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# Role of Tonoplast Proton Pumps and $Na^+/H^+$ Antiport System in Salt Tolerance of *Populus euphratica* Oliv.

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**Abstract** *Populus euphratica* has been used as a plant model to study resistance against salt and osmotic stresses, with recent studies having characterized the tonoplast and the plasma membrane ATPases, and two $Na^+/H^+$ antiporters, homologs of the *Arabidopsis* tonoplast AtNHX1, were published in databases. In the present work we show that *P. euphratica* suspension-cultured cells are highly tolerant to high salinity, being able to grow with up to 150 mM NaCl in the culture medium without substantial modification of the final population size when compared to the control cells in the absence of salt. At a salt concentration of 300 mM, cells were unable to grow but remained highly viable up to 17 days after subculture. The addition of a 1-M-NaCl pulse to unadapted cells did not promote a significant loss in cell viability within 48 h. In tonoplast vesicles purified from cells cultivated in the absence of salt and from salt-stressed cells, vacuolar $H^+$-pyrophosphatase (V-$H^+$-PPase) seemed to be the primary tonoplast proton pump; however, there appears to be a decrease in V-$H^+$-PPase activity with exposure to NaCl, in contrast to the sodium-induced increase in the activity of vacuolar $H^+$-ATPase (V-$H^+$-ATPase). Despite reports that in *P. euphratica* there is no significant difference in the concentration of $Na^+$ in the different cell compartments under NaCl stress, in the present study, confocal and epifluorescence microscopic observations using a $Na^+$-sensitive probe showed that suspension-cultured cells subject to a salt pulse accumulated $Na^+$ in the vacuole when compared with control cells. Concordantly, a tonoplast $Na^+/H^+$ exchange system is described whose activity is upregulated by salt and, indirectly, by a salt-mediated increase of V-$H^+$-ATPase activity.

**Keywords** *Populus euphratica* · Salt tolerance · Tonoplast · $Na^+/H^+$ antiport system · V-$H^+$-PPase · V-$H^+$-ATPase

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## Introduction

The overabundance of sodium in soil and in soil solutions limits plant growth over large parts of the world. Excessive salinity imposes two stress factors on plants: an osmotic component that results from the reduced water availability caused by an increase in osmotic pressure in the soil, and an ionic stress resulting from a solute imbalance, causing changes in the $K^+/Na^+$ ratio and increasing the concentration of $Na^+$ and $Cl^-$ in the cytosol (Blumwald and others 2000). Sodium toxicity is caused mainly by the similarity of the $Na^+$ and $K^+$ ions to plant transporters and enzymes. Plant cells typically maintain a high $K^+/Na^+$ ratio in their cytosol

with relatively high $K^+$, on the order of 100–200 mM, and low $Na^+$ of about 1–10 mM (Higinbotham 1973). Thus, the efficient exclusion of $Na^+$ excess from the cytoplasm and vacuolar $Na^+$ accumulation are the main mechanisms for the adaptation of plants to salt stress. This is typically carried out by transmembrane transport proteins that exclude $Na^+$ from the cytosol in exchange for $H^+$, a secondary transport process which is energy-dependent and driven by the protonmotive force generated by the plasma membrane $H^+$-ATPase (Serrano 1989) and by the vacuolar membrane $H^+$-ATPase (V-$H^+$-ATPase) and $H^+$-pyrophosphatase (V-$H^+$-PPase) (Rea and Sanders 1987; Rea and Poole 1993).

*P. euphratica* is the only tree species that occurs naturally in the semiarid areas from northwestern China to western Morocco and Spain (Browicz 1977). It is a halophytic plant, tolerating salt and drought stresses (Kang and others 1996; Watanabe and others 2000; Chen and others 2002, 2003), and has recently been used as a model to study plant defense mechanisms against salt stress. In *Arabidopsis*, a vacuolar $Na^+/H^+$ antiporter (AtNHX1), a homolog to the yeast antiporter NHX1, was cloned and functionally expressed in *S. cerevisiae* (Gaxiola and others 1999). Since then several NHX homologs have been characterized in plants (Xia and others 2002; Fukuda and others 2004; Wu and others 2004; Saqib and others 2005; Hanana and others 2007). In the case of *P. euphratica*, nucleotide databases display two NHX sequences (*PeNHX1* and *PeNHX2*), but their physiological role remains unknown. In spite of recent work showing that $Na^+$ concentrations in the vacuole are not too different from that in the cytosol (Gu and others 2004; Ottow and others 2005a), microscopic and biochemical data are provided in the present study suggesting the involvement of a salt-inducible $Na^+/H^+$ exchange activity in the tonoplast of *P. euphratica* cultured cells.

## Materials and Methods

### Cell Suspensions and Growth Conditions

Cell suspensions of *Populus euphratica* Oliv. were maintained in 250-ml flasks on a rotary shaker at 100 rpm, in the dark, at 25°C in Murashige and Skoog (MS) medium (Murashige and Skoog 1962), supplemented with 2.5% (w/v) sucrose, 1.1 μM 6-benzylaminopurine (BAP), and 2.7 μM 1-naphthaleneacetic acid (NAA), as described by Gu and others (2004). Cells were subcultured every 15 days by transferring 10-ml aliquots into 50-ml of fresh medium. Growth was monitored by determination of dry weight. Aliquots of 1–5 ml were filtered through preweighed GF/C filters (Whatman, Clifton, NJ). The samples were washed with deionized water and weighed after 24 h at 80°C. Sugar consumption was monitored by high-pressure liquid chromatography (HPLC) with L-arabinose as the internal standard.

### Determination of Cell Viability

Fluorescein diacetate (FDA) staining was applied to estimate cell viability. A concentrated stock solution of FDA (500 μg $\mu l^{-1}$, Sigma, St. Louis, MO) was prepared in dimethyl sulfoxide. For the staining protocol, suspension-cultured cells (1 ml) were incubated with 10 μl of FDA stock solution in the dark for 10 min at room temperature. Cells were observed under a Leitz Laborlux S epifluorescence microscope with a 50-W mercury lamp and appropriate filter settings. Images were acquired with a 3-CCD color video camera (Sony, DXC-9100P), a frame grabber (IMAGRAPH, IMASCAN/Chroma P), and software for image management and archival storage (AxioVision Version 3.0, Carl Zeiss Vision, GmbH).

### Determination of $Na^+$ Accumulation and Intracellular Localization

To determine the intracellular accumulation of $Na^+$, cells were washed twice in MS medium. A concentrated stock solution of Sodium Green (5 mM, Molecular Probes, Eugene, OR, USA) was prepared in DMSO prior to use. One milliliter of a cell suspension was incubated with 10 μM of the fluorescent $Na^+$ probe. After incubation in the dark for 10 min at room temperature, cells were washed twice in MS medium and observed under an Olympus FluoView FV1000 confocal laser microscope with appropriate filter settings. To determine the intracellular localization of $Na^+$ in the vacuole, cells were preincubated with 0.1% (w/v) of Neutral Red 5 min before the addition of the fluorescent $Na^+$ probe. After incubation of both dyes, cells were washed twice in MS medium and observed under a Leica DM5000B fluorescence microscope with appropriate filter settings.

