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Research article

The eelgrass *Zostera marina* antiporter gene (*ZmNa⁺/H⁺*) confers salt stress tolerance to ornamental tobacco (*Nicotiana alata*)

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Abstract

Salinity stress is a main restricting factor for plants' growth and development, resulting from climate change. The ornamental tobacco (*Nicotiana alata*) from the *Solanaceae* family is a bedding plant with attractive flowers suitable for semi-arid and arid lands. To obtain salt tolerance, the Na^+/H^+ antiporter gene was transferred from the halophyte eelgrass (*Z. marina*) grown in saltwater Lake Urmia to *N. alata*. For gene transformation, leaf explants were immersed in a liquid medium containing *Agrobacterium tumefaciens* containing the vacuolar gene *ZmNHX1*. The explants were subcultured on MS medium supplemented with 1 mg L^{-1} benzyl amino purine, 0.1 mg L^{-1} naphthalene acetic acid, 200 mg L^{-1} cefotaxime, and 10 mg L^{-1} kanamycin. After regeneration, salinity stress was applied by 0, 210, 230, and 240 mM NaCl in a completely randomized design with three replications. For the first time, the vacuolar Na^+/H^+ antiporter gene *ZmNHX1* was successfully transferred to ornamental tobacco. Compared to the wild-type plants, the transgenic plants showed higher content of leaf chlorophyll, leaf carotenoid, fresh weight and dry weight of the whole plant, leaf proline content, and leaf catalase activity. After salinity stress, the T revealed greater chlorophyll, carotenoid, anthocyanin, K^+: Na^+ ratio, fresh weight, dry weight, proline amount, catalase, and ascorbate peroxidase activity. The T also demonstrated a lower decline in relative water content and a lesser increase of leaves electrolyte leakage, compared to the WT. The stable expression of *ZmNHX1* obtained from eelgrass confers salinity stress tolerance in ornamental tobacco providing a new window for cultivation of this species in areas exposed to salinity stress and the resulting transgenic ornamental tobacco is suggested for garden flower cultivation in areas exposed to salinity stress.

Keywords: Antiporter gene, *Nicotiana alata*, Ornamental tobacco, Salt stress tolerance, *ZmNHX1*, *Zostera marina*.

Introduction

Ornamental tobacco (*Nicotiana alata* Link & Otto) is a perennial herbaceous plant originating from Southern Brazil and Northern Argentina. It belongs to the *Solanaceae* family with a chromosome number of 2*n*=18 (Knapp *et al.*, 2004). The main features of this garden plant are relatively high photosynthesis efficiency, easy cultivation, fast growth, high seed set, multiple flower colors (white, red, blue, purple, pink, yellow), aromatic flowers, host to a small number of pests and diseases, ease of gene transfer and regeneration of T (Doblin *et al.*, 2000).

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On the other hand, soil salinity is a serious worldwide environmental issue that reduces plant growth, development, and production (Mirzadeh Vaghefi & Jalili, 2020). About 40 % of all arable lands cannot be used because of the salinity problems. In response to various environmental stresses, plants have developed different physiological and biochemical strategies to adapt to or tolerate stress conditions (Moghaieb *et al.,* 2000). Among salinity-tolerant species is the halophyte eelgrass (*Zostera marina*) which this C4 plant grows completely submerged in seawater with a salinity range of 0.5 to 3.3% (Alemzadeh *et al.,* 2006). It is well known that under salt stress, the highly water-soluble salts in the soil (mainly NaCl) cause osmotic stress, which hinders plant roots from absorbing water. Excess Na^+ absorbed into the cytosol of plants results in ion toxicity and increases oxidative stress which further disturbs many important physiological and metabolic processes (Munns & Tester, 2008; Shabala & Cuin, 2008). The presence of salt in the soil and water reduces the osmotic potential, which causes the potential of dehydration in plants and exposes the plants to secondary osmotic stress or drought stress (Mirzaei & Dastoory, 2018). Plants have developed multiple mechanisms to prevent excessive Na^+ accumulation in the cytosol, including restricting the influx of environmental Na^+, increasing the efflux of Na^+ from the cell, and compartmentalizing Na^+ into vacuoles. These processes are largely achieved by some transporters such as Na^+/H^+ antiporters (NHXs) located in the vacuolar and the plasma membranes (Niu *et al.,* 1995; Blumwald *et al.,* 2000; Zhu, 2001). The H^+-ATPase pump is a major protein of the tonoplast in plant species, comprising 6.5-35% of total tonoplast proteins (Jaquinod *et al.,* 2007). The compartmentation of Na^+ in vacuoles not only relieves the toxic effect of Na^+ on the cytosolic metabolism but also reduces the osmotic potential for water uptake and preserves the turgor pressure as an important strategy for salt tolerance of plants (Blumwald *et al.,* 2000; Hasegawa *et al.,* 2000). Therefore, NHX genes encoding vacuole-type NHXs are considered good candidates to be isolated from halophytes and their transfer to salt-sensitive crops could facilitate their cultivation in saline environments (Khan *et al.,* 2015; Mishra & Tanna, 2017). Many vacuolar NHX genes have been isolated from various plant species such as rice (Fukuda *et al.,* 1999), ice plant (Chauhan *et al*., 2000), cotton (Wu *et al.,* 2004), maize (Zörb *et al.,* 2005), wheat (Cao *et al.,* 2011), rapeseed (Wang *et al.,* 2011) and chrysanthemum (Zhang *et al.,* 2012). Vacuolar H^+-ATPase antiporter genes have also been successfully isolated from the eelgrass plant. In previous research, Alemzadeh *et al.* (2006) isolated the *ZmVHA-B1* gene from eelgrass (*Zostera marina*) and later transformed the salt-sensitive mutant yeast cells. Recently, Wang *et al.* (2018) isolated and characterized a novel vacuolar-type KvNHX1 cDNA from the halophyte *Kosteletzkya virginica*.

