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Research article

Multiple compartmentalization of sodium conferred salt tolerance in *Salicornia europaea*

Sulian Lv, Ping Jiang, Xianyang Chen, Pengxiang Fan, Xuchu Wang, Yinxin Li*

Key Laboratory of Plant Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China

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ABSTRACT

Euhalophyte *Salicornia europaea* L., one of the most salt-tolerant plant species in the world, can tolerate more than 1000 mM NaCl. To study the salt tolerance mechanism of this plant, the effects of different NaCl concentrations on plant growth, as well as Na^+ accumulation and distribution at organ, tissue, and subcellular levels, were investigated. Optimal growth and an improved photosynthetic rate were observed with the plant treated with 200–400 mM NaCl. The Na^+ content in the shoots was considerably higher than that in the roots of *S. europaea*. The Na^+ in *S. europaea* cells may act as an effective osmotic adjuster to maintain cell turgor, promoting photosynthetic competence and plant growth. The results from the SEM–X-ray and TEM–X-ray microanalyses demonstrate that Na^+ was compartmentalized predominantly into the cell vacuoles of shoot endodermis tissues. Accordingly, the transcript amounts of *SeNHX1*, *SeVHA-A*, and *SeVP1* increased significantly with increased NaCl concentration, suggesting their important roles in Na^+ sequestration into the vacuoles. Therefore, a multiple sodium compartmentalization mechanism is proposed to enhance further the salt tolerance of *S. europaea*.

1. Introduction

Salinity is one of the major environmental factors that limit plant growth and productivity. This condition interferes with plant growth as it leads to osmotic stress, ion toxicity, and nutrient deficiency [1]. Excessive Na^+ in the cytoplasm not only causes ion imbalance and affects critical biochemical processes [2], but also increases plasma membrane injury, causes malondialdehyde accumulation, and impairs photosynthetic capacity [3,4].

Many mechanisms have evolved in plants as responses to salt stress, such as osmotic adjustment and excessive Na^+ reduction in the cytoplasm. Na^+ extrusion from the cytosol and vacuolar compartmentalization are two means of reducing excessive Na^+ in the cytoplasm. The vacuolar compartmentalization of Na^+ through vacuolar Na^+/H^+ antiporters provides an efficient mechanism for averting the deleterious effects of Na^+ in the cytosol and maintains an osmotic potential through Na^+ (and chloride) accumulation in the vacuole to drive water uptake into cells [5]. Salt tolerance genes may have evolved from halophytes to glycophytes, and halophytes generally use a salt tolerance mechanism similar to that of glycophytes [1,6,7]. Moreover, some euhalophytes can accumulate considerable amounts of Na^+ in their shoots [up to 50% of dry weight (DW)] without drying, remarkably different from glycophytes [6]. Halophytes regulate Na^+ transport more effectively than glycophytes, and many euhalophytes show growth stimulations even upon further NaCl addition [6,8]. Zhao et al. [9] reported that *Kalidium folium* and *Suaeda salsa* both have high Na^+ and Cl^- accumulation capacities, related to the high activities of tonoplast H^+-ATPase and H^+-PPase. However, experimental evidence for the vacuolar compartmentalization of Na^+ is limited [10]. Hajibagheri and Flowers [11] studied the ion distribution within mature root cortical cells of the halophyte *Suaeda maritima* L. Dum. grown in 200 mM NaCl via X-ray microanalysis of the freeze-substituted thin sections. They found that the Na and Cl concentrations in the vacuoles were approximately four-fold of those in the cytoplasm or cell walls. The results from the X-ray analysis of *S. salsa* also revealed that Na^+ and Cl^- were mainly retained in the vacuoles in 100 mM NaCl [12]. However, no data obtained by direct measurement using ion-specific micro-electrodes in the cells of halophytes have been reported to date.

Salicornia europaea L., a succulent euhalophyte in Chenopodiaceae, is widely distributed in coastal and inland salt marshes. *S. europaea* can withstand more than 1000 mM NaCl and is one of the most salt-tolerant plant species in the world [10,13]. Without salt glands or salt bladders, *S. europaea* plants can accumulate considerable amounts of Na^+ in their shoots (up to 50% of DW) [13,14]. In the present study, the effects of different NaCl

* Corresponding author. Tel.: +86 10 62836258; fax: +86 10 82596139.
E-mail address: yxli@ibcas.ac.cn (Y. Li).