### Isolation of Vacuolar Membrane Vesicles

Vacuolar membrane vesicles were isolated from *P. euphratica* suspension-cultured cells by differential centrifugation and sucrose gradient (Façanha and de Meis 1995, 1998; Queirós and others 2009). Cells (40–50 g fresh weight) were harvested, centrifuged at 3000 *g* for 1 min, washed twice with distilled water, and suspended in 100 ml of ice-cold buffer containing 250 mM sucrose, 2 mM EDTA, pH 8.0, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 70 mM Tris-HCl, pH 8.0, 3 mM $MgCl_2$, 100 mM KCl, 0.1% (w/v) BSA, and 0.2% (w/v) polyvinylpyrrolidone (PVPP). The mixture was homogenized with an Ultra-Turrax T25 device (IKA® WERKE, Janke and Kumkel IKA, Germany) for 3 min at 24,000 rpm on ice, and the

homogenate was filtrated through a layer of cheesecloth and centrifuged at 3500 *g* for 10 min. The supernatant was centrifuged once more at 10,000 *g* for 10 min and then at 100,000 *g* for 30 min (Beckman 70Ti). The pellet was resuspended in 8 ml of ice-cold resuspension buffer (15% [v/v] glycerol, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, and 20 mM Tris-HCl, pH 7.5). The suspension was layered over a 32 and 46% (w/v) discontinuous sucrose gradient and centrifuged at 80,000 *g* for 3 h in a Beckman SW 28 rotor. In addition to sucrose, the gradient solutions contained 20 mM Tris-HCl buffer (pH 7.6), 1 mM EDTA, 1 mM DTT, and 1 mM PMSF. The vesicles sedimenting at the 0–32% interface were collected, diluted with 4 volumes of resuspension buffer, and centrifuged at 100,000 *g* for 30 min (Beckman 70Ti rotor). The pellet was resuspended in the resuspension buffer described above. The vesicles were then frozen under liquid nitrogen and stored at −80°C until use. Protein concentration was determined by the method of Lowry (Lowry and others 1951), with BSA as the standard.

### Proton-Pumping Activity of Vacuolar ATPase and PPase

Proton-pumping measurements were determined by measuring the fluorescence quenching of ACMA using a Perkin-Elmer LS-5B fluorometer, as described earlier (Queirós and others 2009). The excitation wavelength was set at 415 nm and the emission wavelength was set at 485 nm. After the addition of tonoplast vesicles (30 µg) to 2 ml of buffer containing 10 mM MOPS-Tris, pH 7.2, 2 µM ACMA, 5 mM $MgCl_2$, and 100 mM KCl, the reaction was started by addition of either ATP or PPi at appropriate concentrations, and the rate of the initial fluorescence quenching was recorded. Mg–PPi complex is the actual substrate for V-$H^+$-PPase (Rea and Poole 1985). The optimal concentration of $Mg^{2+}$ in the assay medium was previously adjusted to values between 2 and 5 mM. The addition of 1.5 mM $NH_4Cl$, an $H^+$ uncoupler, abolished the $H^+$ gradient formed by either ATP or PPi hydrolysis. All experiments were performed at 25°C. The $H^+$-pumping activity was measured by the linear initial slope of fluorescence change. The results were analyzed by computer-assisted nonlinear regression analysis (GraphPad Prism software). By this method, $H^+$-pumping kinetics best fitting the experimental initial acidification curves, corresponding to the quenching of ACMA fluorescence, were determined and estimates for the kinetic parameters were then obtained.

### Study of the Activity and Specificity of the Tonoplast $Na^+/H^+$ Antiport System

For measurements of the rate of dissipation of the pH gradient, tonoplast vesicles were energized by the V-$H^+$-PPase with the addition of a saturating concentration of PPi (Queirós and others 2009). After reaching a fluorescence-quenching steady-state, aliquots of the desired salt solutions were added and the initial rates of fluorescence recovery were recorded. The time used to calculate initial rates of recovery was 15 s. All experiments were performed at 25°C.

### Determination of V-$H^+$-ATPase and V-$H^+$-PPase Hydrolytic Activity

The determination of V-$H^+$-ATPase and V-$H^+$-PPase hydrolytic activity was conducted as described earlier (Queirós and others 2009). Briefly, the reaction was started by the addition of 60 µg of tonoplast membrane to 1.5 ml of reaction medium containing 50 mM MOPS-Tris (pH 7.2), 3 mM $MgSO_4$, 100 mM KCl, and the substrate (ATP or PPi) at the desired concentration. After 10 and 20 min, 0.5 ml was added to ice-cold 3% TCA, mixed, and the release of Pi from either ATP or PPi was determined colorimetrically (Fiske and Subbarow 1925). For the determination of V-$H^+$-ATPase activity, Pi release was measured with and without $KNO_3$ and the difference between these two activities was attributed to the V-$H^+$-ATPase.

## Results

### Growth in Batch Cultures with NaCl and Impact of Salt on Cell Viability

*Populus euphratica* suspension-cultured cells were cultivated in the dark, at 25°C, in MS medium with 2.5% (w/v) sucrose, in the absence of salt and in the presence of 150 and 300 mM NaCl. In all experimental conditions extracellular sucrose was completely hydrolyzed within 5 days and growth/maintenance occurred along with glucose and fructose consumption (not shown). Biomass production and cell viability were monitored at the end of the growth period (Fig. 1a). Cell viability was assessed by fluorescein diacetate (FDA). FDA is permeable to the intact plasma membrane and is converted into a green fluorescent dye, fluorescein, by a function of internal esterases, showing green color in viable cells. Cells cultivated without salt reached a biomass value of 11 mg $ml^{-1}$ dry weight (DW) 17 days after subculture, whereas cells grown in the presence of 150 mM NaCl reached a maximal population size of 9 mg $ml^{-1}$ DW. In both cases, growth arrest occurred only after monosaccharide depletion (not shown) and cell viability remained close to 100%. In the case of cell cultures supplemented with 300 mM NaCl, only 15% of both glucose and fructose were consumed (not shown) and the final population size was very low, suggesting that the assimilated carbon and energy source was being redirected not

