al., 1994).

SEM-EDX was used on frozen sections of leaves and roots harvested from 3 week-old hydroponically grown plants. Samples were mounted in slots of copper holders and fixed with OCT Compound (BDH, Poole, UK), dipped into a bath of slush nitrogen prior to transfer under vacuum into the cryo-preparation chamber (CT1500, Oxford Instruments, Oxfordshire, UK) attached to the SEM (DSM 960, Zeiss, Thornwood, NY). The chamber temperature was left to rise from -163 to -90°C and set for 10 min for ice sublimation before sputter-coating with gold (2 min). Samples were analyzed with an ATW detector interfaced with a Link ISIS analyser (Oxford Instruments) under the following condition: accelerating voltage, 15 kV; take-off angle 42°; collecting time of X-ray counts, 100 sec; working distance between sample and detector, 24 mm. Measurements were performed by focusing on exposed vacuoles of specific cells.

**Ion transport assays.** Vesicles of the yeast plasma membrane were produced by the two-phase partitioning as described previously (Martinez-Atienza et al., 2007). The purity of vesicle preparations was tested by measuring ATP hydrolysis in the presence of inhibitors of mitochondrial (azide), vacuolar (nitrate) and plasmalemma (vanadate)
ATPases. The relative sensitivity of total ATPase activity to these inhibitors demonstrated that vesicle preparations were highly enriched in plasma membrane. 

Na⁺/H⁺ exchange was monitored by the quinacrine fluorescence quenching method. An inside-acid proton gradient (ΔpH) across vesicle membranes was established after the addition of ATP. Sodium chloride was added once ΔpH reached a steady-state and fluorescence recovery (i.e. dissipation of the ΔpH) was recorded with a fluorescence spectrophotometer (Hitachi F-2500). To determine initial rates of Na⁺/H⁺ exchange, the change of relative fluorescence was measured after the first 30 seconds after the addition of sodium salts. Specific activity was calculated by dividing the initial rate of fluorescence recovery, expressed as a ratio of the preformed pH gradient, by the mass of plasma membrane protein in the reaction and time (ΔF mg⁻¹ min⁻¹, where ΔF=F₃₀−F₀ / Fₘₐₓ−Fₘᵢₙ). The change of pH value was measured at excitation and emission wavelengths of 430 and 500 nm respectively.

Visualization of Na⁺ ions. One week-old seedlings were stained and observed by confocal microscopy (TCS SP2 RBB; Leica, Wetzlar, Germany) after salt treatment on media containing 1.1% type A agar (Sigma-Aldrich, St Louis, MO). Staining to reveal Na⁺ content was performed as described (Mazel et al., 2004; Leshem et al., 2006; Meier et al., 2006). Roots were either stained with 20µM CoroNa-Green AM (Invitrogen) in the presence of final concentration 0.02% pluronic acid (Invitrogen) for 3h, or incubated on a filter paper soaked with media containing 10µM CoroNa-Green for 18h to stain the sodium ion in the root stele. For visualizing the stele of roots, FDA (Invitrogen) replaced CoroNa-Green AM in some experiments as positive controls of dye penetration. Where indicated, 2.5 µg/mL propidium iodide (Invitrogen) or 5µM FM4-64 (Invitrogen) was added after incubation with CoroNa-Green AM.
Supplemental Data

The following supplementary material is available for this article online:

**Supplemental Figure S1.** Phylogenic relationships of SOS1 homologs from various species.

**Supplemental Figure S2.** Comparison of *SOS1* mRNA abundance in mature *Arabidopsis* and *Thellungiella* plants.

**Supplemental Figure S3.** Phenotypes of mature *ThSOS1* RNAi plants.

**Supplemental Figure S4.** Comparison of sodium uptake under mildly saline conditions.

**Supplemental Figure S5.** Relative *SOS1* mRNA level in the seedling roots used for microscopy.

**Supplemental Figure S6.** Confocal planes of CoroNa-Green staining of the root tip region under salt stress.

**Supplemental Table S1.** List of RT-PCR primers.
ACKNOWLEDGEMENTS

We thank Drs. Qingqui Gong, Shisong Ma and Valeriy Poroyko for discussions, and Drs. Francoise Quigley and Valeriy Poroyko for Thellungiella SOS1 cDNAs.
(Figure Legends)

Figure 1. Transcript abundance of SOS1 in *Arabidopsis* and *Thellungiella*.