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concentrations on plant growth as well as Na^+ accumulation and distribution at organ, tissue, and subcellular levels, were investigated to examine the salt tolerance mechanism in *S. europaea*.

2. Results

2.1. S. europaea plants showed optimal growth and improved photosynthetic performance under moderate NaCl concentration

Thirty-day old *S. europaea* plants were treated with 0, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 mM NaCl. All plants grew well after 21 d treatment with different NaCl concentrations. *S. europaea* treated with 200–600 mM NaCl grew better than the control (plants under 0 mM NaCl). However, in contrast to the control, plant growth was significantly inhibited under higher NaCl concentrations 800–1000 mM (Fig. 1A). Similar but more significant morphological changes were observed when the treatment duration was prolonged to 3 months (Fig. 1B).

As shown in Fig. 1, after 21 d salt treatment, all the physiological parameters investigated, such as shoot height, shoot diameter, dry weight and water content, initially increased, then they decreased with increasing salt concentration. Shoot height and DW increased markedly with an increase in NaCl from 100 to 400 mM, and then linearly decreased at NaCl concentrations above 400 mM (Fig. 1C, E). The values of these parameters became lower than the control when the NaCl concentration was over 700 mM (Fig. 1C, E), indicating plant growth suppression. After the salt treatments, shoot diameter increased significantly compared with the control plants (Fig. 1D).

Moderate NaCl concentration improved the water content of *S. europaea* shoots (Fig. 1F). The water content in the control plants was approximately 89%, and maximum water content (approximately 93%) was observed in the 200–300 mM NaCl-treated plants. However, the water content decreased markedly in plants treated with at least 400 mM NaCl concentration. The water content decreased to approximately 75% when treated with an excessively high NaCl concentration (800–1000 mM) (Fig. 1F).

Table 1
Effect of NaCl treatments on the photosynthetic performance of *Salicornia europaea* plants. *A*, net rate of photosynthesis; *gs*, stomatal conductance; *Tr*, transpiration rate. Values are means ± SD ($n = 3$). Values followed by different letters within each row are significantly different at $P \leq 0.05$.

NaCl concentration (mM)	*A* ($\mu mol\ m^{-2}\ s^{-1}$)	*gs* ($mol\ m^{-2}\ s^{-1}$)	*Tr* ($mmol\ m^{-2}\ s^{-1}$)
0	4.22 ± 0.54 c	0.18 ± 0.04 b	2.47 ± 0.25 b
200	9.44 ± 0.84 a	0.37 ± 0.04 a	5.07 ± 0.45 a
400	5.91 ± 0.43 b	0.21 ± 0.02 b	2.97 ± 0.32 b
800	3.49 ± 0.48 d	0.10 ± 0.02 c	2.15 ± 0.15 b

The net photosynthetic rate (*A*), stomatal conductance (*gs*), and transpiration rate (*Tr*) in *S. europaea* were maximal in the plants treated with 200 mM NaCl. *A* increased from 4.22 $\mu mol\ m^{-2}\ s^{-1}$ in the control to 9.44 $\mu mol\ m^{-2}\ s^{-1}$ in the plants treated with 200 mM NaCl. Then, *A* decreased gradually with increasing salt concentration. However, 800 mM NaCl decreased markedly the photosynthetic rate of *S. europaea* compared with the control (Table 1). A similar trend in the changes in *gs* and *Tr* under NaCl treatment was observed.

2.2. Shoots accumulated more Na^+ than roots

S. europaea accumulates substantial amounts of Na^+ in shoot tissues (Fig. 2). Na^+ content in shoots increased significantly with an increase in NaCl concentration from 0 to 800 mM, and this nutrient reached 295.24 ± 14.79 mg g^{-1} DW under 800 mM NaCl (Fig. 2), approximately one third of that of the plant shoots.

Compared with the shoots, the roots accumulated considerably less Na^+ (Fig. 2). In the control plants, approximately 7 mg g^{-1} DW of Na^+ accumulation was detected in the roots, whereas this value was approximately 75 mg g^{-1} DW in the shoots. The increase in salt concentration resulted in increased Na^+ content in the roots. The highest Na^+ content in the roots was 61.94 ± 2.77 mg g^{-1} DW in the 600 mM NaCl-treated plants, which was 253.25 ± 13.61 mg g^{-1} DW in the shoots (Fig. 2). Hence, *S. europaea* shoots accumulated more Na^+, which was approximately 4- to 26-fold of that in roots.