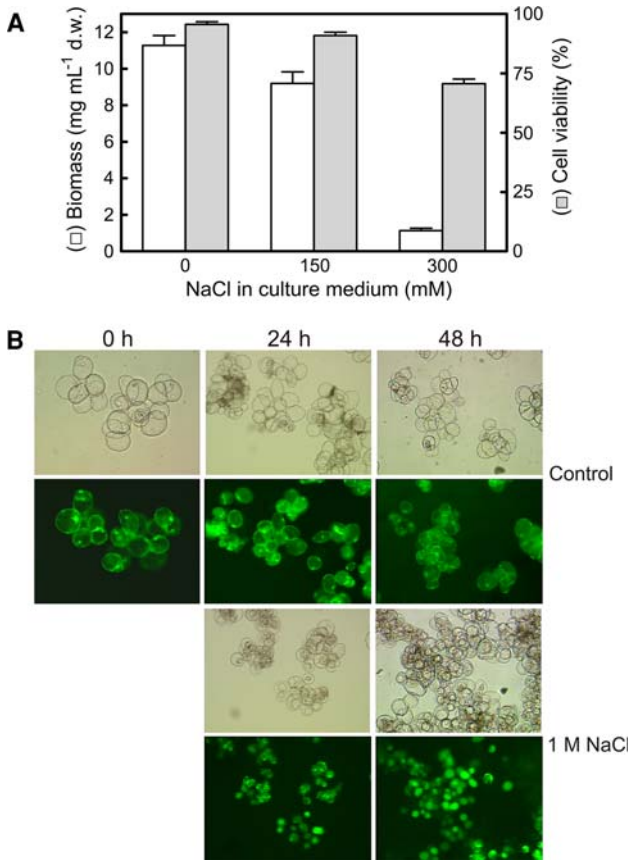


**Fig. 1** Final population size and cell viability of *P. euphratica* suspension-cultured cells 17 days after subculture in mineral media with 2.5% (w/v) sucrose in the absence of salt and in the presence of 150 and 300 mM NaCl (error bars denote SE, $n = 2$) (**a**) and viability assays after the addition of 1 M NaCl to cells cultivated in the absence of salt (**b**). Fluorescence was measured after incubation with fluorescein diacetate (FDA). (Color figure online)

toward cell growth and proliferation but instead to the maintenance of homeostasis in the presence of a high NaCl concentration such as the biosynthesis of compatible solutes and/or the activation of the plasma membrane and tonoplast proton pumps and antiport systems. The surprising observation that *P. euphratica* cell suspensions remained highly viable 17 days after subculture in media with 300 mM NaCl (71% viability) led us to assess in more detail their capacity to resist salt upon addition of NaCl pulses up to a 1-M concentration. Cells were cultivated in media with 2.5% (w/v) sucrose without salt, aliquots were collected at the mid-exponential growth phase, and 0.5-1 M of NaCl was added.

Figure 1b shows that *P. euphratica* suspension cells remained close to 100% viable 24 h after a 1-M-NaCl pulse, although when compared to the control cells they displayed a smaller size and a denser cytoplasm. Forty-eight hours after the salt pulse, cells started to form aggregates and viability decreased to around 70%. Control cells remained viable throughout the experiment.

## Activity of Tonoplast Proton Pumps and $Na^+/H^+$ Exchange

Tonoplast vesicles were isolated from *P. euphratica* suspension cells in the absence of salt and in the presence of 50 and 150 mM NaCl. In these tonoplast fractions both the ATPase hydrolytic and $H^+$-pumping activities, at pH 7.2 (pH optimum of V-$H^+$-ATPase), were inhibited less than 5% by 0.1 mM vanadate (inhibitor of P-ATPase). Also, the F-ATPase (mitochondrial and plastid) inhibitor azide (2 mM $NaN_3$) hardly inhibited ATPase hydrolytic activity, at the same pH condition. Conversely, 50 mM nitrate (in the form of $KNO_3$) inhibited almost completely both the ATPase hydrolytic and $H^+$-pumping activities. These results strongly suggest that the vesicles used in this study consist mainly of vacuolar membrane.

Figure 2 shows the PPi-dependent (Fig. 2a) and ATP-dependent (Fig. 2b) $H^+$-pumping activities across tonoplast vesicles, as measured by the fluorescence quenching of ACMA, determined at substrate-saturating concentrations. Both $NH_4Cl$ and CCCP (not shown) promptly recovered ACMA fluorescence, demonstrating that a pH gradient had been generated. In this biological system, the V-$H^+$-PPase is able to generate a pH gradient threefold greater than the V-$H^+$-ATPase at tenfold less substrate concentration: the $V_{max}$ value for the V-$H^+$-PPase $H^+$ pumping was 936 $\Delta$%F $min^{-1}$ $mg^{-1}$ protein (Fig. 2c) and $V_{max}$ for V-$H^+$-ATPase was 248 $\Delta$%F $min^{-1}$ $mg^{-1}$ protein (Fig. 2b). Conversely, the $K_m$ value of V-$H^+$-PPase $H^+$ pumping, estimated from the Michaelis–Menten plot (Fig. 2c), was determined to be 3.9 μM, whereas $K_m$ for V-$H^+$-ATPase was 0.65 mM ATP (Ma and others 2002). Figure 2d shows the dependence of the initial velocities of PPi hydrolysis by V-$H^+$-PPase on the substrate concentration. From the Michaelis-Menten plot the following kinetic parameters were estimated: $V_{max} = 172$ nmol PPi $min^{-1}$ $mg^{-1}$ protein, $K_m = 45.8$ μM PPi. By comparing $H^+$-pumping activity with hydrolytic activity, we may observe that there is a higher coupling efficiency ($H^+$ pumping/PPi hydrolysis) at lower substrate concentrations. Following the analysis of the initial velocities of $H^+$ pumping at saturating PPi concentrations (Fig. 2a), in tonoplast vesicles from suspension-cultured cells subject to 50 mM NaCl, $V_{max}$ of the V-$H^+$-PPase decreased around twofold to 528 $\Delta$%F $min^{-1}$ $mg^{-1}$ protein, whereas in tonoplast vesicles from cells cultivated in the presence of 150 mM NaCl, a recovery to levels close to the control, 771 $\Delta$%F $min^{-1}$ $mg^{-1}$ protein, was observed.