(a) Transcript abundance was compared by semi-quantitative RT-PCR between *Arabidopsis* and *Thellungiella* for SOS1. Actin (ACT2/8), ribulose bisphosphate carboxylase small chain 1A (RBCS1A), and elongation factor 1-alpha (EF1α) were used as references (Czechowski *et al.*, 2005). Treatment of 350mM NaCl to *Arabidopsis* was not included because this stress condition is lethal.

(b) Quantification of SOS1 transcripts by real time RT-PCR with error bars indicating S.D. from six repeats. All primers were designed against regions where the genes of the two species showed perfect identity to generate amplicons of identical length with >95% sequence identity between the two species.

Figure 2. Complementation of the yeast AXT3K mutant with ThSOS1

(a) The yeast strain AXT3K (∆ena1-4 ∆nha1 ∆nhx1) transformed with either AtSOS1 or ThSOS1 was grown on media supplemented with designated concentration of salt.

(b) Co-expression of AtSOS1 or ThSOS1 with Arabidopsis SOS2 and SOS3.

(c) Na⁺/H⁺ exchange activity in plasma membrane vesicles. Formation of pH gradient, acidic inside, was initiated with ATP. Addition of NaCl started Na⁺/H⁺ exchange and fluorescence recovery. The reaction was terminated by adding 25 mM (NH₄)₂SO₄ that dissipated the pH gradient. One representative experiment is shown.
(d) Initial rates of Na\(^+\)/H\(^+\) exchange (means and SE, n=3); units are percent change of fluorescence (\(\Delta F\)) per minute and milligram of protein.

**Figure 3. Ectopic expression of ThSOS1 in Arabidopsis.**

(a) Comparison of SOS1 mRNA abundance between Arabidopsis wild type (Col0) and representative transgenic lines expressing ThSOS1 (lines 29 and 30) and AtSOS1 (lines 1-1 and 7-1) by RT-PCR and qRT-PCR. Error bars represent S.D. of four independent experiments.

(b) Salt tolerance phenotypes of the transgenic plants. Seedlings with two true leaves were incubated for 7d in media containing 200mM NaCl and subsequently rescued to media without NaCl. Picture shown were taken 5d after the rescue. *Percentages of seedlings which produced new leaves were counted. Results were from five biological repeats (n=20 in each repeat). **Statistically similar at p<0.01 (Tukey test)

**Figure 4. Phenotype of ThSOS1-RNAi plants under salt stress.**

(a) Schematic representation of the vector for RNAi expression.

(b) Ten-day old seedling incubated vertically for 8d in the presence of salt. Line 11 constituted a vector control.

(c) Root growth during 8d of incubation in NaCl concentration indicated. Error bars indicate S.D. of 9 to 12 seedlings. Within each concentration, bars with different letters are significantly different with p<0.01 (Tukey test).

(d) SOS1 expression in plants 3 weeks old determined by qRT-PCR with actin as a control. Error bars indicate S.D. of six repeats.
Figure 5. Growth, water and ion contents of wild type and thsos1-4 seedling shoots.

Shoots of seven seedlings grown in the presence of 200mM NaCl were pooled. Control was from seedlings incubated for four days without salt stress. Error bar indicates S.D. of four independent replicates. Bars with different letters are significantly different at p<0.05 (Tukey test).

(a) Average fresh weight of the shoot.

(b) Water content was calculated as (fresh weight - dry weight) / fresh weight × 100 (%).

(c, d) Sodium and potassium contents of shoots, respectively.

Figure 6. Comparison of Na⁺ efflux characteristic between wild type and thsos1-4

Hydroponically grown plants (30d old) were subjected to stepwise increase of NaCl in growth medium to 150mM over 2 weeks. After the removal of sodium from the media, ion concentration in shoots and roots was monitored for 72 hrs. Anova test probed all measurement were significantly variable over time, except for the thsos1-4 root K⁺ (p=0.065).

(a) Na⁺ in shoots; (b) K⁺ in shoots; (c) Na⁺ in the roots; (d) K⁺ in the root.

Figure 7. Imaging of Na⁺ in wild type and thsos1-4 seedling roots.

