Salt and Drought Tolerance of Transgenic Tobacco Expressing SsNHX1 Gene from Suaeda: A Postprint

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Date: 2017-11-10T00:00:00+00:00

Abstract

Tobacco is an important model plant and economic crop, and both salt stress and drought are environmental factors that cause significant damage to its growth, development, yield, and quality. To improve the salt and drought tolerance of tobacco, this study employed Agrobacterium-mediated genetic transformation to overexpress the vacuolar Na+/H+ antiporter gene SsNHX1 from Suaeda salsa in tobacco, and conducted phenotypic characterization and biochemical index detection for salt and drought tolerance of the transgenic tobacco, aiming to obtain SsNHX1 transgenic tobacco with desirable salt and drought tolerance phenotypes. Phenotypic analysis revealed that the salt resistance of SsNHX1-overexpressing lines L1 and L5 was significantly enhanced compared with the wild type, manifested as the ability to maintain vigorous growth under salt stress conditions without inhibition of root elongation. The SsNHX1-overexpressing lines accumulated more Na+ and K+ in leaves and roots, with a faster increase rate in Na+ content and a slower decrease rate in K+ content, and could maintain higher leaf relative water content and chlorophyll content, as well as lower malondialdehyde content and relative electrical conductivity. Drought stress experiments demonstrated that the overexpressing lines suffered less drought stress damage and rapidly resumed normal growth after re-watering. Meanwhile, the malondialdehyde content and relative electrical conductivity of the overexpressing lines were significantly lower than those of the wild type, while maintaining higher leaf relative water content and chlorophyll content. These results indicate that overexpression of the SsNHX1 gene in tobacco reduced damage to roots and cell membranes caused by salt stress and drought stress, and by regulating ion content and reducing cellular osmotic potential, maintained higher leaf relative water content and chlorophyll content, ultimately enhancing the salt and drought resistance of tobacco.

Full Text

Overexpression of Suaeda salsa SsNHX1 Gene Enhanced Salt and Drought Tolerance of Transgenic Tobacco

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Abstract

Tobacco (*Nicotiana tabacum* L.) is an important model and economic crop, and both salt and drought stress severely affect its growth, development, yield, and quality. To enhance tobacco' s tolerance to these stresses, we overexpressed the vacuolar Na /H antiporter gene *SsNHX1* from the halophyte *Suaeda salsa* in tobacco via *Agrobacterium*-mediated genetic transformation. Phenotypic and biochemical analyses were conducted to evaluate the salt and drought tolerance of the transgenic lines. Phenotypic characterization revealed that the *SsNHX1* overexpression lines L1 and L5 exhibited significantly improved salt tolerance compared to wild-type plants, maintaining vigorous growth and uninhibited root elongation under salt stress. The transgenic lines accumulated higher levels of Na and K in both leaves and roots, with a more rapid increase in Na content and a slower decline in K content. They also maintained higher relative leaf water content and chlorophyll levels while showing lower malondialdehyde content and relative conductivity. Under drought stress, the overexpression lines displayed less severe symptoms and recovered rapidly after rewatering, with significantly lower malondialdehyde content and relative conductivity than wild-type plants, while maintaining higher relative water content and chlorophyll levels. These results demonstrate that *SsNHX1* overexpression reduces damage to roots and cell membranes under salt and drought stress, maintains higher leaf water content and chlorophyll levels by regulating ion content and lowering cellular osmotic potential, and ultimately enhances tobacco' s salt and drought tolerance.

Keywords: *SsNHX1* gene; genetic transformation; *Nicotiana tabacum* L.; salt tolerance; drought tolerance; phenotype; biochemical indices

Introduction

Tobacco (*Nicotiana tabacum* L.) is an important model and economic plant, and both salt damage and drought are environmental factors that significantly harm its growth, development, yield, and quality. Genetic engineering approaches can effectively improve plant resistance to these stresses.

