Arabidopsis AINTEGUMENTA mediates salt tolerance by trans-repressing SCABP8

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ABSTRACT

The Arabidopsis AINTEGUMENTA (ANT) gene, which encodes an APETALA2 (AP2)-like transcription factor, controls plant organ cell number and organ size throughout shoot development. ANT is thus a key factor in the development of plant shoots. Here, we have found that ANT plays an essential role in conferring salt tolerance in Arabidopsis. ant-knockout mutants presented a salt-tolerant phenotype, whereas transgenic plants expressing ANT under the 3SS promoter (3SS:ANT) exhibited more sensitive phenotypes under high salt stress. Further analysis indicated that ANT functions mainly in the shoot response to salt toxicity. Target gene analysis revealed that ANT bound to the promoter of SOS3-LIKE CALCIUM BINDING PROTEIN 8 (SCABP8), which encodes a putative Ca²⁺ sensor, thereby inhibiting expression of SCABP8 (also known as CBL10). It has been reported that the salt sensitivity of scabp8 is more prominent in shoot tissues. Genetic experiments indicated that the mutation of SCABP8 suppresses the ant-knockout salt-tolerant phenotype, implying that ANT functions as a negative transcriptional regulator of SCABP8 upon salt stress. Taken together, the above results reveal that ANT is a novel regulator of salt stress and that ANT binds to the SCABP8 promoter, mediating salt tolerance.

KEY WORDS: AINTEGUMENTA (ANT), SOS3-LIKE CALCIUM BINDING PROTEINS, SCABP8, CALCINEURIN B-LIKE10, Salt stress, Trans-reression, Shoot, Arabidopsis

INTRODUCTION

Soil salinity is a major abiotic stress that decreases plant growth, ranking among the leading factors that limit agricultural productivity. High concentrations of NaCl can arrest plant development and result in plant death. Salt stress triggers several interacting events in plants, including the inhibition of enzymatic activity in metabolic pathways, decreased uptake of nutrients in roots, decreased efficiency of carbon use, and the denaturation of protein and membrane structures.

The Salt Overly Sensitive (SOS) pathway is thought to play essential roles in conferring salt tolerance in Arabidopsis thaliana. SOS3, the first gene derived from this family that was cloned, was obtained through genetic screening of salt-sensitive mutants and map-based cloning (Liu and Zhu, 1998). SOS3 shows physical interaction with and activation of SOS2, a protein kinase (Halfter et al., 2000), thus forming a SOS2–SOS3 complex that in turn activates SOS1, a plasma membrane Na+/H+ antiporter (Shi et al., 2000; Qiu et al., 2002). Although Ca²⁺ is not required for SOS2 and SOS3 to interact in this process, Ca²⁺ is thought to increase the phosphorylation of the synthesized peptide substrate p3 through the SOS2–SOS3 complex (Halfter et al., 2000). SCABP8/CBL10 as a putative Ca²⁺ sensor interacts with the protein kinase SOS2 to protect Arabidopsis shoots from salt stress by modulating ion homeostasis (Quan et al., 2007; Kim et al., 2007). Similar functions in biochemical and cellular tests are fulfilled by SCABP8 and SOS3; however, they must also perform distinct regulatory functions in the salt stress response because they cannot replace each other in genetic complementation experiments, for example (Quan et al., 2007).

The mutations in SOS1, SOS2 and SOS3 all decrease the Na+/H⁺ exchange activity, and a constitutively active SOS2 enhances Na+/H⁺ exchange activity in an SOS1-dependent and SOS3-independent manner (Qiu et al., 2002). The root and shoot growth of sos1 and sos2 mutants is more significantly inhibited than that of sos3 under mild salt stress (Zhu et al., 1998). However, SCABP8/CBL10 mainly protects Arabidopsis shoots from salt stress (Quan et al., 2007; Kim et al., 2007). Consistent with their function, the expression of SOS1 and SOS2 is detected in both root and shoot tissues, whereas the expression of SCABP8 is mainly detected in shoot tissues (Liu et al., 2000; Shi et al., 2002; Quan et al., 2007; Kim et al., 2007).