Zhang & Blumwald (2001) reported for the first time the expression of a vacuolar NHX of *Arabidopsis* in tomato plants that significantly enhanced their tolerance to salt stress. Transgenic wheat with AtNHX1 showed increased shoot and root dry weight under salt stress, and transgenic maize with AtNHX1 exhibited improved germination under salt stress (Xue *et al.,* 2004; Yin *et al.,* 2004). Transgenic rice with AgNHX1 showed salt tolerance and exhibited increased survival of seedlings under salt stress (Ohta *et al.*, 2002). T with AgNHX1 (*Atriplex gmelini*), SsNHX1 (*Suaeda salsa*), or SaNHX1 (*Spartina anglica*) showed enhanced salt tolerance up to 300-400 mM NaCl, compared to glycophyte counterparts (Ohta *et al.*, 2002; Zhao *et al.,* 2006; Lan *et al.*, 2014). Overexpression of KvNHX1 enhanced the salt tolerance in transgenic tobacco lines with better growth, more chlorophyll, higher antioxidant enzyme activities, and osmoregulation capability by accumulating more proline, $Na^{+,}$ and K^+ in the leaves than the wild type under salt stress. Transgenic *Arabidopsis* with TaNHX1, SsNHX1, and MsNHX1 genes showed greater salt tolerance as compared to wild-type plants (Brini *et al.,* 2007; Li *et al.,* 2007; Bao-Yan *et al.,* 2008). Transgenic tobacco with GhNHX1 and BnNHX1 showed improved plant growth under salt stress (Wang *et al.,* 2004; Wu *et al.,* 2004). Mushke *et al.* (2019) showed that the overexpression of wheat TaNHX2

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gene in transgenic sunflowers conferred improved salinity stress tolerance and growth performance. Soliman *et al.* (2009) transferred a vacuolar Na^+/H^+ antiporter (AtNHX1) gene into tobacco leaf discs. Transgenic tobacco plants expressing the AtNHX1gene were able to grow in the presence of 150 to 300 mM NaCl and accumulated significantly at high concentrations of sodium ions in the leaves. In another study, constitutive expression of AtNHX1 enhanced salt tolerance in soybeans for over 6 generations, suggesting a great potential use of AtNHX1 for improving salt tolerance in plants by genetic engineering (Li *et al.*, 2010). Physiological and biochemical studies revealed the contribution of Na^+/H^+ antiporters in sequestrating intracellular ions (Na^+), pH regulation, and K^+ homeostasis in plants (Gaxiola *et al.*, 1999; Leidi *et al.*, 2010; Vijayargiya *et al.*, 2011). The expression of TaNHX1 (Brini *et al.*, 2007) and TaNHX3 (Lu *et al.*, 2014) in tobacco enhanced plant tolerance to high salt stress conditions. The wheat vacuolar Na^+/H^+ antiporter gene TaNHX2 facilitates the compartmentalization of Na^+ from the cytosol into the vacuole and significantly improves salt tolerance under high salinity environments (Yu *et al.*, 2007).

This study aimed to make use of the salt tolerance gene available in the halophyte eelgrass (*Z. marina*) grown in Lake Urmia, the largest salt lake in the Middle East and the six-largest saltwater lake on Earth. Although several Na^+/H^+ antiporter genes have been successfully transferred to tobacco model plants, no vacuolar NHX gene transformation is reported for the ornamental tobacco species *N. alata*. Therefore, for the first time in this study, the vacuolar antiporter NHX gene is transferred to the ornamental tobacco plant, and the salinity tolerance of T is investigated for possible use as garden flowers in saline environments.

Materials and Methods

Agrobacterium growth, selection of transgene, and regeneration

A pTZ57RT vector containing the vacuolar Na^+/H^+ antiporter gene (*NHX1*) was kindly provided by Dr. Abbas Alemzadeh from the Department of Plant Genetics and Production, College of Agriculture, Shiraz University. The *ZmNHX1* gene was previously isolated from eelgrasses (*Z. marina*) grown in Lake Urmia, North West of Iran (Alemzadeh *et al*., 2006). To confirm the presence of the *ZmNHX1* gene (1721bp) in the construct, the recombinant plasmids were digested with *EcoRI* and *BamHI*. The digested part carrying the desired gene was checked using agarose gel electrophoresis. The *Agrobacterium tumefaciens* strain GV3101 containing the binary vector 35SS *ZmNHX1* was used in this study. The vacuolar Na^+/H^+ antiporter target gene was positioned under the control of a 35SS promoter and 35S terminator. The kanamycin resistance gene (*nptII)*, helped in the selection of transformed ornamental tobacco explants.

The recombinant plasmid was transformed into competent cells of *Agrobacterium* strain GV3101 by the freeze–thaw method (Sambrook & Russel, 2001). A recombinant single colony of GV3101 containing *ZmNHX1* gene was selected and cultured in 5 ml liquid LB medium supplemented with 100 µg ml^{-1} carbenicillin, 25 µg ml^{-1} gentamycin, 75 mg L^{-1} kanamycin and 25 µg ml^{-1} rifampicin and incubated in a shaker-incubator at 28 °C, 180 rpm for 7-8 h until bacteria spectrophotometry of 600 nm spectrum reached the optical density of 0.6. Then, bacteria were centrifuged by 6000 g for 10 min at 4 °C. The supernatant was removed and the bacterial cells were suspended in MS liquid medium.

Suitable primers for the vacuolar sodium/proton antiporter gene were designed by Gene Runner software according to the sequence of *ECOR I* and *BamHI* enzyme cleavage sites in the pTRAKC vector. To increase digestion efficiency by restriction enzymes, four additional sequences will be added to both sides of the cut sites as enzyme anchors. The sequences of these primers were:

F: 5' ACAAGAATTCATGGATTTGGGTGTGATTACGGAGATGGC 3' and

R: 5' ACAAGGATCCCTAAAATGGATGCACACTTGGTTCCTCTGTT 3'

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DNA fragment amplification was performed using a Bio-Rad thermal cycler using specific primers. The PCR product was then purified with T4 DNA ligase enzyme and cloned into a T Easy vector. Cloned pTRAKC and T vectors were digested with *ECORI* and *Bam HI* enzymes and then purified. Finally, the antiporter gene was cloned into the pTRAKC vector. The heat shock method was used for transferring the vector to *Escherichia coli* strain Top10. Cloning was confirmed by PCR reactions, enzymatic digestion, and subsequent DNA sequencing.

