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Chapter 3. Differences in proton pumping and Na\(^+\)/H\(^+\) exchange at the leaf cell tonoplast between a halophyte and a glycophyte

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Chapter 3. Differences in proton pumping and Na\(^+/H^+\) exchange at the leaf cell tonoplast between a halophyte and a glycophyte

Abstract

The tonoplast Na\(^+/H^+\)-antiporter and the tonoplast H\(^+\)-pumps are essential components of salt tolerance in plants. The objective of this study was to investigate the transport activities of the tonoplast Na\(^+/H^+\)-antiporter and the tonoplast V-H\(^+\)-ATPase and V-H\(^+\)-PPase in a highly tolerant salt accumulating halophyte, Salicornia dolichostachya, and to compare these transport activities with activities in the related glycophyte Spinacia oleracea. Vacuolar membrane vesicles were isolated by density gradient centrifugation, and proton transport and hydrolytic activity of both H\(^+\)-pumps were studied. Furthermore, the Na\(^+/H^+\)-exchange capacity of the vesicles was investigated by ACMA fluorescence. Salt treatment induced V-H\(^+\)-ATPase and V-H\(^+\)-PPase activity in vesicles derived from S. oleracea, whereas V-H\(^+\)-ATPase and V-H\(^+\)-PPase activity in S. dolichostachya was not affected by salt treatment. Na\(^+/H^+\)-exchange capacity followed the same pattern, i.e. induced in response to salt treatment (0 and 200 mM NaCl) in S. oleracea and not influenced by salt treatment (10 and 200 mM NaCl) in S. dolichostachya. Our results suggest that S. dolichostachya generates a high tonoplast H\(^+\)-gradient already at low external salinities, which is likely to contribute to the high cellular salt accumulation of this species at low external salinities. At high external salinities, S. dolichostachya showed improved growth compared to S. oleracea, but V-H\(^+\)-ATPase, V-H\(^+\)-PPase and Na\(^+/H^+\)-exchange activities were comparable between the species, which might imply that S. dolichostachya more efficiently retains Na\(^+\) in the vacuole.
3.1. Introduction

Salinity negatively affects growth in the vast majority of plant species. The decrease in growth is caused by both the osmotic component of salinity and the direct toxic effects of high levels of Na\(^+\) inside the plant. At the same time Na\(^+\) can be used as a 'cheap osmolyte' in plant adaptation to the low water potential of the saline external environment (Flowers and Colmer 2008). To avoid the toxic effects of salinity it is crucial for plants to keep cytoplasmic Na\(^+\) concentrations low. Inside the cytoplasm Na\(^+\) negatively interferes with pivotal cellular processes, such as enzyme functioning. The cytoplasmic Na\(^+\) concentration which is considered toxic is debatable, but likely it is above 200 mM (Flowers and Yeo 1986; Britto and Kronzucker 2010; Flowers et al. 2014). An important mechanism to keep cytoplasmic Na\(^+\) concentrations low is compartmentalisation of Na\(^+\) in the vacuole (Carden et al. 2003; Kronzucker et al. 2006).

Some highly tolerant salt accumulating halophytes require salt for normal growth and development, and have their growth optimum at external NaCl concentrations between 100 and 300 mM (Flowers and Colmer 2008; Katschnig et al. 2013). The fact that high Na\(^+\) levels, when in the cytoplasm, are detrimental for all plants, including these highly tolerant salt accumulating halophytes, implies evolution of an enhanced capacity for Na\(^+\) compartmentalisation inside their cells (Flowers and Colmer 2008). Vacuolar Na\(^+\) concentrations can be as high as 1200 mM (Flowers 1985). To maintain osmotic equilibrium within their cells, salt-accumulating halophytes must have evolved, together with an efficient intracellular Na\(^+\) compartmentalisation and retention system, the capacities to synthesize and accumulate compatible solutes in their cytoplasm. Glycophytes, in contrast to salt-accumulating halophytes, show strong growth reductions correlated with increased intracellular Na\(^+\) concentrations. It appears that intracellular Na\(^+\) compartmentalisation is less successful in glycophytes compared with highly tolerant salt accumulating halophytes.

The vacuolar Na\(^+\) compartmentalisation capacity may depend on the activity of the Na\(^+\), K\(^+\)/H\(^+\) antiporter, and/or the steepness of the H\(^+\) gradient created by one or both of the tonoplast H\(^+\)-pumps. Sequestration of Na\(^+\) into the vacuole is assumed to be effected by the tonoplast Na\(^+\), K\(^+\)/H\(^+\)-antiporter (NHX1) (Apse et al. 1999; Gaxiola et al. 1999), which transports Na\(^+\) or K\(^+\), dependent on the prevailing concentration, against the ΔpH into the vacuole (Venema et al. 2002). The selectivity of this Na\(^+\), K\(^+\)/H\(^+\)-antiporter is dependent, besides Na\(^+\) and K\(^+\) concentrations, on regulation by the calmodulin-like protein 15, which is in turn dependent on the pH (Yamaguchi et al. 2005). The Na\(^+\), K\(^+\)/H\(^+\)-antiporter uses the energy gradient created by
the two tonoplast proton pumps, H\textsuperscript{+}-ATPase and H\textsuperscript{+}-PPase, to transport Na\textsuperscript{+} into the vacuole. Besides Na\textsuperscript{+} transport into the vacuole, retention of Na\textsuperscript{+} in the vacuole is also likely to be an important mechanism in maintaining low cytoplasmic Na\textsuperscript{+} concentrations (Bonales-Alatorre et al. 2013).