AINTEGUMENTA (ANT), which encodes a transcription factor of the APETALA2 (AP2)-domain family, has been found only in plant systems (Mizukami and Fischer, 2000). Like AP2, ANT contains two AP2 domains that are homologous with the DNA-binding domain of ethylene-response-element binding proteins (Mizukami and Fischer, 2000). ANT as a transcription factor is strongly expressed in many dividing tissues in the plant, in which it plays central roles in developmental processes, such as shoot and flower meristem maintenance, organ size and polarity, flower initiation, ovule development, floral organ identity, as well as cell proliferation (Horstman et al., 2014).

Here, we report that ANT mainly protects Arabidopsis shoots from salt stress and show that SCABP8 is a downstream target of ANT in the regulation of salt stress because the mutation of SCABP8 suppresses the ant-knockout salt-tolerant phenotype, implying that SCABP8 plays a predominant role in inhibiting ANT-induced salt tolerance. Thus, ANT is a novel regulator of salt stress, binding to the SCABP8 promoter in order to mediate salt tolerance.

RESULTS

ANT expression patterns

The Arabidopsis ANT gene, encoding an AP2-like transcription factor, controls plant organ cell number and organ size throughout shoot development (Mizukami and Fischer, 2000). Using histochemical GUS staining to detect expression of GUS under the ANT promoter (ANT-pro:GUS), ANT was mainly detected in the
tissues undergoing active division (including young leaf blades, leaf veins, stem veins, stamens, pistils, meristem tissue and vascular cylinder) but not in mature tissue or root tissue (including the mature zone of roots and mature leaves) (Fig. 1A–H; also see Mizukami and Fischer, 2000; Elliott et al., 1996; Klucher et al., 1996). In the roots, ANT was only expressed in the root tips and vascular cylinder (Fig. 1G,H). Thus, it appears that ANT mainly functions in shoot tissue; this result is consistent with the role of ANT in controlling plant organ cell number and organ size throughout shoot development (Mizukami and Fischer, 2000). In this work, we found that the ant-knockout mutant, but not plants ectopically expressing ANT under the control of the constitutive 35S promoter (35S:ANT), exhibited a tolerant phenotype under high salt stress (Fig. 2). To examine whether the expression of ANT is induced by treatment with NaCl, total RNA was extracted from wild-type plants that had been treated with 75 mM NaCl or 100 mM NaCl for 3 and 6 h. The transcription of ANT was substantially lower at 3 and 6 h after treatment in comparison with that of the control plants (supplementary material Fig. S1A).

ant-knockout and 35S:ANT show different responses to high sodium salt stress

To further determine whether ANT is involved in the regulation of the salt stress response, ant-knockout (stock, SALK-022770) and 35S:ANT plants were analyzed. Reverse-transcriptase (RT)-PCR analyses indicated that the 35S:ANT lines over-accumulated ANT transcripts compared with the wild type, whereas ANT had been eliminated in the ant-knockout (SALK-022770) lines (see supplementary material Fig. S1B).

Seeds of the ant-knockout mutant, 35S:ANT and wild-type (Col-0) lines were sown on solid Murashige and Skoog (MS) medium with 100 mM NaCl for 30 days. The salt tolerance of the ant-knockout plants was maintained throughout their development from seedlings to mature plants, whereas a number of 35S:ANT transgenic plants were dry and dead compared with the wild type (Col-0) (supplementary material Fig. S2A). The ant-knockout exhibited longer primary roots, whereas the 35S:ANT plant showed shorter primary roots than the wild type (supplementary material Fig. S2A,B). The number of ant-knockout lateral roots was increased, whereas the number of 35S:ANT lateral roots was not different to that of the wild type (supplementary material Fig. S2A,C). Consequently, although the overall weight of fresh roots of the ant-knockout mutant was only ~1.6 times greater than that of the wild type (supplementary material Fig. S2E), the weight of fresh shoots in the ant-knockout mutant was increased ~2.9 times over that of the wild type (Col-0), and its leaves were also larger (supplementary material Fig. S2A,D). However, the seedling weight of ant-knockout, 35S:ANT and wild-type plants was not different on medium without salt (supplementary material Fig. S3).

