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Generating salt-tolerant *Nicotiana tabacum* and identification of stress-responsive miRNAs in transgenics

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Abstract: Identification of vacuolar Na^+/H^+ antiporters facilitates the basis of salt stress tolerance mechanisms. Na^+ accumulation into the vacuole is crucial for the avoidance of cytoplasmic Na^+ toxicity. In this study, we show that the introduction of *AtNHX1* into tobacco generated more tolerant plants when compared to wild-type plants. Transgenic tobacco plants exhibited higher germination rates in the presence of increasing salt concentrations compared to wild-type plants. In addition, proline levels were higher under salt stress conditions in both the shoots and the roots of transgenics compared to wild-type plants. Increase in malondialdehyde production during lipid peroxidation by salinity was lower in transgenic plants compared to the controls. Being important and newly discovered determiners of plant stress responses, microRNAs (miRNAs) are noncoding small RNAs and essential indicators of plant stress response mechanisms. For further identification of stress responses, the expression levels of growth and abiotic stress-related miRNAs (miR319a, miR319b, miR159b, miR398a, and miR398b) were quantified. miR319a, miR319b, and miR159b expression levels were higher in wild-type plants, and miR319a and miR159b expression was restored in transgenic plants. There was a slight decrease in the expression levels of miR398a and miR398b of wild-type plants. However, the recovery of the miR398a and miR398b expressions was especially significant in transgenic plants. Furthermore, an investigation of miRNAs in transgenic plants can help to understand the stress tolerance mechanisms of the plants.

Key words: *AtNHX1,* tobacco, salt stress, miRNA

1. Introduction

Plants are constantly exposed to various biotic and abiotic stresses (Fujita et al., 2006). Abiotic stress factors include water deficiency (drought), extreme heat or cold (freezing, chilling, or heat shock), sudden changes in temperature, lack of oxygen in the soil due to flooding, formation of reactive oxygen radicals, exposure to strong light, and elevated salt concentrations (salinity) (Li et al., 2011). Among these factors, salinity imposes two effective stresses on plant tissues: 1) high solute concentrations in soil leads to water deficiency and stress, and 2) altered Na^+/K^+ ratios and deleterious Na^+ and Cl^- ion concentrations lead to ion-specific stresses. Salt-affected plants are characterized by dwarf shoots accompanied by thick and dark green leaves, which normally are water-storing parts that act as water reservoirs for plants (Kotuby-Amacher et al., 1997). Consequently, high salinity limits the use of agricultural lands and decreases their productivity (Flowers et al., 1997).

Plant cells generally respond to salt stress by increasing Na^+ efflux at the plasma cell membrane and sodium accumulation in the vacuole (Xu et al., 2009). Na^+ and H^+ are the most common ions, and they play an important role in critical regulatory events such as cell bioenergetics and maintenance of proton concentrations. If the concentration of these two ions is too high or too low, normal physiological activities and growth properties of cells are adversely affected (Tester and Davenport, 2003). In plants, both vacuolar and plasma membrane Na^+/H^+ antiporters function in the removal of Na^+ from the cytoplasm to prevent such toxic effects (Blumwald, 2000; Dong et al., 2011). Interestingly, NaCl exposure is predicted to induce the exchange activity of these transport proteins (Garbarino and Dupont, 1988). Maintaining a high cytoplasmic Na^+/K^+ ratio is also an efficient mechanism to develop salt tolerance. Moreover, Na^+ ions accumulate in vacuoles to avoid cytoplasmic Na^+ toxicity (Xu et al., 2009). In parallel, vacuolar Na^+ serves as a necessary osmoticum for cellular H_2O homeostasis by facilitating water uptake from the soil.

The *Arabidopsis thaliana AtHNX1* gene codes for a vacuolar Na^+/H^+ antiporter, which is a salt tolerance determinant (Sottosanto et al., 2004). Under salt stress,

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the Na^+/H^+ antiporter provides accumulation of Na^+ in the vacuoles, and thus a low cytoplasmic concentration of Na^+ can be achieved (Xu et al., 2009). Overexpression of the *Arabidopsis thaliana AtNHX1* gene enhances salt tolerance in transgenic *Arabidopsis* plants, which are able to grow in soil watered with up to 200 mM NaCl (Wu et al., 2005). Several studies have shown the effectiveness of *AtNHX1* expression for salt tolerance establishment. For example, transgenic tomato plants overexpressing *AtNHX1* can produce seeds with higher growth rate and flowering abilities, even when exposed to 200 mM NaCl (Zhang and Blumwald, 2001). Transgenic cotton grown in high-salt soil shows higher photosynthetic activity and fiber production (He et al., 2005). Transgenic *Petunia hybrida* (Xu et al., 2009) and common buckwheat (Chen et al., 2008) have also been found to enhance salt tolerance.

As important and newly discovered players of plant stress responses, microRNAs (miRNAs) appear to play a crucial role in both abiotic and biotic stress responses in plants. Abiotic stress factors, such as drought, salt, cold, heat, light, and oxidative stress, are already known to regulate many plant genes. Altered expression of miRNAs upon abiotic stresses is an interesting observation considering their roles in developmental processes with as of yet unknown potential mRNA targets and functions in plant cells (Frazier et al., 2011; Yin et al., 2012).

In this study, we further tested the salt tolerance potential of the *AtNHX1* antiporter in tobacco plants. Following the construction of successful salt-tolerant transgenic lines, we investigated the microRNA-related stress responses in these transgenic plants.

2. Materials and methods

2.1. Plant materials and bacterial strains

In the tissue culture and transformation studies, seeds of *Arabidopsis thaliana* ecotype Columbia, seeds of *Nicotiana tabacum* L. 'Samsun', and leaves of *Nicotiana tabacum* were used. Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal media, containing MS micro-macro elements and vitamins supplemented with 3% sucrose and 0.8% plant agar, were used in all plant tissue culture studies. Cultures were incubated at 24 ± 2 °C under fluorescent light with a 16/8-h (light/dark) period. *Escherichia coli* strain DH5α (Taylor et al., 1993) cells were used as competent cells. The EHA105 (Hood, 1993) strain of *Agrobacterium tumefaciens* was used in plant transformation studies. pCVB1, carrying a cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (Nos) terminator, was used as a vector for the transformation of *Nicotiana tabacum*.