Roots of 5d-old wild type (a, d, f) and thsos1-4 (b, e, g) seedling were stained with CoroNa-Green AM after 24 hours of 150mM NaCl treatment, and observed under a con-focal microscope.

(a, b) Examples of CoroNa-Green staining of the root tip region. Boxes indicate the
cells whose vacuolar fluorescence intensities were measured for comparison in (c).

(c) Comparison of CoroNa-Green fluorescence intensities. Five individual plants were measured for each line. Error bar indicates S.D. of 10 cortex cells from each individual plant.

(d, e) Magnifications of (a) and (b) with FM4-64 added to visualize the membrane and endocytosis. Shown are representative pictures of confocal planes from surface to center at 1um interval at 30 min after FM4-64 treatment. Arrows in (d) indicates the internalized FM4-64 dye.

(f, g) Confocal planes of root tip, elongation zone and root hair zone at the plane showing epidermis and cortex cells. Propidium iodide was added to stain the damaged cells, indicated by the arrow in (g).

Figure 8. Localization of Na\(^+\) in wild type and \(thsos1-4\) root after prolonged stress.

Shown are confocal planes of the root at the center plane stained with CoroNa-Green and propidium iodide.

(a-d) Arrows indicate the position where growth under salt stress started. Wild type (a, b) and \(thsos1-4\) (c, d) after 2 days (a, c) and 4 days (b, d) in 150mM NaCl.

(e, f) Confocal plane of wild type (e) and \(thsos1-4\) (f) root hair zone after 4 days in 100mM NaCl. The yellow arrows signify the direction to the shoot. In panel (f), arrow 1 indicates plasmolyzing cells and 2, higher accumulation of sodium in the adjacent cells. Both images were scanned at 25% higher gain in both channels compared to others in the figure.

(g-j) Confocal plane showing epidermis (ep), cortex (co), endodermis (en), pericycle
(pe) layers and xylem vessel (xy) of wild type (g, i) and thsos1-4 (h, j) after 2 (g, h) or 4 (i, j) days in 150mM NaCl.

**Figure 9. SEM-EDX analysis for ion measurement in mature plants.**

Wt and *thsos1-4* plants were grown hydroponically for 3 weeks and treated with 250mM NaCl for 2 days. Relative contents of K+ and Na+ in the indicated cell types were determined by EDX analysis as describe in the method section.

(a) cross-section of leaf.
(b) close-up of the boxed region in (a), showing central vein of leaf.
(c) cross-section of root.
(d) close-up of the boxed region in (c).

Cell types identified are leaf palisade (pa), lagunar mesophyll (me), xylem parenchyma (xp) and root cortex (ct).

(e) Samples from both lines were processed simultaneously and the quantitation was performed on 4-6 cells from each tissue type in 2-3 plants of each genotype. Values presented are percent of total counts. Within pairwise comparisons, means followed by letters were statistically different at P<0.1 (a), P<0.01 (b) or P<0.001 (c), respectively (Fisher’s LSD test).
Literature Cited.

Adams P, Nelson DE, Yamada S, Chmara W, Jensen RG, Bohnert HJ, Griffiths H (1998) Growth and development of *Mesembryanthemum crystallinum* (Aizoaceae). New Phytologist 138: 171-190

Amtmann A (2009) Learning from Evolution: *Thellungiella* Generates New Knowledge on Essential and Critical Components of Abiotic Stress Tolerance in Plants. Molecular Plant 2: 3-12

Berthomieu P, Conejero G, Nublat A, Brackenbury WJ, Lambert C, Savio C, Uozumi N, Oiki S, Yamada K, Cellier F, Gosti F, Simonneau T, Essah PA, Tester M, Very AA, Sentenac H, Casse F (2003) Functional analysis of AtHKT1 in *Arabidopsis* shows that Na⁺ recirculation by the phloem is crucial for salt tolerance. Embo J 22: 2004-2014

Bressan RA, Zhang C, Zhang H, Hasegawa PM, Bohnert HJ, Zhu JK (2001) Learning from the *Arabidopsis* experience. The next gene search paradigm. Plant Physiol 127: 1354-1360

Cheng NH, Pittman JK, Zhu JK, Hirschi KD (2004) The protein kinase SOS2 activates the *Arabidopsis* H⁺/Ca²⁺ antiporter CAX1 to integrate calcium transport and salt tolerance. J Biol Chem 279: 2922-2926

