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Co-overexpressing a Plasma Membrane and a Vacuolar Membrane Sodium/Proton Antiporter Significantly Improves Salt Tolerance in Transgenic Arabidopsis Plants

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The Arabidopsis gene *AtNHX1* encodes a vacuolar membrane-bound sodium/proton (Na^+/H^+) antiporter that transports Na^+ into the vacuole and exports H^+ into the cytoplasm. The Arabidopsis gene *SOS1* encodes a plasma membrane-bound Na^+/H^+ antiporter that exports Na^+ to the extracellular space and imports H^+ into the plant cell. Plants rely on these enzymes either to keep Na^+ out of the cell or to sequester Na^+ into vacuoles to avoid the toxic level of Na^+ in the cytoplasm. Overexpression of *AtNHX1* or *SOS1* could improve salt tolerance in transgenic plants, but the improved salt tolerance is limited. NaCl at concentration >200 mM would kill *AtNHX1*-overexpressing or *SOS1*-overexpressing plants. Here it is shown that co-overexpressing *AtNHX1* and *SOS1* could further improve salt tolerance in transgenic Arabidopsis plants, making transgenic Arabidopsis able to tolerate up to 250 mM NaCl treatment. Furthermore, co-overexpression of *AtNHX1* and *SOS1* could significantly reduce yield loss caused by the combined stresses of heat and salt, confirming the hypothesis that stacked overexpression of two genes could substantially improve tolerance against multiple stresses. This research serves as a proof of concept for improving salt tolerance in other plants including crops.

Keywords: Arabidopsis • Combined stresses • Genetic engineering • Heat stress • Salt stress • Sodium/proton antiporter.

Abbreviations: AtNHX1, *Arabidopsis thaliana* sodium/hydrogen exchanger 1; AVP1, Arabidopsis pyrophosphate-energized vacuolar membrane proton pump 1; MDA, malondialdehyde; MS, Murashige and Skoog; N lines, *AtNHX1*-overexpressing plants; NS lines, *AtNHX1*/*SOS1*-overexpressing plants; ROS, reactive oxygen species; S lines, *SOS1*-overexpressing plants; SOS1, salt overly sensitive 1; WT, wild type.

Introduction

Salts provide crucial benefits to soils, permitting the optimal growth of crops. Soil characteristics including pH, structural stability and nutritional value are all dependent upon the critical salt balance. However, excessive salts in soil, especially NaCl, can be detrimental to plants, as most consumed plants are glycophytes, a group of organisms incapable of tolerating high salinity in soil. Unfortunately, due to poor agricultural practices, many soils on our planet are already affected by high salinity. Furthermore, because of industrial expansion and land erosion, arable land is becoming saltier and therefore more difficult for cultivating crops. Consequently, salinity is becoming one of the major environmental stresses that cause huge losses in agricultural productivity worldwide. Climate change poses an additional threat to agriculture that will certainly change the face of crop production over the coming decades. Improving salt tolerance in crops will reduce the loss caused by salinity and provide food security to the increasing population on our planet.

Among the most common salts, table salt NaCl causes severe stress to plants. High concentrations of Na^+ and Cl^- ions from table salt cause membrane hyperpolarization and impose two forms of stress on plants that interfere with many metabolic pathways including inhibition of enzyme activities and protein biosynthesis (Munns and Tester 2008). One result of high concentrations of Na^+ in soil is poor water uptake in plant root systems. The second stress resulting from altered Na^+, K^+ and Cl^- homeostasis in soil is the induction of ion-specific stress responses (Blumwald et al. 2000, Zhu 2003), which include production of reactive oxygen species (ROS) and damage to cellular membranes (Mittler 2006, Munns and Tester 2008). The Na^+/H^+ antiporters located on plasma and vacuolar membranes play a critical role in maintaining Na^+ homeostasis by pumping Na^+ from the cytoplasm into either vacuoles or the extracellular space (Blumwald et al. 2000, Zhu 2003). These proteins are fueled by the H^+ electrochemical gradient generated by vacuolar membrane- and plasma membrane-bound proton pumps, preserving low Na^+ concentrations in the cytoplasm (Blumwald et al. 2000, Munns and Tester 2008).

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Given the growing restrictions on land availability and crop yield loss caused by climate change, improvements must be made to sustain humanity's escalating agricultural consumption. Though traditional breeding methods have worked to increase crop yield over the last 10,000 years, such methods are not sufficient to meet the estimated need for a 60% increase in global crop production by the year 2050 (Tester and Langridge 2010, FAO 2015). Efforts in engineering plants for improved salt resistance have been made, but little success has been achieved regarding crops (Munns 2005, Zhang and Shi 2013). Most research groups focused on using genetic engineering strategies to modulate the expression of single genes to increase salt tolerance in plants. As an example, AtNHX1 (*Arabidopsis thaliana* sodium/hydrogen exchanger 1) is a vacuolar membrane-bound Na^+/H^+ antiporter that sequesters Na^+ ions into plant vacuoles (Blumwald et al. 2000). In doing so, cytoplasmic toxicity of Na^+ is avoided and intracellular osmotic potential is maintained to support water uptake. In addition, AtNHX1 transports K^+ into the vacuole, benefiting downstream nutrient uptake and salt tolerance (Leidi et al. 2010). Therefore, increased expression of *AtNHX1* should provide increased salt tolerance in transgenic plants. Indeed this hypothesis has been proven by numerous transgenic studies. To date, *AtNHX1* has been overexpressed in Arabidopsis (Apse et al. 1999), Brassica (Zhang et al. 2001), rice (Chen et al. 2007), cotton (He et al. 2005), buckwheat (Chen et al. 2008), tomato (Leidi et al. 2010), peanut (Asif et al. 2011, Banjara et al. 2012) and tobacco (Zhou et al. 2011), and in all cases, transgenic plants displayed increased salt tolerance.

On the Arabidopsis plasma membrane there exists a Na^+/H^+ antiporter called SOS1 (salt overly sensitive 1) that transports Na^+ across the plasma membrane from the cytoplasm to the apoplastic space in exchange for H^+ (Shi et al. 2002). In doing so, SOS1 decreases the cytotoxicity of excessive Na^+ in plant cells. Along with AtNHX1, SOS1 is a member of a gene family that includes 27 putative Na^+/H^+ antiporters (Zhang and Shi 2013). The SOS1 protein was shown to be involved in Na^+/H^+ exchange activity in purified plasma membrane vesicles, indicating that SOS1 is important for Na^+ and K^+ balance in plant cells (Qiu et al. 2002, Qiu et al. 2003, Olias et al. 2009). Overexpression of *SOS1* improves salt tolerance in transgenic Arabidopsis and tobacco plants (Shi et al. 2003, Yang et al. 2009, Yue et al. 2012). However, transgenic plants overexpressing either *AtNHX1* or *SOS1* could not tolerate NaCl concentrations higher than 200 mM, as none of the published works reported using a higher concentration of NaCl to analyze *AtNHX1*- or *SOS1*-overexpressing plants. Our own experiences working with *AtNHX1*-overexpressing plants indicated that *AtNHX1*-overexpressing cotton could only tolerate NaCl at or below 200 mM (Pasapula et al. 2011), and *AtNHX1*-overexpressing peanut could only tolerate NaCl at or below 150 mM (Banjara et al. 2012); a higher concentration of NaCl would lead to no seed production after salt treatment. Therefore, to increase salt tolerance further in transgenic plants, other approaches must be employed. One of the approaches is to stack genes with functions that contribute to increasing salt tolerance in plants.

There are several salt signaling pathways in plants, and the SOS pathway (Na^+ efflux pathway) and the AtNHX1 pathway (Na^+ sequestration into vacuole) are the two best studied (Zhu 2002, Munns 2005, Munns and Tester 2008). The SOS pathway is made up of three components, SOS1, SOS2 and SOS3 (Qiu et al. 2002, Quintero et al. 2002). SOS2 is a protein kinase that can phosphorylate SOS1, thereby activating SOS1 on the plasma membrane (Liu et al. 2000, Quintero et al. 2002). SOS2 can also phosphorylate AtNHX1 on vacuolar membranes and activate AtNHX1 (Qiu et al. 2002, Zhu 2003). Therefore, SOS2 is a regulator for AtNHX1, linking the two salt signaling pathways. SOS3 is a calcium-binding protein that responds to changes in cellular calcium concentration upon salt stress and regulates SOS2 (Liu and Zhu 1998, Halfter et al. 2000, Ishitani et al. 2000, Mahajan et al. 2008). Yang et al. (2009) explored the possibility of creating more salt-tolerant plants by co-overexpressing components of the SOS pathway or with *AtNHX1*. Their results were not positive, as stacking genes in the same pathways would not lead to higher salt tolerance, and stacking *AtNHX1* with *SOS3* failed to activate both salt signaling pathways simultaneously. Other efforts in stacking genes to obtain higher salt tolerance were either not successful or not conclusive (Zhao et al. 2006, Liu et al. 2010, Bhaskaran and Savithramma 2011, Gouiaa et al. 2012, Liu et al. 2012), because most studies did not provide quantitative analyses by comparing transgenic plants with stacked genes with transgenic plants with a single gene side by side; therefore, it is difficult to judge if there were true improvements in salt tolerance in transgenic plants with stacked genes.

Transporting Na^+ from the cytoplasm into the vacuole or out of the cell is among the most effective methods for cellular salt tolerance (Munns and Tester 2008, Zhang and Shi 2013). Since AtNHX1 and SOS1 work co-operatively to maintain minimal Na^+ ions in the cytoplasm, simultaneous overexpression of these two genes may confer an additive salt tolerance that would exceed that conferred by overexpression of a single gene. Even though *A. thaliana* is not considered a crop plant, its characteristics as a glycophyte and dicotyledon make it an ideal model plant for proof-of-concept studies. AtNHX1 and SOS1 have different mechanisms of action, but they share similar physiological function and originate from the same gene family. In this study, these two genes were arranged on the same DNA construct and both genes were put under the control of the strong constitutive 35S promoter, and introduced into the wild-type (WT) *A. thaliana*. Results from analyzing *AtNHX1/SOS1*-co-overexpressing plants indicate that co-overexpression of these two genes substantially improves salt tolerance in transgenic Arabidopsis plants. Not only could *AtNHX1/SOS1*-co-overexpressing plants now tolerate NaCl up to 250 mM, a concentration that is usually lethal for WT glycophytes, but *AtNHX1/SOS1*-co-overexpressing plants display impressive tolerance against combined stresses of heat and salt. Our result hints at the possibility that this approach might be applicable in improving crop production in places where salinity and high temperature are already a real challenge in agriculture.

