ORIGINAL ARTICLE

Expression of an *Arabidopsis* sodium/proton antiporter gene (*AtNHX1*) in peanut to improve salt tolerance

Manoj Banjara · Longfu Zhu · Guoxin Shen · Paxton Payton · Hong Zhang

Abstract Salinity is a major environmental stress that affects agricultural productivity worldwide. One approach to improving salt tolerance in crops is through high expression of the *Arabidopsis* gene *AtNHX1*, which encodes a vacuolar sodium/proton antiporter that sequesters excess sodium ion into the large intracellular vacuole. Sequestering cytosolic sodium into the vacuoles of plant cells leads to a low level of sodium in cytosol, which minimizes the sodium toxicity and injury to important enzymes in cytosol. In the meantime, the accumulation of sodium in vacuoles restores the correct osmolarity to the intracellular milieu, which favors water uptake by plant root cells and improves water retention in tissues under soils that are high in salt. To improve the yield and quality of peanut under high salt conditions, *AtNHX1* was introduced into peanut plants through *Agrobacterium*-mediated transformation. The *AtNHX1*-expressing peanut plants displayed increased tolerance of salt at levels up to 150 mM NaCl. When compared to wild-type plants, *AtNHX1*-expressing peanut plants suffered less damage, produced more biomass, contained more chlorophyll, and maintained higher photosynthetic rates under salt conditions. These data indicate that *AtNHX1* can be used to enhance salt tolerance in peanut.

Keywords Salt tolerance · Sodium/proton antiporter · Photosynthesis · Photosynthetic parameters

Introduction

Salinity is a soil condition characterized by a high concentration of soluble salts. About 20% of the world's cultivated land and nearly half of the irrigated lands are unsuitable for growing crops because of contamination with high levels of salt (Rhoades and Loveday 1990). High salinity in the soil makes it harder for plant roots to extract water, and a high concentration of salt within plant cells can be toxic to the cellular enzymes (Munns and Tester 2008). Most salt-affected land arises through natural causes, from the accumulation of salts over long periods of time in arid and semiarid zones (Rengasamy 2002). Sodium chloride is an abundant and soluble salt released in soil by the degradation of native saline rocks (Szabolcs 1989).

Peanut (*Arachis hypogaea* L.) is a glycophytic plant whose growth and yield are severely inhibited by high salinity. It is the twelfth most valuable cash crop in the United States, with a farm value of over one billion US dollars. Texas is ranked second among the peanut-growing states, and it accounts for approximately a quarter of all peanuts grown in the United States (http://www.peanutsusa.com).

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Salinity and drought are the two major abiotic stresses that limit agricultural productivity in the southwestern USA, including Texas. Increasing peanut tolerance of salt stress will likely improve its yield, which will have a major impact on the economy of Texas as well as peanut production areas in other arid and semi-arid regions of the world.

Salinity tolerance is a complex multigenic trait that may involve a large number of genes, and these genes are likely responsible for (1) encoding proteins to sequester Na^+ into the vacuole or efflux Na^+ to the apoplast, (2) synthesizing compatible solutes, and (3) radical-scavenging enzymes (Parida and Das 2005). Excess sodium ion (Na^+) in cytosol may be excluded to the apoplastic space via the plasma membrane (Zhu 2003; Shi et al. 2002) or sequestered into the large intracellular vacuole via the tonoplast membrane (Apse et al. 1999; Blumwald 2000) by active mechanisms. Accumulation of ions in the vacuole acts as an osmoticum to maintain water flow into the cell (Glenn et al. 1999; Gaxiola et al. 2002). Apse et al. (1999) demonstrated that overexpressing a single *Arabidopsis* gene, *AtNHX1*, which encodes a vacuolar membrane-bound sodium/proton (Na^+/H^+) antiporter, could confer a high salt tolerance on transgenic plants. It was thought that the proton electrochemical gradient (PEG) across the tonoplast membrane is the driving force for Na^+ to flow into vacuoles via the activity of the Na^+/H^+ antiporter, which is likely the molecular basis for the *AtNHX1* overexpression that leads to increased salt tolerance in transgenic plants (Blumwald 2000). However, recent studies indicate that increased K^+ homeostasis, cytosolic pH regulation, vesicle trafficking, and protein targeting are all likely to contribute to the increased salt tolerance in *AtNHX1*-overexpressing plants (Rodríguez-Rosales et al. 2009; Leidi et al. 2010). Transgenic plants that express *AtNHX1*, such as *Arabidopsis* (Apse et al. 1999), tomato (Zhang and Blumwald 2001), rapeseed (Zhang et al. 2001), cotton (He et al. 2005), tobacco (Duan et al. 2009), tall fescue (Tian et al. 2006), *Petunia hybrida* (Xu et al. 2009), and soybean (Li et al. 2010), all displayed increased salt tolerance. Since overexpression of *AtNHX1* in native and heterologous systems leads to the same phenotype—increased salt tolerance, this approach should work in most plants, including peanut. Therefore, we expressed *AtNHX1* in peanut in order to increase its salt tolerance. As we expected, *AtNHX1*-expressing peanut plants produced higher biomass in greenhouse conditions than wild-type plants under 150 mM NaCl treatment. Also, the physiological and biochemical parameters of transgenic peanut plants are better than wild-type control plants grown under greenhouse conditions, indicating that *AtNHX1* can indeed increase salt tolerance in peanut.