2.2. RNA isolation and cloning of *AtNHX1*

Total RNA was extracted from whole *A. thaliana* plants by TRIzol (Invitrogen), as described previously (de Graaff, 1988). RNA concentration and purity were determined by NanoDrop ND1000 (Thermo Scientific). The RevertAid First Strand cDNA Synthesis Kit (Fermentas) was used for cDNA synthesis from 1 µg of DNase-treated RNA samples. Lack of DNA contamination was confirmed by PCR. The *AtNHX1* coding sequence was PCR-amplified using *Pfu* DNA polymerase (Fermentas) and cloned into the pCVB1 plasmid (provided as pPCVB-RFP by Dr Ferenc Nagy, BRC, Szeged, Hungary, and reconstructed by Dr Feyza Selçuk via the excision of RFP fragment). *Xba*I and *Bam*HI recognition site-harboring cloning primers were as follows (restriction sites are in bold letters and recognition aiding sites are underlined):

AtNHX1-F, 5'-TGC**TCTAGA**GCAATGTTG-GATTCTCTAGTGTCG-3' and AtNHX1-R, 5'-CGC**G-GATCC**GCGTCAAGCCTTACTAAGATCAGG-3'.

Cloned inserts were confirmed by DNA sequencing (İontek Company, İstanbul, Turkey). Isolated plasmids were then used in *Agrobacterium* transformation.

2.3. Transformation and regeneration

The pCVB1 construct was transferred to *Agrobacterium tumefaciens* according to the leaf disc gene transformation method (Öktem et al., 1994). First, *Nicotiana tabacum* plants were grown on MS medium at 24 °C. *Agrobacterium* cells, transformed with plasmid, were grown overnight in 250 mL of yeast extract broth medium with 100 mg/L rifampicin and 100 mg/L ampicillin at 28 °C with 200 rpm shaking. Following dilution of the culture 10 times with liquid MS, sterile leaves were cut aseptically into leaf discs and put into the diluted bacterial solution. After incubation, leaf discs were washed with liquid MS and blotted on sterile paper. They were then placed on MS medium containing 1 mg/L benzylaminopurine and 0.1 mg/L naphthalene acetic acid. After 2 days of cocultivation, the explants were washed with liquid MS supplemented with 500 mg/L augmentin for the elimination of *Agrobacterium* cells. The leaves were dried by blotting and were transferred to petri plates containing MS, 500 mg/L augmentin, 100 mg/L ampicillin, and selection agent (phosphinothricin (PPT), 5 mg/L) for selection and regeneration. Plates were incubated at 25 °C for a 16-h light, 8-h dark period, and the medium was refreshed every 10 days.

Following callus formation and shoot growth, shoots were transferred into jars to induce root formation. Plantlets were transferred to soil and grown in a growth chamber at 24 ± 1 °C with a 16-h light and 8-h dark period. After the emergence of flower buds, the plants were covered with nylon bags to prevent cross-pollination. Seeds of putative transgenic plants (T1 generation) were collected when completely dry.

2.4. Confirmation of putative T_0 transgenic plants

Genomic DNA from control plants and 7 independent putative transgenic lines was isolated according to the CTAB (cetyltrimethylammonium bromide) DNA

extraction method (Saghai-Maroof et al., 1984). Genome integration was tested with *AtNHX1* gene-specific primers.

Following 3 and 6 weeks of transformation, the regeneration ability of the putative transgenic and wild-type plants was tested under different salt concentrations (0 mM, 50 mM, 100 mM, 150 mM, 200 mM, and 250 mM NaCl) by weighing fresh calluses. The putative transgenic plants were also regenerated on MS containing 5 mg/L PPT under different concentrations of NaCl.

2.5. Salt tolerance test

Seeds of putative transgenic (T1 generation) and control tobacco plants were surface-sterilized and germinated on 0 mM, 50 mM, 100 mM, 150 mM, 200 mM, and 250 mM NaCl containing MS. The plates were incubated at 25 ± 2 °C with 16-h light and 8-h dark cycles. Photographs of the plates were taken (data not provided). Germinating and surviving plants were counted.

2.6. Malondialdehyde and proline measurement

Lipid peroxidation was determined by calculating the malondialdehyde (MDA) content in 0.2 g fresh weight (FW) of shoots and roots of plants under 200 mM salt stress conditions. The concentration of MDA was calculated using 532 nm absorbance and the extinction coefficient of 155 mM^{-1} cm^{-1} (Ohkawa et al., 1979). Unspecific turbidity correction was done by subtracting the 600 nm absorbance value. For proline concentration detection in control and 200 mM salt-stressed plants, 0.2 g of shoot and root were homogenized in 3% (w/v) sulfosalicylic acid (Bates et al., 1977). Proline concentration was calculated from the absorbance at 520 nm by establishing a proline standard curve. Proline concentration was defined as μmol/g FW. Data represent the average of three experiments with three replicates, and data were analyzed via paired-samples t-test.

2.7. Expression analysis of stress-related miRNAs

Transgenic (line 7) and wild-type tobacco seeds were germinated for 6 weeks. Plants were grown in MS medium containing 150 mM NaCl for 48 h. Total RNA was extracted using TRIzol (Invitrogen) (de Graaff, 1988). RNA concentration and purity were determined by NanoDrop ND1000 (Thermo Scientific). The RevertAid First Strand cDNA Synthesis Kit (Fermentas) was used for cDNA synthesis from 2 μg of DNase-treated RNA samples. Lack of DNA contamination was confirmed by PCR. For RT-qPCR, SYBR Green Mastermix (Roche Applied Science) was used using the Rotor Gene 6000 (Corbett, QIAGEN) cycler. Reactions (20 μL) were performed with 300 nM specific primer pairs to amplify miRNA precursor sequences. miRNA precursors were amplified using the following primer sets:

miR319a (product size: 176 bp), F: 5'-AGAGAGA-GCTTCCTTGAGTCC-3',

R: 5'-AGAGGGAGCTCCCTTCAGTC-3'

miR319b (product size: 172 bp), F: 5'-AGAGA-GCTTTCTTCGGTCCAC-3',

R: 5'-AGGGAGCTCCCTTCAGTCCAAG-3'

miR159b (product size: 196 bp), F: 5'-GGAAGAGCTCCTTGAAGTTC-3',

R: 5'-GAGAGATGAAGAGCTCCCTTC-3'

miR398a (product size: 105 bp), F: 5'-AAGGAGTG-GCATGTGAACAC-3',

R: 5'-GGGAGATTCAAAGGGGTGAC-3'

miR398a (product size: 116 bp), F: 5'-AAGGAGTG-GCATGTGAACAC-3',

R: 5'-AAGAGCTCAGCAGGGGTGAC-3'

Fold change for miRNA levels was normalized against the reference gene *18S rRNA* (Brunner et al., 2004) and amplified (product size: 243 bp) with the following primer set: F: 5'-AAACGGCTACCACATCCAAG-3', R: 5'-CCCATCCCAA GG TTCAACTA-3'.