Gene transfer and regeneration of explants

Ornamental tobacco seeds were surface sterilized with 70% ethanol for 30 seconds and were then treated with 30% sodium hypochlorite with a drop of Tween 80 for 10 min, then washed 3 times with sterile distilled water. Sterilized seeds were later cultured on hormone-free MS medium and were kept at 25 °C with a photoperiod of 16 h light/8 h darkness. After three weeks, discs with a diameter of 1 cm were separated from young ornamental tobacco leaves as explants for subsequent gene transfer. Afterward, they were immersed in a liquid medium (MS, vitamin, BAP, sucrose, acetosyringone) containing *Agrobacterium* for 60 seconds. The leaf pieces were blotted onto sterile filter papers for a short time and were inversely cultured on co-cultivation MS medium for 48h at the same growing condition described above. The explants were sub-cultured on MS medium, supplemented with 1 mg L^{-1} benzyl amino purine (BAP), 0.1 mg L^{-1} naphthalene acetic acid (NAA), 200 mg L^{-1} cefotaxime and 10 mg L^{-1} kanamycin. The regenerated plants grown on media supplemented with antibiotics and control plantlets were cultured in sterile peat: perlite (1:1) soil mixture and transferred to the greenhouse with 25° C, 60-75% relative humidity, and 16 h light/8 h dark cycle (Figure 1).

Salt stress treatment

After plants' establishment, salinity stress treatments at different levels of 0, 210, 230, and 240 mM NaCl were performed in a completely randomized design with three replications.

Molecular analysis of T

PCR reactions

To identify and amplify the antiporter gene, DNA from transgenic and non-transgenic seedlings was extracted by the CTAB method (Murray & Thompson 1998). PCR was performed using specific primers for the target gene (*ZmNHX1*) as follows:

F: 5′ ACAAGAATTCATGGATTTGGGTGTGATTACGGAGATGGC 3′

R: 5′ ACAAGGATCCCTAAAATGGATGCACACTTGGTTCCTCTGTT 3′

The amplification reaction was carried out with an initial denaturation step at 94 °C for 5 min, followed by denaturation at 94 °C for 1 min (25 cycles), annealing at 70 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min.

RNA isolation and Reverse Transcription PCR (RT-PCR)

The expression of transgene (*ZmNHX1*) was assessed by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from T0 transgenic tobacco plants using a Qiagen kit. RT-PCR amplification was performed using the SinaClone First Strand cDNA Synthesis Kit (SinaClon, Iran). Primer sequences, RT-PCR reaction programs, and amplicon sizes were the same as the PCR program.

Physiological and biochemical characterization of T

Chlorophyll and carotenoid contents

Total chlorophyll, chlorophyll a and chlorophyll b, and leaf carotenoids were measured based on Hicox & Israeistam (1979) method. 100 mg of the leaves were placed in a flask and 7 ml of DMSO (dimethyl sulfoxide) was added on them and incubated for 60 min at 65 ° C. The extract was filtered and the volume of the extract was increased to 10 ml by adding DMSO. Finally, using a spectrophotometer (Epoch

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BioTek, USA), the extract absorption was read at 645 and 663 nm. DMSO was used as blank. Chlorophyll content was calculated using the Shoaf & Lium (1976) formula.