Materials and methods

Peanut transformation

The recombinant vector *35S-AtNHX1* (He et al. 2007) was introduced into the *Agrobacterium tumefaciens* strain GV3101 and used to transform wild-type peanut (Flavor runner 458). The transformation protocol described by Sharma and Anjaiah (2000) was followed.

Isolation of genomic DNAs and polymerase chain reaction analysis

The Powerplant™ DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) was used to isolate genomic DNA from fresh leaves of greenhouse-grown peanut plants. The concentration and purity of the genomic DNA were determined by a NanoDrop® (ND-1000 spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA).

Polymerase chain reaction (PCR) was carried out with a thermocycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany) to amplify a fragment of the *AtNHX1* gene using EcnoTaq® DNA polymerase (Lucigen Corporation, Middleton, WI, USA). Primers NHX-1 and NHX-2 were designed based on the known sequences of *AtNHX1*. The 25 µl PCR reaction mixture contained 5 µl genomic DNA (0.1 µg/µl), 2.5 µl 10× EconoTaq reaction buffer [10 mM Tris–HCl (pH 9.0), 500 mM KCl, 15 mM $MgCl_2$, and 1% Triton X-100], 2 µl dNTP (2.5 mM concentration for each of the four different deoxyribonucleotides), 1 µl forward primer NHX-1 (0.1 µg/µl), 1 µl reverse primer NHX-2 (0.1 µg/µl), 0.25 µl thermotolerant EconoTaq DNA polymerase enzyme, and 13.25 µl H_2O. The amplification reactions were carried out under the following conditions: an initial denaturation at 94°C for 4 min, followed by 35 denaturation cycles at 94°C for 1 min, annealing at 52°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 10 min. The amplified products were segregated by electrophoresis on 0.8% agarose gels with ethidium bromide and visualized under UV light. The nucleotide sequences of NHX-1 and NHX-2 are as follows: NHX-1, 5′-TGATTGGGCTAGGCACTG-3′; NHX-2, 5′-CAGCTTCGTGGTTTAGGTGA-3′.

Isolation of total RNAs and reverse-transcription PCR analysis

About 0.1 g of fresh leaf tissues from each peanut plants was ground into a fine powder using a mortar and pestle in liquid N_2, then mixed with 1 ml of TRIzol reagent (Invitrogen,

Carlsbad, CA, USA) in a 1.5 ml microcentrifuge tube. After incubating the sample at room temperature for 5 min, 200 μl chloroform were added, followed by shaking and vortexing. The samples were again incubated at room temperature for 3 min, then the supernatant was transferred to a new tube and centrifuged at $3,800 \times g$ for 20 min at 4°C. An equal volume of isopropyl alcohol was mixed with the sample and placed at −20°C for 45 min, and then centrifuged for 20 min at $13,100 \times g$ and 4°C. The supernatant was discarded, and after washing the pellet with 1 ml of 75% ethanol, the sample was air dried. The pellet was dissolved in 90 μl of 0.5% sodium dodecyl sulfate (SDS), applying heat for 10 min at 55°C. The samples were then centrifuged at $1,200 \times g$ for 2 min at room temperature, and the supernatant was collected and transferred to a fresh tube. 1/10 of the sample volume of 3 M sodium acetate along with three times ice-cold 100% alcohol were then added. The sample was mixed by inversion and placed at −80°C for 2 h before being centrifuged at $13,100 \times g$ for 20 min at 4°C. The pellet was washed in 75% alcohol, air dried, and re-suspended in H_2O. It was re-dissolved by heating at 55°C for 10 min, then centrifuged for 2 min at $1,200 \times g$, and the supernatant was placed in a new sterile microcentrifuge tube. The RNA concentration and purity were measured using the NanoDrop® (ND-1000 spectrophotometer).

The cDNAs were synthesized from 1.0 μg of DNase-treated total RNAs using Superscript reverse transcriptase (SuperScript®VILOTM cDNA synthesis kit, Invitrogen). The *AtNHX1*-specific primers, NHX-3 and NHX-4, were used for reverse transcription (RT)-PCR. Amplification conditions were: 3 min at 95°C initially, followed by 40 cycles of 1 min at 95°C, 45 s at 60°C, 45 s at 72°C. The amplified products were segregated by electrophoresis on 0.8% agarose gels and visualized under UV light. The actin gene was used as the internal standard (the oligonucleotides for the actin gene are Actin-F1 and Actin-R1). The sequences of the oligonucleotides used are shown below: NHX-3, 5′-CAGGGTTTCAAGTAAAAAAG-3′; NHX-4, 5′-CATACCAAACACCACTGTG-3′; Actin-F1, 5′-GAGGAGAAGCAGAAGCAAGTTG-3′; Actin-R1, 5′-AGACAGCATATCGGCACTCATC-3′.