The hydrolytic activity of V-$H^+$-PPase (measured at 0.1 mM PPi) followed a pattern similar to the $H^+$ pumping, with a slight decrease in vesicles from cells cultivated in the presence of 50 mM NaCl when compared with the control, and a subsequent recovery of hydrolytic activity in

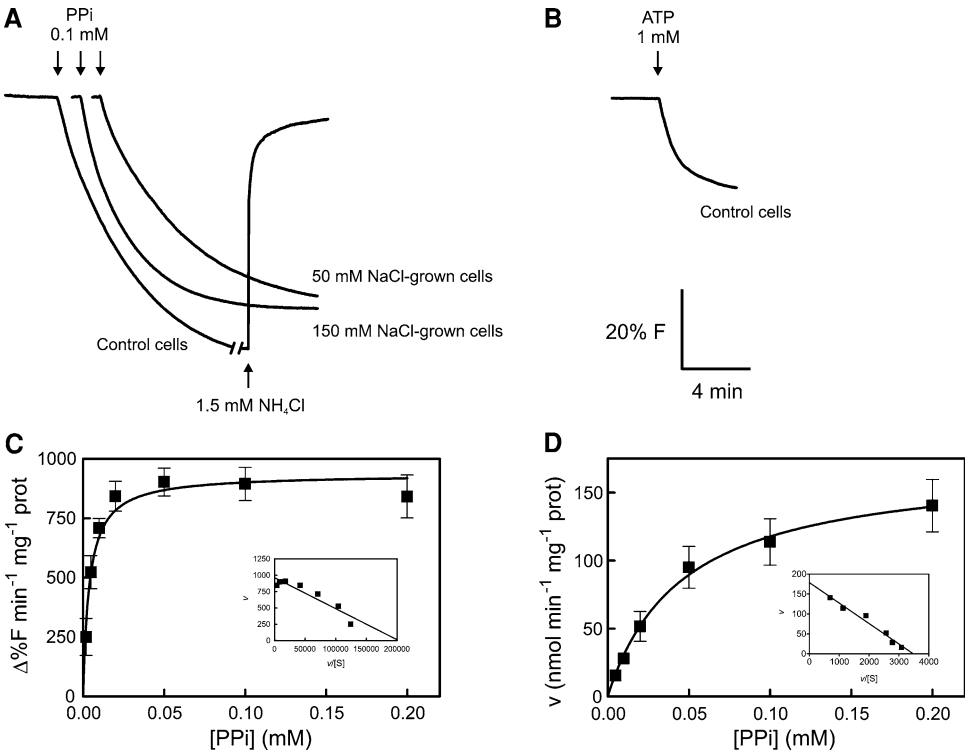


**Fig. 2** Proton-pumping activity of V-$H^+$-PPase in tonoplast vesicles isolated from *P. euphratica* suspension-cultured cells grown in the absence of salt (*control*) and in the presence of 50 and 150 mM NaCl (**a**) and of V-$H^+$-ATPase in suspension cultures grown in the absence of salt (**b**). The accumulation of $H^+$ inside the vesicles was determined by measuring the fluorescence quenching of ACMA (Façanha and de Meis 1998). Addition of 1.5 mM $NH_4Cl$ promoted the recovery of fluorescence. Initial velocities of proton pumping (**c**) and hydrolytic activity (**d**) of *P. euphratica* V-$H^+$-PPase. Insets: Eadie–Hofstee plot of the initial $H^+$ pumping rates and PPi hydrolysis, respectively. Error bars denote SE, $n = 3$

vesicles from cells grown with 150 mM NaCl (Fig. 3). However, the most dramatic change occurred in the V-$H^+$-ATPase hydrolytic activity (measured at 1 mM ATP), which increased in vesicles from cells treated with salt, with the highest value measured in vesicles from cells grown in the presence of 150 mM NaCl (Fig. 3). This confirmed the results of Ma and others (2002), whose studies showed that *P. euphratica* cell suspensions respond to salt stress by increasing both the V-$H^+$-ATPase hydrolytic and $H^+$-pumping activities.

It has been hypothesized that V-$H^+$-PPase may be inhibited directly by salt due to the similarity of $Na^+$ to $K^+$, a cofactor of the enzyme (Rea and Poole 1993). In the present work, the inhibition of *P. euphratica* V-$H^+$-PPase by NaCl was inferred by the effect of different salt concentrations added before activation of the proton pump with 0.1 mM PPi (saturating concentration). The initial velocities of proton pumping were recorded in the first seconds and compared with the value obtained in the absence of salt, an estimate of $V_{max}$ of the enzyme. As can be seen in Fig. 4,

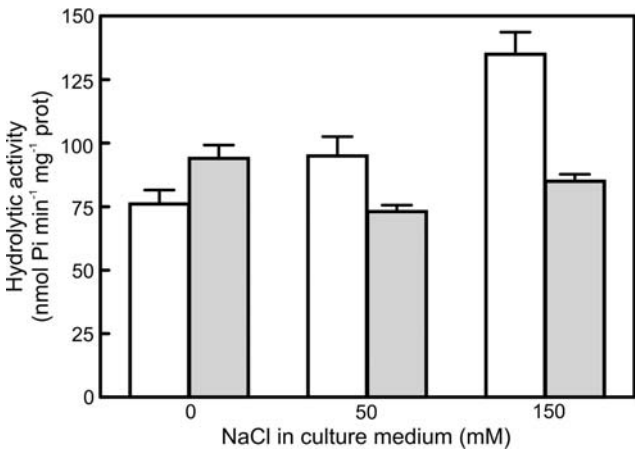


**Fig. 3** Hydrolytic activity of V-$H^+$-ATPase (□) and V-$H^+$-PPase (■) in tonoplast vesicles isolated from *P. euphratica* suspension cells cultivated in the absence of salt and in the presence of 50 and 150 mM NaCl. Error bars denote SE, $n = 2$

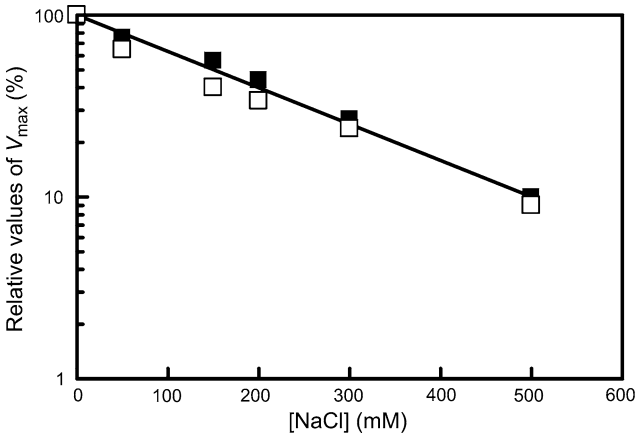


**Fig. 4** Effect of NaCl on proton-pumping activity of the *P. euphratica* V-$H^+$-PPase: dependence of $H^+$-pumping activity on NaCl concentration in the reaction mixture in tonoplast vesicles isolated from cells cultivated in the absence of salt (■) and in the presence of 150 mM NaCl (□)

NaCl decreased $H^+$ pumping and the inhibition kinetics obeyed an exponential relation. The concentration necessary to reduce the $V_{max}$ of proton pumping by 50% ($C_{50}$) allowed expression of $Na^+$ toxicity on V-$H^+$-PPase and the values $C_{50} = 158$ mM and $C_{50} = 124$ mM were estimated in vesicles from cells cultivated in the absence and in the presence of 150 mM NaCl, respectively.