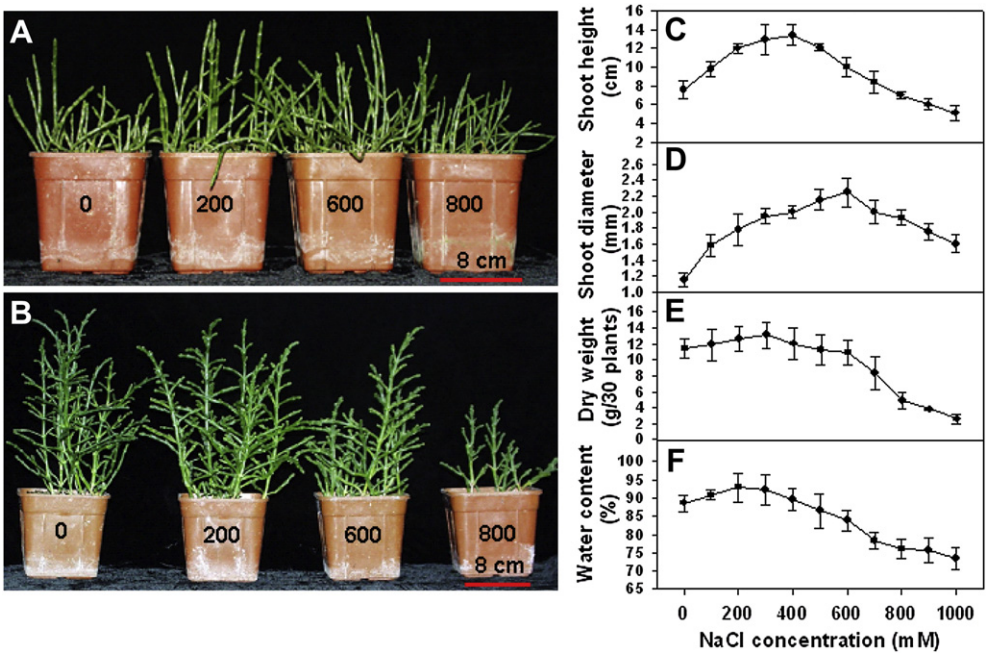

Fig. 1. Growth and physiological changes of *S. europaea* under different NaCl concentrations. Thirty-day old plants were treated with 0–1000 mM NaCl. The growth of *S. europaea* plants treated with 0, 200, 600, and 800 mM NaCl (from left to right) for 21 d (A) and 3 months (B) is shown. The shoot height (C), shoot diameter (D), DW (E), and shoot water content (F) of *S. europaea* plants were measured after 21 d treatment. Values are means ± SD ($n = 25$ for C and D, $n = 3$ for E and F).

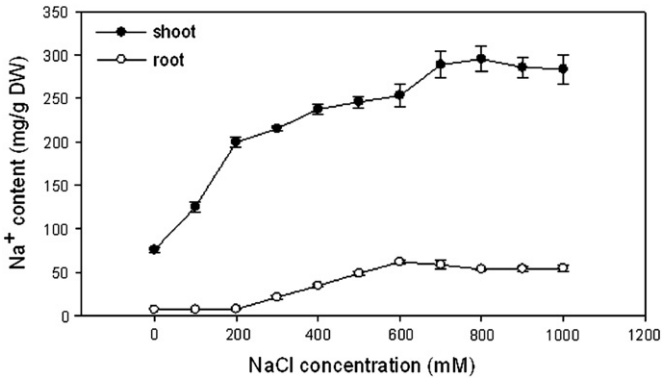

Fig. 2. Na^+ content in shoots and roots of *S. europaea* under different NaCl concentrations. After 21 d treatment with 0–1000 mM NaCl, Na^+ content in shoots and roots of *S. europaea* were determined. Values are means ± SD ($n = 3$).