Salt tolerance tests

Peanut seeds of both *AtNHX1*-expressing plants and wild-type plants were germinated and grown in a greenhouse for 21 days prior to the salt treatment, which was conducted in incremental manner, starting with 30 mM NaCl for 6 days, and then applying 60, 90, 120, and 150 mM for 6 days, respectively (Fig. 2). The temperature and humidity were maintained at 28 ± 2°C and 50 ± 10%, respectively, throughout the growth period in the greenhouse. The experiment was repeated twice.

Plant growth and biomass measurement under salt treatment in a greenhouse

The fresh aboveground and underground biomasses of transgenic and wild-type control plants were determined after the 150 mM NaCl treatment. Roots were analyzed after gently flushing the soil with water and drying it with a paper towel. Subsequently, the dry aboveground and underground biomasses were weighed after drying the shoots and roots for 15 days under light in the greenhouse.

Leaf chlorophyll measurement

The relative amount of chlorophyll was measured using a portable chlorophyll meter (SPAD-502PLUS, Konica Minolta, Japan), which calculates a numerical SPAD value upon measuring the absorbance of leaves in the red and near-infrared regions that is proportional to the amount of chlorophyll present in the leaves. At each evaluation, the content was measured at the center of each leaflet of every primary leaf, and the average was used for analysis.

Gas-exchange measurements

Photosynthesis, stomatal conductance, and transpiration were measured using a portable photosynthesis system (LI-COR 6400, Li-Cor, Inc., Lincoln, NE, USA) at an ambient CO_2 concentration of 400 μmol mol^{-1}, 60% relative humidity, 28°C, and a light intensity of 1,500 μmol m^{-2} s^{-1}. The instrument was stabilized as per the manufacturer's guidelines. Before taking any measurements, the steady state levels of the reference CO_2 and the reference H_2O were observed, and the infra-red gas analyzers (IRGAs) were matched manually. The mean of five measurements obtained for each sample was used for analysis.

AC_i curve measurement and estimation of derived parameters

The same photosynthesis system (LI-COR 6400, Li-Cor, Inc.) was used to measure the response of the CO_2 assimilation rate (A) to changes in the internal CO_2 concentration (C_i) in order to characterize the photosynthetic performance of *AtNHX1*-expressing and wild-type peanut plants. The AC_i curves were plotted at a saturated photosynthetic photon flux density (PPFD) of 2,000 μmol m^{-2} s^{-1}, and the reference and sample IRGAs were matched automatically before taking each measurement. The curves were initiated at 0 Pa CO_2 and increased step-wise to 200 Pa CO_2, as described in He et al. (2007). Photosyn Assistant software (ver. 1.1.2; Dundee Scientific, Dundee, UK) was used to estimate the photosynthetic parameters

such as V_{cmax} (the maximum rate of carboxylation by Rubisco) and J_{max} (the light-saturated rate of maximum electron transport). Afterward, these parameters were used
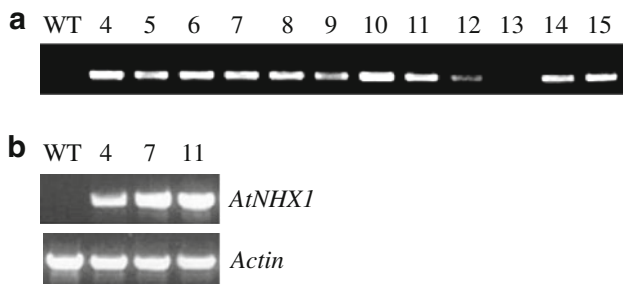

Fig. 1 Molecular analysis of wild-type and *AtNHX1*-expressing peanut plants. **a** Analysis of putative *AtNHX1*-expressing peanut plants by PCR. Genomic DNAs from the wild-type plant and 12 putative transgenic lines were amplified by PCR using *AtNHX1*-specific primers. *WT* wild-type; *4–12, 14* and *15* eleven independent transgenic peanut lines harboring *AtNHX1*; *13*, a peanut line deriving from tissue culture that does not contain *AtNHX1*. **b** RT-PCR analysis of wild-type and *AtNHX1*-expressing plants. *WT* wild-type; *4, 7,* and *11* three independent transgenic lines expressing *AtNHX1* transcripts. The actin gene was used as the internal control
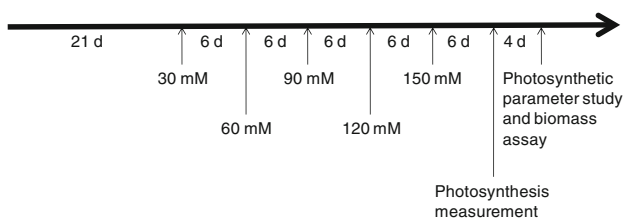

Fig. 2 Salt treatment scheme. NaCl was added at 30 mM on day 22, and the salt concentration was increased by 30 mM incrementally every 6 days. The final salt concentration was 150 mM, and all physiological analyses were conducted 6 and 10 days after 150 mM salt treatment

to estimate A_{sat} (net photosynthesis at saturating PPFD) and A_{max} (photosynthetic capacity at saturating PPFD and saturating atmospheric CO_2), based on the description provided in van Gestel et al. (2005).