Results

Creation and molecular characterization of *AtNHX1/SOS1*-overexpressing plants

To overexpress *AtNHX1* and *SOS1* simultaneously, we created a transforming vector that harbors both genes (**Supplementary Fig. S1**). Then we used this construct to transform WT Arabidopsis Columbia (Col-0) using the 'floral dip' method (Clough and Bent 1998). Fifty-six independent transgenic plants were obtained, and the T_2 plants were sown on Murashige and Skoog (MS)–kanamycin plates to select transgenic plants that gave a 3 : 1 ratio of resistantance to sensitivity, which were potential single T-DNA insertion events. We then isolated total RNAs from 39 putative single T-DNA insertion lines for RNA blot analysis. Based on the RNA blot data (**Supplementary Fig. S2**), we were able to identify *AtNHX1/SOS1*-overexpressing plants (NS lines), *AtNHX1*-overexpressing plants (N lines) and *SOS1*-overexpressing plants (S lines) (**Supplementary Fig. S2**). From these high expression plants, we selected three NS lines (NS-1, NS-2, and NS-3), one N line and one S line for further analyses. Because *SOS1* transcript was reported to be more stable under saline conditions (Shi et al. 2003, Chung et al. 2008), we re-ran the RNA blot analysis to confirm that the selected lines did overexpress transgene transcripts at high levels under conditions with and without NaCl treatment (**Fig. 1A**). NS lines expressed both transgene transcripts, the N line expressed the transgene *AtNHX1* transcript that is slightly shorter than the endogenous *AtNHX1* transcript due to shorter untranslated regions, and the S line expressed the transgene *SOS1* transcript that migrated to the same place as the endogenous transcript (**Fig. 1A**). We went on obtaining homozygous lines for these transgenic plants up to the T_3 generation, then we isolated genomic DNAs from these lines for DNA blot analysis. We used a DNA fragment from the neomycin phosphotransferase gene *NPTII* as the probe in the DNA blot analysis, because it is part of the T-DNA in the construct (coding for kanamycin resistance); we expected to see DNAs from true transgenic plants hybridizing to the probe. Indeed, we observed no band from WT Arabidopsis plants and one to two bands from transgenic plants (**Fig. 1B**). It appears that the N line, the S line and the NS-3 line contain just one copy of the T-DNA, and NS-1 and NS-2 contains two copies of the T-DNA.

AtNHX1/SOS1-overexpressing plants are more salt tolerant than *AtNHX1*- or *SOS1*-overexpressing plants

To test if *AtNHX1/SOS1* co-overexpression could lead to increased tolerance to salt stress, we analyzed these NS lines (i.e. NS-1, NS-2 and NS-3) by comparing them with WT plants, the N line and the S line. Plants were sown on MS plates (Murashige and Skoog 1962) that contained 75, 100 and 150 mM NaCl, respectively. Plants were left to grow vertically. Root length was then measured for each line. At NaCl concentrations of 75 and 100 mM, NS lines produced the longest roots, and the WT produced the shortest roots (**Supplementary Fig. S3**). Without NaCl in the medium,

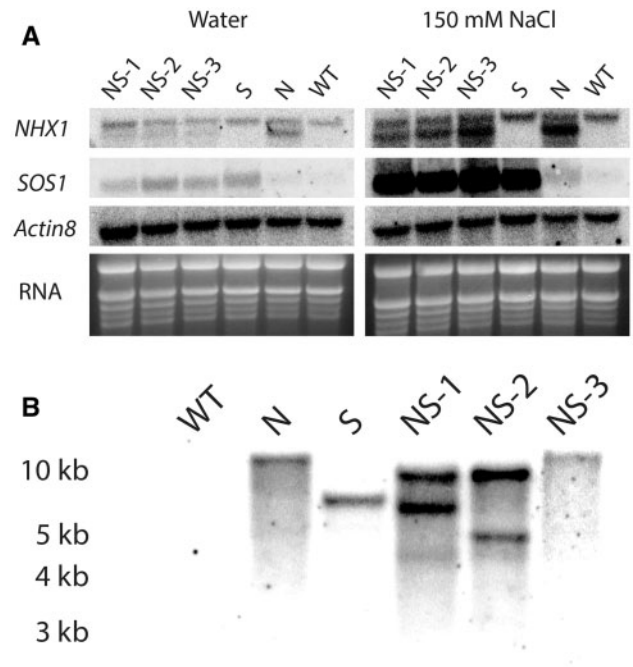

Fig. 1 Molecular analysis of *AtNHX1/SOS1*-overexpressing plants. (A) RNA blot analysis of *AtNHX1/SOS1*-overexpressing plants. RNAs were isolated from plants treated with water (left) and with NaCl solution (right). WT, wild-type plants; N, an *AtNHX1*-overexpressing line; S, a *SOS1*-overexpressing line; NS-1, NS-2 and NS-3, three independent *AtNHX1/SOS1*-overexpressing plants. The probes used are labeled on the left. The gene *actin8* and the ethidium bromide-stained RNA gel were used as the RNA loading controls. (B) DNA blot analysis of *AtNHX1/SOS1*-overexpressing plants. The restriction enzyme *Eco*RI was used to digest genomic DNAs from control plants and transgenic plants. The DNA probe used was the neomycin phosphotransferase gene (*NPTII*) that is used as the selective marker in the T-DNA region, and the molecular weight markers are labeled on the left.

there was no difference in root growth among these plants (**Fig. 2**). At 150 mM NaCl, root lengths of NS lines were significantly longer than those of single gene overexpression plants (N and S lines), which in turn were longer than those of WT plants (**Fig. 2**). At NaCl concentrations of 175 and 20 mM, N lines showed only marginal if any improvement in salt tolerance compared with the WT, as most N lines were chlorotic like WT plants, whereas S lines displayed a slightly better phenotype as about half of them were still green (**Supplementary Fig. S4**). However, all NS plants not only remained green, but also grew a little (**Supplementary Fig. S4**). It is clear that *AtNHX1/SOS1* co-overexpression confers the highest resistance to salt stress, whereas single gene overexpression such as *AtNHX1* overexpression or *SOS1* overexpression confers medium resistance to salt stress.

To test if *AtNHX1/SOS1* co-overexpression could affect plant response to osmotic stress, we analyzed how these plants would respond to high concentration of mannitol in the medium. Plants were sown on MS plates supplemented with 300 mM mannitol and grown for 7 d, then the lengths of plant roots were measured. We did not detect major differences between transgenic plants and WT plants, and no differences

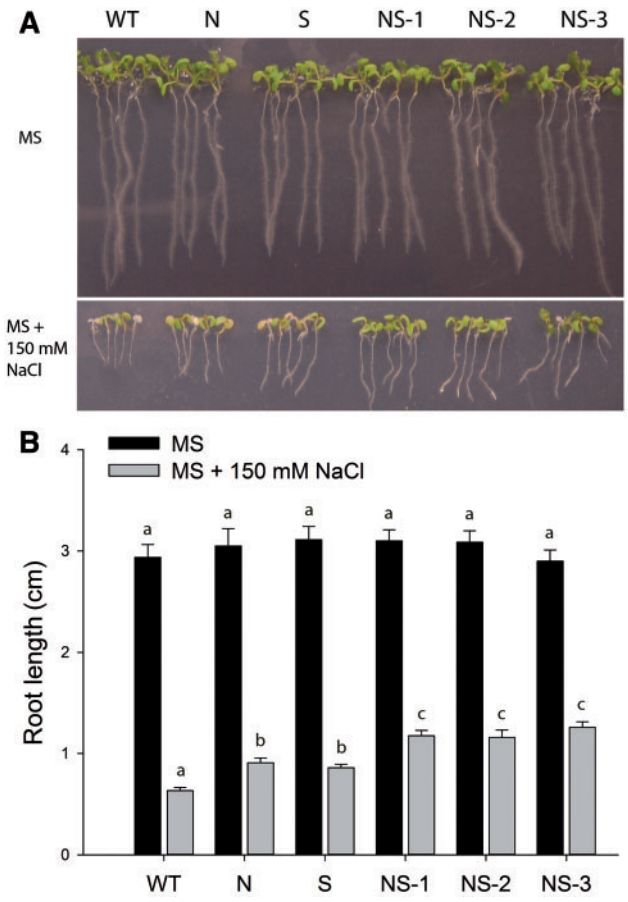

Fig. 2 Analysis of the root growth of *AtNHX1*/*SOS1*-overexpressing plants in the absence or presence of salt. (A) Phenotypes of control and transgenic plants in the absence (MS) or presence of salt (MS + 150 mM NaCl) on day 5. WT, wild-type plants; N, an *AtNHX1*-overexpressing line; S, a *SOS1*-overexpressing line; NS-1, NS-2 and NS-3, three independent *AtNHX1*/*SOS1*-overexpressing plants. MS, Murashige and Skoog medium. (B) Quantitative analyses of the root lengths shown in (A). The results shown are the means ± SE, $n = 12$ plants. Statistical significances was determined using ANOVA at $P < 0.05$. Different letters indicate significant differences in comparison with the WT or with N and S, respectively.

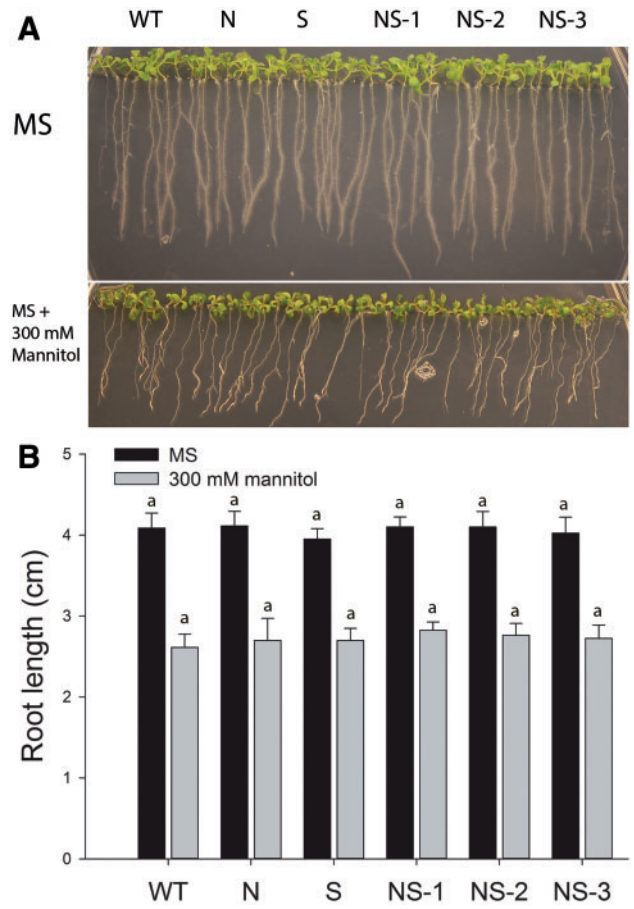

Fig. 3 Analysis of the root growth of *AtNHX1*/*SOS1*-overexpressing plants in the absence or presence of mannitol. (A) Phenotypes of control and transgenic plants in the absence (MS) or presence of mannitol (MS + 300 mM mannitol) on day 7. WT, wild-type plants; N, an *AtNHX1*-overexpressing line; S, a *SOS1*-overexpressing line; NS-1, NS-2 and NS-3, three independent *AtNHX1*/*SOS1*-overexpressing plants. MS, Murashige and Skoog medium. (B) Quantitative analyses of the root lengths shown in (A) The results shown are the means ± SE. a, no significant difference found in comparison with the WT; $n = 10$ plants.

were found between NS and single gene-overexpressing plants (**Fig. 3**).