Plants employ various mechanisms to tolerate salt and drought, including osmotic adjustment and compartmentalization of toxic ions. Na compartmentalization is a key strategy for alleviating salt stress damage caused by osmotic potential imbalances [1]. The Na /H antiporter (NHX) is the primary carrier protein responsible for Na efflux and compartmentalization [2] and serves as an important determinant of salt tolerance through Na sequestration [3-5], though other regulatory mechanisms such as reverse transcriptase also play roles [6-7]. Vacuolar NHX proteins primarily function to maintain pH homeostasis in membrane systems, control K and Na balance, and regulate leaf development. *Arabidopsis thaliana* AtNHX1 is a typical vacuolar NHX; its overexpression confers salt tolerance to yeast *nhx1* mutants [8] and increases vacuolar NHX activity, with both effects contributing to elevated NHX protein levels [9]. AtNHX1 sequesters Na into vacuoles via the tonoplast [9-10] and catalyzes Na /H antiport in reconstituted proteoliposomes [11]. Under salt stress, vacuolar Na concentration increases with AtNHX1 expression levels, demonstrating that plant vacuolar NHX functions in Na compartmentalization [9]. This provides a foundation for studying NHX protein function and applying these proteins to improve plant salt tolerance. Overexpression of AtNHX1 has enhanced salt tolerance in various plants including *Arabidopsis* [12], maize (*Zea mays* L.) [13], rapeseed (*Brassica napus* L.) [14], cotton (*Gossypium herbaceum* L.) [15], tomato (*Lycopersicon esculentum* Mill.) [16], rice (*Oryza sativa* L.) [17], tobacco [18], and poplar (*Populus* spp.) [19].

The *SsNHX1* gene from the halophyte *Suaeda salsa* L. encodes a highly active Na /H antiporter. Under salt stress, *SsNHX1* expression increases, with higher expression in roots than in leaves [19]. Compared to wild-type plants, transgenic rice overexpressing *SsNHX1* shows stronger salt tolerance, indicating that *SsNHX1* plays an important role in salt tolerance in *S. salsa* [20]. *SsNHX1* transgenic *Arabidopsis* can maintain normal germination rates and complete its entire life cycle under high-salt conditions, whereas wild-type *Arabidopsis* shows stunted growth and development.

Tobacco is a major economic crop in China, but its production has long been severely affected by drought and salt damage. Since *SsNHX1* transformation of *Arabidopsis* and rice has demonstrated clear salt-tolerant effects, this study transformed tobacco with *SsNHX1* and evaluated the salt and drought tolerance of transgenic tobacco through phenotypic characterization and biochemical assays to obtain *SsNHX1* transgenic tobacco lines with good salt and drought tolerance.

Materials and Methods

1.1.1 Tobacco Seedling Culture in Nutrient Solution Seeds of wild-type tobacco ('Wisconsin 38') and T generation *SsNHX1* transgenic lines L1, L2, L3, L4, and L5 were germinated in darkness at 28°C for 3 days. Seedlings were then transferred to Hoagland nutrient solution and cultured in a growth chamber (14 h light/10 h dark, 18–25°C, 60–80% relative humidity) with daily solution changes.

1.1.2 Tobacco Seedling Culture in Soil Seeds of wild-type tobacco and T generation *SsNHX1* transgenic lines (L1, L2, L3, L4, L5) were sown in plastic pots containing a 3:1 mixture of regular soil and nutrient soil. Plants were grown in a growth chamber (14 h light/10 h dark, 18°C/25°C, 60–80% relative humidity) and transplanted at the 3–4 leaf stage.

1.2 Molecular Identification of SsNHX1 Overexpressing Transgenic Tobacco Hydroponically grown tobacco seedlings at the 5–6 leaf stage were treated with Hoagland nutrient solution containing 5 g · L^{1} NaCl for 24 h. Leaf DNA was extracted and RNA was isolated using the Trizol method. The *SsNHX1* gene was cloned into the plant expression vector pCPB (modified from pCambia3300) between the XbaI and SmaI sites downstream of the 35S promoter (Fig. 1a [Figure 1: see original paper]). Primers tSsNHX1-F (5 -AGGGAGCAAAGACAAGAG-3) and tSsNHX1-R (5 -TCTTCTATCTGAGCGGAATT-3) were used to detect the *SsNHX1* fragment in transgenic plants. RT-PCR was performed using primers qSsNHX1-F (5 -GTCATTTGGTGGGCTGGTCTC-3) and qSsNHX1-R (5 -TGAAAAGGACAACGGTTATGGTG-3) to screen for high-expression lines.