Statistical analysis

Microsoft® Office Excel 2007 was used for all statistical analysis, which was performed using Student's t test, considering one-tailed, two-sample unequal variance. Significance levels of $P < 0.05$ and $P < 0.01$ (i.e., the null hypothesis is rejected at the 5% or 1% level, respectively) were used in hypothesis testing. P values were calculated from the comparison between wild-type plants and transgenic lines.

Results

Creation and molecular analysis of *AtNHX1*-expressing peanut plants

The *35S-AtNHX1* construct (He et al. 2007) was introduced into the genome of peanut through the *Agrobacterium*-mediated transformation (Sharma and Anjaiah 2000). Eighty independent transgenic lines were generated from tissue culture. To confirm the insertion of *AtNHX1* into the genome of the putative transgenic peanut plants, genomic DNA was extracted from young leaves of 12 independent putative transgenic lines and analyzed using the PCR technique. Our data indicated that 11 independent transgenic lines contained the introduced *AtNHX1* transgene in their genome (Fig. 1a). Subsequently, RT-PCR was performed to identify the transgene transcript in three selected transgenic plants. A PCR product was amplified from all
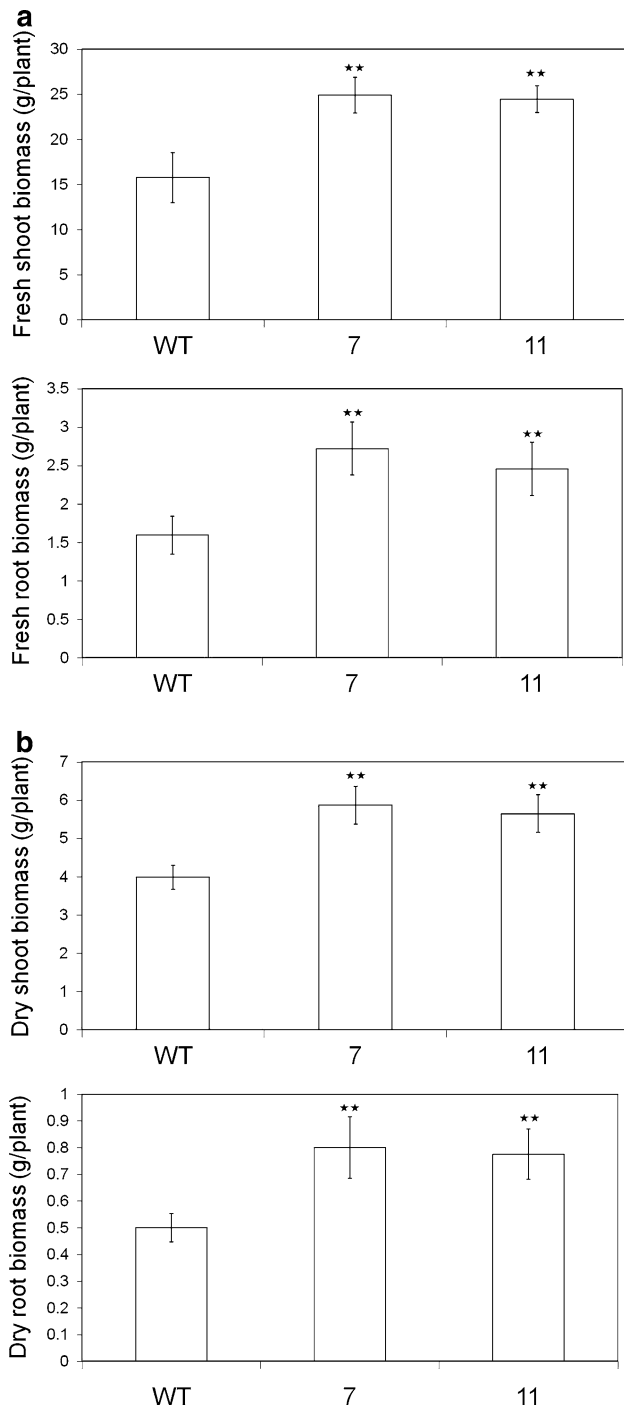