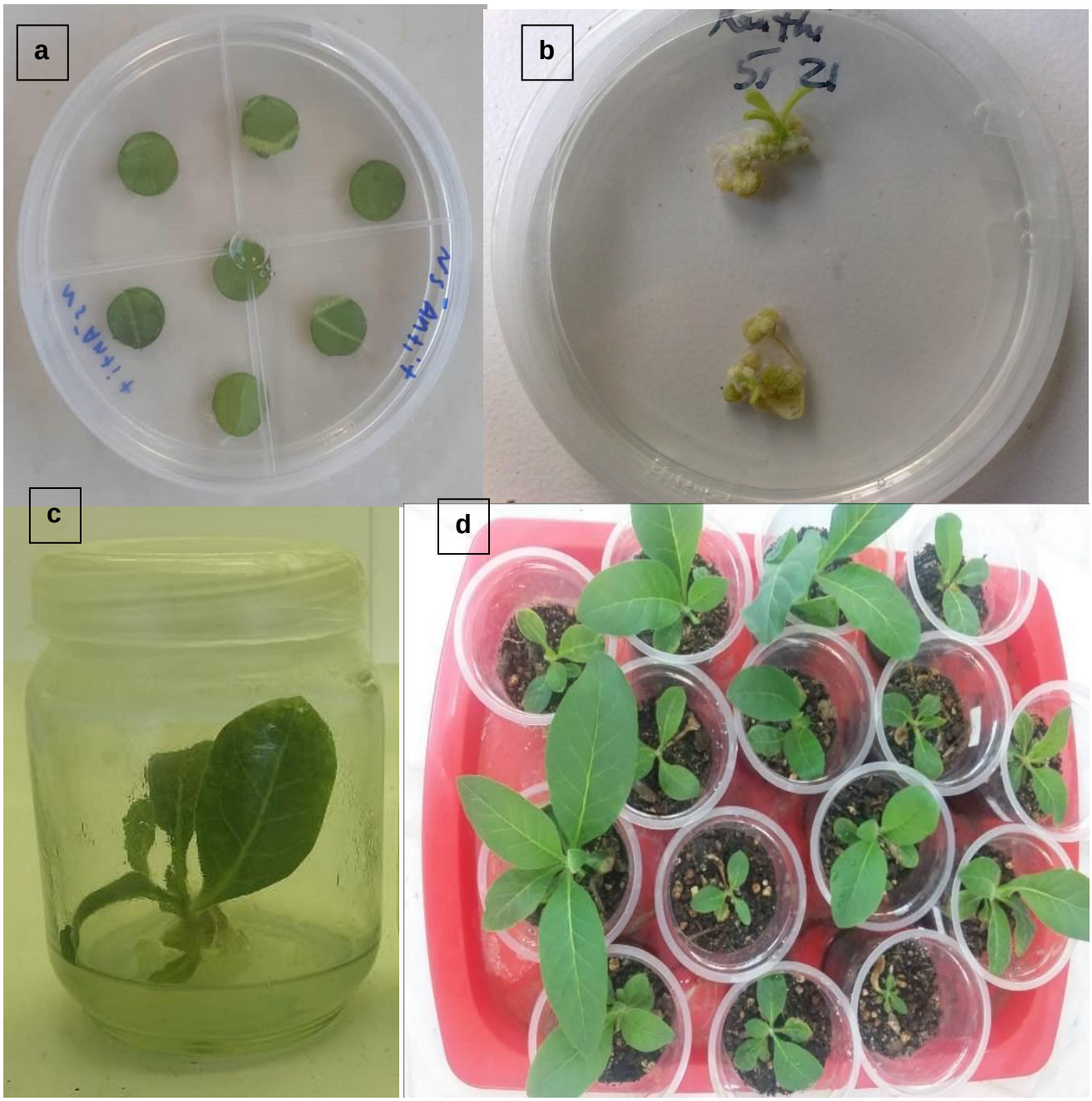


Figure 1- Regeneration and acclimatization of putative transgenic ornamental tobacco plants. a) Leaf discs of putative transgenic ornamental tobacco plant inversely cultured on MS medium. b) Proliferated shoots grown on callus. c) Rooted plantlets ready for transfer to the greenhouse. d) Acclimatized ornamental tobacco plants in peat: perlite (1:1) pot mixture.

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Relative Water Content (RWC)

Leaves with a diameter of one centimeter were weighed and then placed in Petri dishes containing distilled water for 24 h. Excess moisture of the leaves was taken with a paper towel. To determine the dry weight, the leaf pieces were placed in an oven at 70 ° C for 24 h. The relative water content of leaf samples was calculated based on equation 1 proposed by Barrs & Weatherly (1962):

Equation 1- $RWC = [(FW - DW)/ (TW - DW)] \times 100$

FW = fresh weight, TW = turgid weight, DW= dry weight.

Electrolyte leakage

Leaves were placed in test tubes containing 10 ml of double distilled water. Then, test tubes were placed in a water bath at 23 ° C for 4 h and were shaken slowly. The amount of primary ion leakage (C1) was measured using an EC meter and then the test tubes were placed in boiling water (100 ° C) for 15 min and cooled to room temperature. After that second ion leakage (C2) was measured. The percentage of ion leakage was calculated using the equation 2:

Equation 2- $\text{Electrolyte leakage} = C1/C2 \times 100$

Proline content

Proline was quantified using a spectrophotometer (UV-120-20, Japan) by the ninhydrin method according to the procedure of Bates *et al.* (1973). Leaves (100 mg) from treated plants were incubated in 10 ml of 3% sulphosalicylic acid for 48 h. Then 2 ml of leaf extract was applied to assay proline content. Ninhydrin (1.25 g) was dissolved in 30 ml of glacial acetic acid and then 20 ml of 6 M phosphoric acid was added and kept for 24 h at 40 ºC. Then, 2 ml of ninhydrin and 2 ml of glacial acetic acid were added to 2 ml of plant extract and the mixture was boiled at 100 ºC for 1 h in a water bath. Then, the solution was cooled and the reaction was terminated. About 8 ml of toluene was added to the contents and mixed vigorously for a few seconds and OD values for the colored component were measured at 520 nm, using toluene as the blank.

Antioxidant enzymes activity

Catalase (CAT) activity

The activity of catalase (CAT) was measured using the method of Chance & Maehly (1955). The CAT reaction solution (3 ml) contained 50 mM phosphate buffer (pH = 7), 10 mM H_2O_2, and 50 µl enzyme extract. The reaction was initiated by adding enzyme extract. Changes in absorbance of the reaction solution at 240 nm were read by a spectrophotometer device (Epoch BioTek, USA) every 20 s for 3 min.

Ascorbate peroxidase (APX) activity

Leaves (0.1 g) were completely ground with liquid nitrogen, and then 1 ml of 100 mM potassium phosphate buffer (pH = 7) containing 2 mM EDTA, 1 mM ascorbate, and 1% triton X-100 was added. The homogenized solution was centrifuged for 15 min at 10000 rpm at 4 ° C, and 50 µl of supernatant was used for assaying the enzyme. Antioxidant enzymes' activity was measured by a spectrophotometer device (Epoch BioTek, USA). The reaction mixture containing 100 mM potassium phosphate buffer (pH = 7), 1 mM H_2O_2, and 5 mM ascorbate was mixed with 50 µl of enzyme extract. Finally, the absorption decrease at 290 nm was read by the spectrophotometer for 30 s. The extinction coefficient of 2.8 mM^{-1} cm^{-1} was used to calculate the reaction. The specific enzyme activity is expressed as a unit per milligram of protein (Nakano & Asada, 1981).

The amount of anthocyanin

Leaves (0.5 g) were cut and powdered in liquid nitrogen. 10 ml of sodium acetate-HCl (pH 4.5) was added and later it was centrifuged at 6000 rpm for 15 min at 4 °C (Sigma, Germany). The supernatant was transferred to a spectrophotometer (Epoch BioTek, USA) and calculation of light absorbance was done at

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wavelengths of 510 and 700 nm. The anthocyanin concentration (mg l^{-1}) was measured according to the procedure explained by Habibi & Ramezanian (2017).

Sodium and potassium content

One gram of leaf was ground and placed in porcelain crucibles. They were put in a furnace at a temperature of 250 ºC. The temperature was later raised to 500 ºC to obtain the plant's ash. After cooling at room temperature, 5 ml of 2N hydrochloric acid was added to the ashes and mixed and then increased to 10 ml with distilled boiling water. Sodium and potassium content were determined using a flame photometer (Jenway, England). The final value was calculated using the standard curve of potassium and sodium (Chapman & Pratt, 1982).