The activities of the H\textsuperscript{+}-pumps are essential for intracellular Na\textsuperscript{+} sequestration (Flowers and Colmer 2008). However, it is not fully understood if increased sequestration of Na\textsuperscript{+} into the vacuole is achieved by increased activity of the V-H\textsuperscript{+}-ATPase, or the V-H\textsuperscript{+}-PPase, or both, increased activity of the Na\textsuperscript{+}/H\textsuperscript{+}-antiporter, and/or other mechanisms like, for example, by reduced activity of the vacuolar fast- and slow-activating channels (Bonales-Alatorre et al. 2013). Increased activity of the V-H\textsuperscript{+}-ATPase is probably the least likely contributor to increase Na\textsuperscript{+} sequestration into the vacuole (Krebs et al. 2010; Shabala 2013). Highly tolerant salt accumulating halophytes accumulate Na\textsuperscript{+} to very high (1200 mM) intracellular concentrations (Flowers 1985); therefore, they might be useful as model systems to study mechanisms of Na\textsuperscript{+} compartmentalisation inside cells. Knowledge about how salt-accumulating halophytes maintain Na\textsuperscript{+} homeostasis, in comparison with glycophytes, would be useful to increase our current level of understanding of salt tolerance in crop plants.

*Salicornia dolichostachya* is a highly tolerant salt accumulating halophyte of the Amaranthaceae. It can accumulate up to 400 mM of Na\textsuperscript{+} inside its cells without growth reduction (Katschnig et al. 2013). *S. dolichostachya* does not possess any specialized structures for salt storage or removal, such as salt bladders or salt glands. Therefore, it is reasonable to assume that this plant has a high capacity for vacuolar Na\textsuperscript{+} compartmentalisation. The family of the Amaranthaceae also contains less salt tolerant species, such as *Spinacia oleracea*. As the external salt concentration increases, this glycophyte exhibits increased accumulation of Na\textsuperscript{+} in its cells, which is accompanied by growth reduction (Robinson et al. 1983). Therefore, it can be argued that the capacity to compartmentalize and retain Na\textsuperscript{+} in the vacuole is lower in *S. oleracea* than in *S. dolichostachya*.

Na\textsuperscript{+} transport across the tonoplast membrane can be studied in vitro using tonoplast vesicles. This study compared the transport activity of the tonoplast H\textsuperscript{+}-pumps: V-H\textsuperscript{+}-ATPase and V-H\textsuperscript{+}-PPase, and the tonoplast Na\textsuperscript{+}/H\textsuperscript{+}-antiporter in tonoplast vesicles derived from the highly tolerant salt accumulating halophyte *S. dolichostachya*, and compared these activities with the transport activities in tonoplast vesicles derived from the related glycophyte *S. oleracea*. Because of the high capacity of *S. dolichostachya* to accumulate Na\textsuperscript{+}, I hypothesized that the activity of the V-H\textsuperscript{+}-PPase or the V-H\textsuperscript{+}-ATPase, or both, are higher in *S. dolichostachya* than in *S. oleracea*, and this might be accompanied by a higher activity of the Na\textsuperscript{+}/H\textsuperscript{+}-antiporter.
3.2. Materials and Methods

3.2.1. Plant growth and NaCl treatment

Salicornia dolichostachya seeds were collected from a coastal salt marsh at Lutjestrand (Wieringen, Noord-Holland) in The Netherlands. Spinacia oleracea seeds were obtained from a commercial supplier (Tuin plus Service, Holland). Seeds of both species were sown on soil (pot soil; Jongkind, Aalsmeer, The Netherlands) and grown for, respectively, 35 days (S. dolichostachya) and 16 days (S. oleracea). Then seedlings were transferred into individual 1-L polyethylene pots containing a modified half-strength Hoagland solution, composed of (in mM): K\(^{+}\), 3; Ca\(^{2+}\), 2; Mg\(^{2+}\), 0.5; NO\(_{3}\)^{-}, 1.001; HPO\(_{4}\)^{2-}, 1; SO\(_{4}\), 0.516; Cl\(^{-}\), 0.001; H\(_{2}\)BO\(_{3}\), 0.025; Mn\(^{2+}\), 0.002; Zn\(^{2+}\), 0.002; Cu\(^{2+}\), 0.001; Mo\(^{2+}\), 0.001; Fe-Na-EDTA, 0.01, buffered with 2 mM MES, pH 6.0, and in case of S. dolichostachya also 10 mM NaCl. Two weeks after transplanting, NaCl was added in steps of 50 mM per day to the nutrient solution, and NaCl treatments lasted for 8 days after reaching the final salt concentrations. Salt treatments consisted of a control treatment, 0 mM NaCl for S. oleracea and 10 mM NaCl for S. dolichostachya, and a salt treatment of 200 mM NaCl for both species. S. oleracea grows optimally without NaCl in the external medium, while 0 mM NaCl is insufficient to maintain growth in S. dolichostachya (Katschnig et al. 2013). Therefore, the minimum salt concentration applied to S. dolichostachya was 10 mM NaCl. The plants were grown in a randomized block design in a naturally-lit greenhouse with additional lamps (PAR level of the lamps was 250 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) at plant level, 14/10 h light/dark) in March-May 2013 in Amsterdam, The Netherlands. The average temperature was 20±2/16±2 °C day/night and the relative humidity of the air 70±10/90±2 % day/night, respectively.

At harvest, six plants per treatment of each species were rinsed with de-mineralized water and carefully blotted dry. These plants were used for biomass, pH and ion content measurements. After rinsing, the plants were separated in shoots and roots, weighed, and subsamples of shoots and roots were shock-frozen in liquid nitrogen and stored at -20°C or -80°C, or oven dried at 70 °C for 72 h. Plant material stored at -20°C was used to measure pH and oven dried plant material was used to establish dry mass and ion contents.

For the preparation of the tonoplast vesicles, approximately 50 g shoot material (several plants combined) per treatment of each species, replicated four times, was excised and
immediately homogenized in a blender at 4 ºC. The vesicle isolation protocol is described in full in the section: preparation of tonoplast membrane vesicles.

3.2.2. Na\(^+\) and K\(^+\) concentrations

Oven-dried shoot and root material of *S. oleracea* and *S. dolichostachya* was used to determine Na\(^+\) and K\(^+\) contents. The plant material was powdered and heated (~100 ºC) in 5 ml de-mineralized water for 2 h, and thereafter filtered with 4-7 μm cellulose filters (597, Whatman GmbH, Dassel, Germany). Na\(^+\) and K\(^+\) contents were determined with a flame atomic spectrophotometer (Perkin-Elmer 1100B; Perkin Elmer Inc., Waltman, MA, USA) and expressed on a cell water basis to obtain the cellular concentrations in mM.