AtNHX1/*SOS1*-overexpressing plants are most salt tolerant in soil

To test how *AtNHX1*/*SOS1*-overexpressing plants would perform in soil conditions, we conducted salt tolerance test in soils. After growth on MS plates for 12 d, seedlings from NS lines, WT plants, N and S lines were transferred into soil and grown for an additional 12 d, then salt solution was used to irrigate plants. We started using 50 mM NaCl solution to irrigate plants for 4 d, then the concentration was increased to 100 mM for 4 d, to 150 mM for 4 d, to 200 mM for 4 d and finally to 250 mM for 4 d. Before salt treatment, there were no phenotypic differences observed (**Fig. 4A**, **D**); however, after salt treatment at 250 mM for 4 d, WT plants were severely affected, with very little shoot system produced (**Fig. 4E**, **F**). Single gene-overexpressing plants N and S did produce some shoots, but far fewer than NS plants (**Fig. 4E**, **F**). After salt treatment at 250 mM, we measured the heights and biomasses of these plants. Seed yields were measured at the end of 8 weeks. We found that NS plants were the tallest, single gene-overexpressing plants were intermediate and WT plants were the shortest. Interestingly, under well-watered conditions in soils, NS plants were slightly taller than both WT and single gene-overexpressing plants (**Figs. 4B**, **C**, **5A**). The salt treatment had the least impact on the height of NS plants compared with that of other plants (**Fig. 5A**). Similarly, NS plants produced the most biomass, and single gene-overexpressing plants produced less biomass than NS plants, but more biomass than WT plants (**Fig. 5B**). NS plants also produced significantly more seeds than single gene-overexpressing plants, and WT plants produced the least seeds (**Fig. 5C**). To rule out the possbility

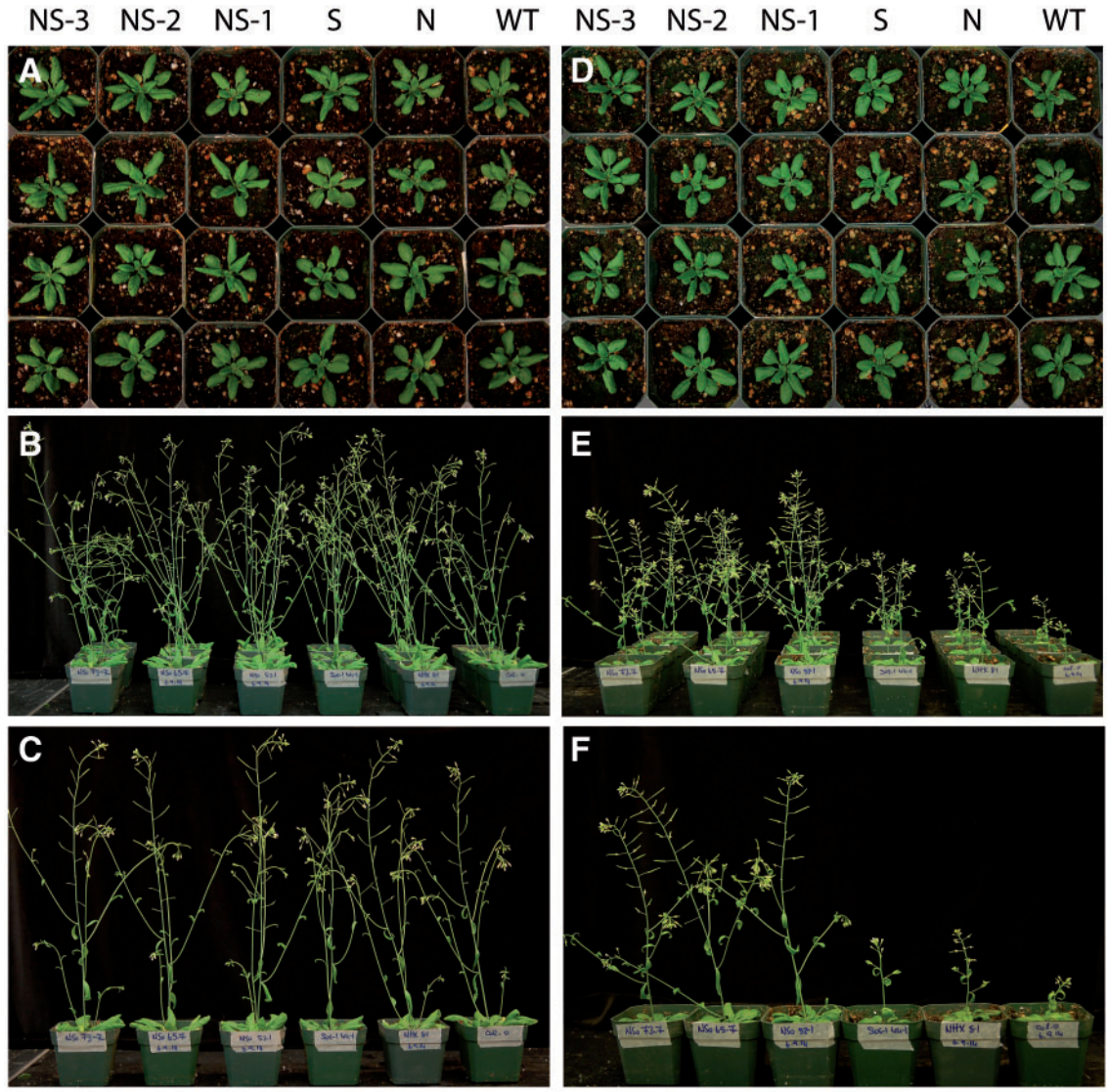

Fig. 4 Phenotypes of control and *AtNHX1*/*SOS1*-overexpressing plants with or without salt treatment. (A–C) Phenotypes of control and *AtNHX1*/*SOS1*-overexpressing plants under normal growth conditions on day 20 (A) and day 45 (B and C). (D) Phenotypes of control and *AtNHX1*/*SOS1*-overexpressing plants on day 20 before salt treatment. (E and F) Phenotypes of control and *AtNHX1*/*SOS1*-overexpressing plants on day 45 after salt treatment. Plants were irrigated with NaCl solution on day 21, starting with 50 mM and rising to 250 mM incrementally at 4 d intervals.

that plants grown in different pots might have been exposed to different concentrations of salt, we grew plants with different genotypes in the same pot and treated these plants with NaCl up to a concentration of 250 mM. Again, we observed similar phenotypes: NS plants displayed the highest tolerance to salt treatment, WT plants the least tolerance, and N and S plants were intermediate (**Fig. 6**).

Seed quality of *AtNHX1*/*SOS1*-overexpressing plants is the best after salt treatment

To assess the impact of salt treatment on seed quality, we analyzed the seed germination rates of seeds harvested from plants that were treated with 250 mM NaCl. All seeds were sown on MS plates and allowed to grow. We found that NS seeds germinated at much higher rates than seeds from single gene-overexpressing plants, and most WT seeds failed to germinate (**Fig. 7A**). On day 7, WT seeds showed no signs of germination, and N and S lines showed a minimal increase in germination, but NS lines reached nearly 30% germination (**Fig. 7B**). Even after 15 d, the germination rates remained the same (data not shown). In contrast, seeds harvested from plants that were grown under normal growth conditions (i.e. no salt treatment) germinated very well (**Fig. 7C**), and they all reached almost 100% germination by day 3 (**Fig. 7D**). In fact, between 40% and 70% of these plants germinated within 24 h after stratification (**Fig. 7D**), yet 0% of seeds coming from salt-treated plants germinated by day 1 (**Fig. 7B**). These experiments indicate that salt treatment severely compromised the quality of WT seeds, leading to almost a complete loss of seed viability. On the other hand, overexpression of either *AtNHX1*

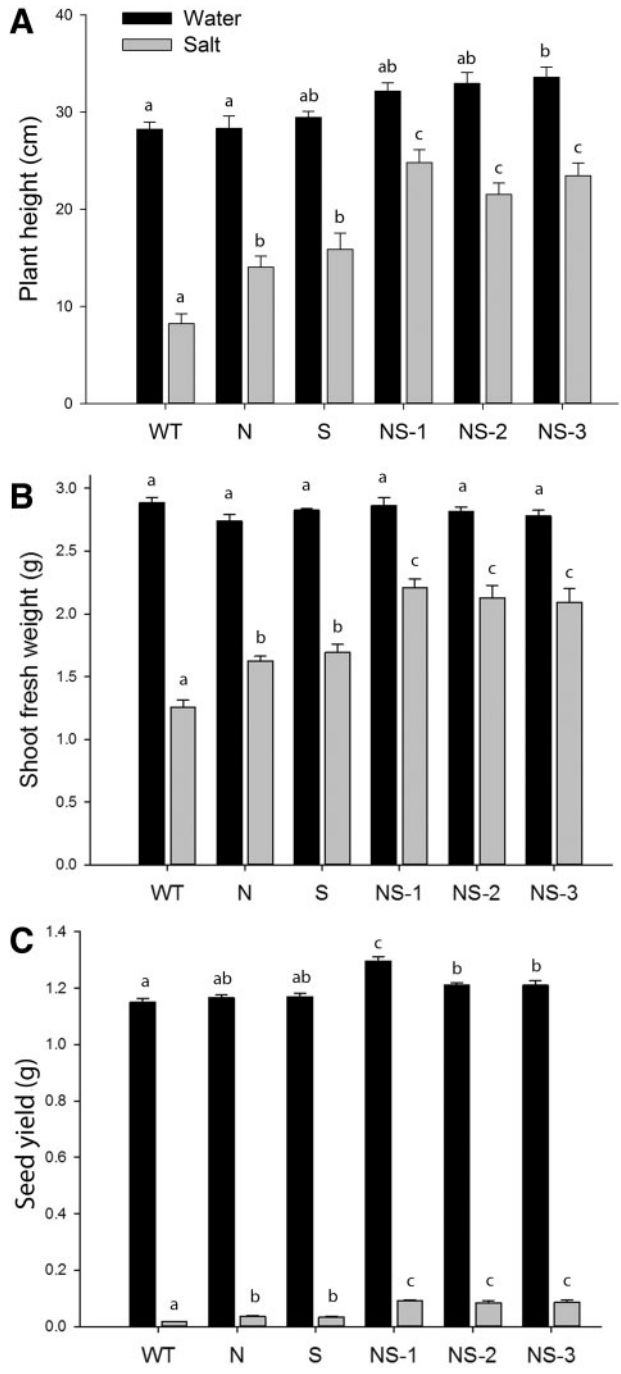

Fig. 5 Analyses of control and *AtNHX1*/*SOS1*-overexpressing plants without or with salt treatment in soils. (A) Plant heights of control and *AtNHX1*/*SOS1*-overexpressing plants without or after 250 mM NaCl treatment on day 45. WT, wild-type plants; N, an *AtNHX1*-overexpressing line; S, a *SOS1*-overexpressing line; NS-1, NS-2 and NS-3, three independent *AtNHX1*/*SOS1*-overexpressing plants. Mean values and the SE were calculated from three independent experiments and, in each experiment, $n = 4$ plants. Statistical significance was determined using ANOVA at $P < 0.05$. Different letters indicate a significant differences in comparison with the WT or with N and S, respectively. (B) Shoot fresh weights of control and *AtNHX1*/*SOS1*-overexpressing plants without or after 250 mM NaCl treatment on day 45. (C) Seed yields of control and *AtNHX1*/*SOS1*-overexpressing plants without or after 250 mM NaCl treatment. Seeds were harvested from 8-week-old plants.

or *SOS1* alone helps in protecting the seed's viability, and co-overexpressing *AtNHX1* and *SOS1* protects seeds most effectively from salt-induced damage.