Chinnusamy V, Zhu J, Zhu JK (2006) Salt stress signaling and mechanisms of plant salt tolerance. Genet Eng (N Y) 27: 141-177

Chung JS, Zhu JK, Bressan RA, Hasegawa PM, Shi H (2008) Reactive oxygen species mediate Na⁺-induced SOS1 mRNA stability in *Arabidopsis*. Plant J 53: 554-565

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. Plant Physiol 139: 5-17

Davenport R, James RA, Zakrissen-Plogander A, Tester M, Munns R (2005) Control of sodium transport in durum wheat. Plant Physiol 137: 807-818

Demidchik V, Maathuis FJ (2007) Physiological roles of nonselective cation channels in plants: from salt stress to signalling and development. New Phytol 175: 387-404

Dhonukshe P, Aniento F, Hwang I, Robinson DG, Mravec J, Stierhof YD, Friml J (2007) Clathrin-mediated constitutive endocytosis of PIN auxin efflux carriers in *Arabidopsis*. Curr Biol 17: 520-527

Dhonukshe P, Baluska F, Schlicht M, Hlavacka A, Samaj J, Friml J, Gadella TW, Jr. (2006) Endocytosis of cell surface material mediates cell plate formation during plant cytokinesis. Dev Cell 10: 137-150

Dinneny JR, Long TA, Wang JY, Jung JW, Mace D, Pointer S, Barron C, Brady SM, Schiefelbein J, Benfey PN (2008) Cell Identity Mediates the Response of *Arabidopsis*
Roots to Abiotic Stress. Science 320: 942-945

Essah PA, Davenport R, Tester M (2003) Sodium influx and accumulation in Arabidopsis. Plant Physiol 133: 307-318

Geldner N, Hyman DL, Wang X, Schumacher K, Chory J (2007) Endosomal signaling of plant steroid receptor kinase BRI1. Genes Dev 21: 1598-1602

Gong Q, Li P, Ma S, Indu Rupassara S, Bohnert HJ (2005) Salinity stress adaptation competence in the extremophile Thellungiella halophila in comparison with its relative Arabidopsis thaliana. Plant J 44: 826-839

Gorham J, Papa R, Aloy-Lleonart M (1994) Varietal differences in sodium uptake in barley cultivars exposed to soil salinity or salt spray. J Exp Bot 45: 895-901

Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol 51: 463-499

Inan G, Zhang Q, Li P, Wang Z, Cao Z, Zhang H, Zhang C, Quist TM, Goodwin SM, Zhu J, Shi H, Damsz B, Charbaji T, Gong Q, Ma S, Fredricksen M, Galbraith DW, Jenks MA, Rhodes D, Hasegawa PM, Bohnert HJ, Joly RJ, Bressan RA, Zhu JK (2004) Salt cress. A halophyte and cryophyte Arabidopsis relative model system and its applicability to molecular genetic analyses of growth and development of extremophiles. Plant Physiol 135: 1718-1737

Katiyar-Agarwal S, Zhu J, Kim K, Agarwal M, Fu X, Huang A, Zhu JK (2006) The plasma membrane Na⁺/H⁺ antipporter SOS1 interacts with RCD1 and functions in oxidative stress tolerance in Arabidopsis. Proc Natl Acad Sci U S A 103: 18816-18821

Kawasaki S, Borchert C, Deyholos M, Wang H, Brazille S, Kawai K, Galbraith D, Bohnert HJ (2001) Gene expression profiles during the initial phase of salt stress in rice. Plant Cell 13: 889-905

Läuchli A, James RA, Huang CX, McCully M, Munns R (2008) Cell-specific localization of Na⁺ in roots of durum wheat and possible control points for salt exclusion. Plant Cell Environ 31: 1565-1574

Leshem Y, Melamed-Book N, Cagnac O, Ronen G, Nishri Y, Solomon M, Cohen G, Levine A (2006) Suppression of Arabidopsis vesicle-SNARE expression inhibited fusion of H₂O₂-containing vesicles with tonoplast and increased salt tolerance. Proc Natl Acad Sci U S A 103: 18008-18013

Li J, Yang H, Peer WA, Richter G, Blakeslee J, Bandypadhyay A, Titapiwantakun B, Undurraga S, Khodakovskaya M, Richards EL, Krizek B, Murphy AS, Gilroy S, Gaxiola R (2005) Arabidopsis H⁺-PPase AVP1 regulates auxin-mediated organ development. Science 310: 121-125