3.2.3. Shoot pH measurements

Shoot pH was measured according to the method of (Perez-Harguindeguy *et al.* 2013). Frozen (~20°C) leaf material (0.15 ml) of *S. oleracea* and *S. dolichostachya* was chopped, and shaken in 1.2 ml demineralised water for one hour. After shaking, the samples were centrifuged, and the supernatant was used to measure the pH. Measurements were performed with a thin SenTix 41 electrode coupled to an Inolab level 2 pH meter (WTW, Weilheim, Germany).

3.2.4. Preparation of tonoplast membrane vesicles

Tonoplast membranes were isolated using two-phase partitioning on a sucrose gradient. All steps were carried out at 4°C and all material was chilled prior to use. Shoot material (approximately 50 g fresh weight per replicate) of *S. dolichostachya* and *S. oleracea* was homogenized with a blender, immediately after excision, in 100 ml extraction buffer. There were four independent isolations per treatment per species. The extraction buffer contained: 250 mM sucrose, 3 mM MgCl\(_2\), 100 mM KCl, 2 mM EDTA pH 8, 70 mM TRIS-HCl pH 8, 0.2% (p/v) polyvinylpyrrolidone (PVPP), 0.1% (p/v) BSA, 2 mM dithiothreitol (DTT) and cOmplete Protease Inhibitor Tablets (Roche). The homogenate was filtered through four layers of miracloth and centrifuged at 10,000x g for 10 min. The pellets were discarded and the supernatant was centrifuged at 100,000x g for 60 min. The microsomal pellet was resuspended in resuspension buffer, containing glycerol 15% (v/v), 1 mM EDTA pH 7.5, 20 mM TRIS-HCl pH 7.5, 1mM DTT and cOmplete Protease Inhibitor Tablets (Roche). This suspension was
layered on top of a discontinuous sucrose gradient existing of 15 ml 32% and 10 ml 46% (w/v) sucrose, additionally containing 1 mM EDTA pH 7.5, 20 mM TRIS-HCl pH 7.5 and 1mM DTT. The sucrose gradient was centrifuged in a swing-out rotor at 80,000x g for 3 h. Thereafter, the tonoplast-enriched fraction at the 0/32% interface was collected, diluted with resuspension buffer and centrifuged at 100,000x g for 30 min. The resulting pellet was collected and resuspended in resuspension buffer. Subsequently, 100 µl aliquots were frozen in liquid nitrogen and stored at -80ºC. Protein concentrations were determined by the method of (Bradford 1976) using BSA as a standard.

3.2.5. H⁺-transport

The formation and dissipation of a proton gradient across the membranes of vesicles derived from salt-grown (200 mM NaCl) and control-grown (0 mM NaCl in S. oleracea and 10 mM NaCl in S. dolichostachya) plants was monitored by fluorescence quenching and recovery of 9-amino-6-chloro-2-methoxyacridine (ACMA) upon ATP or pyrophosphate (PP_i) supply. Reactions were carried out at 22 ºC in quartz cuvettes containing 1.5 ml reaction medium (10 mM MOPS-TRIS pH 7.0, 100 mM KCL, 2 μM ACMA and 2.5 mM MgCl₂). After addition of the required amount of protein to the reaction medium, cuvettes were placed in the dark chamber of a fluorescence spectrometer (AB2 Luminescence spectrometer SLM-Aminco, Bowman) and stirred for 5 min preceding the fluorescence readings. Fluorescence was measured at an excitation wavelength of 415 nm and an emission wavelength of 485 nm. When quenching leveled, the formation of the proton gradient by the V-H⁺-ATPase or V-H⁺-PPase was initiated by the addition of the required amount of ATP (0.05-1.5 mM) or PP_i (0.005-0.25 mM), respectively. Fluorescence quenching was allowed to proceed until a stable value had been reached. The initial rate of fluorescence quenching was calculated as a percentage of the rate of fluorescence quenching after dissipation of the pH gradient by 4.5 mM NH₄Cl at the end of each measurement. I also corrected each measurement for the orientation of the vesicles. Kinetic analysis was performed fitting a Michaelis-Menten curve through the data points. Curve fitting of the concentration-dependent transport data was performed with non-linear least square (nls) model fitting.
3.2.6. ATP hydrolysis

The rate of ATP hydrolysis of vesicles derived from salt-treated (200 mM NaCl) and control-grown (0 mM NaCl S. oleracea and 10 mM NaCl S. dolichostachya) plants was determined. To determine the rates of ATP hydrolysis, the release of inorganic phosphate (P$_i$) was measured. Sample protein (6 µg) was added to 175 µl reaction medium. The reaction medium consisted of 50 mM MOPS-TRIS pH 7.0, 250 mM sucrose, 1 mM dithiothreitol (DTT), 50 mM KCl and 3 mM MgCl$_2$. The reaction was started by addition of 75 µl 3.3 mM ATP (final concentration 1 mM) or 75 µl 5 mM PP$_i$ (final concentration 0.15 mM) After 30 minutes incubation at 37°C, reactions were stopped by the addition of 27.5 µl of 30% (w/w) trichloroacetic acid (TCA) and 30 µl 10 % sodium dodecyl sulfate (SDS). P$_i$ was measured according to the spectrophotometric method of Murphy and Riley (1962). Hydrolytic activity was expressed as nmol P$_i$ mg$^{-1}$ protein min$^{-1}$.