The ability of $Na^+$ to dissipate a pre-established pH gradient was used to study the involvement of a $Na^+/H^+$ exchange transport system in tonoplast vesicles from *P. euphratica*. The ΔpH was generated by V-$H^+$-PPase because it showed a greater efficiency in generating and maintaining a pH gradient across tonoplast vesicles than the V-$H^+$-ATPase and NaCl may stimulate the activity of V-$H^+$-ATPase (Rea and Poole 1985). After the pH gradient reached a steady state, aliquots of salt were added to achieve concentrations of 50–400 mM in the assay medium; this falls within the range used to study the *Arabidopsis* $Na^+/H^+$ exchanger AtNHX1 in acid-loaded lipid vesicles (Venema and others 2002), and the rate of fluorescence recovery was recorded. As can be seen in Fig. 5, the addition of NaCl to tonoplast vesicles caused the dissipation of a preformed pH gradient, measured as the recovery of ACMA fluorescence. Moreover, the initial rates of fluorescence recovery were very pronounced in vesicles isolated from salt-grown suspension cultures (Fig. 5b, c) than in vesicles from cells cultivated without salt (Fig. 5a), with the following values calculated upon the addition of 400 mM NaCl to the reaction medium in control and in 50 and 150 mM NaCl-grown cells: 63.7 Δ%F $min^{-1}$ $mg^{-1}$ protein, 166 Δ%F $min^{-1}$ $mg^{-1}$ protein (2.6-fold increase), and 392 Δ%F $min^{-1}$ $mg^{-1}$ protein (6.1-fold increase), respectively. In tonoplast vesicles isolated from 150 mM NaCl-grown cells, fluorescence recovery increased almost linearly for NaCl concentrations up to 400 mM; however, when $Na_2SO_4$ was used in the range of 100–600 mM, an evident saturation was observed and an apparent $K_m$ of 373 mM $Na^+$ was estimated (Fig. 5e).

The possibility that part of the fluorescence recovery observed after salt addition may be due to the direct inhibition of V-$H^+$-PPase proton-pumping activity by $Na^+$, as shown above, cannot be discarded. However, this effect is similar in membranes from control cells and from salt-stressed cells (Fig. 4), therefore not influencing the overall interpretation of the results. In the same way, we cannot exclude the fact that part of the inhibition of V-$H^+$-PPase proton pumping after salt addition may be attributed to the dissipation of the proton gradient through the $Na^+/H^+$ antiport system, although the initial velocities of proton pumping were measured in the first few seconds after activation of the enzyme, when the ΔpH magnitude might not be sufficient to measure antiport activity.

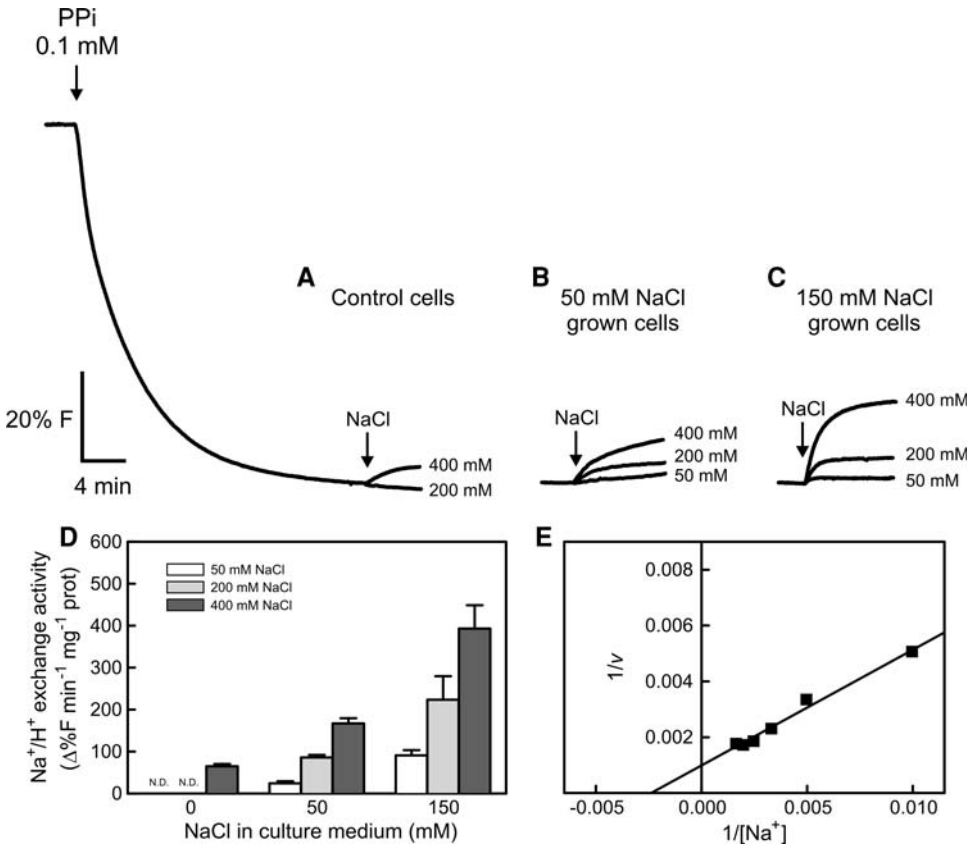


**Fig. 5** Dissipation of the PPi-dependent $H^+$ gradient upon addition of 50–400 mM NaCl (final concentrations) to tonoplast vesicles isolated from *P. euphratica* suspension-cultured cells grown in the absence of salt (**a**) and in the presence of 50 mM (**b**) and 150 mM NaCl (**c**). Initial velocities of fluorescence recovery upon addition of NaCl to tonoplast vesicles (**d**). Lineweaver–Burk plot of the initial velocities of $Na^+$-induced proton dissipation upon addition of aliquots of $Na_2SO_4$ to tonoplast vesicles of 150 mM NaCl grown cells (**e**). Error bars denote SE, $n = 3$

To investigate the ion specificity of the measured $Na^+/H^+$ exchange activity, different salts were added after the ΔpH had been generated by the V-$H^+$-PPase. Vesicles isolated from suspension-cultured cells grown in the presence of 150 mM NaCl were used where the exchange activity was highest. Besides NaCl (427 Δ%F $min^{-1}$ $mg^{-1}$ protein), other sodium salts ($Na_2SO_4$ and Na-gluconate, 502 and 557 Δ%F $min^{-1}$ $mg^{-1}$ protein, respectively) were also able to dissipate the pre-established pH gradient with similar initial velocities, and lithium (in the form of LiCl, 304 Δ%F $min^{-1}$ $mg^{-1}$ protein), a cation with a similar charge and ionic radius, also dissipated the ΔpH, although with a lower initial velocity (Fig. 6a). The addition of KCl had an opposite effect, increasing the fluorescence quenching of ACMA (Fig. 6b), probably because of the stimulating effect of $K^+$ on V-$H^+$-PPase (Rea and Poole 1985) and/or the accumulation of $Cl^-$ in the vacuole via anion channels. The addition of chloride had the same effect (Fig. 6b), possibly through the stimulation of V-$H^+$-PPase $H^+$ pumping to compensate for the electrical depolarization of the tonoplast vesicles caused by the accumulation of the negatively charged chloride anions.