The following conditions were used for precursor miRNA PCRs for miR319a: incubation at 95 °C for 10 min and 40 cycles of 94 °C for 15 s, 58 °C for 40 s, and 72 °C for 25 s. For miR319b, the conditions used were incubation at 95 °C for 10 min and 40 cycles of 94 °C for 15 s, 57 °C for 40 s, and 72 °C for 25 s; for miR159b, incubation at 95 °C for 10 min and 40 cycles of 94 °C for 15 s, 55 °C for 40 s, and 72 °C for 25 s; for miR398a, incubation at 95 °C for 10 min and 40 cycles of 94 °C for 15 s, 54 °C for 40 s, and 72 °C for 25 s; for miR398b, incubation at 95 °C for 10 min and 40 cycles of 94 °C for 15 s, 53 °C for 40 s, and 72 °C for 25 s.

For relative quantification, the $\Delta\Delta C_T$ formula was used (Livak and Schmittgen, 2001). Three independent biological replicates with 3 technical replicas per experiment were used for each qRT-PCR. One-way ANOVA with Tukey's multiple comparison posttest was performed using GraphPad Prism (San Diego, CA, USA).

3. Results

3.1. Cloning of the *AtNHX1* gene

The *Arabidopsis thaliana AtNHX1* coding sequence (1614 bp, NM_122597) was amplified and cloned into *Xba*I and *Bam*HI sites of the pCVB1 vector under the control of the CaMV 35S promoter. *Nicotiana tabacum* leaves were then transformed with pCVB1-*AtNHX1* by *Agrobacterium*. Using this direct gene transfer method, we generated 7 putative transgenic lines and confirmed expression of *AtNHX1* by RT-PCR (see Supplementary Figure 1, on the journal's website).

3.2. Regeneration test under salt stress

Putative transgenic plants were regenerated on MS containing 5 mg/L PPT with increasing NaCl concentrations (0, 50, 100, 150, 200, 250 mM). Following callus formation, regeneration efficiency of the wild-type control and transgenic plants was determined by fresh callus weight after 3 and 6 weeks to assess salt stress response (Figures

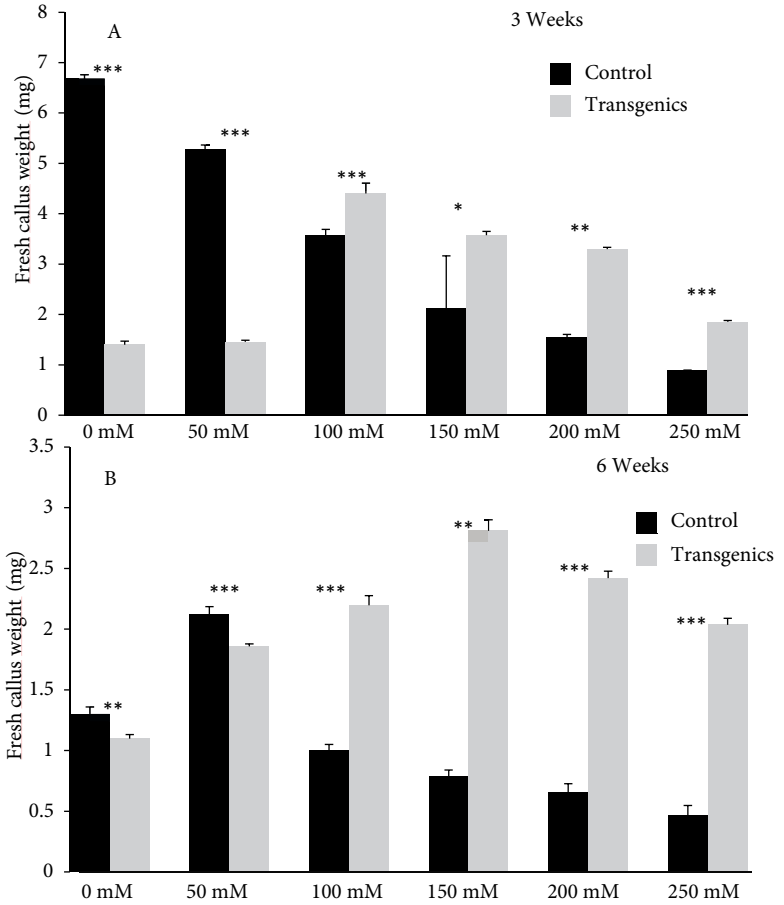

Figure 1. Regeneration ability of (A) 3-week-old and (B) 6-week-old control (black bars) and transgenic plantlets (gray bars) under different salt concentrations (0, 50, 100, 150, 200, and 250 mM NaCl) based on fresh callus weight. Data represent the average of five experiments with three replicates. Vertical bars indicate ±standard error of mean (SEM). Data were analyzed by paired-samples t-test (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

1A and 1B). Due to increasing salt concentrations, fresh callus weight of wild-type plants decreased as an expected indication of osmotic stress and water loss (Munns, 2002). On the other hand, fresh callus weight of transgenic plants was significantly higher than that of control plants at high salt (100 mM and up) concentrations after 3 and 6 weeks. At low salt concentrations (0 and 50 mM), transgenic plants had lower fresh callus weight compared to the control plants, especially in the early regeneration phase (3 weeks), possibly due to the burden of the transformation process.