Lunde C, Drew DP, Jacobs AK, Tester M (2007) Exclusion of Na⁺ via sodium ATPase
(PpENA1) ensures normal growth of Physcomitrella patens under moderate salt stress. Plant Physiol 144: 1786-1796

Martinez-Atienza J, Jiang X, Garcia-Deblas B, Mendoza I, Zhu JK, Pardo JM, Quintero FJ (2007) Conservation of the salt overly sensitive pathway in rice. Plant Physiol 143: 1001-1012

Mazel A, Leshem Y, Tiwari BS, Levine A (2004) Induction of salt and osmotic stress tolerance by overexpression of an intracellular vesicle trafficking protein AtRab7 (AtRabG3e). Plant Physiol 134: 118-128

Meier SD, Kovalchuk Y, Rose CR (2006) Properties of the new fluorescent Na⁺ indicator CoroNa Green: comparison with SBFI and confocal Na⁺ imaging. J Neurosci Methods 155: 251-259

Munns R, James RA, Lauchli A (2006) Approaches to increasing the salt tolerance of wheat and other cereals. J Exp Bot 57: 1025-1043

Murphy AS, Bandyopadhyay A, Holstein SE, Peer WA (2005) Endocytotic cycling of PM proteins. Annu Rev Plant Biol 56: 221-251

Oh DH, Gong Q, Ulanov A, Zhang Q, Li Y, Ma W, Yun DJ, Bressan RA, Bohnert HJ (2007) Sodium stress in the halophyte Thellungiella halophila and transcriptional changes in a thsos1-RNA interference line J Integr Plant Biol 49: 1484-1483

Ottow EA, Brinker M, Teichmann T, Fritz E, Kaiser W, Brosche M, Kangasjarvi J, Jiang X, Polle A (2005) Populus euphratica displays apoplastic sodium accumulation, osmotic adjustment by decreases in calcium and soluble carbohydrates, and develops leaf succulence under salt stress. Plant Physiol 139: 1762-1772

Pardo JM, Cubero B, Leidi EO, Quintero FJ (2006) Alkali cation exchangers: roles in cellular homeostasis and stress tolerance. J Exp Bot 57: 1181-1199

Peng YH, Zhu YF, Mao YQ, Wang SM, Su WA, Tang ZC (2004) Alkali grass resists salt stress through high [K⁺] and an endodermis barrier to Na⁺. J Exp Bot 55: 939-949

Qiu QS, Guo Y, Dietrich MA, Schumaker KS, Zhu JK (2002) Regulation of SOS1, a plasma membrane Na⁺/H⁺ exchanger in Arabidopsis thaliana, by SOS2 and SOS3. Proc Natl Acad Sci U S A 99: 8436-8441

Quintero FJ, Ohta M, Shi H, Zhu JK, Pardo JM (2002) Reconstitution in yeast of the Arabidopsis SOS signaling pathway for Na⁺ homeostasis. Proc Natl Acad Sci U S A 99: 9061-9066

Reichardt I, Stierhof YD, Mayer U, Richter S, Schwarz H, Schumacher K, Jurgens G (2007) Plant cytokinesis requires de novo secretory trafficking but not endocytosis. Curr Biol 17: 2047-2053

Ren ZH, Gao JP, Li LG, Cai XL, Huang W, Chao DY, Zhu MZ, Wang ZY, Luan S, Lin HX
A rice quantitative trait locus for salt tolerance encodes a sodium transporter. Nat Genet 37: 1141-1146

Rus A, Lee BH, Munoz-Mayor A, Sharkhuu A, Miura K, Zhu JK, Bressan RA, Hasegawa PM (2004) AtHKT1 facilitates Na⁺ homeostasis and K⁺ nutrition in planta. Plant Physiol 136: 2500-2511

Shabala L, Cuin TA, Newman IA, Shabala S (2005) Salinity-induced ion flux patterns from the excised roots of Arabidopsis sos mutants. Planta 222: 1041-1050

Shi H, Ishitani M, Kim C, Zhu JK (2000) The Arabidopsis thaliana salt tolerance gene SOS1 encodes a putative Na⁺/H⁺ antiporter. Proc Natl Acad Sci U S A 97: 6896-6901