Many studies have shown that vacuolar $Na^+/H^+$ antiporter activity is enhanced by salt stress, but there has been very few direct links made between this increased activity and $Na^+$ sequestration into the vacuole. To determine whether $Na^+$ accumulates inside the vacuole in *P. euphratica* suspension cells, both control and cells subject to 150-mM-NaCl treatment for 24 h were stained with the florescent $Na^+$ probe Sodium Green. In Fig. 7a it can be seen that control cells show only fluorescence on the periphery, whereas green fluorescence in 150-mM-treated

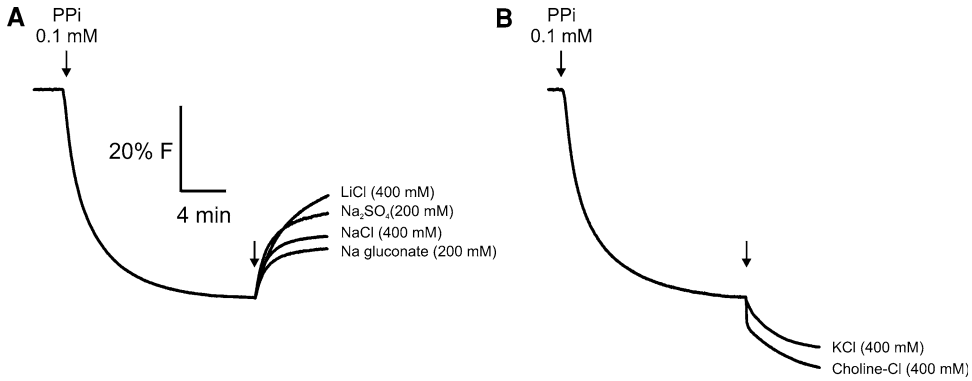


**Fig. 6** Ion specificity of the tonoplast $H^+$-coupled $Na^+$ exchanger in vesicles isolated from *P. euphratica* suspension-cultured cells grown in the presence of 150 mM NaCl

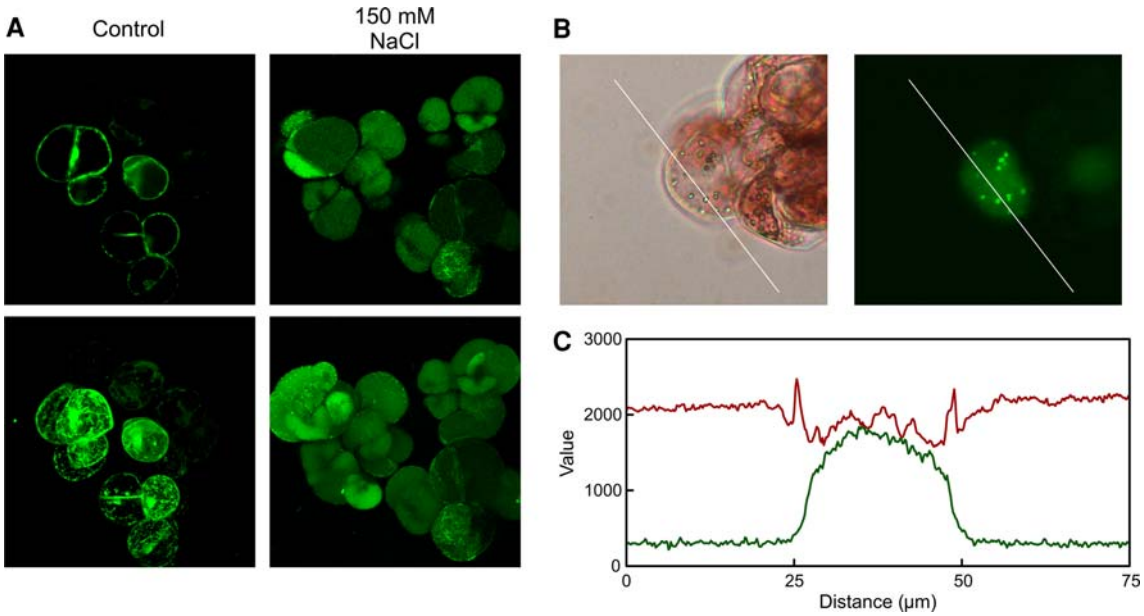


**Fig. 7** **a** $Na^+$ accumulation in *P. euphratica* suspension cells. Cells were stained with Sodium Green and observed under a confocal microscope. *Top row*: single section; *bottom row*: maximum Z projection of 20 sections covering approximately 30 μm. **b** Colocalization of Sodium Green fluorescence and Neutral Red in *P. euphratica* suspension cells subject to a 150-mM-NaCl pulse for 24 h, observed under a fluorescence microscope. **c** Pixel intensity plot along the line shown in **b** of Sodium Green (*green/lower line*) and Neutral Red (*red/upper line*). (Color figure online)

cells seems to be distributed throughout the insides of most cells, suggesting $Na^+$ compartmentalization inside the vacuole. To more accurately colocalize the green $Na^+$ fluorescence to the vacuole, aliquots of salt-stressed cells were double-stained with Sodium Green and Neutral Red, a pH-sensitive dye that stains in red acidic organelles such as the vacuole. Figure 7b and c show that the green fluorescence colocalizes with the vacuoles stained by Neutral Red.

## Discussion

The following evidence shows that *P. euphratica* cells are highly tolerant to high salinity in the extracellular medium: (1) cells were able to grow with up to 150 mM NaCl in the culture medium with a slight reduction of the final population size compared with the control cells in the absence of salt; (2) when the salt concentration in the culture medium was doubled to 300 mM, cells were unable to grow but remained highly viable up to 17 days after subculture; and (3) pulses of 1 M NaCl did not promote a significant loss in cell viability 48 h after addition to unadapted cells. Gu and others (2004) also studied the impact of salt on the growth of *P. euphratica* cell suspensions and reported that 479 mM NaCl induced extensive cell death after four subcultures. The effect of salt on cell viability was also studied in suspension-cultured cells obtained from the moderately salt-tolerant tree *Olea europaea* cultivated with either sucrose or mannitol as the sole carbon and energy source (Conde and others 2007b). Cells growing with sucrose were unable to survive a 500-mM salt pulse after 24 h, whereas cells grown in a medium supplemented with mannitol showed an increased viability. It was proposed that the higher resistance to salt is due at least in part to the intracellular accumulation of this osmoprotectant via a polyol/$H^+$ symport system whose activity is increased by NaCl that transcriptionally upregulates OeMaT1 (*Olea europaea* mannitol transporter 1). Although it is recognized that suspension-cultured cells may not be close to normal physiological conditions, they provide a convenient experimental system that has already yielded much useful information on several key physiological, biochemical, and molecular processes such as sugar transport, gene expression, and plant salt-stress tolerance (Vera-Estrella and others 1999; Xia and others 2002; Conde and others 2006, 2007a, b, c).