AtNHX1/*SOS1*-overexpressing plants display healthier biochemical characteristics

AtNHX1/*SOS1*-overexpressing plants produced more biomass and remained green despite 250 mM NaCl in soils, indicating that these plants maintained relatively normal cellular metabolism under high salt conditions. To explore the biochemical basis of the superior phenotype of NS plants under 250 mM NaCl, we examined the total chlorophyll (Chl) contents (*a* and *b*) in the leaves of plants that were treated with 250 mM NaCl. The contents of total Chl in WT and transgenic plants were markedly decreased after salt treatment; however, the decrease in NS plants was significantly lower than those in single gene-overexpressing plants and WT plants (**Fig. 8A**), which explains why NS plants were still green after treatment with 250 mM NaCl for 8 d whereas WT plants became almost chlorotic. Single gene-overexpressing plants showed a phenotype that was better than WT plants, but worse than NS plants.

Salt stress can induce production of ROS, which can cause extensive damage to plant tissues and especially cellular membranes (Munns 2005, Zhang and Shi 2013). One indicator of damage caused by ROS is the production of malondialdehyde (MDA), a lipid peroxidation product (Draper and Hadley 1990). We measured the content of MDA in plants that were treated with 250 mM NaCl. It was found that NS plants produced very little MDA after salt treatment, whereas single gene-overexpressing plants produced moderate levels of MDA, and WT plants produced far more MDA than NS plants (**Fig. 8B**). There was no difference in the amount of MDA among these plants grown under well-watered conditions, indicating that the reduced production of MDA in transgenic plants after salt treatment was due to the expression of transgenes, and co-overexpressing *AtNHX1* and *SOS1* led to the least production of MDA.

Because NS plants produced very little MDA, this indicated that these plants might have the highest capacity for removing ROS among all plants being analyzed under salt stress conditions. One of the ROS scanvengers in plants is anthocyanin, commonly known as the stress pigment (Abogadallah 2010). We found that NS plants produced much more anthocyanin pigment than N, S and WT plants after salt treatment (**Fig. 8C**), indicating that NS plants have the greatest potential to scanvenge ROS, which explains why leaves from NS plants remain green and NS seeds are least damaged after salt treatment. The single gene-overexpressing plants N and S produced more anthocyanin than WT plants, explaining why these plants performed better than WT plants, but not as well as NS plants after salt treatment.

AtNHX1/*SOS1*-overexpressing plants confer the highest tolerance against combined stresses of salt and heat

To determine the effect of multiple stresses on plants, we analzyed how *AtNHX1*/*SOS1*-overexpressing plants would respond

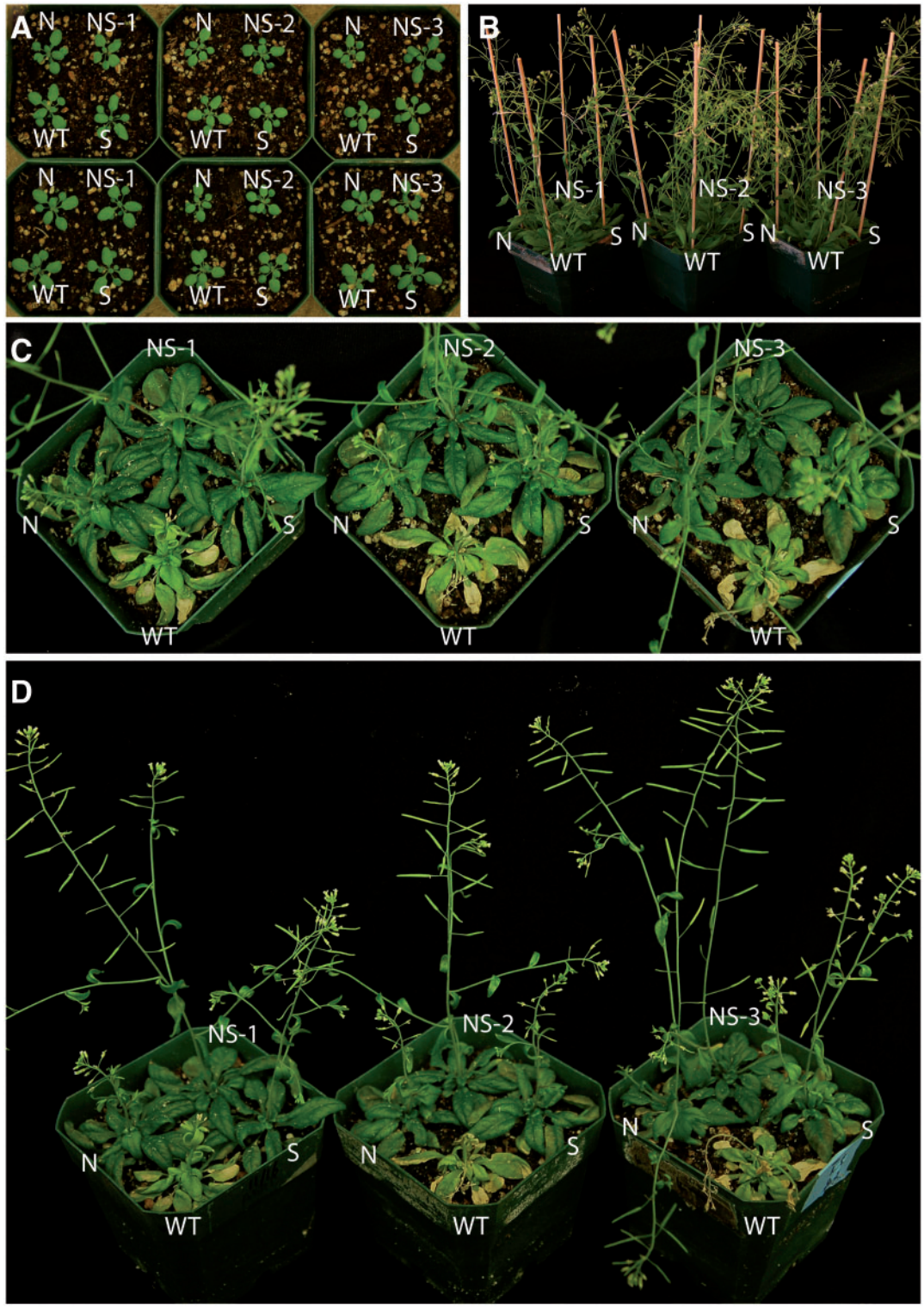

Fig. 6 Phenotypes of control and *AtNHX1/SOS1*-overexpressing plants with or without salt treatment. (A and B) Phenotypes of control and *AtNHX1/SOS1*-overexpressing plants under normal growth conditions on day 21 (A) and day 43 (B), respectively. (C and D) Phenotypes of control and *AtNHX1/SOS1*-overexpressing plants on day 43 after salt treatment. Plants were irrigated with NaCl solution on day 21, starting with 50 mM and rising to 250 mM incrementally at 4 d intervals.

to the combined stresses of heat and salt on MS plates as well as in soils. Arabidopsis seeds were sown on MS plates that contained 100 mM NaCl, and were grown vertically at 28°C for 9 d before root lengths of these plants were measured. We found that NS plants produced the longest roots and WT plants produced the shortest root length under the combined stress conditions (**Fig. 9A**). A temperature of 28°C is considered moderately high for Arabidopsis, and it slightly reduced root growth

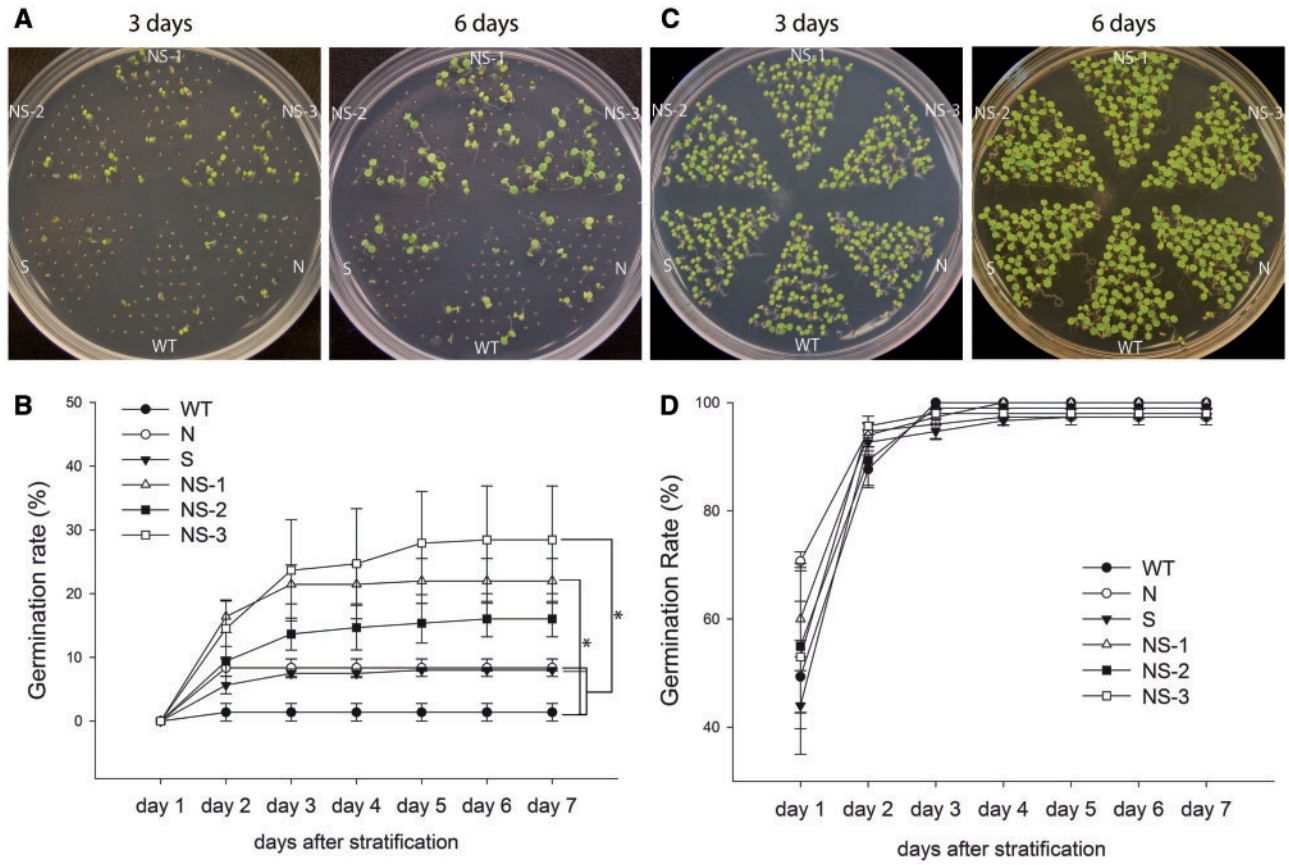

Fig. 7 The seed quality of *AtNHX1*/*SOS1*-overexpressing plants is better than those of single gene-overexpressing plants and WT plants after salt treatment. (A) Seed germination on an MS plate from seeds that were harvested from plants treated with 250 mM NaCl on day 3 and day 6. WT, wild-type plants; N, an *AtNHX1*-overexpressing line; S, a *SOS1*-overexpressiong line; NS-1, NS-2 and NS-3, three independent *AtNHX1*/*SOS1*-overexpressing plants. (B) Germination rates of seeds in (A). (C) Seed germination on an MS plate from seeds that were harvested from normally grown plants on day 3 and day 6. (D) Germination rates of seeds in (C). The experiments were repeated three times, and 45 seeds were used for each experiment. The results shown are the means ± SE. An asterisk indicates a significant difference in comparison with the WT or with N and S, respectively. Statistical significance was determined using the germination rate on day 7 by pairwise *t*-test at $P < 0.05$.