Fig. 3 Phenotypes of wild-type and *AtNHX1*-expressing peanut plants after 150 mM NaCl treatment. *WT* wild-type; *7* and *11* two independent *AtNHX1*-expressing peanut lines. *Arrows* in WT indicate chlorotic and necrotic leaves

Fig. 4 Biomasses of wild-type and *AtNHX1*-expressing peanut plants after 150 mM NaCl treatment. **a** Fresh shoot weight and fresh root weight. **b** Dry shoot weight and dry root weight. *WT* wild-type; *7* and *11* two independent *AtNHX1*-expressing peanut lines. Values are mean ± SD ($n = 4$ plants). *Double stars* indicate statistical significance at $P < 0.01$

Fig. 5 Relative amounts of chlorophyll present in wild-type and *AtNHX1*-expressing peanut plants after 150 mM NaCl treatment. *WT* wild-type; *7* and *11* two independent *AtNHX1*-expressing peanut lines. Values are mean ± SD ($n = 4$ plants). *Double stars* indicate statistical significance at $P < 0.01$

AtNHX1-expressing peanut plants are more salt tolerant than wild-type plants

To test the salt tolerance of *AtNHX1*-expressing peanut plants, we selected transgenic lines 7 and 11 for detailed study under greenhouse conditions. The salt treatment was designed as follows: NaCl was added to peanut plants on day 22 at 30 mM (Fig. 2), then the concentration was increased incrementally every 6 days. When the salt concentration reached 150 mM NaCl on day 42, the phenotypic differences between the wild-type and *AtNHX1*-expressing peanut plants were noticeable. The *AtNHX1*-expressing peanut plants were bigger than the control wild-type plants (Fig. 3); also, there were large numbers of chlorotic leaves in wild-type peanut plants in comparison to *AtNHX1*-expressing transgenic plants (Fig. 3). On the other hand, when grown under normal conditions, there were no apparent phenotypic differences between the wild-type and *AtNHX1*-expressing peanut plants (data not shown).

To quantify the phenotypic differences between wild-type and *AtNHX1*-expressing lines grown under saline conditions, the fresh and dry biomasses of the aboveground and underground portions of the peanut plants were measured. The fresh shoot masses of *AtNHX1*-expressing peanut plants were higher than those of the wild-type plants, and the differences in the fresh root masses were even larger between the transgenic and control plants (Fig. 4a). In addition to the fresh masses, dry mass analysis also confirmed that the *AtNHX1*-expressing lines exhibited better shoot and root growth than the wild-type peanut plants (Fig. 4b).

To measure the relative chlorophyll content in peanut leaves, we used a chlorophyll meter to analyze the SPAD values (Fig. 5). The SPAD reading indicates the plant nitrogen status, which is an indicator of healthy growth and development, protein synthesis, and potential good yields

three putative transgenic peanut plants, but not from the wild-type peanut (Fig. 1b), indicating that the *AtNHX1* transgene was indeed expressed in these transgenic plants.
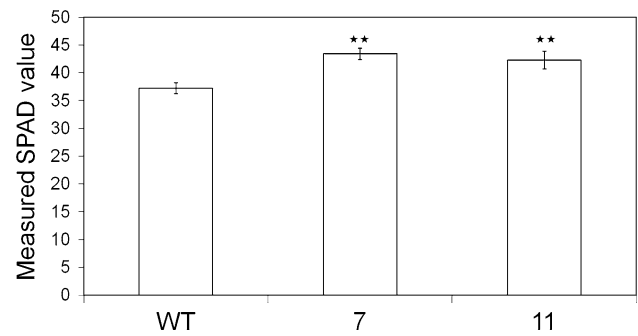
of plants (Evans 1983). Our data indicate that the leaves of NaCl-treated *AtNHX1*-expressing peanut plants have a higher nitrogen content and hence more chlorophyll than wild-type peanut plants.

AtNHX1-expressing peanut plants maintain higher photosynthetic rates than wild-type plants under salinity conditions

The photosynthetic performance of *AtNHX1*-expressing peanut plants was analyzed under normal and salt stress conditions. Net CO_2 assimilation rate, stomatal conductance, and transpiration rate were examined using a LI-COR 6400 photosynthesis system. There were no significant differences in photosynthetic parameters between wild-type peanut plants and transgenic lines expressing *AtNHX1* under normal conditions (data not shown), while *AtNHX1*-expressing peanut plants showed better photosynthetic rates (Fig. 6a), stomatal conductance (Fig. 6b), and transpiration rates (Fig. 6c) under salt conditions.