Maintenance of internal cellular ion homeostasis is important for all living organisms. Salt stress creates ion imbalances, causing inhibition of $K^+$ uptake by roots and thus changing the internal $Na^+/K^+$ ratio. In plants, this ratio can be restored either by pumping excess $Na^+$ out of the cell by means of a SOS1-like (Shi and others 2000) and/or NhaD-like (Ottow and others 2005a) $Na^+/H^+$ antiporter at the plasma membrane level, or by sequestration of $Na^+$ and $Cl^-$ into the vacuole. Besides detoxifying the cytoplasm, this accumulation can allow plants to use NaCl as an osmoticum, driving water into the cells. As referred to in the Introduction, the *Arabidopsis* vacuolar $Na^+/H^+$ antiporter (*AtNHX1*), homolog to the yeast antiporter NHX1, was cloned, and its expression in *S. cerevisiae* Δ*nxh1* mutants restored its phenotype (Gaxiola and others 1999). In addition, overexpression of this gene in *Arabidopsis* resulted in plants able to grow in soil watered with up to 200 mM NaCl (Apse and others 1999). Since then, several NHX genes have been characterized in other plants such as cotton (Wu and others 2004), wheat (Saqib and others 2005) beet (Xia and others 2002), grape berry (Hanana and others 2007), and rice (Fukuda and others 2004). In this study we present biochemical data corroborating the involvement of $Na^+/H^+$ exchange activity in *P. euphratica* cell suspensions at the tonoplast level, whose salt-inducible activity increased several-fold in NaCl-treated cell suspensions over cells cultivated in the absence of salt. The data obtained are particularly relevant because two sequences for *P. euphratica* NHX genes (*PeNHX1*, AJ853472 and *PeNHX2*, DQ414512) are already published in nucleotide databases that may be responsible for the observed biochemical activity.

The electrogenic $H^+$ pumps V-$H^+$-ATPase and V-$H^+$-PPase are major components of the vacuolar membrane of plant cells (Maeshima 2001). Up to now, vacuolar membranes prepared from all plant species exhibited V-$H^+$-PPase activity in addition to V-$H^+$-ATPase activity, with the noticeable exception of lemon in which the V-$H^+$-PPase is absent (Müller and others 1996). We also found that in *P. euphratica* suspension cells, both V-$H^+$-ATPase and V-$H^+$-PPase generate and maintain the electrochemical gradient across the vacuolar membrane. In this system, V-$H^+$-PPase seems to be able to generate and maintain across the vacuolar membrane a higher pH gradient than V-$H^+$-ATPase, at PPi concentrations in the micromolar range. This is similar to the germinating tissue of mung bean (Nakanishi and Maeshima 1998) and cowpea (Otoch and others 2001), in which the V-$H^+$-PPase is the main $H^+$ pump due to the high availability of PPi, which is produced as a by-product of several metabolic processes. V-$H^+$-PPase also appears to be the main $H^+$ pump acidifying the vacuole of grape berry mesocarp cells as measured in tonoplast vesicles from grape tissues (Terrier and others 2001) and in tonoplast vesicles and intact vacuoles from cell suspensions developed from grape berry callus (CSB cells) (unpublished results). Suspension-cultured cells, growing exponentially and thus having a high metabolic rate, probably also have a high concentration of PPi and therefore show a higher V-$H^+$-PPase activity than V-$H^+$-ATPase activity (Pfeiffer 1998).

It has been described that the activity of both V-$H^+$-ATPase and V-$H^+$-PPase can be regulated by salt.

Induction of protein synthesis by salt has been described as an important mode of regulation of V-$H^+$-ATPase activity, despite some authors proposing that post-translational modifications of V-$H^+$-ATPase can also occur in response to salt (Barkla and Pantoja 1996). The hydrolytic and $H^+$-pumping activity of V-$H^+$-ATPase increased twofold in the halophyte *Suaeda salsa* treated with 200 mM NaCl compared to control plants (Qiu and others 2007). The same result was found in *Mesembryanthemum crystallinum*, in which both V-$H^+$-ATPase $H^+$-transport activity and ATP hydrolytic activity were found to be twofold higher in vesicles isolated from the leaves of a plant treated with 200 mM NaCl compared with the activity measured in control plants of the same age (Barkla and others 1995). With respect to *P. euphratica*, studies performed previously by Ma and others (2002) showed that cell suspensions treated with 50 mM NaCl increased both the hydrolytic activity and the $H^+$-pumping activity of the tonoplast V-$H^+$-ATPase compared with those of control cells. Results obtained in our work correlate well with those results, with a 1.8-fold increase in V-$H^+$-ATPase hydrolytic activity in cells grown in the presence of 150 mM NaCl compared with the control (Fig. 3).

Besides evidence suggesting that V-$H^+$-PPase may be regulated developmentally or by tissue-specific signals, several studies have also focused on the regulation of V-$H^+$-PPase activity by growth in NaCl (Barkla and Pantoja 1996). In contrast with the general sodium-induced increase in V-$H^+$-ATPase activity, there appears to be a decrease in V-$H^+$-PPase activity with exposure to NaCl, as was observed in tonoplast vesicles from barley roots and *M. crystallinum.* This appears to be the case with *P. euphratica* V-$H^+$-PPase, with a slight decrease in both $H^+$-pumping and hydrolytic activities in cells cultivated in the presence of 50 or 150 mM NaCl compared with the control. However, exceptions to this $Na^+$-induced decrease in V-$H^+$-PPase activity have also been described in NaCl-adapted cells of *A. pseudoplatanus* and NaCl-treated *Daucus carota* cells in which the activity of V-$H^+$-PPase increases over control cells (Barkla and Pantoja 1996). We could also observe in our lab that the activities of both V-$H^+$-ATPase and V-$H^+$-PPase are twice as high in 150-mM-NaCl-adapted cells of *Solanum tuberosum* than in the unadapted cell line (Queirós and others 2009).