3.2.7. Purity of the tonoplast fraction and ATPase latency

The purity of the membrane fractions was estimated by measuring the degrees of inhibition of ACMA-fluorescence quenching by 50 mM KNO$_3$ and 0.1 mM vanadate (Na$_3$VO$_4$) to estimate the contributions of the vacuolar V-type H$^+$-ATPase and the plasma membrane P-type H$^+$-ATPase, respectively. To assess if the observed fluorescence quenching was due to an H$^+$-gradient established over the vesicle membranes and to calculate the initial rate of fluorescence quenching, 4.5 mM NH$_4$Cl was added at the end of each measurement.

To establish the sidedness of the vesicles, I measured the release of inorganic phosphate (P$_i$) after ATP hydrolysis both with and without 0.06% (w/v) Triton X-102 in the reaction medium (Meszaros et al. 2012). A volume corresponding with 6 µg of protein was added to 175 µl reaction medium, consisting of 50 mM MOPS-TRIS pH 7.0, 250 mM sucrose, 1 mM dithiothreitol (DTT), 50 mM KCl and 3 mM MgCl$_2$. The reaction was started by addition of 75 µl 3.3 mM ATP. After 30 minutes incubation at 37°C, reactions were stopped by the addition of 27.5 µl of 30% (w/w) trichloroacetic acid (TCA) and 30 µl 10 % sodium dodecyl sulfate (SDS). P$_i$ was measured according to the spectrophotometric method of Murphy and Riley (1962). The percentage of right-side out (outside-out) vesicles was calculated as the amount of P$_i$ released in the essay without Triton X-102 divided by the amount of P$_i$ released in the essay with Triton X-102.
3.2.8. Na⁺/H⁺-exchange

The rate of Na⁺/H⁺-exchange was measured as the dissipation of a pre-established pH gradient. The pH gradient required for identification of Na⁺/H⁺-exchange was generated by the V-H⁺-ATPase. The formation of the proton gradient by the V-H⁺-ATPase was initiated by the addition of 1 mM ATP. The method and reaction media were the same as described in the section H⁺-transport assays. Fluorescence quenching was allowed to proceed until a stable value had been reached, then different concentrations of NaCl (5–250 mM) were added to the reaction medium. The maximal recovery of fluorescence quenching at 180 sec after NaCl addition was recorded. The rate of Na⁺/H⁺-exchange was expressed as a percentage of the initial quenching.

3.2.9. Statistical analysis

Normality and homogeneity of the data was checked. Student’s t-test was used to assess the effect of salt treatment on ash-free dry mass, shoot Na⁺ and shoot K⁺ accumulation. One-way ANOVA’s with Tukey’s post hoc test were used to assess the effect of salt treatment and species on shoot pH, hydrolytic activity of the V-H⁺-ATPase and the tonoplast Na⁺/H⁺-exchange capacity, 2-way ANOVA’s were used to examine the species x salt treatment interaction effect on these parameters. Kinetic parameters with standard errors of the ATP-dependent and PPi-dependent H⁺-translocating activity were calculated with non-linear least square (nls) model fitting in R (R Core Team 2012).
3.1. Results

3.3.1. Characterization of the plant material

3.3.1.1. Plant ash-free dry mass

The 200-mM NaCl salt treatment had a negative effect on growth of Spinacia oleracea and a positive effect on growth of Salicornia dolichostachya (Fig. 1). Ash-free dry mass was, as a result of the salt treatment, reduced by approximately 30% in S. oleracea and approximately tripled in S. dolichostachya compared to the control treatment.

![Figure 1](image)

**Figure 1.** Ash-free dry mass of Spinacia oleracea and Salicornia dolichostachya after 8 days of growth. Plants were exposed for 8 days to 0 and 200 mM NaCl for S. oleracea, and 10 and 200 mM NaCl for S. dolichostachya. Values shown are means ± S.E.M. with 6 replicate plants per treatment. Note that the ordinates of the two species have different scales. The treatment effect was statistically significant for both species (Student’s t-test, *p*<0.05).

3.3.1.2. Shoot Na\(^+\) and K\(^+\) concentrations

Shoot Na\(^+\) concentrations were comparable in S. oleracea and S. dolichostachya after 8 days of growth at 200 mM of NaCl; however, at low external salinity Na\(^+\) concentrations they were much higher in S. dolichostachya than in S. oleracea (Fig. 2). Shoot K\(^+\) concentrations decreased with salt treatment in both S. oleracea and S. dolichostachya.
3.3.1.3. Shoot pH

Shoot pH decreased with increased salinity by about 0.6 units in *S. oleracea*, but had no effect on shoot pH in *S. dolichostachya* (Fig. 3).

**Figure 2.** Na$^+$ and K$^+$ concentrations in the shoot of *Spinacia oleracea* and *Salicornia dolichostachya* after 8 days of growth. Plants were exposed for 8 days to 0 and 200 mM NaCl for *S. oleracea*, and 10 and 200 mM NaCl for *S. dolichostachya*. Values shown are means ± S.E.M. with 6 replicate plants per treatment. The treatment effect was statistically significant for shoot Na$^+$ and shoot K$^+$ concentrations for both species (Student’s t-test, *p* < 0.05).

**Figure 3.** Shoot pH of *Spinacia oleracea* and *Salicornia dolichostachya* after 8 days of growth. Plants were exposed for 8 days to 0 and 200 mM NaCl for *S. oleracea*, and 10 and 200 mM NaCl for *S. dolichostachya*. Values shown are means ± S.E.M. with 6 replicate plants per treatment. Different letters indicate a statistically significant treatment effect on leaf pH (1-way ANOVA, *p* < 0.05). Interaction effect: 2-way ANOVA, species x treatment *p* < 0.05.
3.3.2. Characterization of the membrane fractions

H⁺-translocating activity and hydrolytic activity of the V-H⁺-ATPase and the V-H⁺-PPase were determined in vesicles derived from shoots of salt-treated (200 mM NaCl) and control-grown S. dolichostachya (10 mM NaCl) and S. oleracea (0 mM NaCl). To characterize the V-H⁺-ATPase and the V-H⁺-PPase activities in S. dolichostachya and S. oleracea, the hydrolysis of ATP and the magnitude of ATP- and PPᵢ-dependent ∆pH formation were determined. The formation of a transmembrane H⁺-gradient was measured by fluorescence quenching of the surface charge density probe ACMA. After addition of ATP or PPᵢ, fluorescence quenching was recorded and allowed to proceed until it reached a stable value. Addition of NH₄Cl resulted in an instantaneous collapse of the pH gradient and full recovery of the fluorescence signal (Fig. 4). Recovery of the fluorescence signal by the uncoupler NH₄Cl demonstrated that fluorescence quenching was induced by an established pH gradient generated by H⁺-translocation of the H⁺-ATPase and the H⁺-PPase.