in all plants, but it did not cause significant differences in root growth between transgenic and WT plants (**Fig. 9B**). The NaCl concentration of 100 mM is moderately toxic to Arabidopsis plants, and it caused a large reduction in root growth among all plants; however, NS plants produced the longest root systems, and WT plants produced the shortest root systems (**Fig. 9B**). Yet, the combined stress of heat and salt caused the largest reduction in root growth among all plants (**Fig. 9B**), indicating that multiple stresses can indeed cause greater damage to plants. Interestingly, the root lengths of NS plants were almost the same as those from WT plants that were treated with salt only, suggesting that *AtNHX1*/*SOS1* co-overexpression significantly reduced the damage caused by combined stresses of heat and salt.

To test how *AtNHX1*/*SOS1*-overexpressing plants would perform in soil conditions, homozygous seeds from different lines and the WT were grown on MS plates for 7 d, then seedlings were transferred into soil and grown under normal conditions for another two and a half weeks. Then we moved plants to the greenhouse that was set at 28°C and irrigated plants with increasing concentrations of NaCl starting from 50 mM and rising up to 150 mM (every 4 d). Prior to imposing heat and salt stresses, all plants were healthy and roughly similar in size, but after 2 weeks under combined stress conditions, dramatic phenotypic differences appeared: NS lines performed the best as they produced robust shoot systems with dense lateral branching and large rosette leaves, the N line performed similarly to the S line and both lines outperformed the WT plants with regard to plant height and lateral branching (**Fig. 10**). Under normal conditions (22°C and irrigation with water), these plants were indistinguishable, and the heat stress alone did not affect plant shoot height much (**Figs. 10D**, **11A**). Salt stress alone caused a substantial reduction in plant height for all plants, yet NS plants displayed the smallest reduction and WT plants displayed the greatest reduction in plant height (**Figs. 10F**, **11A**). When both stresses were applied, again NS plants displayed the smallest reduction in shoot height, and WT plants displayed the greatest reduction in shoot height (**Figs. 10H**, **11A**).

Without abiotic stress treatment, transgenic plants and WT plants produced similar numbers of siliques and a similar amount of seeds (**Fig. 11B**, **C**). However, with heat treatment,

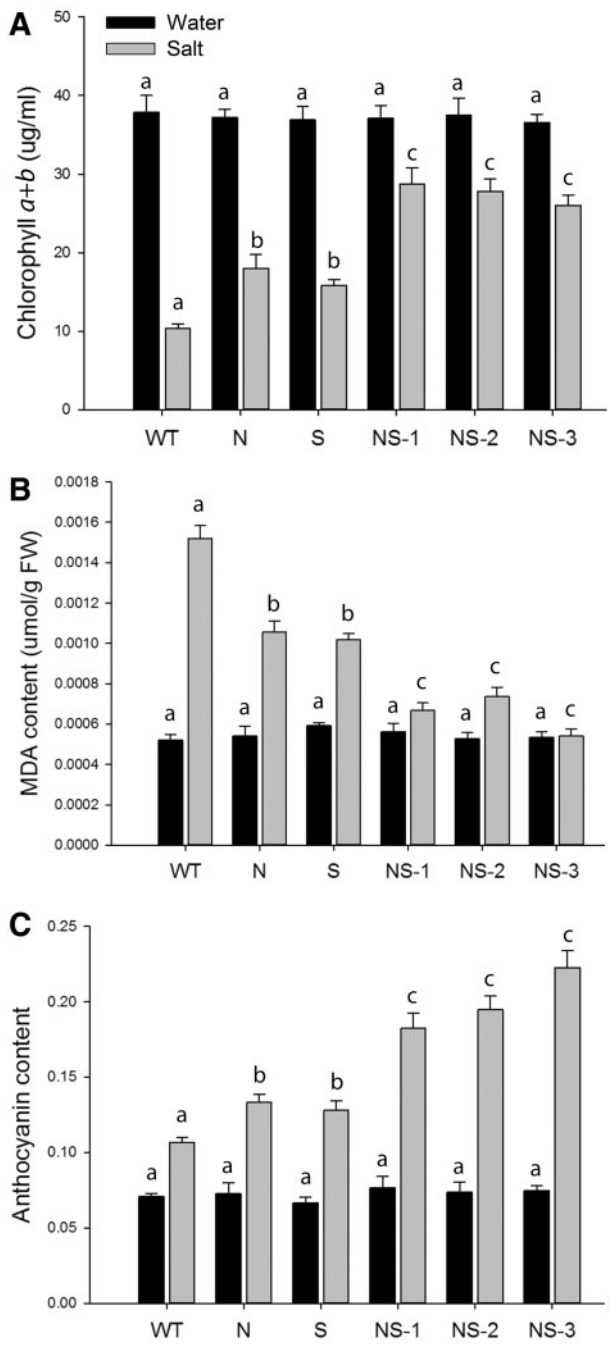

Fig. 8 Biochemical responses of control and *AtNHX1*/*SOS1*-overexpressing plants to salt treatment. Total Chl *a* and *b* (A), MDA (B) and anthocyanin (C) contents of control and *AtNHX1*/*SOS1*-overexpressing plants without or after 250 mM NaCl treatment. WT, wild-type plants; N, an *AtNHX1*-overexpressing line; S, a *SOS1*-overexpressing line; NS-1, NS-2 and NS-3, three independent *AtNHX1*/*SOS1*-overexpressing plants. The results shown are the means ± SE. Statistical significance was determined using ANOVA at $P < 0.05$. Different letters indicate significant differences in comparison with the WT or with N and S, respectively.

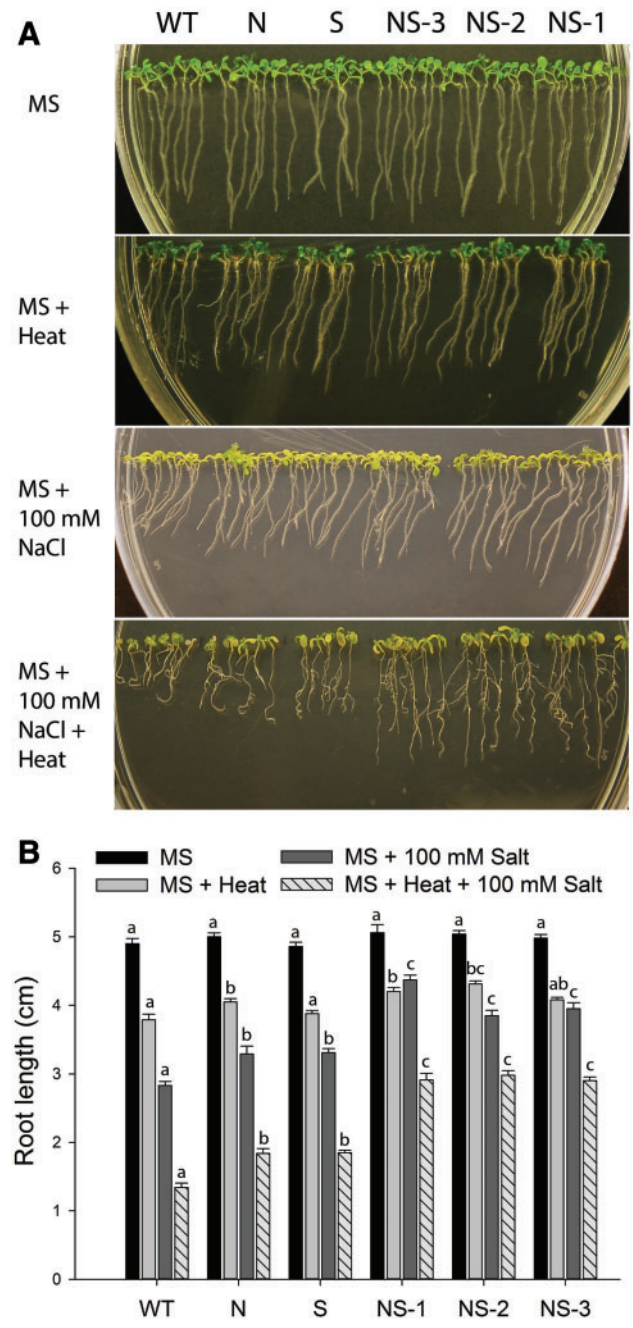

Fig. 9 Phenotypes (A) and root length analyses (B) of control and *AtNHX1*/*SOS1*-overexpressing plants without or after stress treatments on MS plates. The salt stress (100 mM NaCl), heat stress (28°C) and the combined stresses (100 mM NaCl + 28°C) were used in these experiments. WT, wild-type plants; N, an *AtNHX1*-overexpressing line; S, a *SOS1*-overexpressing line; NS-1, NS-2 and NS-3, three independent *AtNHX1*/*SOS1*-overexpressing plants. The results shown are the means ± SE. Statistical significances was determined using ANOVA at $P < 0.05$. Different letters indicate significant differences in comparison with the WT or with N and S, respectively.