Next, we investigated whether any morphology changes occurred in the control and transgenic plants. As expected, there were no morphology changes under no/low salt conditions. At 50 mM, 100 mM, and 150 mM NaCl concentrations, wild-type control plant leaves started to turn pale green and yellow, whereas transgenic plant leaves remained green (Figure 2). Some morphological differences, such as color change at the tips and edges of the leaves of the control and transgenic plants, were seen, especially with increasing salt concentrations. When shooting efficiencies were observed, shooting efficiency of wild-type control plants visibly declined with increasing salt concentrations (150 mM and 200 mM NaCl), whereas the transgenic plant shoots developed within 2–3 weeks, even at higher salt concentrations. For concentrations higher than 150 mM NaCl, wild-type control plants did not initiate roots and started to wither sooner (data not shown), whereas transgenic plants were still able to form proper calluses (Figure 2) and roots (data not provided) at up to 200 mM NaCl. Inhibition of shoot and root growth (Sottosanto et al., 2007), leaf expansion, and number of leaves (He et al., 2007) is a common response to salt stress.

Subsequently, the shoots of transformed plants were cut from their bases and transferred to root-inducing media containing 5 mg/L PPT. Root formation was observed in

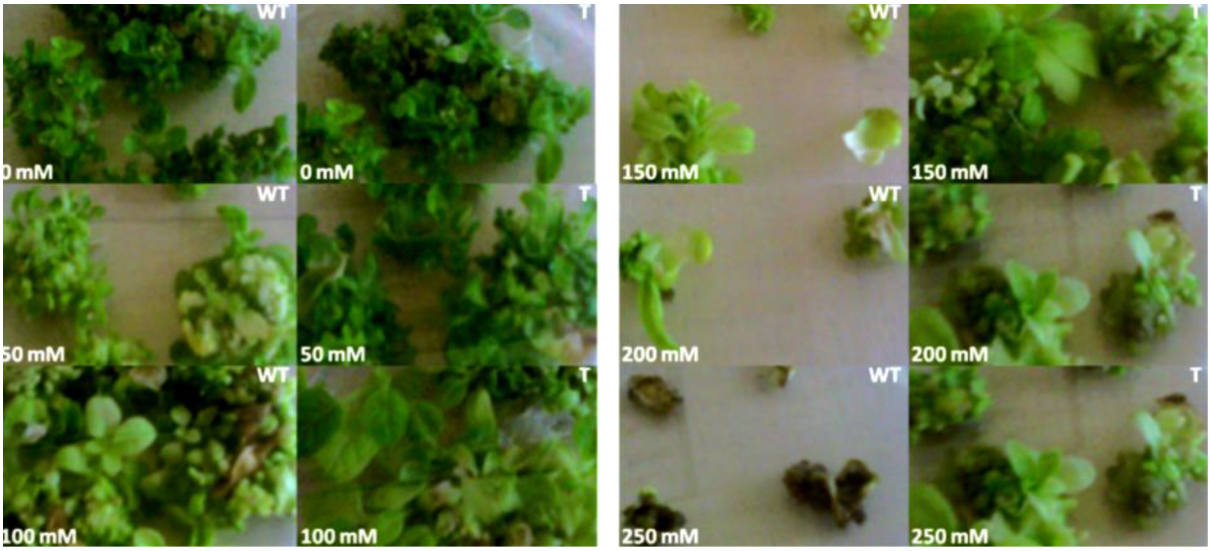

Figure 2. Three-week-old wild-type and transformed tobacco leaf discs on plates containing different concentrations of salt (0, 50, 100, 150, 200, 250 mM NaCl) (W: wild, T: transgenic).

jars within 2–3 weeks (data not shown). To test whether our transgenic lines transmitted salt tolerance ability to the next generations, regenerated plantlets obtained from all 7 putative transgenic plants were transferred from jars to soil. The transfer of 7 putative transgenic plant explants to the soil induced the regeneration of T_1 seeds. All 7 transgenic lines were able to produce seeds. It is noteworthy that, consistent with earlier observations, wild-type control plants reached seed-producing size faster than the transgenic plants (Supplementary Figure 2, on the journal's website).

Following the collection of seeds, transgenic plants were further tested for improved tolerance to salt. Seeds of independent transgenic lines and control plants were germinated on MS petri plates under different salt concentrations (0, 50, 100, 150, 200, and 250 mM NaCl). After 6 weeks, the total number of germinated seeds was counted and documented (Figure 3). No significant change was detected at low salt concentrations (50 and 100 mM). However, at 150 mM salt concentration, germination efficiency of transgenic plants was approximately 35% higher than that of wild-type control plants. At 200 mM salt

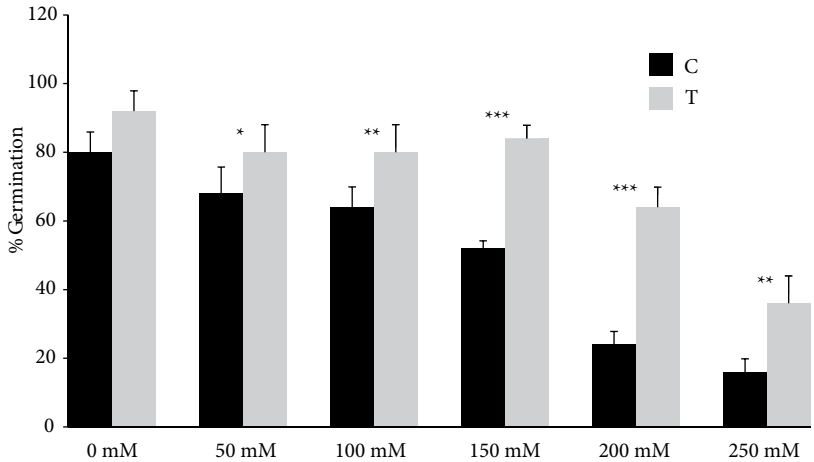

Figure 3. The percentage of germinated 3-week-old wild-type (black bars) and transgenic (gray bars) plants on NaCl (0, 50, 100, 150, 200, and 250 mM) selective medium. Data represent the average of three experiments with three replicates. Vertical bars indicate ±standard deviation (SD). C: Control, T: transgenic. Data were analyzed via paired-samples t-test (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

concentration, germination efficiency of wild-type plants significantly decreased to 22%, whereas transgenic plants were still able to germinate 40% more than the controls. Hence, the germination ability of transgenic seeds in salt media confirmed the salt tolerance of the transformed plants. At very high (250 mM) salt concentration, although still higher than wild-type controls, transgenic plant germination efficiencies decreased compared to 150 and 200 mM salt concentrations.