The purity of the tonoplast fractions was estimated by measuring the recovery of a pre-established pH gradient in the presence of inhibitors that differentiate specific types of H⁺-ATPases. The V-H⁺-ATPase is sensitive to nitrate and insensitive to vanadate (Na₃VO₄). Vanadate is an inhibitor of the P-type H⁺-ATPase. V-H⁺-ATPase activity, measured as nitrate-sensitive and vanadate insensitive H⁺-translocating activity, was observed in membrane fractions collected at the 0/32% interface of the continuous sucrose gradient. In these fractions, the recovery of the pre-established pH gradient generated by the V-H⁺-ATPase after addition of vanadate was small, and addition of nitrate led to approximately 95% recovery of the fluorescence signal (Fig. 4). Membrane fractions isolated from S. dolichostachya and S. oleracea showed similar fluorescence-recovery responses in the presence of the inhibitors. Moreover, the degrees of inhibition were similar in salt-treated and control plants indicating that the salt treatment did not affect the composition of the isolated membrane fractions. Only tonoplast vesicles with an outside-out orientation (cytoplasmic-side out) participate in H⁺-ATPase and H⁺-PPase dependent transport. Therefore, I determined the percentage of outside-out orientated vesicles by measuring the hydrolytic activity of the V-H⁺-ATPase in medium with and without the detergent Triton X-102. The percentage outside-out vesicles was 57% ± 3.2 and 54.3% ± 1.0 in S. oleracea derived vesicles of 0 and 200 mM NaCl treated plants, respectively, and 33.3% ± 3.6 and 31.4% ± 2.2 in S. dolichostachya derived vesicles of 10 and 200 mM NaCl treated plants, respectively (Table 1). Because of the differences in orientation of the isolated membrane fractions in S. dolichostachya and S. oleracea, I corrected all the H⁺-
translocating activity and hydrolytic activity values of the V-H⁺-ATPase and the V-H⁺-PPase for the percentage of outside-out orientated vesicles.

**Table 1.** H⁺-ATPase activity, measured as release of inorganic phosphate (Pᵢ), of tonoplast vesicles derived from shoots of *Spinacia oleracea* or *Salicornia dolichostachya* and grown with 200 mM NaCl or control salinity (0 mM NaCl for *S. oleracea* and 10 mM NaCl for *S. dolichostachya*) in the external medium. The vesicles were intact (-Triton X-102) or collapsed by 0.06% (w/v) Triton X-102 (+Triton X-102). Values shown are means ± S.E.M. with 4 replicates per treatment. Different letters indicate a statistically significant effect on orientation of the vesicles between the four different treatment-species combinations (1-way ANOVA, *p* < 0.05).

| Species            | Treatment NaCl (mM) | ATPase activity (nmol Pi mg protein⁻¹ m⁻¹) -Triton X-102 ± S.E.M. | +Triton X-102 ± S.E.M. | Outside-out (%) |
|--------------------|---------------------|---------------------------------------------------------------|------------------------|-----------------|
| *S. oleracea*      | 0                   | 1129.9 ±109.0                                               | 2012.9 ±196.2         | 57.0 ±3.2        |
|                    | 200                 | 1676.1 ±92.3                                               | 3089.2 ±151.4         | 54.3 ±1.0        |
| *S. dolichostachya*| 10                  | 1016.5 ±142.4                                              | 3009.8 ±194.3         | 33.3 ±3.6        |
|                    | 200                 | 842.4 ± 54.5                                               | 2726.3 ±184.3         | 31.4 ±2.2        |

**Figure 4.** Characteristics of the H⁺-ATPase and H⁺-PPase dependent pH gradient. H⁺-translocation was determined by measuring the level of fluorescence quenching of ACMA. Formation of the pH gradient was initiated by adding 1 mM ATP or 0.15 mM PPᵢ. When ATP-dependent fluorescence quenching reached a stable value, 50 mM KNO₃ or 0.1 mM Na₃VO₄ was added. Five μg of protein was used per assay. Recordings of vesicles derived from shoots of 200 mM grown *Spinacia oleracea* are shown.
3.3.3. Na⁺/H⁺-exchange

The effect of Na⁺ (range 0-250 mM) on the dissipation of a pre-established pH gradient created by the V-H⁺-ATPase was tested in tonoplast membrane vesicles derived from shoots of salt grown-treated (200 mM NaCl) and control-grown S. dolichostachya (10 mM NaCl) and S. oleracea (0 mM NaCl). The dissipation of the pre-established pH gradient was measured as recovery of the ACMA-fluorescence signal. Addition of NaCl resulted in dissipation of the pre-established H⁺-gradient. Additions of higher concentrations of Na⁺ resulted in a stronger recovery of the fluorescence signal (Fig 5A). When ATP was omitted from the reaction medium, Na⁺ uptake was very low and independent of vesicle origin (data not shown).