To further characterize the salt insensitivity of the 35S:ANT and ant-knockout mutant plants, 5-day-old seedlings were transferred to solid MS medium with NaCl (75 mM NaCl and 100 mM NaCl) for 15 days. On solid MS medium with both 75 mM NaCl and 100 mM NaCl, the length of the ant-knockout primary roots was increased, whereas the length of the 35S:ANT primary roots was not different to that of the wild type (Fig. 2A,B). On MS medium with 75 mM NaCl, the number of ant-knockout lateral roots increased, whereas the number of 35S:ANT lateral roots was not significantly different to that of the wild type (Fig. 2A,C). However, on MS medium with 100 mM NaCl, the number of ant-knockout lateral roots increased, whereas the number of 35S:ANT lateral roots was reduced relative to that of the wild type (Fig. 2A,C). Consequently, the overall weight of fresh roots in the ant-knockout mutant increased relative to that of the wild type (Col-0) by only ~1.16- and ~1.22-fold on MS medium with 75 mM NaCl and 100 mM NaCl, respectively (Fig. 2A,E), whereas the weight of fresh shoots in the ant-knockout mutant was ~1.44 and ~1.53 times greater on MS medium with 75 mM NaCl and 100 mM NaCl, respectively (Fig. 2A,D). Moreover, the weight of 35S:ANT fresh shoots was decreased on MS medium with 100 mM NaCl (Fig. 2A,D). However, the seedling weight of ant-knockout, 35S:ANT and wild-type plants was not different on medium that lacked salt (supplementary material Fig. S3).

To confirm that ANT is involved in the regulation of salt stress, we assayed whether transforming the mutant with ANT genomic DNA (ant-knockout+35S:ANT) could render the ant-knockout less sensitive to salt, similar to wild type. Five-day-old ant-knockout, ant-knockout+35S:ANT, 35S:ANT and Col-0 seedlings were transferred to solid MS medium with 100 mM NaCl for 10 days. On solid MS medium without NaCl, the weight of the 15-day-old shoots did not differ (supplementary material Fig. S3A,B). However, on MS medium with 100 mM NaCl, the weight of 15-day-old ant-knockout shoots increased, whereas that of corresponding 35S:ANT plants declined (supplementary material Fig. S3A,B). Furthermore, the weight of shoots of ant-knockout+35S:ANT and Col-0 seedlings did not differ (supplementary material Fig. S3A,B), indicating that the ant-knockout mutant phenotype can be rescued.

Taken together, our findings indicate that ANT functions predominantly in the shoot response to salt toxicity.
The growth defects of ant-knockout and 35S:ANT seedlings are not specific to the Na⁺ response

To determine whether the growth defects of the ant-knockout mutant and 35S:ANT seedlings are specific to the Na⁺ response; these seedlings were treated with different kinds of salt. Upon treatment with 100 mM KCl, ant-knockout and 35S:ANT seedlings were less sensitive and more sensitive relative to wild type, respectively (Fig. 3A–D). Similarly, upon treatment with 10 mM LiCl, ant-knockout mutant and 35S:ANT seedlings also appeared to be less sensitive and more sensitive relative to wild type, respectively (Fig. 3A–D). Furthermore, ant-knockout mutant and 35S:ANT seedlings that had been treated with a combination of both 100 mM KCl and 10 mM LiCl exhibited less-sensitive and more-sensitive phenotypes relative to those observed upon single treatment with 100 mM KCl or 10 mM LiCl, respectively (supplementary material Fig. S4). However, the weight of ant-knockout, 35S:ANT and wild-type seedlings did not differ on medium that lacked salt (supplementary material Fig. S4).

ANT negatively modulates SCABP8 at the level of transcription

To detect the downstream target genes of ANT, the expression levels of the genes involved in the salt stress response were analyzed by using quantitative RT-PCR analyses. The SOS pathway plays an essential role in the salt tolerance of Arabidopsis thaliana. Thus, SOS1, SOS2, SOS3 and SCABP8 were the first genes examined. Transcripts of SCABP8 were much more abundant in ant-knockout plants, whereas transcripts of SCABP8 were lower in 35S:ANT plants compared with those in the wild type (Fig. 4A). We also observed that the expression levels of SOS1, SOS2 and SOS3 were not significantly different between ant-knockout, 35S:ANT and wild-type plants (Fig. 4A). Moreover, ANT transcript abundance was similar between wild-type, sos1, sos2, sos3 and scabp8 plants (Fig. 4B). Therefore, ANT negatively modulates SCABP8 expression at the transcript level. Plant NHX1, NHX4, NHX5 and NHX6 transporters are involved in salt tolerance (Hernandez et al., 2009; Bassil et al., 2011). These findings demonstrate that the expression levels of the genes encoding these transporters were not altered in 35S:ANT seedlings relative to those in wild type (Fig. 4G), indicating that ANT specifically regulates SCABP8.