Statistical analysis

The statistical significances of differences between mean values were assessed by analysis of variance and Duncan's multiple range tests ($p \leq 0.05$).

Results

Molecular analysis

Molecular analysis by RT-PCR showed the 1623 bp DNA fragment for the Na^+/H^+ antiporter gene in the transgenic plant (T). RT-PCR analysis approved that the Na^+/H^+ antiporter gene fragment was detected in agrobacterium-transformed plants but not in WT plants. Furthermore, RT-PCR analysis showed that the Na^+/H^+ antiporter gene was expressed in T (Figure 2).

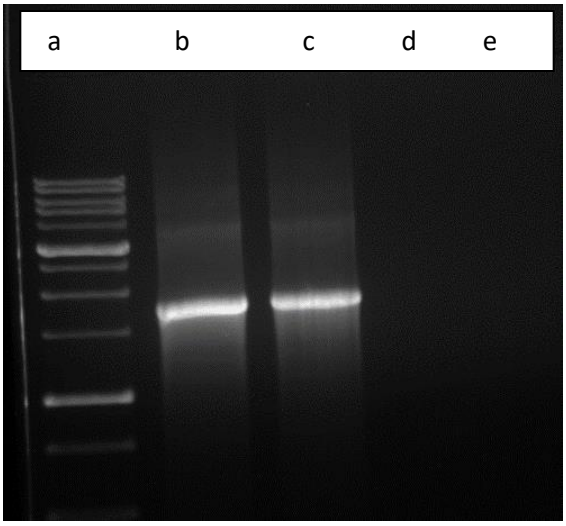


Figure 2- Gel electrophoresis of the RT-PCR products taken from the wild type and putative transgenic ornamental tobacco plant. From left to right: a) 1 Kbp DNA size marker. b) Positive control*. c) DNA sample of the putative transgenic plant (T1). The arrow indicates the 1623 bp band of the antiporter gene. d) Negative control. e) DNA sample of a wild-type plant (WT).
Positive control*= The positive control was obtained from the PCR of the pTRAKC vector containing the antiporter gene, whose primers were designed on the cutting enzymes on both sides of the gene.

Physiological properties of tobacco T subjected to salt stress

Chlorophyll and carotenoid contents

In most transgenic plants, chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid content were significantly increased, compared to wild-type (WT) plants. However, under saline conditions chlorophyll

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a, chlorophyll b, total chlorophyll, and carotenoid of all plants were reduced, whereas T showed a slighter decrease (Table 1).

Table 1- The effect of NaCl salt stress on photosynthesis pigments of wild type and transgenic *N. alata*.

Chlorophyll a (mg. g^{-1}F.W)	Control	210 mM	230 mM	240 mM
WT	0.45^{b}	0.43^{b}	0.40^{c}	0.36^{d}
T 1	0.50^{ab}	0.48^{ab}	0.42^{bc}	0.39^{c}
T 2	0.43^{b}	0.43^{b}	0.40^{c}	0.38^{c}
T 3	0.58^{a}	0.53^{a}	0.52^{a}	0.51^{ab}
T 4	0.52^{a}	0.49^{ab}	0.44^{b}	0.42^{bc}
T 5	0.56^{a}	0.55^{a}	0.53^{a}	0.50^{ab}

Chlorophyll b (mg. g^{-1} F.W)	Control	210 mM	230 mM	240 mM
WT	0.25^{b}	0.23^{b}	0.20^{c}	0.16^{d}
T 1	0.30^{ab}	0.28^{ab}	0.22^{bc}	0.19^{c}
T 2	0.23^{b}	0.23^{b}	0.20^{c}	0.18^{c}
T 3	0.38^{a}	0.33^{a}	0.32^{a}	0.31^{ab}
T 4	0.32^{a}	0.29^{ab}	0.24^{b}	0.22^{bc}
T 5	0.36^{a}	0.35^{a}	0.33^{a}	0.30^{ab}

Total chlorophyll (mg. g^{-1} F.W)	Control	210 mM	230 mM	240 mM
WT	0.70^{b}*	0.66^{b}	0.60^{c}	0.52^{d}
T 1	0.80^{ab}	0.76a^{b}	0.64b^{c}	0.58^{c}
T 2	0.66^{b}	0.66^{b}	0.60^{c}	0.56^{c}
T 3	0.96^{a}	0.86^{a}	0.84^{a}	0.82^{ab}
T 4	0.84^{a}	0.78^{ab}	0.66^{b}	0.64^{bc}
T 5	0.92^{a}	0.90^{a}	0.86^{a}	0.80^{ab}

Carotenoid (mg. g^{-1} F.W)	Control	210 mM	230 mM	240 mM
WT	0.35^{b}	0.31^{b}	0.31^{b}	0.28^{c}
T 1	0.40^{a}	0.41^{a}	0.38^{a}	0.34^{b}
T 2	0.41^{a}	0.39^{a}	0.34^{b}	0.31^{b}
T 3	0.39^{a}	0.39^{a}	0.35^{b}	0.34^{b}
T 4	0.38^{a}	0.35^{b}	0.32^{b}	0.32^{b}
T 5	0.40^{a}	0.39^{a}	0.38^{a}	0.35^{b}

* Means followed by the same letters are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

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Fresh and dry weight

The fresh and dry weights of most T were significantly increased, compared to WT plants. When *N. alata* plants were exposed to salt stress, the fresh and dry weights of all plants were decreased. As can be seen in Figure 3, the T 3 plant survived after exposure to 230 mM salt stress while the wild-type plant died after 45 days (Figure 3). The fresh weight and dry weight of WT were decreased by 61% and 59% at 240 mM NaCl, while T 3 showed a reduction of 30% and 27%, respectively (Table 2).