Figure 5. A) Na⁺/H⁺-exchange capacity of membrane vesicles derived from shoots of the halophyte Salicornia dolichostachya grown at 200 mM NaCl. The Na⁺/H⁺-exchange capacity of the vesicles was measured as the level of recovery of ACMA-fluorescence quenching of a pre-established pH gradient created by the V-H⁺-ATPase after 1 mM ATP addition. 2.5 μg of protein was used per assay. B) Na⁺/H⁺-exchange capacity of membrane vesicles derived from shoots of the halophyte Salicornia dolichostachya and the glycophyte Spinacia oleracea. The Na⁺/H⁺-exchange capacity of the vesicles was measured as the level of recovery of ACMA-fluorescence quenching by addition of 250 mM NaCl of a pre-established pH gradient created by the V-H⁺-ATPase. Plants were grown under control conditions (10 mM NaCl for S. dolichostachya and 0 mM NaCl for S. oleracea) or saline conditions (200 mM NaCl for both species) for 8 days. Data points are means ± S.E.M. of three independent membrane isolations per treatment. Different letters indicate a statistically significant treatment effect on Na⁺/H⁺-exchange capacity (1-way ANOVA, p < 0.05). Interaction effect: 2-way ANOVA, species x treatment p < 0.05.
Addition of 250 mM NaCl to vesicles derived from 200 mM NaCl grown *S. oleracea* resulted in a recovery of the fluorescence signal approximately to the level observed before ATP addition (Fig. 5B). The level of recovery of the fluorescence signal was significantly lower in 0-mM-NaCl grown *S. oleracea*, than in 200-mM-NaCl grown *S. oleracea*. Na⁺/H⁺-exchange in *S. dolichostachya* did not differ between vesicles derived from salt-treated (200 mM NaCl) and non-salt treated (10 mM NaCl) plants.

3.3.4. Activity of the V-H⁺-ATPase and the V-H⁺-PPase of *S. dolichostachya* and *S. oleracea* in response to salt treatment

The H⁺-translocating activity of the V-H⁺-ATPase in vesicles derived from *S. oleracea*, measured as the rate of fluorescence quenching in the first 120 s following the addition of ATP, was approximately 2-fold higher in salt-grown plants (200 mM NaCl) than in control-grown (0 mM NaCl) plants (Fig. 6). The hydrolytic activity of the V-H⁺-ATPase, measured as the release of Pᵢ, showed the same pattern as the H⁺-translocating activity; it was higher in salt-grown *S. oleracea* (200 mM NaCl) than in control-grown *S. oleracea* (0 mM NaCl) (Fig. 7). In contrast to *S. oleracea*, the H⁺-translocating activity of the V-H⁺-ATPase in vesicles derived from *S. dolichostachya* did not differ between salt-grown (200 mM NaCl) and control-grown (10 mM NaCl) plants (Fig. 6). Accordingly, the hydrolytic activity of the V-H⁺-ATPase showed the same pattern as the H⁺-translocating activity in that it did not differ between vesicles derived from salt-grown (200 mM NaCl) and control-grown (10 mM NaCl) *S. dolichostachya* (Fig. 7).

The H⁺-translocating activity of the V-H⁺-PPase in vesicles derived from both *S. oleracea* and *S. dolichostachya* followed the same pattern as the H⁺-translocating activity of the V-H⁺-ATPase. Vesicles derived from salt-grown (200 mM NaCl) *S. oleracea* had a greater H⁺-translocating activity than vesicles derived from control-grown (0 mM NaCl) plants, and, in contrast to *S. oleracea*, the H⁺-translocating activity of the V-H⁺-PPase in vesicles derived from *S. dolichostachya* did not differ between salt-grown (200 mM NaCl) and control-grown (10 mM NaCl) plants (Fig. 8).
Figure 6. $\text{H}^+$-translocating activity of the tonoplast $\text{V-H}^+$-ATPase of vesicles derived from shoots of the halophyte *Salicornia dolichostachya* (circles) and the glycophyte *Spinacia oleracea* (squares). The $\text{H}^+$-translocating activity of the V-\text{H}^+\text{-ATPase}$ was determined by measuring the level of fluorescence quenching of ACMA. Plants were grown under control conditions (10 mM NaCl for *S. dolichostachya* and 0 mM NaCl for *S. oleracea*, open symbols) or saline conditions (200 mM NaCl both species, closed symbols) for 8 days. Data points are means ± S.E.M. of three independent membrane isolations per treatment. Kinetic analysis was performed by fitting a Michaelis-Menten curve through the data points. Kinetic parameters with standard errors were calculated with non-linear least square (nls) model fitting in R.

**Figure 7.** Hydrolytic activity of the tonoplast $\text{V-H}^+$-ATPase of vesicles derived from shoots of the halophyte *Salicornia dolichostachya* and the glycophyte *Spinacia oleracea*. The hydrolytic activity of the V-\text{H}^+\text{-ATPase}$ was determined by measuring the amount of inorganic phosphate ($P_i$) released. Plants were grown under control conditions (10 mM NaCl for *S. dolichostachya* and 0 mM NaCl for *S. oleracea*, open symbols) or saline conditions (200 mM NaCl both species, closed symbols) for 8 days. Data points are means ± S.E.M. of four independent membrane isolations per treatment. Different letters indicate a statistically significant treatment effect on hydrolytic activity (1-way ANOVA, $p < 0.05$). Interaction effect: 2-way ANOVA, species x treatment $p < 0.05$. 

| Treatment NaCl (mM) | Hydrolytic activity (μmol P mg⁻¹ protein h⁻¹) | *S. oleracea* | *S. dolichostachya* |
|---------------------|--------------------------------------------|---------------|---------------------|
| 0                   | a                                          | b             | ab                  |
| 200                 | b                                          | b             | ab                  |
| 10                  | a                                          | b             | ab                  |
| 200                 | b                                          | b             | ab                  |
Figure 8. H⁺-translocating activity of the tonoplast V-H⁺-PPase of vesicles derived from shoots of the halophyte *Salicornia dolichostachya* (circles) and the glycophyte *Spinacia oleracea* (squares). The H⁺-translocating activity of the V-H⁺-PPase was determined by measuring the level of fluorescence quenching of ACMA. Plants were grown under control conditions (10 mM NaCl for *S. dolichostachya* and 0 mM NaCl for *S. oleracea*, open symbols) or saline conditions (200 mM NaCl for both species, closed symbols) for 8 days. Data points are means ± S.E.M. of three independent membrane isolations per treatment. Kinetic analysis was performed fitting a Michaelis-Menten curve through the data points. Kinetic parameters with standard errors were calculated with non-linear least square (nls) model fitting in R.