To study the effects of salinity on photosynthetic parameters, CO_2 assimilation rates (A) with respect to internal CO_2 concentrations (C_i) were obtained from *AtNHX1*-expressing lines and wild-type plants grown under salt conditions (Table 1). To estimate the photosynthesis-limiting parameters V_{cmax} (maximum rate of carboxylation by Rubisco) and J_{max} (light-saturated rate of maximum electron transport), the data from the CO_2 response curves were passed to the Photosyn Assistant software (ver. 1.1.2; Dundee Scientific). Next, photosynthesis on wild-type and transgenic plants was characterized by using the data derived from Photosyn Assistant software to estimate A_{sat} (net photosynthesis at saturating PPFD) and A_{max} (photosynthetic capacity at saturating PPFD and saturating atmospheric CO_2). The transgenic peanut lines grown under 150 mM NaCl had significantly higher V_{cmax}, J_{max}, A_{sat}, and A_{max} values than wild-type plants (Fig. 7). The higher V_{cmax} value for *AtNHX1*-expressing peanut plants indicates that under low CO_2 conditions, the photosynthesis of the transgenic lines was limited to a lesser degree by the Rubisco activity than the wild-type plants. Similarly, the higher J_{max} value indicates that at high C_i, the photosynthesis of the transgenic lines was less limited by the rate of regeneration of the CO_2 acceptor and ribulose bisphosphate compared to wild-type plants.

Discussion

Salinity is a major environmental stress that reduces crop yields, yet the increasing population and warming environment are increasing demands for improvements in agricultural productivity, which makes it necessary to
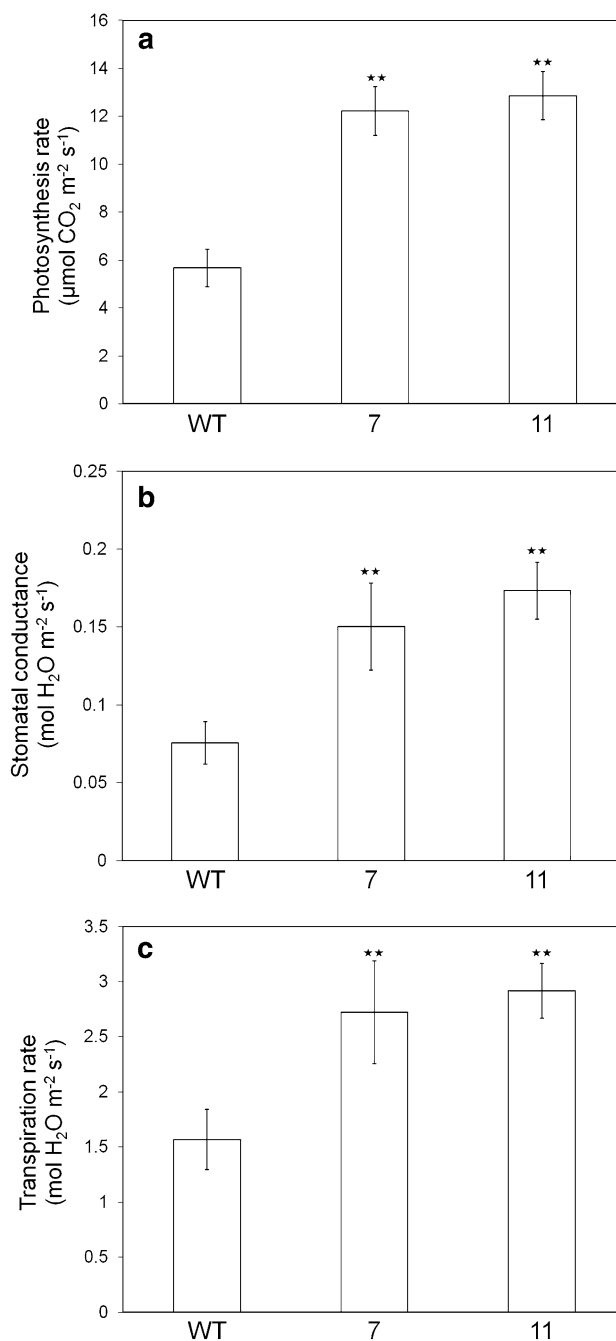

Fig. 6 Gas-exchange performances of wild-type and *AtNHX1*-expressing peanut plants after 150 mM NaCl treatment. **a** Photosynthetic rate measurement. **b** Stomatal conductance measurement. **c** Transpiration rate measurement. *WT* wild-type; *7* and *11* two independent *AtNHX1*-expressing peanut lines. Values are mean ± SD ($n = 4$ plants). *Double stars* indicate statistical significance at $P < 0.01$

generate salt-tolerant crops that can produce the increasing amounts of food required. Along with traditional breeding strategies, genetic engineering can be employed to improve crop yield and quality. Advances in plant biotechnology have made it possible to identify genes that may be

Table 1 CO_2 assimilation rates (A) with respect to the internal CO_2 concentrations (C_i) of wild-type and *AtNHX1*-expressing peanut plants after 150 mM NaCl treatment