1.3 Salt Tolerance Analysis of SsNHX1 Transgenic Tobacco Under Salt Stress Hydroponically cultured seedlings of *SsNHX1* transgenic lines L1 and L5 and wild-type tobacco were treated with 0, 2.5, 5.0, and 7.5 g · L^{1} NaCl. Each line had 3 plants per treatment, with 3 replicates per salt concentration. Treatments lasted 7 days with solution changes every 2 days. After treatment, roots were scanned using a root scanner to observe seedling growth and measure root length. Na and K contents in roots and leaves of wild-type and transgenic lines were determined using a flame photometer.

1.4 Drought Tolerance Analysis of SsNHX1 Transgenic Tobacco Under Drought Stress Soil-grown seedlings of *SsNHX1* transgenic lines L1 and L5 and wild-type plants at the 9–10 leaf stage were subjected to drought stress by withholding water for 12 days, followed by 3 days of rewatering. Each line had 3 plants per treatment with 3 replicates per time point. Samples were taken from the middle portion of leaves at 0, 3, 6, 9, and 12 days of water withholding and at 1 and 3 days after rewatering to observe growth status.

1.5 Physiological and Biochemical Index Determination of SsNHX1 Transgenic Tobacco Under Salt and Drought Stress Wild-type and *SsNHX1* transgenic lines L1 and L5 were assessed for physiological and biochemical indices after salt and drought stress treatments, including relative water content (RWC) [21], chlorophyll content [22], malondialdehyde (MDA) content [23], and relative conductivity [24]. Data were analyzed using SPSS 13.0 software and plotted using Microsoft Excel.

Results

2.1 Molecular Identification of SsNHX1 Overexpressing Transgenic Tobacco After *SsNHX1* transformation, tobacco transgenic lines overexpressing the gene under the 35S promoter were obtained (Fig. 1a). PCR analysis of *SsNHX1* overexpression lines L1, L2, L3, L4, L5 and wild-type plants revealed clear target bands in all five transgenic lines but not in wild-type plants, confirming *SsNHX1* integration into these transgenic lines (Fig. 1b). For subsequent phenotypic analysis and physiological measurements under salt and drought stress, RT-PCR was performed on the five transgenic positive lines and wild-type tobacco. Lines L1, L4, and L5 showed the highest expression levels, and L1 and L5 were selected for further experiments (Fig. 1c).

Fig. 1 Schematic of the expression vector pCPB-35S-SsNHX1 and molecular detection of *SsNHX1*-transgenic tobacco plants.
a: The *SsNHX1* open reading frame (ORF) was inserted between the CaMV 35S promoter and nopaline synthase terminator (Nos) regions. The *bar* gene was used as the selective marker.
b: PCR detection of transgenic tobacco plants; M: DNA marker DL2000; (−): H O; (+): plasmid DNA; WT: wild-type tobacco plant transformed with empty vector; L1–L5: independent transgenic tobacco lines.
c: Expression analysis of *SsNHX1* in transgenic tobacco lines and WT plants.

2.2 Phenotypic Analysis of SsNHX1 Transgenic Tobacco Under Salt Stress As shown in Fig. 2a [Figure 2: see original paper], no significant phenotypic differences were observed between wild-type and transgenic tobacco before salt stress treatment. After 7 days of treatment with $2.5\ \text{g}\cdot\text{L}^{1}$ NaCl, growth and development of both wild-type and transgenic tobacco remained unaffected. However, under $5\ \text{g}\cdot\text{L}^{1}$ NaCl stress, wild-type tobacco growth was significantly inhibited compared to transgenic lines, showing reduced root number, shorter root length, and fewer, smaller leaves. At $7.5\ \text{g}\cdot\text{L}^{1}$ NaCl, wild-type tobacco leaves began yellowing and rotting by day 5, eventually leading to plant death. In contrast, although growth of transgenic lines L1 and L5 gradually slowed with increasing salt concentration, they maintained growth status and demonstrated superior salt tolerance compared to wild-type plants.