NS lines produced slightly more siliques and seeds than both wild-type plants and N and S plants (**Fig. 11B**, **C**). With salt treatment, NS lines produced substantially more siliques and seeds than N and S plants that in turn produced more siliques and seeds than WT plants (**Fig. 11B**, **C**). Finally, with both heat and salt stresses, the yield differences between NS plants and all other plants were even greater (**Fig. 11B**, **C**). These results are similar to those obtained from experiments conducted using

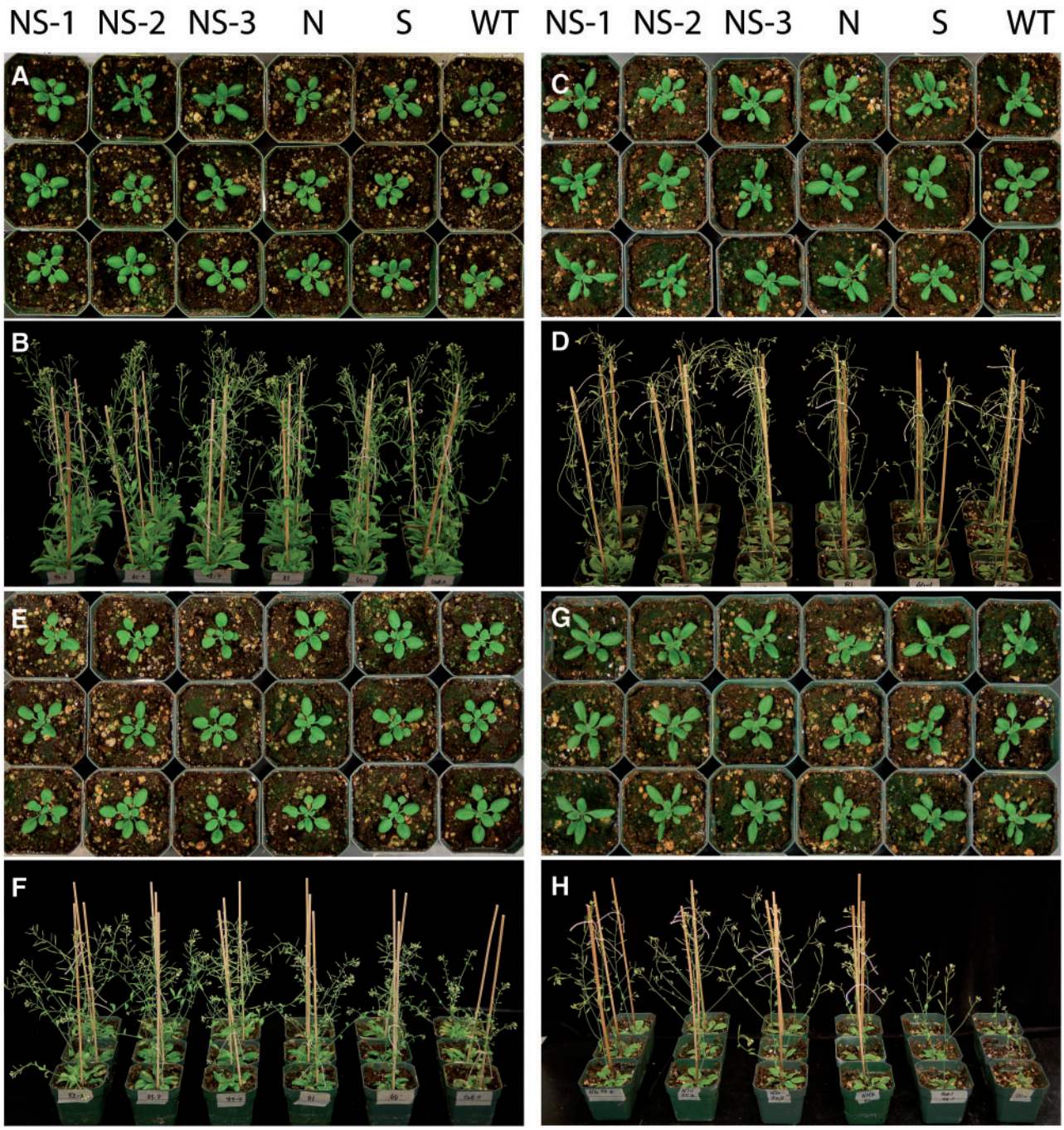

Fig. 10 Phenotypes of control and *AtNHX1/SOS1*-overexpressing plants before and after stress treatments in soils. (A, C, E and G) Three-week-old plants before stress treatments. (B) Plants without stress treatment on day 42. (D) Plants after being treated with heat (28°C) for 21 d. (F) Plants after being treated with salt (50, 100 then 150 mM NaCl incrementally) for 21 d. (H) Plants after being treated with heat plus salt (28°C + 50, 100 then 150 mM NaCl). WT, wild-type plants; N, an *AtNHX1*-overexpressing line; S, a *SOS1*-overexpressing line; NS-1, NS-2 and NS-3, three independent *AtNHX1/SOS1*-overexpressing plants.

MS plate-grown plants, confirming that *AtNHX1/SOS1* co-overexpression indeed significantly improves stress tolerance, especially under multiple stress conditions.

AtNHX1/SOS1-overexpressing plants contain higher Na^+, K^+ and Cl^- ions than wild-type plant

To analyze the effect of *AtNHX1/SOS1* co-overexpression on the ion contents in transgenic plants, we analyzed Na^+, K^+ and Cl^- contents in *AtNHX1/SOS1*-co-overexpressing plants, reference lines and WT plants. We found that *AtNHX1/SOS1* co-overexpression caused Na^+ and Cl^- contents to increase after salt treatment, but the levels of Na^+ and Cl^- did not reach the levels of these ions found in *AtNHX1*-overexpressing plants (**Fig. 12A**, **B**). *AtNHX1* overexpression in transgenic plants leads to increased levels of Na^+ and Cl^- in plant tissues (Zhang and Blumwald 2001), whereas *SOS1* overexpression

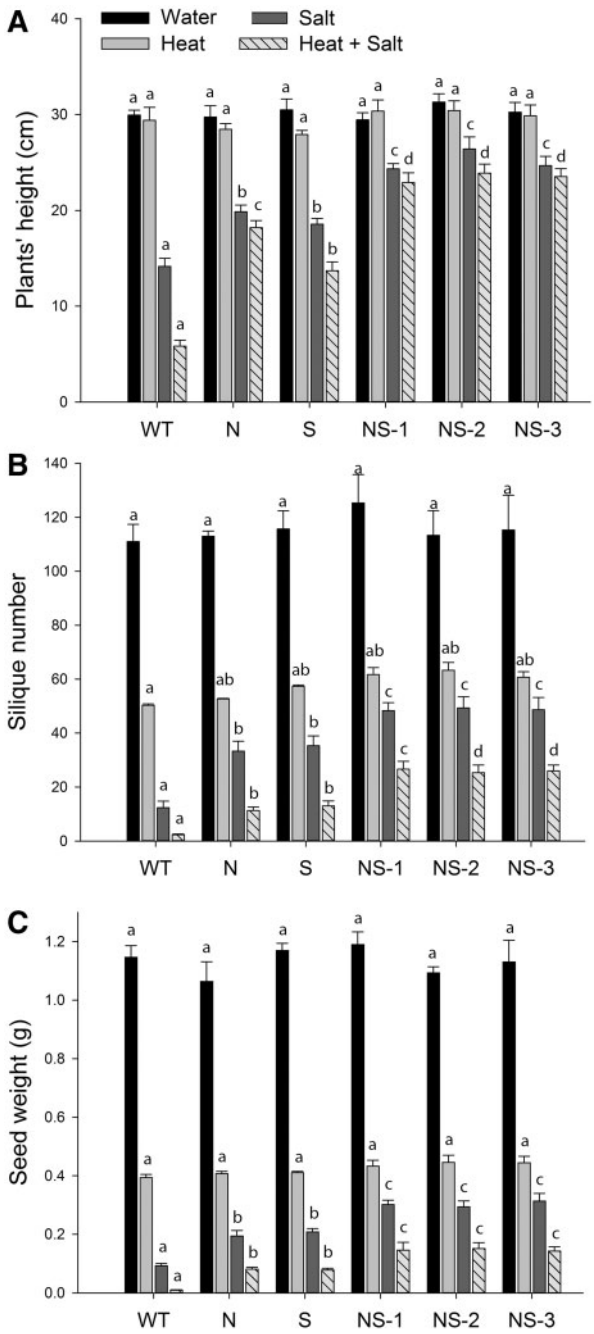

Fig. 11 Plant heights (A), silique numbers (B) and seed weights (C) of control and *AtNHX1*/*SOS1*-overexpressing plants without or with stress treatments in soils. The heat stress (28°C), salt stress (50, 100 then 150 mM NaCl incrementally) and the combined stresses (28°C + 50, 100 then 150 mM NaCl) were used in these experiments. WT, wild-type plants; N, an *AtNHX1*-overexpressing line; S, a *SOS1*-overexpressing line; NS-1, NS-2 and NS-3, three independent *AtNHX1*/*SOS1*-overexpressing plants. The figure is representative of three independent experiments. Values represent means ± SE ($n = 3$). Different letters indicate significant differences in comparison with the WT or with N and S, respectively, at $P < 0.05$.

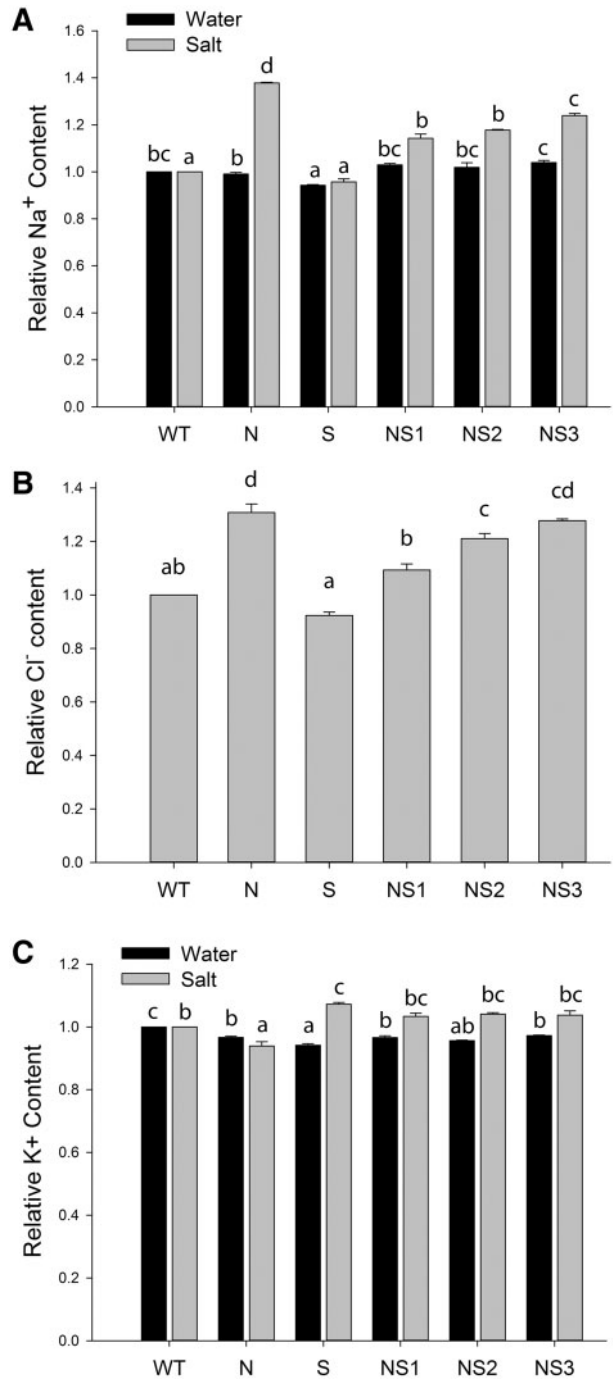

Fig. 12 Relative contents of sodium, chloride and potassium in *AtNHX1*/*SOS1*-co-overexpressing and control plants. WT, wild-type plants; N, an *AtNHX1*-overexpressing line; S, a *SOS1*-overexpressing line; NS-1, NS-2 and NS-3, three independent *AtNHX1*/*SOS1*-co-overexpressing plants. Mean values and the SE were calculated from three independent experiments and, in each experiment, $n = 12$ plants. Statistical significance was determined using ANOVA at $P < 0.05$. Different letters indicate significant differences in comparison with the WT or with N and S, respectively.