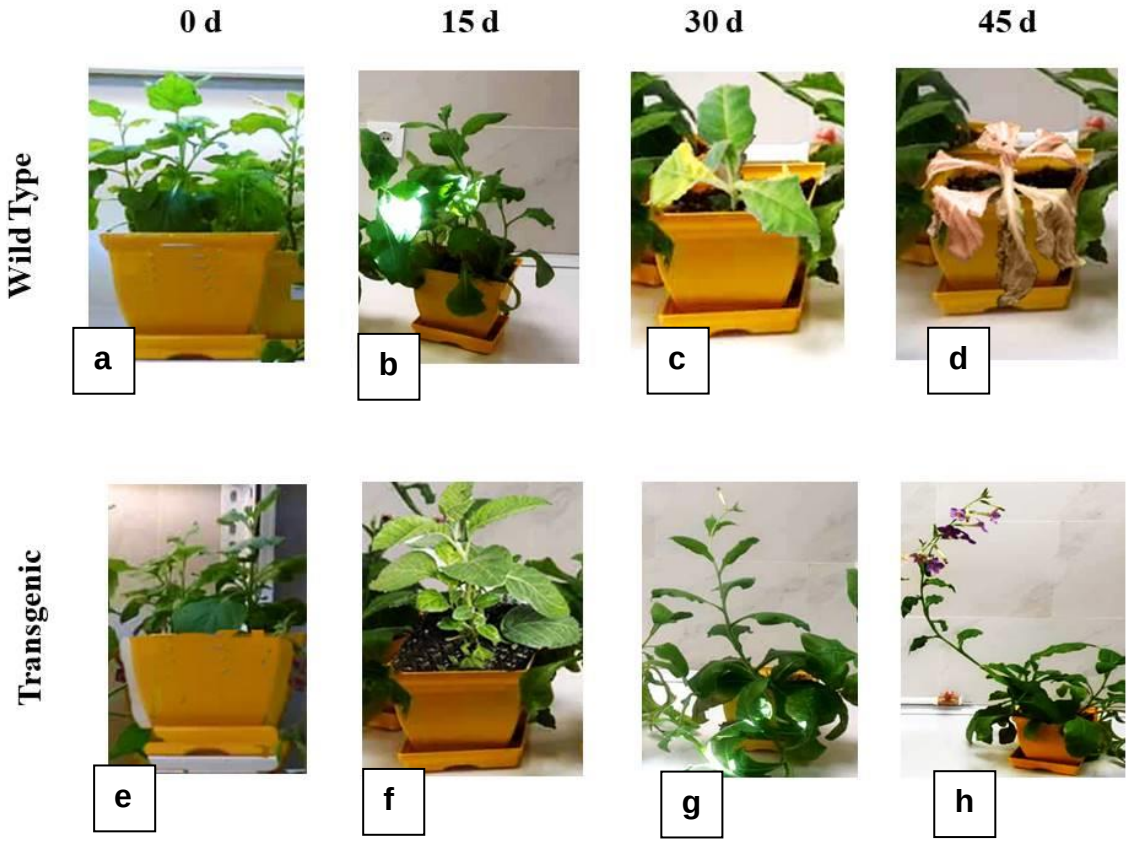


Figure 3- The effect of 230 mM NaCl on the growth of wild-type (top) and transgenic ornamental tobacco plants (bottom). a-d) The growth of wild-type plants was reduced after 15 days, while it died after 45 days. e-h) The growth of transgenic plants continued under salt stress. It started flowering after 30 days and survived afterward.

Sodium and potassium content

The Na^+ and K^+ amount of WT and T were similar at unstressed conditions. After that NaCl was applied, and Na^+ content increased in both wild and T. The wild-type plant showed a 12-fold rise in Na^+ absorption at the 240 mM NaCl treatment, while T showed a lower 10-fold increase. Under maximum salt stress conditions, the absorption of K^+ in WT was reduced significantly by 58%, while T showed about of 42% reduction. The T demonstrated a greater K^+: Na^+ ratio, in comparison with WT plants (Table 3).

Total anthocyanin content

The anthocyanin content of WT and T were similar under normal conditions. However, under salinity stress conditions, T produced more anthocyanin content compared to WT plants. The anthocyanin content

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of T showed a 100% increase under 240 mM salt treatment, while WT plants just showed a 33% increase (Table 4).

Table 2- The effect of NaCl salt stress on the fresh and dry weights of wild type and T of *N. alata*.

Fresh weight (g)	Control	210 mM	230 mM	240 mM
WT	18^{b}*	12^{cd}	10^{d}	7^{d}
T 1	19^{b}	13^{c}	12^{cd}	12^{cd}
T 2	23^{a}	17^{b}	17^{b}	15^{c}
T 3	23^{a}	17^{b}	16^{bc}	16^{bc}
T 4	22^{a}	16^{bc}	15^{c}	14^{c}
T 5	21^{a}	15^{c}	15^{c}	14^{c}
Dry weight (g)	Control	210 mM	230 mM	240 mM
WT	0.17^{b}	0.13^{cd}	0.11^{d}	0.07^{e}
T 1	0.19^{b}	0.14^{c}	0.12^{d}	0.12^{d}
T 2	0.22^{a}	0.17^{b}	0.16^{bc}	0.14^{c}
T 3	0.22^{a}	0.16^{bc}	0.16^{bc}	0.16^{bc}
T 4	0.21^{a}	0.16^{bc}	0.14^{c}	0.13^{cd}
T 5	0.21^{a}	0.14^{c}	0.14^{c}	0.13^{cd}

* Means followed by the same letters are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

Table 3- The effect of NaCl treatments on Na^+ and K^+ content of wild-type and transgenic *N. alata*.