ANT associates with the SCABP8 promoter both in vitro and in vivo

To investigate the binding of ANT to the SCABP8 promoter, we performed nucleotide sequence analysis and found that the two AP2 domains in ANT were selectively associated with the consensus sequence 5′-gCAC(A/G)N(A/T)TcCC(a/g)ANG(c/t)-3′ (lowercase letters indicate somewhat less-conserved positions that are present in at least 65% of the selected sites) (Nole-Wilson and Krizek, 2000;
Our nucleotide sequence analysis also found that the promoters of the SCABP8 gene contained conserved sequence motifs (Fig. 5 and Fig. 4C). This portion of ANT corresponds to amino acid residues 276–456 of full-length ANT, comprising the two AP2 repeats and the linker region between them, and will be referred to as ANT-AP2R1R2 (Nole-Wilson and Krizek, 2000). Glutathione S-transferase (GST) was fused to the DNA-binding domain of ANT-AP2R1R2, and the protein was expressed in Escherichia coli and purified by using the GST tag. A gel mobility shift assay was performed to assay whether the recombinant ANT-AP2R1R2 protein could associate with the SCABP8 promoter. When the ANT-AP2R1R2 protein and the probes to the ANT-binding consensus sequences were added, a shift was detected as a result of DNA binding; no band was detected when only the probes to the ANT-binding consensus sequences were added (Fig. 5A,B). When unlabeled probes of the ANT-binding consensus sequences were added to the reaction mixture, the DNA-binding was abolished (Fig. 5B). Furthermore, the GST–ANT-AP2R1R2 protein was unable to bind to mutated DNA probes (Fig. 5C,D). These data indicate that the ANT-AP2R1R2 DNA-binding site binds to the SCABP8 promoter in vitro.

To further investigate the possible binding in vivo, we used transgenic shoots expressing ANT with a C-terminal hemagglutinin (HA) tag under the control of the constitutive 35S promoter (35S: ANT-HA construct) for a chromatin immunoprecipitation (ChIP) analysis. The use of the S1 region (−1456 to −1152 bp) of the primer resulted in greater amounts of PCR product than the use of the S2 (−1152 to −852 bp) or S3 (−348 to −48 bp) regions (Fig. 4D,E). These data clearly indicate that ANT binds to the SCABP8 promoter in vivo, which is required to suppress SCABP8 expression.