3.4. Discussion

Since high concentrations of Na\(^+\) (>200 mM) in the cytoplasm are assumed to be toxic, the storage of Na\(^+\) is assumed to be restricted to the vacuole (Flowers and Yeo, 1986). The vacuolar H\(^+\)-pumps and the vacuolar Na\(^+\), K\(^+\)/H\(^+\)-antiporter are essential for Na\(^+\) sequestration into the vacuole. Over-expressing the vacuolar Na\(^+\), K\(^+\)/H\(^+\)-antiporter NHX1 has been argued to increase the salt tolerance of transgenic plants (Apse et al. 1999; Zhang and Blumwald, 2001), which suggested NHX1 as a promising target to improve salt tolerance in plants. However, the reported effects of over-expression of NHX1 on salt tolerance in transgenic plants are far from convincing thus far (Rozema and Schat, 2013). Therefore, the question to what extent the tonoplast Na\(^+\), K\(^+\)/H\(^+\)-exchange capacity differs between a highly salt-tolerant salt accumulating halophyte, such as Salicornia dolichostachya, and a related glycophyte is interesting.

I investigated Na\(^+\)/H\(^+\)-exchange activity in tonoplast membrane vesicles derived from shoots of S. dolichostachya, and compared this to activities in tonoplast membrane vesicles derived from shoots of the related glycophyte Spinacia oleracea. Dissipation of the pH gradient after addition of 200 mM NaCl (Fig. 5B) was the same in vesicles derived from both control-grown (10 mM NaCl) and salt-treated (200 mM NaCl) S. dolichostachya. In contrast, dissipation of the pH gradient in S. oleracea was influenced by the salt treatment: the dissipation was higher in vesicles derived from salt-treated (200 mM NaCl) S. oleracea than in control-grown S. oleracea (0 mM NaCl). This salt-induced stimulation of the Na\(^+\)-H\(^+\)-exchange capacity, as observed in S. oleracea, has often been reported for glycophytic species (Vera-Estrella et al. 2005; Queiros et al. 2009). In some species, no activity of the tonoplast Na\(^+\)-H\(^+\)-exchanger could be detected at all (Medicago media, Staal et al. 1991), or only after exposure to salinity (Garbarino and Dupont 1988). The inducibility of the tonoplast Na\(^+\)/H\(^+\)-exchanger might be related to stress perception. S. oleracea does experience stress at the salt concentration used for salt treatment in this experiment (200 mM NaCl), as can be seen by the reduction in biomass in this species (Fig. 1). A growth reduction in S. oleracea in response to salt treatment is already apparent at low (10 mM NaCl) external salinity (Chapter 4). A low external Ca\(^{2+}\) concentration can cause a decline in cytoplasmic K\(^+\) concentrations (Shabala et al. 2006) and since the Na\(^+\)/Ca\(^{2+}\) ratio was not kept constant while applying the salt treatment, the effect of NaCl in our experiments might have been more severe than if a constant Na\(^+\)/Ca\(^{2+}\) ratio had been used. In contrast to S. oleracea, S. dolichostachya does not experience salt stress at external salinities lower and equal to its growth optimum, but is stimulated by Na\(^+\) instead. The
high activity of the tonoplast Na\(^+\)/H\(^+\)-exchanger in *S. dolichostachya* observed in this study is consistent with the observation that rates of Na\(^+\) accumulation in halophytic species can be extremely high, even under low levels of salt exposure (Flowers and Yeo, 1986; Cheeseman 1988). Therefore, it is likely that the high activity of the tonoplast Na\(^+\)/H\(^+\)-exchanger in *S. dolichostachya* in response to the 10-mM NaCl treatment reflects the constitutive nature of Na\(^+\) accumulation in this species. Our results are in line with the conclusion of Shabala and Mackay (2011) that tonoplast Na\(^+\)/H\(^+\)-exchangers are often constitutive in halophytes and inducible by salt treatment in salt-tolerant glycophytes (Shabala and Mackay 2011). However, the Na\(^+\)/H\(^+\)-exchanger has also been reported to be induced by 200 mM NaCl (control treatment 5 mM NaCl) in *Salicornia bigelovii*, with salt tolerance and salt accumulation strategies comparable to *S. dolichostachya* (Parks et al. 2002). It is possible that the difference in inducibility of the Na\(^+\)/H\(^+\)-exchanger observed between the study of Parks et al. (2002) and our study is caused by the differences in control salinity. It might be that the 10 mM NaCl treatment was enough to cause an induction of the activity of the tonoplast Na\(^+\)/H\(^+\)-exchanger in *S. dolichostachya*, and that 10 mM NaCl treatment would cause an induction of the Na\(^+\)/H\(^+\)-exchanger in *S. oleracea*. However, because *S. oleracea*, in contrast to *S. dolichostachya*, accumulates very low concentrations (less than 10-fold) of Na\(^+\) in its leaves in response to 10 mM NaCl treatment (Chapter 4), an induction of the Na\(^+\)/H\(^+\)-exchanger up to the level of *S. dolichostachya* is not likely.