Shi H, Lee BH, Wu SJ, Zhu JK (2003) Overexpression of a plasma membrane Na⁺/H⁺ antiporter gene improves salt tolerance in Arabidopsis thaliana. Nat Biotechnol 21: 81-85

Shi H, Quintero FJ, Pardo JM, Zhu JK (2002) The putative plasma membrane Na⁺/H⁺ antiporter SOS1 controls long-distance Na⁺ transport in plants. Plant Cell 14: 465-477

Shoji T, Suzuki K, Abe T, Kaneko Y, Shi H, Zhu JK, Rus A, Hasegawa PM, Hashimoto T (2006) Salt stress affects cortical microtubule organization and helical growth in Arabidopsis. Plant Cell Physiol 47: 1158-1168

Soldati T, Schliwa M (2006) Powering membrane traffic in endocytosis and recycling. Nat Rev Mol Cell Biol 7: 897-908

Sun F, Zhang W, Hu H, Li B, Wang Y, Zhao Y, Li K, Liu M, Li X (2008) Salt modulates gravity signaling pathway to regulate growth direction of primary roots in Arabidopsis. Plant Physiol 146: 178-188

Sutter JU, Sieben C, Hartel A, Eisenach C, Thiel G, Blatt MR (2007) Abscisic acid triggers the endocytosis of the Arabidopsis KAT1 K⁺ Channel and its recycling to the plasma membrane. Curr Biol 17: 1396-1402

Taji T, Seki M, Satou M, Sakurai T, Kobayashi M, Ishiyama K, Narusaka Y, Narusaka M, Zhu JK, Shinozaki K (2004) Comparative genomics in salt tolerance between Arabidopsis and aRabidopsis-related halophyte salt cress using Arabidopsis microarray. Plant Physiol 135: 1697-1709

Tester M, Davenport R (2003) Na⁺ tolerance and Na⁺ transport in higher plants. Ann Bot (Lond) 91: 503-527

Vera-Estrella R, Barkla BJ, Garcia-Ramirez L, Pantoja O (2005) Salt stress in Thellungiella halophila activates Na⁺ transport mechanisms required for salinity tolerance. Plant Physiol 139: 1507-1517

Volkov V, Amtmann A (2006) Thellungiella halophila, a salt-tolerant relative of Arabidopsis thaliana, has specific root ion-channel features supporting K⁺/Na⁺ homeostasis under
salinity stress. Plant J 48: 342-353

Wang B, Davenport RJ, Volkov V, Amtmann A (2006) Low unidirectional sodium influx into root cells restricts net sodium accumulation in *Thellungiella halophila*, a salt-tolerant relative of *Arabidopsis thaliana*. J Exp Bot 57: 1161-1170

Yamaguchi T, Aharon GS, Sottosanto JB, Blumwald E (2005) Vacuolar Na⁺/H⁺ antiporter cation selectivity is regulated by calmodulin from within the vacuole in a Ca²⁺- and pH-dependent manner. Proc Natl Acad Sci U S A 102: 16107-16112
Figure 1

(a) NaCl (mM) effects on SOS1 mRNA levels in shoot and root of Arabidopsis (At) and Thale Cress (Th) plants. Relative mRNA levels were measured by qPCR and normalized to ACT2/8 and RBCS1A. EF1α was used as an internal control.

(b) Bar graph showing the relative SOS1 mRNA levels in shoots and roots of Arabidopsis (At) and Thale Cress (Th) plants grown under different NaCl concentrations (0, 200, 350 mM).
Figure 1. Transcript abundance of SOS1 in *Arabidopsis* and *Thellungiella*.
(a) Transcript abundance was compared by semi-quantitative RT-PCR between *Arabidopsis* and *Thellungiella* for SOS1. Actin (ACT2/8), ribulose biphosphate carboxylase small chain 1A (RBCS1A), and elongation factor 1-alpha (EF1α) were used as references (Czechowski *et al.*, 2005). Treatment of 350mM NaCl to *Arabidopsis* was not included because this stress condition is lethal.
(b) Quantification of SOS1 transcripts by real time RT-PCR with error bars indicating S.D. from six repeats. All primers were designed against regions where the genes of the two species showed perfect identity to generate amplicons of identical length with >95% sequence identity between the two species.
Figure 2
Figure 2. Complementation of the yeast AXT3K mutant with ThSOS1
(a) The yeast strain AXT3K (Δena1-4 Δnha1 Δnhx1) transformed with either AtSOS1 or ThSOS1 was grown on media supplemented with designated concentration of salt.
(b) Co-expression of AtSOS1 or ThSOS1 with Arabidopsis SOS2 and SOS3.
(c) Na⁺/H⁺ exchange activity in plasma membrane vesicles. Formation of pH gradient, acidic inside, was initiated with ATP. Addition of NaCl started Na⁺/H⁺ exchange and fluorescence recovery. The reaction was terminated by adding 25 mM (NH₄)₂SO₄ that dissipated the pH gradient. One representative experiment is shown.
(d) Initial rates of Na⁺/H⁺ exchange (means and SE, n=3); units are percent change of fluorescence (∆F) per minute and milligram of protein.
Figure 3