leads to decreased Na^+ content in transgenic plants (Shi et al. 2003), which can be explained by the functions of these two enzymes: AtNHX1 sequesters Na^+ into the vacuole whereas SOS1 transports Na^+ out of cells. The higher levels of Na^+ and Cl^- in *AtNHX1*/*SOS1*-co-overexpressing plants than in WT plants might be due to *AtNHX1* overexpression. Interestingly, the content of K^+ was reported to be lower in *AtNHX1*-overexpressing plants than that in WT plants

(Apse et al. 1999, Zhang and Blumwald 2001), yet in *AtNHX1/SOS1*-co-overexpressing plants we found that the K^+ content increased in both *AtNHX1/SOS1*-co-overexpressing and *SOS1*-overexpressing plants after salt treatment (**Fig. 12C**); therefore, the increased K^+ content in *AtNHX1/SOS1*-co-overexpressing plants might be due to *SOS1* overexpression. Our data are consistent with earlier reports that overexpression of *CsSOS1* in *Chrysanthemum crassum* led to decreased Na^+ content and increased K^+ content after salt treatment (An et al. 2014), overexpression of a hyperactive form of TdSOS1 in Arabidopsis made plants retain less Na^+ and more K^+ during mild salt treatment (Feki et al. 2014), and both *SOS1* and *SbSOS1* could help exclude Na^+ and retain K^+ under salt stress conditions in transgenic tobacco (Yadav et al. 2012, Yue et al. 2012).

Discussion

In this report, we demonstrate that co-overexpression of a vacuolar membrane-bound Na^+/H^+ antiporter and a plasma membrane-bound Na^+/H^+ antiporter could further improve salt tolerance in transgenic plants. We first demonstrate that *AtNHX1/SOS1*-co-overexpressing plants are clearly more salt tolerant than either *AtNHX1*-overexpressing or *SOS1*-overexpressing plants (**Figs. 2–6**; **Supplementary Figs. 3**, **4**). Interestingly, under well-watered conditions in soil, NS plants were slightly taller than both WT and single gene-overexpressing plants (**Fig. 5A**). The taller NS plants might also contribute to better performance under salt stress, as they were healthier. However, this should not be the major reason, because under salt treatment NS plants showed the least reduction in height and biomass compared with NS plants grown under normal conditions (**Fig. 5A**). This increased tolerance was specific to salt only, as these *AtNHX1/SOS1*-co-overexpressing plants responded to osmotic stress such as 300 mM mannitol similarly to the WT, *AtNHX1*-overexpressing and *SOS1*-overexpressing plants (**Fig. 3**). The better performance of these *AtNHX1/SOS1*-co-overexpressing plants might be partially due to their better biochemical response to salt stress, as they are able to maintain more Chl and produce more anthocyanin pigments (**Fig. 8A**, **C**). Presumably more Chl allows *AtNHX1/SOS1*-co-overexpressing plants to have higher photosynthetic rates under salt conditions, and more anthocyanin pigment allows *AtNHX1/SOS1*-co-overexpressing plants to remove ROS more effectively under salt conditions, consequently producing less MDA, the degradation product of lipid peroxidation caused by ROS (**Fig. 8B**). Furthermore, the seeds harvested from *AtNHX1/SOS1*-co-overexpressing plants after severe salt stress germinated at much higher frequencies than WT, *AtNHX1*-overexpressing and *SOS1*-overexpressing plants (**Figs. 7A**, **B**), whereas seeds from normally grown plants displayed similar germination rates (**Fig. 7C**, **D**), indicating that salt causes less damage to seeds from *AtNHX1/SOS1*-co-overexpressing plants than seeds from WT, *AtNHX1*-overexpressing and *SOS1*-overexpressing plants.

Overexpression of either *AtNHX1* or *SOS1* confers increased salt tolerance in transgenic plants (Apse et al. 1999, Shi et al. 2003), which has also been demonstrated in other plants (Zhang and Blumwald 2001, Zhang et al. 2001, He et al. 2005, Banjara et al. 2011, Yue et al. 2012). These studies indicated a potential for the use of *AtNHX1* and *SOS1* to create salt-tolerant crops. However, the increased salt tolerance by overexpressing these two genes separately is limited in transgenic plants. We and others observed that *AtNHX1*-overexpressing plants and *SOS1*-overexpressing plants could only tolerate NaCl concentrations up to 200 mM, and treatment with higher concentrations of NaCl would kill *AtNHX1*-overexpressing plants and *SOS1*-overexpressing plants, leading to no seeds being produced. In nature, saline conditions are widespread, and salt concentrations higher than 200 mM are common. It appears that simply overexpressing *AtNHX1* or *SOS1* alone cannot make plants tolerate a higher concentration of salt. Therefore, other approaches must be employed, and stacking genes that contribute to salt tolerance is one of them. Yang et al. (2009) successfully co-overexpressed *AtNHX1* with *SOS3*, *SOS2* with *SOS3*, and *SOS1* with *SOS2* and *SOS3* in transgenic Arabidopsis plants, but unfortunately they were not able to increase salt tolerance further in transgenic plants. All these transgenic plants, overexpressing either two genes (*AtNHX1* + *SOS3* or *SOS2* + *SOS3*) or three genes (*SOS1* + *SOS2* + *SOS3*), displayed similar salt tolerance to *SOS1*-overexpressing plants (Yang et al. 2009). This study suggested that simultaneously overexpressing genes in the same salt signaling pathway could not confer higher salt tolerance. Although the AtNHX1 pathway and the SOS pathway could cross-talk via SOS2, as SOS2 can regulate AtNHX1's activity on the vacuolar membrane, there does not appear to be a synergy between overexpression of *AtNHX1* and *SOS3*.

We have tested the effect of co-overexpression of a vacuolar proton pump AVP1 and a vacuolar Na^+/H^+ antiporter AtNHX1 in transgenic cotton, and we obtained substantially improved salt tolerance with *AVP1/AtNHX1*-co-overexpressing cotton plants (Shen et al. 2015). We demonstrated that by co-overexpression of *AVP1* and *AtNHX1*, we could significantly improve salt tolerance and drought tolerance in transgenic cotton, and improve fiber yield in laboratory, greenhouse and field conditions (Shen et al. 2015). *AVP1/AtNHX1*-co-overexpressing cotton outperformed *AVP1*-overexpressing cotton and *AtNHX1*-overexpressing cotton in every condition that we tested. We confirmed the hypothesis that co-overexpression of *AVP1* and *AtNHX1* could further improve salt tolerance and drought tolerance proposed by Gaxiola et al. (2002). Unfortunately the *AVP1/AtNHX1*-co-overexpressing cotton plants were created by crossing *AVP1*-overexpressing cotton and *AtNHX1*-overexpressing cotton, and *AVP1/AtNHX1*-co-overexpressing cotton plants that we used for studies were F_1 plants of the cross between *AVP1*-cotton and *AtNHX1*-cotton. It would be very difficult to bring these two genes into the homozygous state if we need to introduce them into a commercial cotton variety. Therefore, it will be an advantage to put the genes of interest in the same DNA construct for plant transformation if the purpose is to introduce these genes into crops that are in production, instead of just for demonstration of the proof of concept. Independent of our work, Bao et al. (2014, 2015) demonstrated that co-expression of *ZxNHX* and

ZxVP1-1, an Na^+/H^+ antiporter gene and a H^+ pump gene from the xerophyte plant *Zygophyllum xanthoxylum*, enhances salt and drought tolerance in transgenic *Lotus corniculatus*. All these findings support the idea that co-overexpression of multiple genes in the same construct would be a worthwhile approach in making crops more salt tolerant.

As well as the major contribution that it is possible to increase salt tolerance further in transgenic plants by stacking genes that contribute to salt tolerance in different salt signaling pathways, we made another important discovery that co-overexpression of *AtNHX1* and *SOS1* could significantly improve plant tolerance to multiple stresses. The temperature of 28°C reduced Arabidopsis growth slightly (**Figs. 9**, **10**), and it caused little difference between WT plants and transgenic plants. The 100 mM salt concentration caused more reduction in Arabidopsis root growth, and it caused the largest reduction in WT plants, and less reduction in *AtNHX1*-overexpressing and *SOS1*-overexpressing plants. With both stresses, *AtNHX1/SOS1*-co-overexpressing plants performed significantly better than *AtNHX1*-overexpressing and *SOS1*-overexpressing plants, and the WT plants showed the greatest reduction in root growth. Our results confirmed earlier discoveries that multiple stresses could cause greater damage to plants (Mittler 2006) than a single stress applied separately, and co-overexpression of *AtNHX1* and *SOS1* could substantially reduce the damage caused by the combined stresses of moderate high temperature and salt concentration. A recent report showed that the combined effect of heat and salt stresses led to a certain degree of resistance to salt stress, instead of generating the expected additive detrimental effects (Rivero et al. 2014). In our case, we clearly see that the combined stresses cause greater damage to all plants including WT and transgenic plants (**Figs. 9**, **10**). However, *AtNHX1/SOS1*-co-overexpressing plants displayed the highest resistance to the combined stresses of heat and salt. In soil conditions, *AtNHX1/SOS1*-co-overexpressing plants completed their life cycle, and produced viable seeds, whereas the WT plants were mostly dead (**Fig. 10**). Although the molecular mechanism of the increased salt tolerance in *AtNHX1/SOS1*-co-overexpressing plants is not clear at this time, as we do not know if the Na^+ concentration is further reduced in the cytoplasm of *AtNHX1/SOS1*-co-overexpressing plants, we do provide the proof of concept that co-overexpression of *AtNHX1* and *SOS1* would substantially improve salt tolerance in transgenic plants and provide better protection against combined stresses of heat and salt. This work should be applicable in improving agriculturally important crops such as rice, maize, wheat and cotton in the future.

Materials and Methods

Plant materials and growth conditions

For plate-grown plants, Arabidopsis seeds were sterilized by washing in 75% ethanol for 5 min, then 15% sodium hypochlorite for 8 min followed by rinsing with sterile H_2O. Sterilized seeds were then stratified in 0.07% agar for 4 d at 4°C. Following stratification, seeds were planted on MS solid medium (Murashige and Skoog 1962) vertically or horizontally (per experimental design) and grown under continuous light (100 μmol m^{-2} s^{-1}) at 22°C.

For soil-grown plants, stratified Arabidopsis seeds were germinated on MS plates for 12 d and then transferred to Sunshine soil mix (SunGro Horticulture) in 2.5, 3 or 4 inch pots. Plants were grown in a growth chamber (Conviron) with long-day conditions (16 h light/8 h darkness at 22/16°C cycle, respectively, and light strength of 150 μmol m^{-2} s^{-1}). Seedlings and leaves intended for molecular and biochemical analyses were harvested from plate- or soil-grown plants, frozen in liquid nitrogen and stored at −80°C until use.