Wild-type		7		11	
$C_i \pm$ SD (Pa)	$A \pm$ SD (μmol CO_2 m^{-2} s^{-1})	$C_i \pm$ SD (Pa)	$A \pm$ SD (μmol CO_2 m^{-2} s^{-1})	$C_i \pm$ SD (Pa)	$A \pm$ SD (μmol CO_2 m^{-2} s^{-1})
12.48 ± 13.63	−1.34 ± 0.44	6.08 ± 1.71	−1.41 ± 1.15	8.64 ± 2.14	−1.24 ± 0.32
7.83 ± 3.63	−0.51 ± 0.34	5.59 ± 0.07	−0.23 ± 0.67	5.42 ± 0.34	−0.31 ± 0.24
10.01 ± 5.29	−0.43 ± 0.32	6.39 ± 0.28	0.39 ± 1.04	6.91 ± 0.79	−0.25 ± 0.12
12.97 ± 5.06	−0.23 ± 0.15	7.86 ± 0.12	1.48 ± 0.64	8.25 ± 0.25	0.46 ± 0.22
14.04 ± 3.13	0.09 ± 0.29	10.06 ± 0.13	3.28 ± 0.58	10.35 ± 0.49	1.45 ± 0.48
18.04 ± 2.87	0.25 ± 0.32	13.2 ± 0.46	5.06 ± 0.49	12.2 ± 0.70	2.7 ± 0.55
20.4 ± 1.38	0.54 ± 0.35	14.1 ± 0.40	6.96 ± 0.43	14.1 ± 1.04	4.11 ± 0.92
28.27 ± 1.38	1.09 ± 0.29	19.44 ± 2.03	10.89 ± 2.68	20.8 ± 0.86	7.11 ± 1.09
43.3 ± 7.18	1.69 ± 0.79	25.7 ± 9.93	14.13 ± 5.34	26.07 ± 2.54	9.61 ± 1.33
52.44 ± 16.37	1.91 ± 0.91	32.9 ± 7.00	16.74 ± 4.83	29.74 ± 0.96	9.87 ± 1.24
62.57 ± 18.69	2.44 ± 0.63	39.6 ± 5.33	16.37 ± 4.89	35.67 ± 5.61	9.41 ± 0.78
81.47 ± 21.13	2.42 ± 1.04	45 ± 4.79	15.44 ± 3.56	41.57 ± 8.21	9.16 ± 0.55
101.6 ± 27.87	2.64 ± 1.03	51.57 ± 9.25	15.54 ± 3.27	58.17 ± 2.65	8.89 ± 0.89
131 ± 35.52	3.15 ± 0.47	71.8 ± 9.29	15 ± 3.56	79.67 ± 1.66	

7 and *11* two independent *AtNHX1*-expressing peanut lines. Values are mean ± SD (*n* = 3 plants)

valuable for improving crops (Wang et al. 2003; Chin-nusami et al. 2004). *AtNHX1* is one of these genes, as it can be used to improve salt tolerance in crops (Zhang and Blumwald 2001; Zhang et al. 2001; He et al. 2005; Duan et al. 2009; Tian et al. 2006; Xu et al. 2009; Li et al. 2010).

In this study, we aimed to improve salt tolerance in peanut by introducing *AtNHX1* into peanut through a *Agrobacterium*-mediated transformation (Sharma and Anjaiah 2000). Eighty independent putative transgenic lines were generated, and PCR analysis of 12 putative lines confirmed the incorporation of *AtNHX1* into the genomes of 11 transgenic peanut lines (Fig. 1a), indicating a high transformation efficiency of this transformation protocol. RT-PCR data indicated that the *AtNHX1* transcript was expressed in all three transgenic lines tested (Fig. 1b). Two of these three *AtNHX1*-expressing lines, 7 and 11, were selected for detailed physiological studies. When compared to wild-type controls, *AtNHX1*-expressing peanut plants displayed better growth under salt conditions. For example, they suffered less damage (Fig. 3) and produced more biomass (Fig. 4) after 150 mM NaCl treatment. The leaf area and number of leaves of *AtNHX1*-expressing peanut plants are larger than those of wild-type plants after salt treatment (data not shown). In addition to the increased photosynthetic surface area, transgenic lines had higher chlorophyll content than wild-type plants under salt conditions (Fig. 5). Furthermore, *AtNHX1*-expressing peanut plants maintained higher photosynthetic rates, higher stomatal conductances, and higher transpiration rates (Fig. 6).

While CO_2 assimilation rate (A) was measured using internal CO_2 concentration (C_i) values, A values for *AtNHX1*-expressing peanut plants showed greater improvements than those of wild-type plants grown at 150 mM NaCl (Table 1). Carboxylation efficiency by Rubisco and electron transport rate are two photosynthetic characteristics that were enhanced for the *AtNHX1*-expressing plants at low and high CO_2, respectively (Fig. 7a, b). Also, the transgenic peanut lines showed improved photosynthesis under saturated light and saturated CO_2 conditions than wild-type plants under salt-stressed conditions (Fig. 7c, d). Therefore, we believe that the increased biomass produced by *AtNHX1*-expressing plants is likely due to the multiple effects of large photosynthetic surface area, better photosynthetic rate, and higher chlorophyll content. Overall, our data are consistent with other *AtNHX1*-overexpression studies in *Arabidopsis*, rapeseed, tomato, cotton, tobacco, tall fescue, *Petunia hybrid*, and soybean (Apse et al. 1999; Zhang and Blumwald 2001; Zhang et al. 2001; He et al. 2005; Duan et al. 2009; Tian et al. 2006; Xu et al. 2009; Li et al. 2010).