2.3. Na was accumulated predominantly in the endodermis tissues

A high resolution technique is required to study *in situ* the ion distribution in plants. X-ray microanalysis combined with scanning electron microscopy (SEM) can provide a sensitive technique to study diffusible elements at tissue level. Transverse sections of *S. europaea* shoots were scanned via X-ray microanalysis, from the outermost to the middle tissues, in the order of exodermis cells, endodermis cells, and the stelar parenchyma (Fig. 3A). Correspondingly, the whole Na element location in the shoot cross-section of 200 mM NaCl-treated plants was presented (Fig. 3B). The typical spectra of the SEM–X-ray microanalysis collected from the exodermis cells (Fig. 3C), endodermis cells (Fig. 3D), and stele tissues (Fig. 3E) were also presented. These spectra were then converted to data by the professional software in the JSM-6300 computer. Counts per second (CPS) for sodium element of the three treatments were converted into relative element weights (Fig. 3F).

For all the examined plants, the relative weight of Na was highest in the endodermis tissues, followed by those in the exodermis tissues, and the least value was detected in the stelar tissues (Fig. 3F). The relative weight of Na in the shoots of the 200 mM NaCl-treated plants was 19.4 ± 0.8, 24.5 ± 3.2, and 21.7 ± 1.9 in the stele tissues, endodermis cells, and exodermis cells, respectively.