**ANT acts genetically upstream of SCABP8**

To assay whether ANT acts genetically upstream of SCABP8 in regulating the salt stress response, we performed a double-mutant analysis by combining ant-knockout with scabp8 lines (to generate ant-knockout-scabp8) that were insensitive and supersensitive to high salt stress, respectively. We selected the genotypes from the segregating F3 population by PCR genotyping. Mutation of SCABP8 significantly suppressed the insensitive phenotype of the ant-knockout line under high salt stress (Fig. 6). The length of the ant-knockout primary roots obviously increased, whereas the lengths of the ant-knockout-scabp8 and scabp8 primary roots were significantly reduced relative to that of the wild type on solid MS medium with NaCl (75 mM NaCl and 100 mM NaCl) (Fig. 6A,B). The overall weights of fresh shoots and roots in the ant-knockout mutants were increased, whereas those in the ant-knockout-scabp8 and scabp8 mutants were decreased relative to those of the wild type on MS medium with 75 mM NaCl and 100 mM NaCl, respectively (Fig. 6A,C,D). Taken together, the above results indicate that ANT acts genetically upstream of SCABP8 in regulating the salt stress response (Fig. 7).
ANT, encoding a transcription factor of the APETALA2 family, is involved in cell proliferation and growth control (Elliott et al., 1996; Klucher et al., 1996; Eshed et al., 1999; Krizek, 1999; Mizukami and Fischer, 2000; Nole-Wilson and Krizek, 2000). Here, we have shown that ant-knockout mutants exhibit a salt-tolerant phenotype, whereas transgenic plants harboring the 35S:ANT construct exhibit a more sensitive phenotype under high salt stress. Under normal growth conditions in the glasshouse, 35S:ANT exhibited a greater cell number and larger organ size (seeds, leaves, flowers, large root systems and higher weight) (Mizukami and Fischer, 2000), at the same time, 35S:ANT plants were hypersensitive to salt stress. Thus, a regulator of plant development might be involved in modulating environmental stress. Arabidopsis AN3 (also known as GIF1), a transcription co-activator, is an important regulator of leaf and light-induced root development (Kim and Kende, 2004; Horiguchi et al., 2005; Meng, 2015). Very recently, we have found that AN3 controls water-use efficiency (WUE) and drought tolerance by regulating stomatal density and improving root architecture through the trans-repression of YODA (YDA) (Meng and Yao, 2015). The Arabidopsis E3 SUMO ligase SIZ1 regulates both plant growth and drought tolerance, that is, the null mutant siz1-3 shows smaller organ size owing to reduced expression of genes involved in brassinosteroid biosynthesis and signaling, at the same time, mutant seedlings of...
siz1-3 exhibit significantly lower tolerance to drought stress (Catala et al., 2007). Loss-of-function of PROTEIN S-ACYL TRANSFERASE10 (PAT10) leads to pleiotropic growth defects, including smaller leaves, dwarfism and sterility, whereas pat10 mutants exhibit hypersensitivity to salt stresses (Zhou et al., 2013). NHX1 (K+/H+) and NHX2 are vacuolar proteins, and they control vacuolar pH and K+ homeostasis, and salt tolerance (Bassil et al., 2011). Thus, the role of NHX1 and NHX2 is to regulate K+/H+ exchange, and this exchange modulates cell expansion in rapidly elongating tissues—such as filaments and hypocotyls—and plays unique and specific roles in flower development (Bassil et al., 2011). We speculate that, similar to NHX1 and NHX2, ANT is involved in regulating either plant growth or development through regulation of the SOS system, in which SCABP8/CBL10 as a putative Ca2+ sensor interacts with the protein kinase SOS2 to protect Arabidopsis shoots from salt stress by modulating ion homeostasis (Quan et al., 2007; Kim et al., 2007). The connection between plant development and environmental stress could open up future research to provide further insights into the mechanism that links development and stress.

On MS medium supplemented with 100 mM NaCl, sos3, sos2, sos1 and scabp8 mutants exhibited severe growth suppression in both shoot and root tissues; however, nearly complete growth arrest occurred only in the roots of sos3 and the shoots of 35S:ANT and scabp8 plants (Zhu et al., 1998; Quan et al., 2007; Fig. 2). At a lower NaCl concentration (75 mM), the root growth in the sos3 mutants was significantly suppressed (Zhu et al., 1998), whereas the shoot growth in the 35S:ANT and scabp8 mutants was more inhibited (Quan et al., 2007; Fig. 2). These findings tell us that salt tolerance in plants under extreme conditions is necessary for the functional integration of all tissues but also that SOS3, ANT and SCABP8 are needed for salt tolerance in a tissue-specific manner. Their respective expression patterns were consistent with these findings, as assayed by using promoter–GUS fusions (Fig. 1; also see Quan et al., 2007). SOS3 was only expressed in root tissues, particularly in root tips, whereas ANT and SCABP8 were preferentially expressed in shoot tissues. Thus, SCABP8 and ANT mainly protect shoot tissues, whereas SOS3 protects root tissues from salt stress, suggesting that tissue specificity could be important for plants coping with environmental stress.

Although the expression of SCABP8 was upregulated upon salt stress, the expression of ANT was downregulated, indicating that ANT and SCABP8 differ in their transcriptional regulation in response to salinity. Functional analyses, as well as the specific Na+ sensitivity of the sos1, sos2, sos3 and scabp8 mutants, indicate that SOS3 and SCABP8 have overlapping functions in the activation of SOS2 and its downstream target, SOS1 (Fig. 7; also see Quan et al., 2007). Thus, we conclude that ANT directly binds to the SCABP8 promoter in order to regulate the SOS signaling cascade in the salt stress response.