The ΔpH needed for Na\(^+\)-transport over the tonoplast membrane is generated by the V-H\(^+\)-ATPase and the V-H\(^+\)-PPase (Apse et al. 1999; Gaxiola et al. 2007). In our study, both the H\(^+\)-translocating activity and the hydrolytic activity of the V-H\(^+\)-ATPase (Fig. 6 and Fig. 7) followed the same pattern in response to salinity as the Na\(^+\)/H\(^+\)-exchange activities (Fig. 5B), both were upregulated in *S. oleracea*, whereas they were high and not influenced by salt treatment in *S. dolichostachya*. The inducibility of the H\(^+\)-pumps in *S. oleracea* and the constitutive activity of the H\(^+\)-pumps in *S. dolichostachya* in response to the salt treatment were reflected in the total shoot pH of the two species. The salt treatment decreased total shoot pH in *S. oleracea*, whereas it did not affect total shoot pH in *S. dolichostachya* (Fig. 3). Therefore, the total shoot pH seems to reflect changes in the vacuolar pH, such as previously demonstrated for the total petal pH in *Petunia hybrida* (Quattrocchio et al. 2006). Salt-induced H\(^+\)-translocating activity or hydrolytic activity of the V-H\(^+\)-ATPase has been reported for several glycophytic and halophytic species (Ayala et al. 1996; Barkla et al. 2002; Vera-Estrella et al. 2005). Also protein levels and transcript levels of subunits of the gene coding for the V-H\(^+\)-ATPase have been reported to increase in response to NaCl treatment (Ratajczak et al. 1994; Golldack and
Dietz 2001). However, such effects were not found in other studies (cf Binzel and Ratajczak 2002). In studies of mutants, Krebs et al. (2010) found that the V-H\(^+\)-ATPase is not involved in vacuolar Na\(^+\) sequestration in gamete and embryo development in *Arabidopsis thaliana* (Krebs et al. 2010). In addition, Bose et al. 2014 argued that the ATP pool is greatly reduced under saline conditions due to the restoration of an otherwise depolarized plasma membrane potential and because of the synthesis of organic osmolytes (Bose et al. 2014), which implies that the V-H\(^+\)-PPase will be the main generator of the H\(^+\)-gradient needed for proper functioning of the tonoplast Na\(^+\)-H\(^+\)-exchanger under saline conditions (Bose et al. 2014, Shabala 2013). In our experiment, the H\(^+\)-translocating activity of the V-H\(^+\)-PPase also followed, just like that of the V-H\(^+\)-ATPase, the same pattern as I observed for the tonoplast Na\(^+\)/H\(^+\)-exchanger (Fig. 8). Literature reports on the inducibility of the tonoplast V-H\(^+\)-PPase are highly inconsistent (cf Silva and Geros 2009). The V-H\(^+\)-PPase activity is sometimes reported to increase (Wang et al. 2001; Queiros et al. 2009), but as often reported to decrease (Wang et al. 2000; Otoch et al. 2001), in response to salt treatment. The V-H\(^+\)-PPase is dependent on K\(^+\) for its activation (Rea and Poole 1993). Under saline conditions, K\(^+\) concentrations in the cytoplasm can become low (Cuin et al. 2003; Shabala and Cuin 2007), which would have a negative effect on the functioning of the V-H\(^+\)-PPase. Thus efficient K\(^+\) retention inside the cytoplasm is needed to enable the functioning of the H\(^+\)-PPase, which makes efficient K\(^+\) retention a necessity for transgenic plants over-expressing the Na\(^+\)/H\(^+\)-exchanger (Shabala 2013). This might also contribute to the limited success of increasing salt tolerance in transgenic plants over-expressing the vacuolar Na\(^+\), K\(^+\)/H\(^+\)-antiporter NHX1 (Shabala 2013).

Vesicles derived from both *S. dolichostachya* and *S. oleracea* had similar activities of V-H\(^+\)-ATPase, V-H\(^+\)-PPase and Na\(^+\)/H\(^+\)-exchanger when treated with 200 mM NaCl (Fig. 5-8). Based on these results, it seems unlikely for *S. oleracea* to be limited by its Na\(^+\)/H\(^+\)-transport capacity over the tonoplast. However, *S. dolichostachya* had a better growth compared with *S. oleracea* when treated with 200 mM NaCl, which could imply that Na\(^+\)-compartmentalisation is more efficient in *S. dolichostachya* than in *S. oleracea*. Efficient Na\(^+\)-compartmentalisation is not only dependent on Na\(^+\)-transport into the vacuole, but also on the rate of Na\(^+\) leakage from the vacuole into the cytoplasm. Since salt treatment had comparable effects on Na\(^+\)-H\(^+\)-transport activities in *S. dolichostachya* as in *S. oleracea*, it is possible that *S. dolichostachya* more efficiently retains Na\(^+\) within the vacuole. Retention of Na\(^+\) within the vacuole has been correlated with structural membrane traits such as lipid composition (Leach et al. 1990), but, more likely, also the tonoplast Na\(^+\)-permeable channels could play a major part in Na\(^+\)-retention inside the vacuole. This is illustrated by the finding that regulation of the slow- and fast-
activating vacuolar channels is fundamental for salinity tolerance in Chenopodium quinoa (Bonales-Alatorre et al. 2013). Next to less efficient Na⁺-retention inside the vacuole, it is also possible that S. oleracea, compared with S. dolichostachya, has a lower ability to synthesize and accumulate compatible solutes in its cytoplasm. To maintain water potential equilibrium within the cell compatible solutes are assumed to accumulate in the cytoplasm when Na⁺ is stored in the vacuole (Storey and Wynjones 1979). Thus, the differences in growth between S. oleracea and S. dolichostachya at 200 mM NaCl cannot be explained by differences in Na⁺/H⁺-exchange activity or H⁺-pumping activity, which suggests that Na⁺-retention inside the vacuole is stronger in S. dolichostachya compared with S. oleracea, or that its capacity to maintain water potential equilibrium within the cell is higher.

3.5. Conclusions

Our results showed that the activity of the V-H⁺-ATPase, V-H⁺-PPase and also the Na⁺/H⁺-exchanger was induced by salt treatment in S. oleracea (0 and 200 mM NaCl treatment), whereas in S. dolichostachya these activities were high and unaffected by salt treatment (10 and 200 mM NaCl treatment). This might reflect the fact that S. dolichostachya is an obligate halophyte with a constitutive high salt requirement. Furthermore, S. dolichostachya grew better compared with S. oleracea when treated with 200 mM NaCl, but both species had a similar capacity for Na⁺-influx into the vacuole. This might be taken as an indication that S. dolichostachya had a more efficient system of Na⁺-retention inside the vacuole than S. oleracea, or it could also suggest that the water potential equilibrium within the cell was better regulated in S. dolichostachya than in S. oleracea.