To understand the better growth of transgenic plants in salt-containing media, germination ability of T_1 seeds (line 7) and survival rate of transgenics were calculated by counting the seeds and surviving plants (Table). These calculations were repeated independently three times and their average results are given as percentages in the Table. The germination efficiency and survival rate of T_1 seeds were higher than in the wild-type plants, especially under high salt concentrations (150, 200, and 250 mM NaCl). The germination ability of the seeds in salt-containing media confirmed the evaluation of salt tolerance of transformed plants. There was no statistically significant difference between the germination efficiency of the transgenic and control groups at 0 mM salt concentration ($P = 0.1$). On the other hand, at 50, 100, 150, 200, and 250 mM salt concentrations, the germination efficiencies of the putative transgenic T_1 seeds were statistically higher than the control seeds ($P_{50} = 0.01$, $P_{100} = 0.01$, $P_{150} = 0.01$, $P_{200} = 0.001$, and $P_{250} = 0.01$).

Given the ability of the transgenic plants to survive and germinate under high salt concentrations, we further examined the impact of salt stress on transgenic plants.

3.3. Determination of MDA and proline concentrations

Cell membrane stability is a discriminating factor for stress-tolerant and stress-intolerant plant species (Meloni et al., 2003). MDA is produced by the disruption of polyunsaturated fatty acids of cellular membranes and accumulates in cells due to abiotic stresses (Sharma et al., 2012). To test whether MDA production increased in response to high salt stress in transgenic plants, we grew wild-type and transgenic plants on MS medium with 150 mM NaCl for 15 days. While MDA concentration was higher in the shoots than the roots in both the wild-type and transgenic plants, MDA levels were significantly lower in all transgenic plants under high salt concentrations (150 mM) (Figure 4) compared to the controls. This suggested less lipid peroxidation in transgenic plants under high salt concentrations, implying increased salt tolerance.

To further confirm decreased salt-associated stress in transgenic lines, proline accumulation was measured as an osmoprotectant and an indicator of stress adaptation. As expected, salt stress caused proline accumulation in all transgenic lines (T1–T7) (Figures 5A and 5B), both in the shoots and the roots. Control plants also accumulated proline to a lesser extent.

In short, decreased lipid peroxidation and increased proline levels clearly demonstrated a tolerance phenotype for the transgenic lines. However, in order to further inspect this tolerance response, we sought to investigate the expression of several miRNAs as novel and important plant stress response elements that regulate posttranscriptional gene regulation. In addition, the role of miRNAs, especially in transgenic plants, is of great interest to better understand tolerance mechanisms due to abiotic stresses. Hence, we chose 5 common miRNA genes found to be associated with salt stress (Jagadeeswaran et al., 2009; Sunkar, 2010) and examined their expression levels.

3.4. Salt stress-related miRNA expression

miRNAs are ubiquitous regulators of gene expression (Koyro et al., 2012). Moreover, transcriptional and posttranscriptional regulation of genes during stress conditions is connected to plant development, metabolism, and stress responses. Several miRNAs play a vital role in plant resistance to abiotic and biotic stress. miRNAs mentioned in this study were selected according to their

Table. Percent germination efficiency and % survival rate of wild-type and transgenic plants (line 7) at different salt concentrations (0, 50, 100, 150, 200, and 250 mM NaCl).

Salt concentration	% Germination efficiency		% Survival rate	
	Wild-type	Transgenic	Wild-type	Transgenic
0 mM NaCl	89.1	95.8	94.7	95.6
50 mM NaCl	76.6	80.3	81.3	89.5
100 mM NaCl	61.2	78.9	61.5	84.2
150 mM NaCl	45.8	81	45.6	70
200 mM NaCl	24.2	64.5	28.6	57.1
250 mM NaCl	16.6	38.7	25	33.3

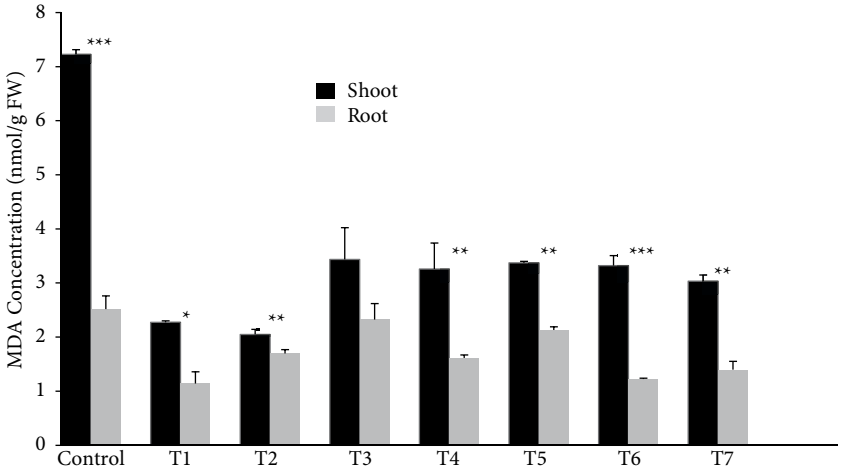

Figure 4. MDA concentration in the shoots (black bars) and roots (gray bars) of 150 mM salt-treated wild-type and transgenic tobacco plants. Data represent the average of three experiments with three replicates. Vertical bars indicate ±SEM. Data were analyzed by paired-samples t-test.