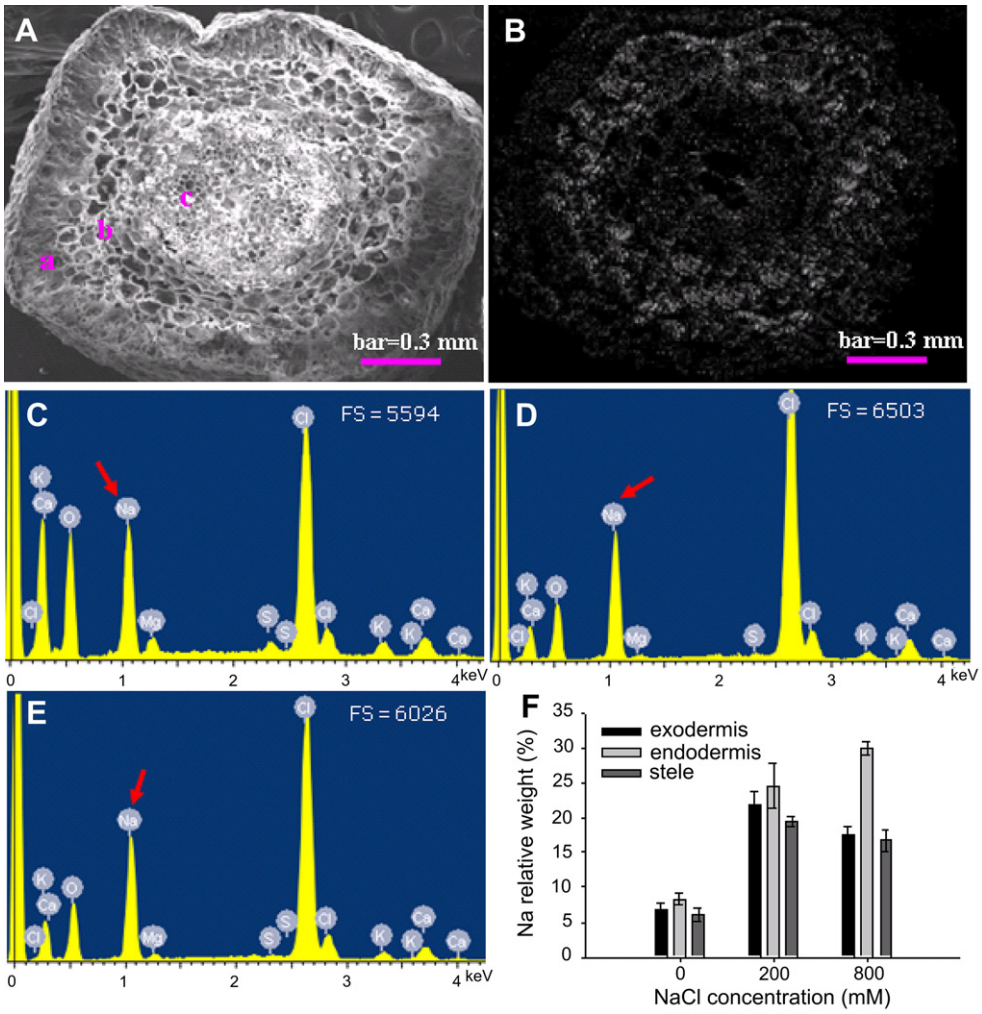

Fig. 3. SEM–X-ray microanalysis of Na element distribution in *S. europaea* shoots at tissue level. The eighth shoot segments from the bottom of *S. europaea* plant were used to determine the distribution of Na via SEM–X-ray microanalysis after 21 d treatment with 200 mM NaCl. (A) A typical freeze-substituted cross-section of *S. europaea* shoots: (a) exodermis; (b) endodermis; and (c) stele. (B) Na element location in shoot cross-section of 200 mM NaCl-treated plants. (C), (D), and (E) are typical spectra of the SEM–X-ray microanalysis from exodermis, endodermis, and stele tissue, respectively. (F) Na element relative content in shoot tissues from plant treated with 200 and 800 mM NaCl for 21 d, with 0 mM NaCl treatment as control. FS, full-length scale. The arrows in figures (C), (D), and (E) indicate the Na positions in the energy spectra. Values are means ± SD ($n = 5$).

2.4. Na was well compartmentalized into vacuoles

The SEM–X-ray microanalysis results reveal that Na accumulated predominantly in the endodermis tissues. X-ray microanalysis coupled with transmission electron microscopy (TEM) was used to determine further the subcellular localization of Na in the shoot endodermis tissues of *S. europaea* treated with 200 mM NaCl for 21 d. The relative Na content in the vacuole, apoplast, cytoplasm, and cell wall was approximately 289, 233, 165, and 54 CPS, respectively (Fig. 4). Na relative content in the vacuole was 1.75-fold of that in the cytoplasm. The results clearly demonstrate that the Na^+ was compartmentalized predominantly into the cell vacuoles of *S. europaea*.

2.5. Expression patterns of genes involved in Na segregation

To test further these experimental results, the expression patterns of genes involved in Na segregation in *S. europaea* shoots under saline conditions were analyzed with real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). These genes included *SeNHX1*, encoding a tonoplast Na^+/H^+ antiporter; *SeVHA-A*, encoding catalytic subunit A of the vacuolar H^+-ATPase; and *SeVP1*, as well as *SeVP2*, encoding the vacuolar H^+-PPase. The transcript amount of *SeNHX1*, *SeVHA-A*, and *SeVP1* increased significantly with the increase in NaCl concentration (Fig. 5). The transcript amounts of *SeNHX1*, *SeVHA-A*, and *SeVP1* were 1.4- to 2.2-fold of those in the control plants under 200 mM NaCl, and these values were 2.3- to 2.9-fold in the case of 800 mM NaCl. However, the *SeVP2* expression was not affected by saline treatment (Fig. 5).

3. Discussion

Salinity is considered as a stress factor for plants, even for most of the halophytes. In the present study, plant growth and the net photosynthetic rate of *S. europaea* were significantly stimulated, rather than inhibited, by 100–400 mM NaCl (Fig. 1 and Table 1). These results suggest that 100–400 mM NaCl did not cause stress to *S. europaea*, instead optimal growth conditions for this plant were observed under these NaCl concentrations. Consistent with the previous studies [10,13], the present results also demonstrate that *S. europaea* is extremely resistant to salinity. The plant survived even after NaCl treatment reaching 800 mM for over 3 months (Fig. 1B).

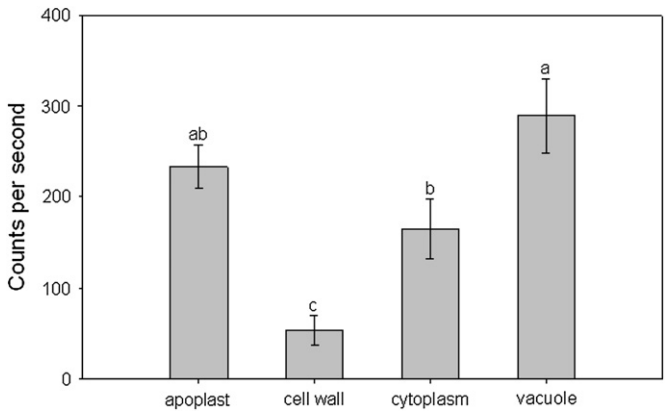

Fig. 4. Subcellular location of Na element in cortex tissues of *S. europaea* shoots. The subcellular location of Na element in the cortex tissues of *S. europaea* shoots was determined via TEM–X-ray microanalysis after 21 d treatment with 200 mM NaCl. Values are means ± SD ($n = 5$). Letters above each bar indicate significant differences at $P \leq 0.05$.