Since salt stress significantly inhibits plant root length, we examined root length of *SsNHX1* transgenic and wild-type tobacco under salt stress. No significant difference in total root length was observed under salt-free conditions, but differences became significant under salt stress (Fig. 2b). As NaCl concentration increased, total root length of wild-type plants decreased markedly, whereas the reduction was less severe in transgenic lines L1 and L5. For example, under 7.5 g · L ^{1} NaCl treatment, root lengths of transgenic lines L1 and L5 were 2.64-fold and 2.72-fold greater than wild-type tobacco, respectively, with significant differences. These results indicate that while salt stress inhibited root growth in all tobacco plants, the inhibitory effect was less severe in transgenic lines, essentially not affecting their root development.

Fig. 2 Phenotype (a) and root length (b) of *SsNHX1*-transgenic tobacco plants and wild-type plants after salt stress treatment.
a: Seedlings were cultured in Hoagland nutrient solution with NaCl gradients of 0, 2.5, 5.0, and 7.5 g · L ^{1} (w/v) until the three-leaf stage.
b: Root length after immersion in 0, 2.5, 5.0, and 7.5 g · L ^{1} NaCl. WT: wild-type tobacco; L1 and L5: *SsNHX1* transgenic tobacco lines. * indicates significant difference. Error bars represent standard deviation.

2.3 Na and K Content in Roots and Leaves of SsNHX1 Transgenic Tobacco Under Salt Stress Excessive Na uptake causes ionic imbalance in plants, particularly inhibiting K absorption and promoting K loss. Under salt stress, vacuolar Na /H antiporters transport Na into vacuoles, reducing K loss [25]. In this study, Na content increased rapidly in both leaves and roots of wild-type and transgenic tobacco after NaCl treatment. For example, under 7.5 g · L ^{1} NaCl treatment, Na in wild-type tobacco leaves increased from 15.84 mg · g ^{1} to 52.53 mg · g ^{1}, while transgenic lines L1 and L5 increased from 18.09 mg · g ^{1} to 60.37 mg · g ^{1} and from 20.04 mg · g ^{1} to 63.35 mg · g ^{1}, respectively. Similarly, Na content in roots of wild-type, L1, and L5 increased significantly to 40.48 mg · g ^{1}, 42.77 mg · g ^{1}, and 53.75 mg · g ^{1}, respectively (Fig. 3a [Figure 3: see original paper], 3b). K content in leaves and roots decreased with increasing NaCl concentration. For instance, after 7.5 g · L ^{1} NaCl treatment, K in wild-type leaves decreased from 79.76 mg · g ^{1} to 46.13 mg · g ^{1}, while transgenic lines L1 and L5 decreased from 79.04 mg · g ^{1} to 58.76 mg · g ^{1} and from 80.17 mg · g ^{1} to 53.03 mg · g ^{1}, respectively, with significant differences. Similarly, K content in roots of wild-type, L1, and L5 decreased to 29.44 mg · g ^{1}, 32.04 mg · g ^{1}, and 38.69 mg · g ^{1}, respectively, with significant differences (Fig. 3c, 3d). The trend of K and Na changes in roots mirrored that in leaves (Fig. 3b, 3d). Compared to wild-type, transgenic tobacco showed slower K decline and faster Na increase with rising salt concentration, resulting in higher K /Na ratios. Na and K contents were higher in leaves than in roots, particularly in transgenic plants, indicating that Na compartmentalization primarily occurs in leaves under stress conditions.

Fig. 3 Na and K contents of wild-type and *SsNHX1*-transgenic tobacco plants under salt stress. WT: wild-type tobacco; L1 and L5: *SsNHX1* transgenic tobacco lines. * indicates significant difference. Error bars represent standard deviation.

2.4 Physiological Indexes of SsNHX1 Transgenic Tobacco Under Salt Stress Salt stress induces various physiological changes in plants, including alterations in relative water content, malondialdehyde content, relative conductivity, and chlorophyll content, which typically correlate with salt stress severity. As shown in Fig. 4a [Figure 4: see original paper], relative water content began decreasing in both wild-type and transgenic tobacco after 6 days of NaCl treatment. From day 9 to day 15, the decline became more pronounced and differences between genotypes became significant. For chlorophyll content, the decrease in wild-type tobacco became more severe over time, dropping to 0.65 mg • g ^{1} at day 15, while transgenic lines L1 (1.51 mg • g ^{1}) and L5 (1.68 mg • g ^{1}) maintained significantly higher levels (Fig. 4b).