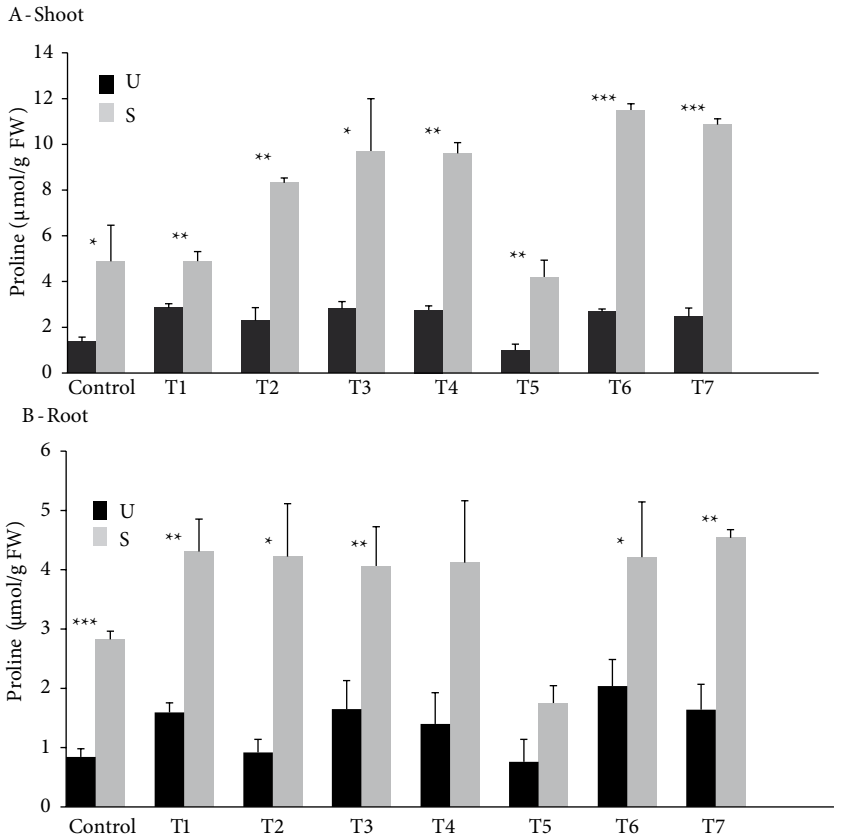

Figure 5. Proline concentrations in the (A) shoots and (B) roots of nontreated (black bars) and 150 mM salt-treated (gray bars) wild-type and transgenic tobacco plants. Data represent the average of three experiments with three replicates. Vertical bars indicate ±SD. Data were analyzed with paired-samples t-test. U: Unstressed, S: stressed.

impact on salt stress responses, based on previous studies (Sunkar and Zhu, 2004; Navarro et al., 2006). Related to the abiotic stress-associated miRNAs, miR398 was the first miRNA directly linked to abiotic stresses (Sunkar et al., 2006). In addition, miR156, miR159, miR319, and miR393 play an important role in response to salt stress in *Arabidopsis*, rice, and poplar (Sunkar and Zhu, 2004; Ding et al., 2009; Jia et al., 2009). On the basis of this information, we investigated the expression levels of miR398a, miR398b, miR319a, miR319b, and miR159b.

Transgenic line 7 was selected for the miRNA expression experiment due to its optimum germination efficiency and growth on MS medium containing 150 mM NaCl (Supplementary Figure 3, on the journal's website). Expression levels of 5 miRNAs in transgenic and control plants were assessed by qRT-PCR. Our wild-type plants demonstrated decreased levels of miR398b after 150 mM NaCl treatment. Interestingly, both miR398a and miR398b expression levels were high in salt-treated transgenic plants compared to wild-type plants (Figures 6A and 6B). miR319a expression increased in salt-treated wild-type tobacco plants, whereas its normal expression was high in untreated tobacco plants and low in salt-treated transgenic plants (Figure 6C). This fluctuating change in