Na^+ and K^+content	Control		210 mM		230 mM		240 mM	
(mg. g^{-1} D.W)	Na^+	K^+	Na^+	K^+	Na^+	K^+	Na^+	K^+
WT	6.1^{e}	41.2^{a*}	74.4^{a}	22.4^{c}	78.1^{a}	21.5^{c}	80.2^{a}	17.1^{d}
T 1	5.8^{e}	40.1^{a}	54.2^{d}	30.1^{b}	58.3^{c}	29.7^{b}	65.5^{b}	24.4^{c}
T 2	6.2^{e}	41.4^{a}	51.4^{d}	31.6^{b}	58.6^{c}	26.2^{c}	59.8^{c}	25.5^{c}
T 3	6.5^{e}	42.2^{a}	53.2^{d}	30.1^{b}	60.5^{c}	29.2^{b}	60.6^{c}	24.7^{c}
T 4	6.8^{e}	41.3^{a}	52.1^{d}	30.5^{b}	59.1^{c}	30.5^{b}	60.3^{c}	24.2^{c}
T 5	5.7^{e}	42.1^{a}	51.5^{d}	32.2^{b}	53.4^{d}	26.1^{c}	58.2^{c}	25.6^{c}

* Means followed by the same letters are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

Table 4- Anthocyanin content of transgenic and WT tobaccos under NaCl salt stress.

Anthocyanin content (mg g^{-1} D.W)	Control	210 mM	230 mM	240 mM
WT	0.81^{d*}	1.00^{c}	1.05^{c}	1.08^{c}
T 1	0.81^{d}	1.41^{b}	1.42^{b}	1.50^{b}
T 2	0.79^{d}	1.49^{b}	1.53^{b}	1.68^{a}
T 3	0.85^{d}	1.54^{b}	1.63^{a}	1.71^{a}
T 4	0.82^{d}	1.51^{b}	1.52^{b}	1.54^{b}
T 5	0.80^{d}	1.54^{b}	1.59^{a}	1.65^{a}

* Means followed by the same letters are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

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Proline content

The proline content of most T was significantly increased, in comparison with WT plants. When salinity stress was applied, the proline content of both WT and T was significantly enhanced. However, at maximum salt stress conditions, the WT showed a 32% increase, while T accumulated more proline content. The T_3 plant indicated an 80% rise in proline amount under 240 mM NaCl treatment (Table 5).

Table 5. The effect of NaCl treatments on proline content of WT and transgenic *N. alata*.

Proline content (µmole. g^{-1} F.W)	Control	210 mM	230 mM	240 mM
WT	140^{d}*	163^{c}	169^{c}	185^{c}
T 1	148^{d}	160^{c}	197^{b}	214^{b}
T 2	142^{d}	199^{b}	245^{a}	245^{a}
T 3	154^{c}	210^{b}	260^{a}	278^{a}
T 4	149^{d}	197^{b}	260^{a}	260^{a}
T 5	152^{c}	206^{b}	223^{b}	245^{a}

* Means followed by the same letters are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

Relative water content and electrolyte leakage

Under normal conditions, the relative water content (RWC) of both WT and T did not show a significant difference. As the NaCl concentration increased to 240 mM, the WT plant indicated a 36% decline in RWC while T displayed a lower 20-25% decline in relative water content (Table 6).

During un-stressful conditions, electrolyte leakage of both WT and T did not show a significant difference. When the highest salt stress concentration was applied, a 119% intensification of electrolyte leakage was seen in WT plants while T showed a lower 51-64% increase in electrolyte extrusion (Table 6).

Table 6. RWC and Electrolyte leakage of WT and transgenic ornamental tobaccos under NaCl salt stress conditions.

RWC (%)	Control	210 mM	230 mM	240 mM
WT	90^{d}*	68^{b}	60^{a}	57^{a}
T 1	91^{d}	80^{c}	72^{b}	68^{b}
T 2	90^{d}	79^{c}	71^{b}	68^{b}
T 3	89^{d}	78^{c}	70^{b}	70^{b}
T 4	91^{d}	81^{c}	74^{b}	72^{b}
T 5	88^{d}	79^{c}	71^{b}	69^{b}
Electrolyte leakage (%)	**Control**	**210 mM**	**230 mM**	**240 mM**
WT	31^{a}*	60^{d}	64^{d}	68^{d}
T 1	29^{a}	41^{b}	45^{bc}	45^{bc}
T 2	31^{a}	44^{bc}	45^{bc}	47^{c}
T 3	28^{a}	38^{b}	41^{b}	44^{bc}
T 4	31^{a}	44^{bc}	46^{c}	47^{c}
T 5	28^{a}	41^{b}	44^{bc}	46^{c}

* Means followed by the same letters are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

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Antioxidant enzymes activity

Under normal conditions, the CAT activity of T was higher than WT plants. The antioxidant enzyme activity of all plants was increased as NaCl salt concentrations were elevated. Although CAT enzyme activity showed a slight increase.

During normal conditions, the APX activity of both WT and T did not show significant differences. As the salinity stress was implemented, the APX enzyme activity of T demonstrated a greater amplification, compared to WT plants. At maximum salt stress of 240 mM, the WT plants showed a 41% rise in the APX enzyme activity, while an 86-99% increase was observed in the T (Table 7).

Table 7- Effect of NaCl salt stress on antioxidant enzyme activity of WT and transgenic *N. alata* plants.

CAT activity (U.mg^{-1} protein)	Control	210 mM	230 mM	240 mM
WT	5.41^{f}*	5.50^{e}	5.56^{e}	5.66^{d}
T 1	5.56^{e}	5.59^{e}	5.64^{d}	5.71^{c}
T 2	5.70^{b}	5.71^{c}	5.85^{b}	6.22^{a}
T 3	5.91^{c}	6.00^{b}	6.01^{b}	6.28^{a}
T 4	5.48^{ef}	5.54^{e}	5.62^{d}	5.64^{d}
T 5	5.51^{e}	5.58^{e}	5.66^{d}	5.78^{c}
APX activity (U.mg^{-1} protein)	Control	210 mM	230 mM	240 mM
WT	1.51^{d}	2.03^{c}	2.10^{c}	2.13^{c}
T 1	1.57^{d}	2.29^{c}	2.65^{b}	3.00^{b}
T 2	1.53^{d}	2.31^{c}	2.71^{b}	3.05^{b}
T 3	1.62^{d}	2.30^{c}	3.06^{b}	3.23^{a}
T 4	1.52^{d}	2.27^{c}	2.81^{b}	3.02^{b}
T 5	1.59^{d}	2.17^{c}	2.51^{c}	2.96^{b}

* Means followed by the same letters are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

Discussion

Saline conditions are considered a major abiotic stress that reduces crops' productivity. In response to these harsh conditions, plants have developed various physiological and phytochemical mechanisms (Qiu, 2012; Bassil *et al.,* 2019). Among various strategies, the halophyte *Z. marina* grown in Urmia salt lake uses the Na^+/H^+ antiporter gene to overcome salt stress. Perhaps this study is the first report on eelgrass gene transfer to ornamental tobacco, which proved successful and the result T showed increased salt tolerance.