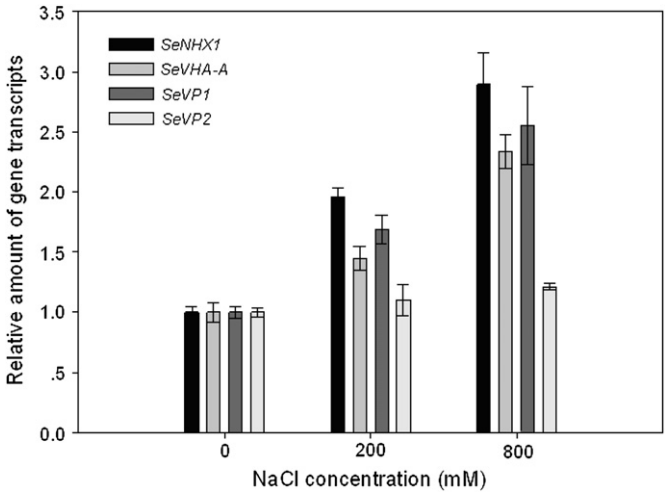

Fig. 5. Expression patterns of genes involved in sodium segregation in shoots of *S. europaea* under different NaCl concentrations. The expression patterns of *SeNHX1*, *SeVHA-A*, *SeVP1*, and *SeVP2* were analyzed by qRT-PCR in shoots of *S. europaea* after 21 d treatment with 200 or 800 mM NaCl. The relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method [33] with α-*tubulin* gene as an internal control. The relative amount of transcripts of these genes in untreated plants was arbitrarily set to 1.0. Each sample was repeated thrice.

Halophytes have shown a tight coupling of plant growth and salt-ion uptake [7]. In contrast to glycophytes, which accumulate low amounts of NaCl [10], *S. europaea*, as a euhalophyte, can accumulate large amounts of Na^+ in its shoots (Fig. 2). A maximum of approximately 300 mg g^{-1} DW of Na^+ content was determined in the 800 mM NaCl-treated *S. europaea* shoots (Fig. 2). This value is much higher than that in glycophytes [10], and even compared with some salt-excreting halophytes [15]. Compared with shoots, roots accumulated far less Na^+ (Fig. 2), indicating that Na^+ predominantly accumulates in the shoot tissues of *S. europaea*. This result is consistent with the reports on *Suaeda altissima* [16] and *Mesembryanthemum crystallinum* [17]. Together with the upregulated SOS1, HKT and HAK function to translocate Na^+ from root to shoot tissues in the halophyte [18–20]. The process of Na^+ loading into the xylem plays the key role in the Na^+ delivery from roots to shoots. Experiments with *S. altissima* showed that Na^+ accumulation in the root xylem of euhalophytes is accomplished by the Na^+/H^+ antiporter located at the plasma membrane of parenchymal cells adjacent to the xylem [21]. A model in which Na^+ storage in the root was decreased and transport to the shoot was enhanced was proposed for the ice plant *M. crystallinum*, with the upregulation of McHKT1 suggested to contribute to Na^+ storage in the leaves [22]. This model may help elucidate the process of Na^+ translocation from roots to shoots in *S. europaea* in the future.

Three mechanisms are available to plant cells for the prevention of excessive Na^+ accumulation in the cytosol [1,23]. These mechanisms include restricting Na^+ by selective ion uptake, storing Na^+ in vacuoles, and exporting Na^+ back to the growth medium or to apoplastic space. High Na^+ concentration in shoots did not lead to a decrease in plant growth and photosynthesis of *S. europaea*, implying that the mechanism of this species is to store Na^+ in vacuoles. This phenomenon was proven by the results of TEM–X-ray microanalysis, clearly demonstrating that Na^+ was compartmentalized predominantly into the vacuoles in *S. europaea* (Fig. 4). One of the transporters responsible for Na^+ transport from cytosol to vacuole is the Na^+/H^+ antiporter in tonoplast, which plays a central role in salinity tolerance [5]. Na^+/H^+ antiport activity is tightly controlled by electrochemical H^+-gradient across the tonoplast, generated by V-H^+-ATPase and V-H^+-PPase [24,25]. In