(a) Relative SOS1 mRNA level

| NaCl (mM) | Col0  | 29   | 30   | 1-1  | 7-1  |
|-----------|-------|------|------|------|------|
| 0         | 0     | 0    | 0    | 0    | 0    |
| 200       | 0     | 0    | 0    | 0    | 0    |

**SOS1**

**ACT2**

(b) Survival rate (%)*

| Line | Survival rate (%)* |
|------|--------------------|
| Col  | 9.8 ± 9.5          |
| 29   | 61.8** ± 6.9       |
| 30   | 98.0 ± 2.7         |
| 1-1  | 67.1** ± 8.1       |
| 7-1  | 64.5** ± 5.3       |
Figure 3. Ectopic expression of ThSOS1 in Arabidopsis.
(a) Comparison of SOS1 mRNA abundance between Arabidopsis wild type (Col0) and representative transgenic lines expressing ThSOS1 (lines 29 and 30) and AtSOS1 (lines 1-1 and 7-1) by RT-PCR and qRT-PCR. Error bars represent S.D. of four independent experiments.
(b) Salt tolerance phenotypes of the transgenic plants. Seedlings with two true leaves were incubated for 7d in media containing 200mM NaCl and subsequently rescued to media without NaCl. Picture shown were taken 5d after the rescue. *Percentages of seedlings which produced new leaves were counted. Results were from five biological repeats (n=20 in each repeat). **Statistically similar at p<0.01 (Tukey test)
Figure 4

(a) Diagram illustrating the construction of the ThSOS1 cDNA fragment (1326-2046) using restriction enzymes Ascl, SwaI, BamHI, and SpeI.

(b) Root growth (cm) at 300 mM NaCl and 200 mM NaCl for Th WT, Line 11 (thsos1-4), Line 4 (thsos1-4), Line 6 (thsos1-6), Th WT, Line 11 (thsos1-4), Line 4 (thsos1-6), and Line 6 (thsos1-6). The images show the root lengths of the different lines under NaCl stress.

(c) Bar graph showing root growth (cm) at different NaCl concentrations (0, 50, 100, 150, 200 mM) for Th WT, Line 11 (thsos1-4), Line 4 (thsos1-4), and Line 6 (thsos1-6). Letters indicate statistical differences.

(d) Bar graph showing relative SOS1 mRNA levels in shoots and roots at 0 and 200 mM NaCl for Th WT, Line 11 (thsos1-4), Line 4 (thsos1-4), and Line 6 (thsos1-6).
Figure 4. Phenotype of *ThSOS1*-RNAi plants under salt stress.
(a) Schematic representation of the vector for RNAi expression.
(b) Ten-day old seedling incubated vertically for 8d in the presence of salt. Line 11 constituted a vector control.
(c) Root growth during 8d of incubation in NaCl concentration indicated. Error bars indicate S.D. of 9 to 12 seedlings. Within each concentration, bars with different letters are significantly different with p<0.01 (Tukey test).
(d) SOS1 expression in plants 3 weeks old determined by qRT-PCR with actin as a control. Error bars indicate S.D. of six repeats.
Figure 5. Growth, water and ion contents of wild type and thsos1-4 seedling shoots.
Shoots of seven seedlings grown in the presence of 200mM NaCl were pooled. Control was from seedlings incubated for four days without salt stress. Error bar indicates S.D. of four independent replicates. Bars with different letters are significantly different at p<0.05 (Tukey test).
(a) Average fresh weight of the shoot.
(b) Water content was calculated as (fresh weight - dry weight) / fresh weight × 100 (%).
(c, d) Sodium and potassium contents of shoots, respectively.
Figure 6. Comparison of Na\(^+\) efflux characteristic between wild type and thsos1-4
Hydroponically grown plants (30d old) were subjected to stepwise increase of NaCl in growth medium to 150mM over 2 weeks. After the removal of sodium from the media, ion concentration in shoots and roots was monitored for 72 hrs. Anova test probed all measurement were significantly variable over time, except for the thsos1-4 root K\(^+\) (p=0.065).
(a) Na\(^+\) in shoots; (b) K\(^+\) in shoots; (c) Na\(^+\) in the roots; (d) K\(^+\) in the root.
Figure 7 (1/2)