The transgenic ornamental tobacco plants under salt stress showed more chlorophyll, carotenoid pigments, and fresh and dry weight of the whole plant than WT. Similarly, in a previous report on sweet potato, T with Na^+/H^+ antiporter gene showed an 11-29% increase in chlorophyll content under 200 mM NaCl stress (Wang *et al.,* 2016). In another study, the transgenic *Jatropha* plants using the *SbNHX1* gene from extreme halophyte *Salicornia brachiata* also had more chlorophyll than the WT plants (Jha *et al.,* 2013). Recently the red seaweed *Kappaphycus alvarezii* antiporter gene (*KaNa$^+$/H$^+$*) transfer to *Nicotiana tabacum* enhanced the chlorophyll and carotenoid pigments under salt stress and they grew better (Kumari *et al.,* 2022), as it can be seen in *N. alata* of the current study as well (Tables 1-2).

An improved K^+/Na^+ accumulation was detected among transgenic tobaccos, compared to WT plants (Table 3). In many plant species, salt tolerance property is associated with lower Na^+ absorption and higher K^+ content in whole plants (Apse *et al.,* 2003; Wu *et al.,* 2004; Dong *et al.,* 2021; Kumari *et al.,*

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2022). In this study, we found that the Na^+ content of the T exposed to various concentrations of NaCl (210, 230, and 240 µM) was lower in comparison to WT plants. It shows the sequestration of Na^+ in vacuoles by the Na^+/ H^+ antiporter gene, which averts the toxic effects of Na^+ on plant cells. This effect can also be seen in previous reports on grapevine and sugar beet (Ayadi *et al.,* 2020; Wu *et al.,* 2019). Furthermore, similar findings were reported by Chen *et al.* (2015) and Long *et al.* (2020) which demonstrated Na^+/H^+ antiporter genes overexpression and salt tolerance of *Arabidopsis* and *Gossypium* T, respectively.

The transgenic ornamental tobacco plants under salt stress showed more anthocyanin pigment than WT. It has been proven before that anthocyanin amounts are increased to counter salt stress and enhance tolerance to this harsh condition in wheat (Li *et al.,* 2022), *Pongamia* (Marriboina *et al.,* 2022) and barley (Ouertani *et al.* 2022). After salt stress, plants respond by producing several proteins associated with secondary metabolism, such as anthocyanin biosynthesis, to protect against ROS injury (Chen *et al.,* 2019).

Furthermore, higher proline amounts (44% to 80%) were found in the transgenic *N. alata* plants under salt stress, than wild-type plants. Compatible solutes such as proline accumulate in taxonomically various plant species in proportion to the external osmolarity, to retain osmotic balance and continue the water inflow (Himabindu *et al.,* 2016). This osmotic protection is a renowned approach implemented for the relief of salinity stress and it also provides a nitrogen source for stress recovery (Saxena *et al.,* 2013). Similar observations were also demonstrated by antiporter gene transfer to plants under 200 mM salt stress (Guo *et al.,* 2020; Kumari *et al.,* 2022), though *N. alata* of the current study faced more severe salinity conditions (240 mM NaCl), which led to a greater increase in the proline content of T (Table 5).

The transgenic ornamental tobacco plants under salt stress indicated greater relative water content (RWC) in comparison to control plants (Table 6). Comparable outcomes were also demonstrated by Guo *et al.* (2020) and Kumari *et al.* (2022). Higher RWC is assumed to be the most suitable tactic to maintain plant water status and it is considered an appropriate physiological measure during cellular water shortage, which explains why T tries to increase more RWC to attain salinity tolerance (Himabindu *et al.,* 2016).

The transgenic *N. alata* plants under salt stress showed less electrolyte leakage in comparison to control WT plants. In previous reports, the transgenic *Jatropha* using the *SbNHX1* gene (Jha *et al.,* 2013) and also transgenic tobacco by the *$KaNa^+/H^+$* antiporter gene (Kumari *et al*., 2022) showed diminished ion extrusion. These reduced amounts of electrolyte indicate less oxidative damage and decreased membrane injury in the T that were exposed to salinity stress.

Greater CAT and APX enzyme activity were observed in the T of ornamental tobaccos compared to wild-type plants (Table 7). Salt stress induces osmotic stress and the accumulation of reactive oxygen species also further hinders plant growth. It is assumed that enhanced antioxidant activity, ion compartmentation and/or exclusion, and osmotic adjustment are used by plants to counter salinity stress (Zhang *et al.,* 2014; Whang *et al.,* 2016; Li *et al.,* 2017). In a previous investigation, the antioxidant enzymes ascorbate peroxidase 1 and catalase 4 were found to be responsible for protection against reactive oxygen species and helped control the damaging superoxide amounts under salinity stress. They diminished the accumulation of ROS inside the plant cells struggling with saline conditions (Li *et al.,* 2015). In a previous report, Kumari *et al.* (2022) revealed that antiporter gene transfer could double, or even triple the CAT and APX enzymes' activity to fend off the saline stress. However, the ornamental tobacco plants of the current study exhibited a slight increase in CAT activity while the APX enzyme was more involved in countering NaCl stress, where its activity indicated a double increase (Table 7).

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Conclusion

In conclusion, the Na^+/H^+ antiporter gene was successfully transferred to ornamental tobacco plants. The T showed improved salinity tolerance and survived after 230 mM NaCl treatment. They grew better and continued flowering under salt stress and revealed more antioxidant activity and higher osmoregulation ability. The antiporter gene obtained from eelgrass has great potential to be used in ornamental plants' genetic engineering programs to enhance their salt tolerance.

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