MATERIALS AND METHODS

Plant materials and growth conditions
sos1, sos2, sos3 and scabp8 mutants in the Col-0 background have been described previously (Quan et al., 2007). ant-knockout (SALK-022770), ant-knockout+35S:ANT and 35S:ANT seeds (Ph2GW7; Col-0 background; homozygote; hygromycin) were kindly provided by Professor H. G. Nam (Daegu Gyeongbuk Institute of Science and Technology [DGIST], Korea). ant-knockout (SALK-022770) is a T-DNA mutant, the single-pass
sequence of which was recovered from the left border of the T-DNA; and this sequence lies within 300 bases of the 3′-end of At4g37750, as has been described by Arabidopsis Biological Resource Center (ABRC). Transgenic plants were generated using the Agrobacterium tumefaciens-mediated floral dip method (Zhang et al., 2006). For ant-knockout and 35S:ANT identification, the primers forward 5′-ATGAAGTCTTTTTGTGATAAT-GA-3′ and reverse 5′-TTCAATCTCTTTCTGATAATTCTC-3′ were used. ant-8 (CS3944) was obtained from ABRC. Double ant-knockout-scabp8 mutants were generated through genetic crosses, and homozygous lines were identified through comparison with the parental phenotype and PCR-based genotyping. The ANT-gene-specific primers were forward 5′-GAGAAGGAAGCAGTGGTTTCT-3′ and reverse 5′-CAGATGTTCGGATCCAATATGG-3′; the SCABP8 gene-specific primers have been described previously (Quan et al., 2007). Plants exhibiting the 35S:ANT phenotype [large rosette leaves and large seeds grown under white light (Mizukami and Fischer, 2000)] in the F2 populations were screened for Dr5:GUS expression in roots. F3 seeds were collected from those exhibiting expression, and lines expressing GUS in all F3 plants were used for subsequent analysis.

The seeds were subjected to 4°C for 3 days and then sown onto solid MS medium that had been supplemented with 1% sucrose. The roots, seeds, leaves and embryos were photographed, and then they were analyzed using ImageJ software. The seedlings grown on agar were maintained in a growth room under 16-h–8-h light–dark cycles with cool white fluorescent light at 21°C±2°C. Plants grown in soil-less medium were maintained in a controlled-environment growth room under 16-h–8-h light–dark cycles with cool white fluorescent light at 21°C±2°C.

Salt sensitivity

Seeds of the mutant and wild-type (Col-0) plants were sterilized in a solution containing 30% sodium hypochlorite and 0.1% Triton X-100 for 10 min, washed five times with sterilized water, and sown on MS medium with 0.8% Phytagel (Sigma-Aldrich). The plates were placed at 4°C for 3 days, and the seeds were then germinated vertically at 21°C±2°C under continual illumination. Five-day-old seedlings with a root length of

Fig. 6. scabp8 mutation suppresses the ant-knockout mutant phenotype. (A) Seedlings were grown on solid MS medium without NaCl for 5 days and were then transferred to solid MS medium with 75 mM NaCl or 100 mM NaCl for 15 days. (B) Bar graph exhibiting the differences in primary root length between the indicated 20-day-old seedlings grown on solid MS medium with 75 mM NaCl or 100 mM NaCl; n=12. (C) Bar graph exhibiting the differences in the weight of fresh shoots between the indicated 20-day-old seedlings grown on solid MS medium with 75 mM NaCl or 100 mM NaCl; n=12. (D) Bar graph exhibiting the differences in root fresh weight between the indicated 20-day-old seedlings grown on solid MS medium with 75 mM NaCl or 100 mM NaCl. Error bars represent s.d.; **P<0.01, *P<0.05; n=12. ant-KO, ant knockout; ant-KOscabp8, ant-KO-scabp8; WT, wild type.