the present study, Na^+ accumulation was in agreement with increased transcript amount of *SeNHX1*, *SeVHA-A*, and *SeVP1* under the NaCl treatments (Fig. 5), suggesting the important roles of Na^+/H^+ antiporter, V-H^+-ATPase, and V-H^+-PPase in the Na^+ influx to vacuole in plant cells. Jha et al. [26] recently reported that the transcript of a Na^+/H^+ antiporter gene (*SbNHX1*) from *Salicornia brachiata* also increased under different NaCl concentrations, consistent with the present results. However, *SbNHX1* transcripts increased from 4- to 12-fold when NaCl concentration reached 500 mM NaCl, significantly higher than the increase in *SeNHX1* transcripts (2.2- and 2.9-fold by 200 and 800 mM NaCl, respectively) (Fig. 5). This phenomenon may be attributed to the different plant species, salt treatments, and duration of the two studies.

Maintenance of greater leaf turgor can lead to the maintenance of greater photosynthetic competence and growth in plants exposed to low soil water potential [27,28]. In the current study, instead of decreasing, the net photosynthetic rate in plants treated with 200 mM and 400 mM NaCl increased remarkably compared with the control (Table 1). Accordingly, the stomatal conductance and transpiration rate in these plants were higher than those in the control plants (Table 1). These results are consistent with the results from another halophyte *S. salsa* [29]. Under saline conditions, the ability to take up and confine Na^+ to shoots lowers the osmotic potential of the aerial plant part. This condition then facilitates water uptake and transport, and lowers the metabolic cost for the osmolyte production [30]. Na^+ in *S. europaea* cell may act as an effective osmotic adjuster to maintain cell turgor, promoting photosynthetic competence and plant growth.

In conclusion, cell vacuoles may be used as Na^+ pools in *S. europaea*. A multi-compartmentalization mechanism of Na^+ in *S. europaea* exists. At the whole plant level, this species can compartmentalize most of Na^+ in its shoots rather than roots. At tissue level, Na^+ is mainly accumulated in the endodermis tissues, whereas at the subcellular level, Na^+ is compartmentalized predominantly into the cell vacuoles. Na^+/H^+ antiporter, V-H^+-ATPase, and V-H^+-PPase may play important roles in Na^+ vacuolar influx. Therefore, the *S. europaea* plant provides a perfect salt-tolerant model, which not only can survive under high salinity, but can also utilize Na^+ to maintain cell turgor, promoting photosynthetic competence and plant growth.

4. Materials and methods

4.1. Plant materials and salt treatments

S. europaea L. seeds were collected from coastal areas of Dafeng City, Jiangsu Province in China (latitude: 33°19′N; longitude: 120°45′E). The plants were grown in a greenhouse at 25/20 °C (day/night) with a photon flux density (PFD) of 1000 μmol m^{-2} s^{-1}, a relative humidity of 50%–60% and a photoperiod of 16/8 h (light/dark) at Beijing Botanical Garden affiliated with Institute of Botany, Chinese Academy of Sciences (latitude: 39°99′N; longitude: 116°20′E). Seeds were sown on vermiculite (Jixiang Gardening Co. Ltd., Beijing, China) damped with tap water in the plastic pots. After germination, seedlings were irrigated weekly with half-strength modified Hoagland's nutrient solution [31] for 30 d. Then, the plants were treated with NaCl by enhancing the NaCl concentration with 100 mM each day. These plants were then irrigated sequentially with half-strength nutrients containing 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 mM NaCl at 3-day intervals. Plants irrigated with half-strength modified Hoagland's nutrient solution served as the control. All physiological and cytological assays were performed after 21 d salt treatment.

4.2. Plant height, shoot diameter, DW, and total water content (TWC) determination

The shoot height and fresh weight (FW) were determined immediately after plant harvesting. The shoot diameter of the middle section at the eighth segment from the bottom was determined. DW was measured after drying for 72 h in an oven at 60 °C. TWC was calculated as follows: $TWC = [(FW - DW)/FW] \times 100$.