Since PEG is generated by vacuolar adenosine triphosphatase (V-ATPase) and H$^+$-pyrophosphatase (Apse et al. 1999; Gaxiola et al. 2002), increased activities of these two enzymes should lead to increased salt tolerance. Indeed, increased expression of *AVP1*, which encodes an H$^+$-pyrophosphatase on the vacuolar membrane, increased salt tolerance in transgenic *Arabidopsis* (Gaxiola et al. 2001). We also confirmed this by overexpressing *AVP1* in cotton,
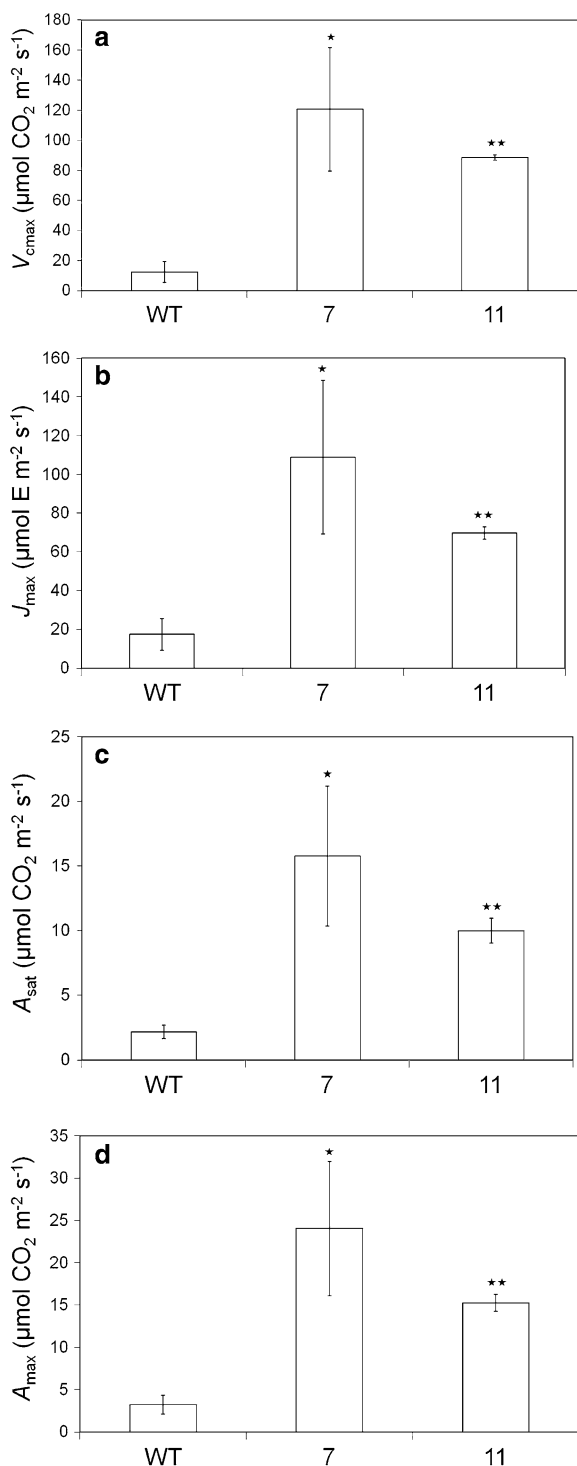
Fig. 7 Estimation of photosynthetic parameters and characteristics for wild-type and *AtNHX1*-expressing peanut plants after 150 mM NaCl treatment. **a** Maximum carboxylation rate by Rubisco (V_{cmax}). **b** Light-saturated rate of maximum electron transport (J_{max}). **c** CO_2-saturated photosynthesis (A_{sat}). **d** Maximum potential photosynthesis (A_{max}). *WT* wild-type; *7* and *11* two independent *AtNHX1*-expressing peanut lines. Values are mean ± SD ($n = 3$ plants). *Double stars* indicate statistical significance at $P < 0.01$; *stars* indicates statistical significance at $P < 0.05$

and obtained increased salt tolerance in transgenic cotton plants (Pasapula et al. 2011). However, based on our observations, and others (Gaxiola et al. 2002), it appears that the increase in salt tolernace caused by *AtNHX1* overexpression is between 150 and 200 mM NaCl. To further improve salt tolerance in transgenic plants, it may be necessary to overexpress both *AVP1* and *AtNHX1* (Gaxiola et al. 2002). We should test this idea to generate higher salt tolerance in peanut in the future.

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