Fig. 7. Salt stress induces downregulation of ANT. ANT associates directly with the SCABP8 promoter and causes the upregulation of SCABP8, thereby inducing salt stress tolerance in the plant. In vitro, SOS3 physically interacts with and activates a Ser/Thr protein kinase, SOS2. Among the downstream targets of the SOS3–SOS2 complex is SOS1, a plasma-membrane–localized Na+/H+ antiporter (Shi et al., 2000; Qiu et al., 2002; Quintero et al., 2002). SCABP8/CBL10, a putative Ca2+ sensor, interacts with the protein kinase SOS2 to protect Arabidopsis shoots from salt stress (Quan et al., 2007).
and 1.5 cm were transferred onto MS medium with salt added as previously described (Quan et al., 2007).

**RT-PCR**

Total RNA was extracted from the tissues indicated in the figures using TRIZOL reagent (Invitrogen), as has been previously described by Yu et al. (2008). SYBR green was used to monitor the kinetics of the PCR products in the real-time RT-PCR analyses, as has been previously described by Yu et al. (2008). To analyze the expression levels of **SOS1**, **SOS2**, **SOS3** and **SCABP8** in Col-0, **ant**-knockout and **3SS:ANT** seedlings, the following primers were used – forward 5′-ATGACGACT-GTAATCGACGCGC-3′ and reverse 5′-CGATTTCCAATGCCGCTT-3′ for **SOS1**; forward 5′-ATGACAAAGAAATGGAAGAGT-3′ and reverse 5′-CACAACACTCCAAATATAT-3′ for **SOS2**; forward 5′-TGGCAATGGCACCACCCGGAT-3′ and reverse 5′-TTTCATGGACC-GGCCTTCG-3′ for **SOS3**; forward 5′-ATTGCCAGACCATCCTGC-3′ and reverse 5′-TGGAGCTTGAACAGCCCGAG-3′ for **SCABP8**. To analyze the expression levels of **NHX1**, **NHX4**, **NHX5** and **NHX6** in Col-0 and 3SS:ANT seedlings, the following primers were used – forward 5′-ATGTTGGATTCCTCTAGTGTCA-3′ and reverse 5′-AGCAAAATTGCG-GAAAAAAGCCTTT-3′ for **NHX1**; forward 5′-ATGGTGATGCCGATTAA-GCACAAT-3′ and reverse 5′-TGGAGAAGATTAATAGAAGAG-3′ for **NHX2**; forward 5′-ATGGAGAAAGTAGATGATCTC-3′ and reverse 5′-TTTAGGTTGAAGACTGAAACCTG-3′ for **NHX3**; forward 5′-ATGC-TGGCGAGGTCGAGAT-3′ and reverse 5′-GAAGAAGAAGCTCAG-GTTGAA-3′ for **NHX6**.

To analyze **ANT** expression in Col-0, **sos1**, **sos2**, **sos3** and **scabp8** seedlings, the primers forward 5′-AGTGCGAGCAGGCAAGGTTG-3′ and reverse 5′-AGGCAACGGAAATCCGCG-3′ were used. To analyze the expression levels of **ANT** in Col-0, **ant**-knockout and **3SS:ANT** seedlings, the following primers were used – forward 5′-CTCTCTCTCTG- TTCCACCTCAC-3′ and reverse 5′-CTCTGCACTTCTTCGTTG-3′ and forward 5′-CTTGAGAGAAGAACGACGT-3′ and reverse 5′-ATACGTCGGGAGTTCCTC-3′ were used for **TUB4**.

**Plasmid constructs**

For the **ANT** promoter analysis, the promoter-GUS construct At4g37750 was created by inserting approximately 2.0-kb promoter fragments into pCB308R, as previously described by Lei et al. (2007). The primers used for **ANT** were P1 5′-ggggacagtgtggaaaaagacgagcttggcttataaatagttgctcacttcgctgtgcaaggaaagatggagttttg-3′ and P2 5′-ggggacagtgtggaaaaagacgagcttggcttataaatagttgctcacttcgctgtgcaaggaaagatggagttttg-3′. To obtain the **ANT**-**H4** plasmid (CB2004), we used the primers forward 5′-ggggacagtgtggaaaaagacgagcttggcttataaatagttgctcacttcgctgtgcaaggaaagatggagttttg-3′ and reverse 5′-ggggacagtgtggaaaaagacgagcttggcttataaatagttgctcacttcgctgtgcaaggaaagatggagttttg-3′. Lowercase letters denote sequences from the connector primers, whereas capital letters denote the sequences of targeted genes.