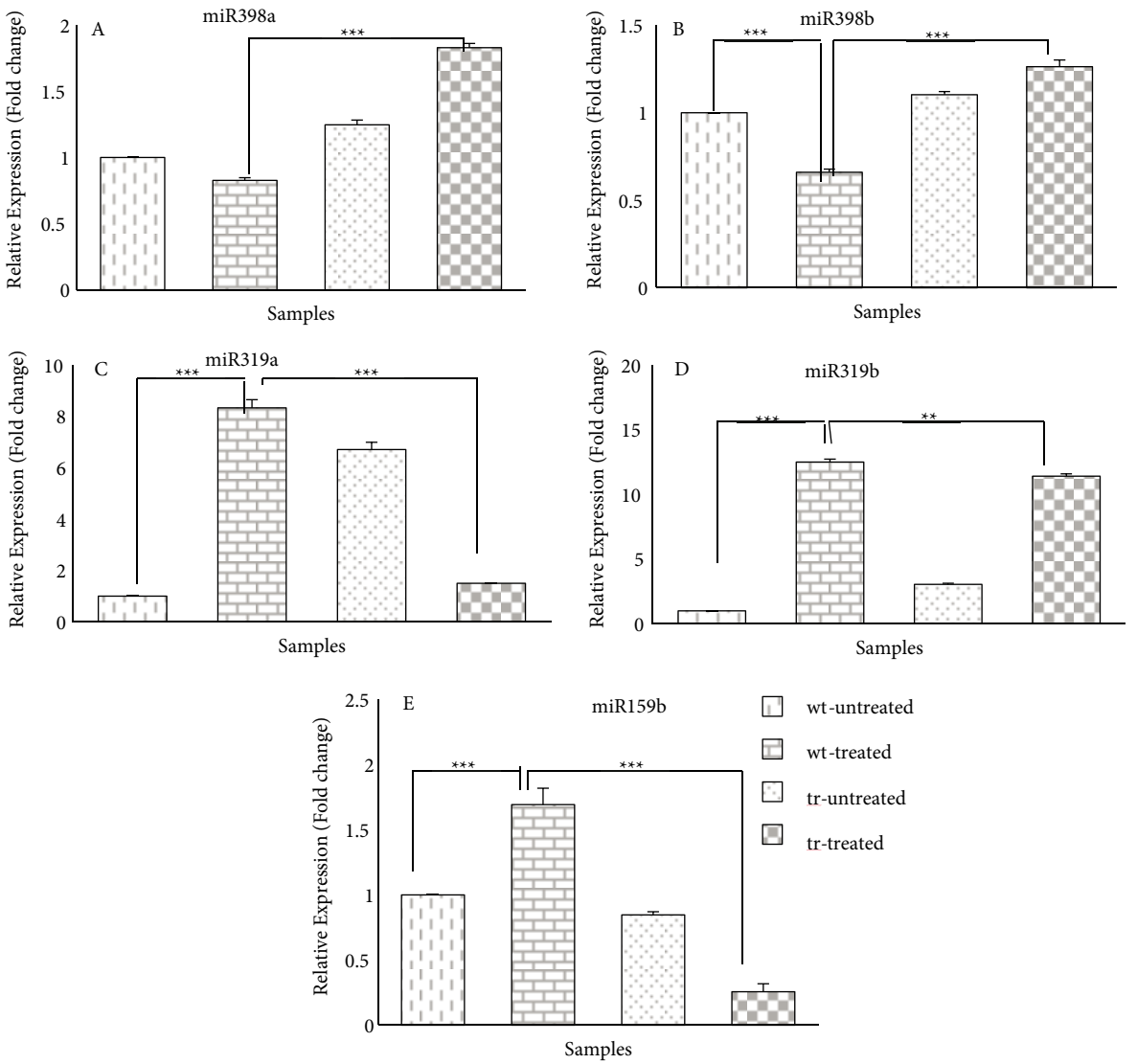

Figure 6. Relative pre-miRNA expression level comparisons of wild-type untreated, wild-type 150 mM salt-treated, transgenic untreated, and transgenic 150 mM salt-treated tobacco plants. (A) Relative expression level of miR398a, (B) relative expression level of miR398b, (C) relative expression level of miR319a, (D) relative expression level of miR319b, (E) relative expression level of miR159b. For relative quantification, the ΔΔCt formula was used (Livak and Schmittgen, 2001). Three independent biological replicates with 3 technical replicas per experiment were used for each qRT-PCR. Fold change for miRNA levels was normalized against the reference gene *18S rRNA*.

miR319a levels can be explained by the possible existence of a dynamic regulatory network for this miRNA. Levels of miR319b expression in salt-treated wild-type and salt-treated transgenic tobacco plants were not as significant as in miR319a (Figure 6D). Although these two miRNAs are expected to target similar mRNAs, it is possible that the expression profiles of these two miRNAs may be tissue- and context-dependent. Another miRNA gene that we investigated was miR159b. miR159b is known to be differentially regulated during callus formation, plant regeneration, and drought stress (Kantar et al., 2011). Our findings showed increased levels of miR159b in wild-type plants treated with high salt concentrations (Figure 6E). miR159b expression was decreased due to salt treatment in transgenic plants.

In summary, it appears that transgenic plants may have a different miRNA expression profile in response to salt stress. It is important to understand which miRNAs may act upstream of salt tolerance pathways so as to be able to manipulate certain miRNAs and engineer more efficient transgenics.

4. Discussion

Plant Na^+/H^+ antiporters are important for salt tolerance (Rodríguez-Rosales et al., 2009). The *AtNHX1* gene codes for a plant vacuolar Na^+/H^+ antiporter (Apse et al., 1999). In the present study, *AtNHX1*-expressing transgenic tobacco plants were generated by *Agrobacterium*-mediated transformation. Transgenic lines were then investigated for salt tolerance and salt stress-related responses, including miRNA expression level changes.

For concentrations higher than 150 mM NaCl, untransformed plants did not initiate roots and started to whither sooner, whereas transgenics were able to form proper calluses and roots up to 200 mM NaCl.

Interestingly, under low salt conditions, minor growth retardation and callus formation problems were observed in transgenic plants (Figures 1A and 1B). Under no salt stress, the presence of *AtNHX1* expression in transgenic plants may alter cytosolic pH values (Martinoia et al., 2006) and cause minor growth retardation. Similar observations were reported for the growth of transgenic *Arabidopsis* plants under nonstress conditions (Aharoni et al., 2003). However, transgenic plants were clearly growing better under high salt concentrations compared to the controls (100 mM, 150 mM, and 200 mM) (Figure 2).

Transgenic plants clearly showed higher resistance to elevated concentrations of NaCl and better growth than the controls. The regeneration ability of transgenic tobacco seeds (Figure 1) was also higher than that of the controls, especially under high salt concentrations (150, 200, and 250 mM) in 3- and 6-week-old plants. There was no statistically significant difference between the germination efficiencies of the transgenic and control groups at 0 mM salt concentration. On the other hand, at 50, 100, 150, 200, and 250 mM salt concentrations, the germination efficiency of the transgenic T_1 seeds (Figure 3) was statistically higher than in the control seeds. Higher germination ability of the transgenic plant seeds under high salt conditions also indicated increased salt tolerance of transgenic lines compared to the controls (Figure 3). All these results suggested that our transgenic tobacco plants grew much better under high saline conditions because of the *AtNHX1* gene.

To further investigate the effect of salt stress on our transgenic plants, lipid peroxidation was investigated in wild-type and transgenic tobacco plants. Environmental stresses may cause upregulation of reactive oxygen species, which can then cause oxidative damage to many cellular components, including membrane lipids. Lipid peroxidation can occur as a result of high salt stress implicated in cellular membranes. It generates a complex variety of products, many of which are reactive electrophiles. MDA is one of the by-products of lipid peroxidation and is hence a marker of the injury caused by stress. Its reaction with thiobarbituric acid to form an intensely colored chromogen makes it a convenient biomarker for determining lipid peroxidation (Ellouzi et al., 2011). Hence, MDA levels were quantified in the shoot and roots of salt-treated wild-type and transgenic plants. MDA levels in all transgenic lines were lower compared to the control plants under 150 mM NaCl salt stress (Figure 4). Accordingly, lower MDA levels are known to correlate with salt tolerance (Xu et al., 2009). Therefore, low levels of MDA were an indication of salt tolerance in our salt-treated transgenic plants.

Plants generally cope with osmotic stress by synthesizing and accumulating certain compatible solutes, which are termed as osmoprotectants. This process is known as osmoregulation. Proline is one of the most common osmolytes, accumulating in response to various environmental stresses in plants (Liu and Zhu, 1997). Proline maintains cell turgor, stabilizes membranes, and maintains protein conformation at low leaf water potentials. Hence, accumulation of proline is a known indicator of stress tolerance due to its role in osmotic adjustment (Li et al., 2011). Proline is also a major source of nitrogen for stress recovery and growth regulation (Sharma and Verslues, 2010). Moreover, proline is known to be involved in reducing photodamage in thylakoid membranes by scavenging and/or reducing the production of singlet oxygen (Reddy et al., 2004). In many plant species, accumulation of proline under stress is correlated with stress tolerance, and its concentration is generally higher

in stress-tolerant plants than in stress-sensitive plants (Ashraf and Foolad, 2007). Consistent with the literature and our earlier findings, proline levels were higher in both the shoots and roots (Figure 5A and 5B, respectively) of transgenic plants compared to the control plants.