4.3. Gas exchange measurements

A portable infrared gas analyzer-based photosynthesis system (LI-6400, Li-Cor Inc., USA) was used to measure *A*, *gs*, and *Tr* after 21 d of NaCl treatment. The photosynthetic PFD was maintained at 1000 μmol m^{-2} s^{-1} by an internal 6400-02BLED source. All the measurements were performed from 09:00 to 10:00 A.M. During the measurements, the air relative humidity was about 60% and the ambient CO_2 concentration about 400 μmol CO_2 mol^{-1}.

4.4. Quantification of Na^+ content

Fresh shoots and roots were washed with distilled water immediately after harvesting, dried at 60 °C for 72 h in an oven, and subsequently ground into fine powder with a mortar and pestle. Approximately 300 mg powder was added in 10 mL of 500 mM HNO_3 and then incubated in 80 °C for 1 h. The Na^+ content in the shoots and roots was measured by an atomic absorption spectrophotometer (PE-5100ZL, USA) after extract filtration.

4.5. SEM–X-ray microanalysis

To determine the diffusible elements at tissue level, X-ray microanalysis coupled with SEM was performed according to the described protocol of Peng et al. [32]. The eighth shoot segments from 200 mM NaCl-treated *S. europaea* were harvested and washed with distilled water thrice. Then, the shoot segments were dipped in 5% agar, inserted to a depth of 1.0 cm in a copper holder, and immediately sliced free-hand with a razor blade to obtain transverse sections, and then frozen in liquid nitrogen. The samples were freeze dried and carbon coated in a high-vacuum sputter coater, then stored in a desiccator. The samples were analyzed in an X-650 scanning electron microscope equipped with an energy-dispersive X-ray detector (EDX-9100, Hitachi, Japan). A map-scan model was performed. The CPS of the sodium peak after subtraction of the background was determined. More than five transverse sections of each treatment were analyzed. Values were expressed as means ± SD ($n = 5$).

4.6. TEM–X-ray microanalysis

To determine further the diffusible elements at subcellular level, an X-ray microanalysis equipped with TEM was performed following the protocol of Zhao et al. [12]. The eighth shoot segments from 200 mM NaCl-treated *S. europaea* were harvested and washed with distilled water thrice. The slices from the middle section were examined with an H-800 transmission electron microscope equipped with an EDX-9100 X-ray analyzer (Hitachi, Tokyo, Japan). The accelerating voltage was 120 kV with a take-off angle of 25°. The counting time for all analyses was 60 s. The data were expressed as CPS of an element peak after subtraction of the background. The analysis was performed for the apoplast, cell wall, cytoplasm, and vacuole. For each micro-area, five replicates were conducted and the results were expressed in means ± SD.

Table 2
Gene specific primers for real-time quantitative reverse transcriptase polymerase chain reaction.

Name	GenBank ID	Forward primer (5′–3′)	Reverse primer (5′–3′)
α-tubulin	AB437373	CAGTGCCTTTGAGCCATCTTC	CTGAATGGTTCGCTTGGTCTT
SeNHX1	AY131235	GGAGAATCGTTGGATGAATGAGTC	GCTTCTTTTTTACCTGGAACCCTG
SeVHA-A	HM490006	CTGGTTCGGATGGTCAAAAGA	GATTGCGGAAAGAAGCACTCA
SeVP1	HM490004	GAGGTGTTTTCTGCCCTTATGTC	ACAGCCTTGTCATCTAACCGAGT
SeVP2	HM490005	GGTTTTGTCTGTTAGTTTTGGTATG	ATTGTGCTAGTTAATCTAATGTGGTT

4.7. Real-time qRT-PCR

The expression patterns of the genes involved in the sodium segregation in *S. europaea* shoots were determined by real-time qRT-PCR after 21 d of salt treatment with 200 or 800 mM NaCl. Total RNA was extracted by Trizol reagent and treated with RNase-free DNase I (Promega). One microgram of RNA was reverse transcribed with the SuperScript III first-strand synthesis system (Invitrogen). qRT-PCR was performed with an Mx3000P™ Real-Time PCR System (Agilent, USA) using SYBR Green Real time PCR Master Mix (Toyobo, Japan). The relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method [33] with *α-tubulin* gene as an internal control. Each sample was repeated thrice. Gene specific primer sequences are shown in Table 2.

4.8. Statistical analysis

The statistical results were expressed as means ± SD. Statistical analysis, one-way ANOVA, and Duncan's multiple range test were performed with 5% level of significance using SPSS software version 12.0.

Acknowledgments

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