**GUS staining**

Using a buffer mix [1 mM X-gluc, 60 mM NaPO₄ buffer, 0.4 mM of K₃Fe(CN)₆/K₄Fe(CN)₆, and 0.1% (v/v) Triton X-100], the samples (transgenic plants harboring and expressing **ANT-Pro:GUS**) were stained and then incubated at 37°C for 8 h. After GUS staining, chlorophyll was removed using washes of 30%, 50%, 70%, 90% and 100% ethanol for approximately 30 min each. GUS staining was performed as previously described by Lei et al. (2007).

**ChiP assay**

Transgenic lines overexpressing 3SS:ANT-HA were used in this assay. ChiP was performed as previously described (Yoo et al., 2010). HA- and green fluorescent protein (GFP)-tag-specific monoclonal antibodies were used for ChiP analysis. The ChiP DNA products were analyzed by using quantitative RT-PCR with primers that had been designed to amplify ~300-bp DNA fragments in the promoter region of **SCABP8**. The following primer sequences were used – S1 P1 5′-ATAAACAATTTTTATATCTCGATA-3′, P2 5′-ATTTTTGACCCTTATTTTGTTTAT-3′, S2 P3 5′-AAGATTGACAC- AAATTTTTC-3′, P4 5′-AATACAAATATATGCGACCATTG-3′; S3 P5 5′-GGTGAGATCCACCTAGTATAT-3′, P6 5′-TGTTCTTATCACAATCTGTA-3′.


**Figure S1.** ANT expression analysis.

A. ANT expression analysis after 0.0 mM NaCl, 75 mM NaCl, 100 mM NaCl treatment for 3 h. The 0.0 mM NaCl treatment was set as 1.0. Quantifications were normalized to the expression of UBQ5. Error bars represent SD (n=3).

B. Molecular identification of 35S:ANT and ant-KO (KO-knock out) mutants. ANT mRNA levels are higher in 35S:ANT transgenic plants relative to wild-type plants, but ANT mRNA levels was not detected in the ant-KO mutant lines (SALK-022770) relative to wild-type plants. TUB4 is as a control.
**Figure S2.** *ant-KO* plants and *35S:ANT* showed resistance and sensitivity to salt stress, respectively.

(A). Representative 30-day-old *ant-KO*, wild-type (Col-0) and *35S:ANT* plants grown on solid MS medium with 100 mM NaCl. Magnification is the same for each image. Bar=2.0 cm.

(B), (C), (D) and (E). Bar graph exhibiting the differences in primary root length (B), lateral root number (C), shoot fresh weight (D) and root fresh weight (E) between the 30-day-old *ant-KO*, wild-type (Col-0) and *35S:ANT* plants grown on solid MS medium with 100 mM NaCl. Error bars represent SD (**P < 0.001, *P < 0.01, *P < 0.05; n=20).
Figure s3. ANT is involved in regulation of salt stress.

(A). Representative seedlings of 15-day-old indicated seedlings grown on solid MS medium without NaCl (a) and with 100.0 mM NaCl (b). Magnification is the same for each image. Bar=2.0 cm.

(B). Bar graph exhibiting the differences in shoot fresh weight in (A). Error bars represent SD (**P < 0.01, *P < 0.05; n=20).
Figure S4. *ant-KO* (KO-knock out) seedlings showed different response from the wild type (Col-0) to KCl+LiCl.

Seedlings were grown on solid MS medium without NaCl for 5 days and were then transferred to solid MS medium with 10 mM LiCl +100 mM KCl for 15 days.

(A). Representative 20-day-old 35S:ANT (a), wild-type (Col-0) (b) and *ant-KO* (c) seedlings grown on solid MS medium with 10 mM LiCl +100 mM KCl for 15 days. Magnification is the same for each image.

(B), (C) and (D). Bar graph exhibiting the differences in primary root length (B), lateral root number (C) and shoot fresh weight (D) between the 20-day-old *ant-KO*, wild-type (Col-0) and 35S:ANT seedlings grown on solid MS medium with 100 mM KCl +10 mM LiCl. Error bars represent SD (*P < 0.05; **P < 0.01; n=16).