After establishing the salt tolerance of our transgenic plants, we focused on the expression of several stress-related miRNAs. miRNAs are considered important novel factors for plant stress tolerance mechanisms via altering the expression patterns of stress-related target proteins (Reyes et al., 2010; Wang et al., 2013). Interestingly, salt stress-responsive miRNA targets are generally transcription factors and functional proteins such as MYBs, superoxide dismutases, and transcription factor family members (TCPs) (Sunkar et al., 2006; Jia et al., 2009). These target proteins play a vital role in the control of growth, protection against formation of reactive oxygen species and floral initiation, and seed production and development. Hence, it is also important to understand miRNA-based regulation of salt tolerance mechanisms.

miRNA398 (miR398) is a well-studied miRNA thought to be directly linked to plant stress regulatory networks (Zhu et al., 2011), as salinity was shown to cause decreased expression of miR398 in *Arabidopsis* (Jagadeeswaran et al., 2009). CSD1 and CSD2, as targets of abiotic stress-responsive miR398 in *Arabidopsis* (Sunkar and Zhu, 2004), are two closely related copper/zinc superoxide dismutases that play a role in the detoxification of superoxide radicals (Sunkar et al., 2006). Therefore, increased expression of miR398a/b may have a protectant role in the transgenic plants.

TCPs are known targets of miR319a during regulation of developmental processes (Li et al., 2013) and morphological adaptation (Zhou et al., 2013). TCPs are known to regulate plant growth and development (Cubas et al., 1999). Hence, miR319a upregulation leads to the downregulation of TCPs and this alters the proliferation and morphogenesis of leaves and roots (Palatnik et al., 2007; Li et al., 2012; Wang et al., 2014). Accordingly, in our case, the decrease in the expression of miR319a in salt-treated transgenic tobacco plants may be a contributing factor to the better growth of transgenic plants under high salt stress conditions. It may also explain why we observed minor growth retardation in our untreated transgenic plants compared to the controls, given the high levels of miR319a in untreated transgenic plants.

miR159b is thought to regulate the expression of *MYB33* and *MYB55* transcription factors that are members of *GAMYB*-like genes. *GAMYB*-like genes play a key role in plant growth, flowering, formation of reproductive organs of flowers, and following seed production (Woodger et al., 2003). Hence, the inhibition of *MYB33* and *MYB55* by miR159b was shown to cause growth inhibition in *Arabidopsis* plants, especially in seeds and anthers (Alonso-Peral et al., 2010). The decreased expression of miR159b may therefore be related to the improved growth of the transgenic plants under salt stress.

miR159, miR398, and miR319 family members are salt stress-responsive miRNAs identified in maize, *Arabidopsis*, and rice (Sunkar and Zhu, 2004; Ding et al., 2009). Changes in the levels of miR159, miR398, and miR319 family members of transgenic tobacco plants are possibly related to their adaptation to salt stress, such as better regeneration ability especially with increasing NaCl, better growth, low MDA, and high proline amounts.

The above-mentioned changes in precursor miRNA levels may be due to transcriptional up- or downregulation in transgenic plants. However, it is also important to consider the possibility of altered miRNA maturation pathways, especially under high salt concentrations. It is known that pre-microRNAs are intermediate products during the microRNA transcription process. A recent study reported that the presence and absence of mature forms of miRNAs depends on the presence and absence of their precursor forms. Therefore, the existence of pre-miRNAs can provide hints of miRNA gene expression regulation (Gan and Denecke, 2013). Decreased or increased maturation rates of precursor structures into mature miRNAs may also cause accumulation or depletion of precursor structures. It is also important to note that altered expression of miRNAs may be causative or consequential of salt treatment. Hence, more research is needed to better delineate their functions in stress-related pathways.

In conclusion, the introduction of *AtNHX1* to tobacco plants in this study enhanced the regeneration ability of transgenic plants under high salt stress conditions. Elevated proline accumulation and decreased MDA contents further confirmed salt tolerance in the transgenic plants. In addition, changes in the expression levels of the stress-associated miRNAs were observed for transgenic plants. These and other miRNAs are likely to help us better understand the responses of transgenic plants against salt and possibly other stresses. Future efforts for identification and characterization of abiotic-stress related miRNAs and their targets will possibly help us manipulate miRNAs to improve stress tolerance in transgenic plants and develop more powerful strategies for struggling against changing environmental conditions.

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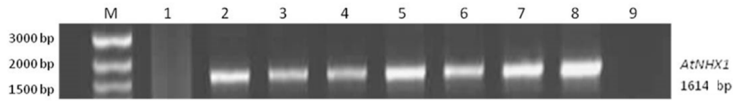
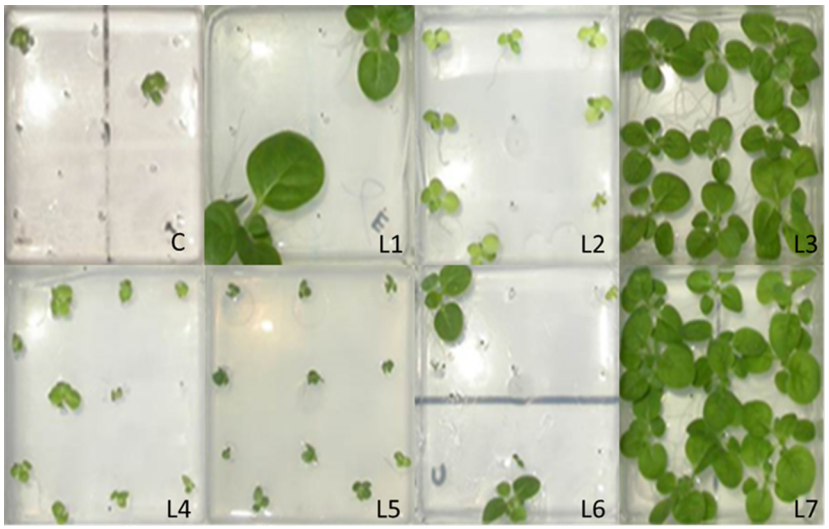
Supplementary Figures



Supplementary Figure 1. RT-PCR analysis of putative T_0 transgenic tobacco plants using specific primers for *AtNHX1* (M: GeneRuler 100-bp DNA Ladder Plus, 1: nontransgenic control tobacco plants, 2–8: transgenic lines, 9: negative control).

Supplementary Figure 2. Regeneration of T_1 seeds of wild-type (WT) and representative transgenic plants (T2, T7) in the soil.



Supplementary Figure 3. Germination and growth of 17-day-old wild-type (WT) and transgenic plants (L1–L7) on MS media containing 150 mM NaCl.