Malondialdehyde content and relative conductivity showed opposite trends to relative water content and chlorophyll content. Both parameters increased with NaCl treatment duration, with higher values in wild-type than in transgenic lines (Fig. 4c, 4d). For example, after 15 days of NaCl treatment, malondialdehyde content in wild-type tobacco was 9.71 nmol • g ^{1}, while transgenic lines L1 and L5 had significantly lower values of 8.75 nmol • g ^{1} and 7.34 nmol • g ^{1}, respectively. Similarly, after 15 days of NaCl treatment, relative conductivity in wild-type plants was 90.68%, while transgenic lines L1 and L5 showed significantly lower values of 75.52% and 77.20%, respectively. These results demonstrate that physiological changes in *SsNHX1* transgenic plants closely correlate with their enhanced salt tolerance phenotype.

Fig. 4 Changes in physiological indexes of *SsNHX1*-transgenic tobacco under salt stress. WT: wild-type tobacco; L1 and L5: *SsNHX1* transgenic tobacco lines. * indicates significant difference. Error bars represent standard deviation.

2.5 Phenotypic Analysis of SsNHX1 Transgenic Tobacco Under Drought Stress Many studies have shown that salt and drought tolerance are associated. As shown in Fig. 5 [Figure 5: see original paper], no obvious differences were observed between *SsNHX1* transgenic and wild-type tobacco after 3 days of water withholding (Fig. 5a, b). However, after 6 days of drought treatment, wild-type tobacco began to wilt while transgenic tobacco continued normal growth (Fig. 5c). At 9 days of water withholding, wild-type tobacco leaves became severely curled and wilted, whereas transgenic lines L1 and L5 showed only mild wilting and yellowing (Fig. 5d). After 12 days of water withholding, both wild-type and transgenic tobacco were severely wilted, but transgenic line L1 showed milder symptoms (Fig. 5e). After 1 day of rewatering, transgenic tobacco leaves, though still visibly chlorotic, quickly recovered to a relatively upright growth state, while wild-type plants remained wilted and failed to recover even after 3 days of rewatering, indicating severe physiological water loss and inability to restore normal growth through self-regulation (Fig.

5f, g).

Fig. 5 Growth phenotypes of wild-type (middle) and transgenic tobacco lines L1 (left) and L5 (right) under drought treatment.
a: Before drought treatment; b: 3 days of drought; c: 6 days of drought; d: 9 days of drought; e: 12 days of drought; f: 0 days of rewatering; g: 3 days of rewatering. WT: wild-type tobacco; L1 and L5: *SsNHX1* transgenic tobacco lines.

2.6 Physiological Indexes of SsNHX1 Transgenic Tobacco Under Drought Stress Like other stress factors, drought stress affects plant physiological changes. The trends in relative water content and chlorophyll content in *SsNHX1* transgenic tobacco under drought stress were similar to those under salt stress, decreasing with treatment duration (Fig. 6a [Figure 6: see original paper], 6b). After 12 days of water withholding, relative water content in wild-type tobacco decreased to 63.40%, while transgenic lines L1 and L5 maintained significantly higher values of 70.56% and 69.02%, respectively. Chlorophyll content differences also reached maximum values at this time, with wild-type at 0.94 mg · g ^{1} compared to 1.77 mg · g ^{1} in L1 and 1.78 mg · g ^{1} in L5, with significant differences. During drought treatment, relative conductivity and malondialdehyde increased in both wild-type and transgenic tobacco, but the increase was more pronounced in wild-type plants. After 12 days of treatment, malondialdehyde content in wild-type tobacco was 4.67 nmol · g ^{1}, while transgenic lines L1 and L5 had significantly lower values of 3.73 nmol · g ^{1} and 3.90 nmol · g ^{1}, respectively (Fig. 6c). Relative conductivity was also higher in wild-type than in transgenic plants; after 12 days of treatment, wild-type relative conductivity was 84.92% compared to 70.72% in L1 and 72.92% in L5 (Fig. 6d).

Fig. 6 Changes in physiological indexes of *SsNHX1*-transgenic tobacco under drought stress. WT: wild-type tobacco; L1 and L5: *SsNHX1* transgenic tobacco lines. * indicates significant differences. Error bars represent standard deviation.