Vector construction and Arabidopsis transformation

Total RNAs of 2-week-old WT Arabidopsis seedlings (Col-0 ecotype) were isolated using a protocol described by Hu et al. (2014), and cDNAs were synthesized using the Moloney murine leukemia virus reverse transcriptase (Promega). The *AtNHX1* gene (1,627 bp) was amplified from a cDNA library by PCR with a Q5 high fidelity polymerase (New England Biolabs) with the primers NHX1-F1 and NHX-R1. After the cDNA fragment was isolated and cleaned from the agarose gel using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research), it was digested with *Bam*HI. The pBI121 vector was digested with *Sac*I and blunt ended with T4 DNA polymerase. Then, the GUS (β-glucuronidase) sequence was removed by digesting the DNA with *Bam*HI. The resulting 12,864 bp binary vector backbone contains the *Cauliflower mosaic virus* (CaMV) 35S promoter, Nos terminator sequence and the selective marker gene *NPTII* (for kanamycin resistance). The processed cDNA fragment described above was ligated into this linear DNA to form an intermediate plasmid pBI121-NHX1.

For cloning the cDNA clone of the *SOS1* gene, we used two PCR primers, SOS1-F1 and SOS1-R1. The PCR product was purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research) and digested with *Xba*I and *Sac*I to obtain the 3.4 kb *SOS1* cDNA fragment ready for ligation. The pBI121 plasmid was modified by inserting a 339 bp *Hind*III fragment from the vector pJG4-5 (Golemis et al. 1996), resulting in a pBI121EE plasmid. The pBI121EE plasmid was digested with *Xba*I and *Sac*I to remove the GUS sequence and ligated with the *SOS1* cDNA fragment described above to form pBI121EE-SOS1. To combine the *SOS1* expression cassette and the *AtNHX1* expression cassette, pBI121EE-SOS1 was partially digested with *Eco*RI to yield a 4,864 bp expressing cassette including a CaMV 35S promoter, the *SOS1* cDNA and the Nos terminator. The pBI121-NHX1 plasmid was then digested by *Eco*RI and ligated with the *SOS1* cassette to yield the final pBI121-NHX1-SOS1 construct. The direction of the *SOS1* cassette in the final construct was checked by enzyme digestion before the construct was transformed into *Agrobacterium* GV3101 for Arabidopsis transformation.

There were 56 independent transgenic plants obtained using the 'floral dip' transformation method (Clough and Bent 1998). The seeds from T_1 plants, called T_2 plants, were screened on MS plates supplemented with kanamycin (30 mg l^{-1}) for segregation analysis. Plants that gave rise to roughly 3 : 1 kanamycin resistance vs. sensitivity were probably single T-DNA insertion plants. To screen for homozygous plants, T_3 plants that gave all kanamycin-resistant progeny were probably homozygous plants. RNAs from T_2 plants were used for RNA blot analysis to select high transgene expression plants. From the 56 initial putative transgenic lines, 39 homozygous lines were identified and analyzed via RNA blot analysis. Six lines that overexpress both transgenes at high levels were identified and used for further physiological analyses, and all of them gave similar results (only 3–5 are presented in the text). Several lines that either overexpress *AtNHX1* or *SOS1* were also identified and these lines were chosen as reference lines for this study (only one line for each gene, N and S, was presented here).

The PCR primers used to create the transforming vector are as follows: NHX1-F1, AGTCGGATCCATGTTGGATTCTCTAGTGTCGAAACTG; NHX1-R1, TCAAGCCTTACTAAGATCAGGAGGG; SOS1-F1, AGTCTCTAGAATGACGAC TGTAATCGACGCG; and SOS1-R1, AGTCGAGCTCTCATAGATCGTTCCTGAA AACGATT

Preparation of radioactive labels

Gene-specific probes used for RNA blot analyses were designed for *AtNHX1*, *SOS1* and *Actin8* that have lengths of 1,445, 1,959 and 502 bp, respectively. The pBI121-NHX1-SOS1 construct was used as the template from which to amplify

AtNHX1 or *SOS1* via PCR. A cDNA library was used to amplify the *Actin8* gene. The probe for DNA blot was made by using the *NPTII* gene fragment as the template, which was amplified by PCR with forward primer 121southern-1F and reverse primer 121southern-2R. PCR products were gel-purified and used for random priming reaction with the DECAprime™ II DNA Labeling Kit (Life Technologies) to label the PCR fragments radiochemically using [α-^{32}P]dATP as the ^{32}P donor.

The PCR primers used for making hybridization probes are as follows: NHX165 FW, 5′ TGATTGGGCTAGGCACTG 3′; NHX7 RW, 5′ AAGATCAGG AGGGTTTCTCTC 3′; SOS1-842-FW, 5′ GGGCTTCTGGTGTTTTGACG 3′; SOS1-2881-RW, 5′ CGTTAGAAGGTGATAATGCGGC 3′; Act8-Forward, 5′ TC ACCACAACAGCAGAGCGGG 3′; Act8-Reverse, 5′ GGACCTGCCTCATCATAC TCGG 3′; 121southern-1F, GATTGAACAAGATGGATTGCACG; and 121southern-2R, CCCGATCATATTGTCGCTCAGG

RNA blot and DNA blot analyses

Total RNAs (10 μg) from WT and transgenic plants were electrophoresed in a 1.2% agarose gel containing 0.4 M formaldehyde (w/v) and transferred to a Hybond-XL membrane (GE Healthcare Amersham). The membrane was pre-hybridized at 64°C for 1 h, and then hybridized with α-^{32} P-labeled probe(s) for 24 h at 64°C. The membrane was washed and incubated in a phosphor screen cassette (Fisher Scientific) for 24 h. The screen was imaged using a Personal Molecular Imager System (BioRad), and the level of *Actin8* or *18S rRNA* was used as the RNA loading control.

A 2 g aliquot of rosette leaves from each of the five transgenic lines and WT plants were used for genomic DNA extraction using the cetyltrimethylammonium bromide (CTAB) DNA extraction method with minor modifications (Paterson et al. 1993). After precipitation in 0.6 vol. of isopropanol, DNA was collected by centrifugation at 10,000×*g* for 5 min at room temperature, followed by draining off the isopropanol solution. After DNA was dissolved into 1 ml of TE buffer, 1 μl of RNase (10 mg ml^{-1}) was used to degrade RNA at 37°C for 2 h. Genomic DNA was then purified with phenol/chloroform extraction and isopropanol precipitation, and finally dissolved in 100 μl of TE buffer (pH 8.0) for measurement of the concentration using the Nanodrop ND-1000. A 20 μg aliquot of DNA from each line was digested with *Eco*RI in a 400 μl reaction volume according to the manufacturer's protocol (NEB). After overnight digestion, DNA was precipitated and dissolved into 33 μl of water, and mixed with 7 μl of 6× loading dye. The resultant 40 μl solution was loaded into a 0.8% agarose gel for electrophoresis overnight. Thereafter, membrane transfer and hybridization were done as previously described (Hu et al. 2014).

Salt tolerance test and osmotic stress experiments

For the root elongation assay, Arabidopsis seeds were directly sown on MS plates supplemented with 0, 75, 100, 150, 175 and 200 mM NaCl, and grown for 10–14 d before root lengths were measured. For salt tolerance test in soils, 3-week-old Arabidopsis plants were irrigated with water or incremental levels of NaCl concentrations of 50, 100, 150, 200 and 250 mM, at 4 d intervals. On day 24 after initiation of salt stress, pictures were taken and then plant materials were sampled for biochemical analyses of anthocyanin, Chl and lipid peroxidation products. Plant fresh weight was measured by weighing the above-ground portion of plants. Plants grown under normal conditions were used as non-stressed controls. For osmotic stress experiments, Arabidopsis seeds were directly sown on MS plates supplemented with 300 mM mannitol, and grown for 7 d vertically before root lengths were measured.

Combined stress experiment

Plants were sown on MS plates supplemented with 100 mM NaCl and grown at 28°C for 9 d before root lengths were measured. Control plants were grown on MS plates at 22°C for 9 d. For soil-grown plants, 3-week-old plants grown continuously at either 22 or 28°C were irrigated with incremental concentrations of NaCl starting from 50 mM up to 150 mM at 4 d intervals. Plants were irrigated with 150 mM NaCl solution until they were 6 weeks old, and then plant fresh weights were measured. Plants grown under the same conditions were harvested at the end of 8 weeks for counting siliques and seeds.

Analyses of chemical compounds

Chl measurements were conducted by using the methanol extraction method (Porra et al. 1989). About 100 mg of fresh leaf samples were used for methanol extraction for 24 h at 4°C. A 1 ml aliquot of the extraction solution was used per plant, and results represent the mean of three biological and three technical replicates. Concentrations of Chl *a* and *b* in 250 mM salt-stressed and well-watered control plants were calculated based on absorption at A_{665} and A_{652}. Molar extinction coefficients were used to convert the absorption values to relative amounts of Chl *a*, *b* and *a* + *b* for each sample (Porra et al. 1989).

Anthocyanin content was determined as described earlier (Solfanelli et al. 2006). Briefly, 200 mg of leaf tissues were ground in methanol-HCl solution along with a two-thirds volume dilution using distilled water. After extraction of the aqueous phase, an equal volume of chloroform was added. The samples were mixed and centrifuged (14,000 r.p.m. for 1 min). Light absorbance in the supernatant was then determined spectrophotometrically at A_{535} in a BioTek Synergy HT Microplate Reader (BioTek Instruments, Inc.). Values represent the mean of six independent replicates.

MDA content of 6-week-old plants was determined by using the thiobarbituric acid–MDA complex analysis method in 100 mg of leaf samples (Heath and Packer 1968). Absorbance readings at A_{532} and A_{600} were collected using a BioTek Synergy HT Microplate Reader (BioTek Instruments, Inc.). The results are presented as μmol of MDA per gram of plant FW, and values represent the mean of three biological and three technical replicates.

Germination rate test

Arabidopsis seeds harvested from plants grown under normal light and temperature conditions in soils after salt treatment (up to 250 mM NaCl) were surface sterilized, sown on MS medium and grown under continuous white light at 22°C. Seed germination was scored by the visibility of radicle emergence over a 7 d period with respect to a pre-determined baseline visibility.

Na^+, K^+, and Cl^- measurements

For the quantification of Na^+, K^+ and Cl^-, 20 mg of dried materials were weighed in 10 ml tubes. A 5 ml aliquot of ultrapure water was added and the tubes were boiled for 3 h at 100°C. Samples were then filtered and 8 ml of ultrapure water was added. For Na^+ and K^+ measurements, 1 ml of the filtrate was diluted with 1 ml of ultrapure water, and the resulting solution was analyzed for Na^+ and K^+ concentrations using an atomic absorption spectrometer (Analyst 200, PerkinElmer). The Cl^- ions were measured by diluting 1 ml of the filtrate with 19 ml of ultrapure water, and the Cl^- ion concentration was measured with the Ion Chromatography Instrument 883 Basic IC plus (Metrohm). Three technical replicates were used for all the measurements. The ion concentrations from transgenic plants were compared with those from WT plants; therefore, the relative ion content is presented.

Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze the difference of means among the WT and different transgenic plants at the 0.05 (5%) level of significance in hypothesis testing.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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