Fluorescence intensity

|                | ThWt     | thsos1-4 |
|----------------|----------|----------|
| 29.68 ± 9.43   | 68.80 ± 16.89 |          |

Downloaded from on August 24, 2017 - Published by www.plantphysiol.org
Copyright © 2009 American Society of Plant Biologists. All rights reserved.
Figure 7. Imaging of Na⁺ in wild type and thsos1-4 seedling roots.
Roots of 5d-old wild type (a, d, f) and thsos1-4 (b, e, g) seedling were stained with CoroNa-Green AM after 24 hours of 150mM NaCl treatment, and observed under a confocal microscope.
(a, b) Examples of CoroNa-Green staining of the root tip region. Boxes indicate the cells whose vacuolar fluorescence intensities were measured for comparison in (c).
(c) Comparison of CoroNa-Green fluorescence intensities. Five individual plants were measured for each line. Error bar indicates S.D. of 10 cortex cells from each individual plant.
(d, e) Magnifications of (a) and (b) with FM4-64 added to visualize the membrane and endocytosis. Shown are representative pictures of confocal planes from surface to center at 1μm interval at 30 min after FM4-64 treatment. Arrows in (d) indicates the internalized FM4-64 dye.
(f, g) Confocal planes of root tip, elongation zone and root hair zone at the plane showing epidermis and cortex cells. Propidium iodide was added to stain the damaged cells, indicated by the arrow in (g).
Figure 8 (1/2)
Figure 8 (2/2)
Figure 8. Localization of Na\(^+\) in wild type and *thsos1-4* root after prolonged stress.
Shown are confocal planes of the root at the center plane stained with CoroNa-Green and propidium iodide.
(a-d) Arrows indicate the position where growth under salt stress started. Wild type (a, b) and *thsos1-4* (c, d) after 2 days (a, c) and 4 days (b, d) in 150mM NaCl.
(e, f) Confocal plane of wild type (e) and *thsos1-4* (f) root hair zone after 4 days in 100mM NaCl. The yellow arrows signify the direction to the shoot. In panel (f), arrow 1 indicates plasmolyzing cells and 2, higher accumulation of sodium in the adjacent cells. Both images were scanned at 25% higher gain in both channels compared to others in the figure.
(g-j) Confocal plane showing epidermis (ep), cortex (co), endodermis (en), pericycle (pe) layers and xylem vessel (xy) of wild type (g, i) and *thsos1-4* (h, j) after 2 (g, h) or 4 (i, j) days in 150mM NaCl.
Figure 9
Figure 9. SEM-EDX analysis for ion measurement in mature plants.

Wt and thsos1-4 plants were grown hydroponically for 3 weeks and treated with 250mM NaCl for 2 days. Relative contents of K+ and Na+ in the indicated cell types were determined by EDX analysis as describe in the method section.

(a) cross-section of leaf.
(b) close-up of the boxed region in (a), showing central vein of leaf.
(c) cross-section of root.
(d) close-up of the boxed region in (c).

Cell types identified are leaf palisade (pa), lagunar mesophyll (me), xylem parenchyma (xp) and root cortex (ct).

(e) Samples from both lines were processed simultaneously and the quantitation was performed on 4-6 cells from each tissue type in 2-3 plants of each genotype. Values presented are percent of total counts. Within pairwise comparisons, means followed by letters were statistically different at P<0.1 (a), P<0.01 (b) or P<0.001 (c), respectively (Fisher’s LSD test).