Discussion and Conclusion

To effectively combat salt and drought stress and maintain ionic balance of Na and K , plants can extrude Na or compartmentalize it into vacuoles, thereby enhancing tolerance to these stresses [26]. In this regulatory process, tonoplast Na /H antiporters play a crucial role, and overexpression of NHX-type genes encoding these proteins can improve salt tolerance in transgenic plants of many species [27]. *SsNHX1* is an NHX-type gene from the halophyte *Suaeda salsa* whose expression increases under salt stress and significantly improves salt tolerance in rice and *Arabidopsis* [28–29]. However, studies on salt and drought resistance of *SsNHX1* in other plants remain scarce. Therefore, this study employed genetic engineering to overexpress *SsNHX1* to improve tobacco’ s salt and drought tolerance.

Plant salt tolerance is closely related to drought tolerance. In many transgenic lines, improved salt tolerance is often accompanied by enhanced drought tolerance. In this study, *SsNHX1* transgenic tobacco showed normal root development under high salt concentrations and strong salt-tolerant phenotypes under increasing salt treatments. Under drought stress, they exhibited strong drought tolerance and largely recovered their pre-stress phenotype after rewatering. These results indicate that both salt and drought tolerance were improved in transgenic tobacco compared to wild-type. Similar results were reported for AtNHX1-transformed *Arabidopsis* and TNHX1-transformed wheat, which showed improved salt and drought tolerance [30–31]. These findings suggest that overexpression of vacuolar NHX genes can enhance the ability of transgenic plants to cope with both salt and drought stress.

Under salt stress, vacuolar NHX proteins maintain Na /K balance. In this study, Na content increased significantly in leaves and roots of *SsNHX1* transgenic tobacco after NaCl treatment, with higher levels in leaves than in roots. This may result from enhanced *SsNHX1* activity directly compartmentalizing Na into vacuoles in tobacco leaves [32–33], consistent with reports that increased tonoplast Na /H antiporter expression enhances Na compartmentalization [34]. However, current technical limitations prevent the separation of vacuoles from cytoplasm [4], so more detailed studies on the precise allocation of Na and K between cytoplasm and vacuole by vacuolar NHX proteins like SsNHX1 are needed. Maintaining lower Na /K ratios can effectively reduce plant osmotic potential to facilitate water uptake, similar to findings in TaNHX2 transgenic tomato [35] and AtNHX1 transgenic petunia [*Petunia hybrida* (J. D. Hooker) Vilmorin] [36]. These results suggest that overexpression of vacuolar *SsNHX1* improves transgenic tobacco salt tolerance, possibly by effectively regulating intracellular osmotic balance through Na compartmentalization.

Compared to *SsNHX1* transgenic tobacco, wild-type plants showed lower relative water content under both salt and drought stress, indicating that transgenic tobacco had improved ability to regulate leaf osmotic pressure and water retention, maintaining appropriate osmotic pressure under these stress conditions. Although chlorophyll content in *SsNHX1* transgenic tobacco decreased under salt and drought stress, it remained higher than in wild-type plants, likely because high Na concentrations and low water content inhibit photosystems I and II, reducing photosynthesis and chlorophyll synthesis rates [37-38]. This result is consistent with studies on AtNHX1 transgenic cotton [14], *SsNHX1* transgenic rice [39], VxNHX [40] and MdNHX1 [41] transgenic *Arabidopsis*, and ThDREB transgenic tobacco [42]. In this study, both MDA content and relative conductivity increased in *SsNHX1* transgenic and wild-type tobacco under salt and drought stress, indicating plasma membrane damage, with more severe damage in wild-type plants. This demonstrates that *SsNHX1* transgenic tobacco experienced reduced plasma membrane damage under salt and drought stress.

In conclusion, this study demonstrates that the tonoplast Na /H antiporter encoded by *SsNHX1* can reduce stress damage in transgenic tobacco by altering Na and K ratios. Overexpression of *SsNHX1* in tobacco enhanced both salt and drought tolerance. The physiological changes reflected by relative water content, malondialdehyde content, relative conductivity, and chlorophyll content in transgenic tobacco were consistent with their improved salt and drought tolerance.

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Note: Figure translations are in progress. See original paper for figures.