According to the data of Barkla and others (1995), V-$H^+$-ATPase $H^+$ transport measured in tonoplast vesicles from the halophyte *M. crystallinum* was stimulated directly by the presence of 50 mM $Cl^-$ in the reaction medium in both control and salt-treated plants, whereas V-$H^+$-ATPase $H^+$ transport of the salt-sensitive *Kalanchoe daigremontiana* was inhibited in the presence this ion (White and Smith 1989). This may be due to an adaptation of salt-tolerant plants to NaCl stress, where a greater permeability of the tonoplast vesicles to $Cl^-$ can allow it to accumulate in the vacuole down its electrical gradient, dissipating an inside-positive membrane potential and thus stimulating the formation of a $\Delta pH$ through V-$H^+$-ATPase and V-$H^+$-PPase activity (Bennett and Spanswick 1983). Chloride channels have already been identified and cloned in plants (Plant and others 1994; Lurin and others 1996), and in yeasts, mutants lacking the gene *GEF1* that encodes a chloride channel are more susceptible to cation toxicity (Gaxiola and others 1998). Our results showed an enhanced ability of V-$H^+$-PPase to create a $H^+$ gradient in the presence of $Cl^-$ (Fig. 6b). This could be due to the accumulation of this anion down its electrical gradient via a CLC-like chloride channel present in the tonoplast of *P. euphratica*. In fact, results by Chen and others (2002) showed that in salt-stressed *P. euphratica*, young root cortical cells accumulated $Cl^-$ in the vacuoles when compared with control plants. In *P. euphratica* suspension-cultured cells subjected to 200 mM NaCl, a higher amount of $Cl^-$ was found in the vacuole than in the cytoplasm and cell wall (Gu and others 2004). Chloride accumulation into the vacuole may allow the maintenance of a higher tonoplast $H^+$ gradient that can be used in cation detoxification and an increase in osmotic pressure (Gaxiola and others 1999).

The $Na^+/H^+$ group of antiporters has long attracted attention in relation to salt tolerance in plants (Maeshima 2001). Our results showed that $Na^+/H^+$ exchange activity is negligible in cells grown without salt; activity is induced abruptly when suspension cells were grown with salt, indicating the important role of the antiporter in $Na^+$ detoxification. Concordantly, preliminary work by Ottow and others (2003) showed a fast and significant increase in *PeNHX1* and *PeNHX2* transcripts, as measured by quantitative RT-PCR, in response to severe salt stress, thus supporting the involvement of a concentrative $Na^+/H^+$ antiporter mediating $Na^+$ uptake into the vacuole. In agreement with these biochemical observations, confocal and epifluorescence microscopy analyses performed in the present study using a $Na^+$-sensitive probe showed that suspension-cultured cells subject to a salt pulse accumulated $Na^+$ in the vacuole when compared with control cells. Distinct conclusions were drawn by Gu and others (2004) who found no significant difference in sequestration of $Na^+$ in the different cell compartments under NaCl stress, although its level increased in all cell compartments as the NaCl stress level increased as measured by electron microscopic dispersive X-ray microanalysis. Nevertheless, it may be noted from the study that at the tested external concentrations of 51, 137, and 225 mM NaCl, there is a consistent increase of about 80, 23, and 24%, respectively, of sodium in the vacuole when compared to the cytosol. In addition, in a study with *P. euphratica* plantlets, it was concluded that there is apoplastic $Na^+$ accumulation, but not vacuolar accumulation, as a

response mechanism against salinity (Ottow and others 2005a). However, 9 weeks after 150-mM-NaCl treatment, plantlets showed a higher accumulation of $Na^+$ in the vacuole than in the cytosol. Therefore, we may not discard the hypothesis that the discrepancy between our conclusion that $Na^+$ is accumulated in the vacuole and those conclusions reported above is due to the different sensitivities of the $Na^+$ detection methods used, and that $Na^+$ accumulation in the vacuole may be tissue-specific or dependent on salt level and/or on the physiological/developmental state of the plant.

In the present work, $Na^+$-induced fluorescence recovery signals were measurable at very high $Na^+$ concentrations, revealing the involvement of a tonoplast $H^+$/cation antiporter with very low affinity ($K_m$ = 373 mM $Na^+$). Similarly, very high concentrations of NaCl (10–500 mM) were used to measure cation-dependent $H^+$ exchange activity of AtNHX1 (Venema and others 2002). This very low-affinity $Na^+/H^+$ exchange system is likely to be physiologically relevant to *P. euphratica* under salt stress because very high NaCl concentrations, in the molar range, have been measured in the apoplast (Ottow and others 2005a) and in the cytosol (Gu and others 2004). The exchanger AtNHX1 from *Arabidopsis* (Venema and others 2002) catalyzes low-affinity $Na^+$ transport of $K_m$ ~45 mM $Na^+$, a value similar to that found in *Mesembryanthemum crystallinum* ($K_m$ = 44–51 mM $Na^+$) (Barkla and others 1995) and in *Solanum tuberosum* ($K_m$ = 40–69 mM $Na^+$) (Queirós and others 2009). The gene *VvNHX1* that encodes a vacuolar cation/$H^+$ antiporter from *V. vinifera* was recently cloned and characterized by Hanana and others (2007). VvNHX1 displays low-affinity $K^+/H^+$ and $Na^+/H^+$ exchange activities ($K_m$ = 12.8 and 40.2 mM, respectively). The possibility that the $Na^+/H^+$ antiporter of *P. euphratica* may accept $K^+$ deserves further investigation. If this were the case in the present study, the $Na^+$-dependent $H^+$ dissipation observed would be much more pronounced in the absence of KCl, which was introduced in the assay medium to stimulate the $H^+$-pumping activity of V-$H^+$-PPase. However, because the addition of 400 mM KCl promoted an additional stimulating effect of $H^+$-pumping activity after the $H^+$ gradient had been generated (Fig. 6b), contrary to the fluorescence recovery signal observed upon NaCl addition (Figs. 5 and 6a), the *P. euphratica* cation/$H^+$ antiport system proposed in this work seems to not transport $K^+$, or at least this cation may be taken up with much lower affinity than $Na^+$.

As a whole, the coordinated activities of the tonoplast $Na^+/H^+$ antiport system proposed in this work, together with the extrusion of salt through sodium antiporters at the plasma membrane level, should contribute to the active reduction of $Na^+$ in the cytosol in *P. euphratica*. The characterization at a molecular level of the *P. euphratica* tonoplast antiporters, as was carried out for the plasma membrane SOS-like (Wu and others 2007) and NhaD-like $Na^+$ antiporters (Ottow and others 2005b), would be a valuable contribution to completing our understanding of salt-stress resistance in *P. euphratica*. Proteomic analysis using highly enriched vacuolar preparations has been undertaken and published for *Arabidopsis* and barley vacuoles. These studies have revealed novel tonoplast transporters and their role in important cell functions, including salinity tolerance (for review see Martinoia and others 2007). Also, osmotic adjustments resulting in a net increase of compatible solutes in the cytoplasm have been reported in *P. euphratica* in response to salt (Watanabe and others 2000), as described in olive cells which dramatically increase mannitol uptake via a salt-induced transcription of the corresponding transporter gene (Conde and others 2007b). An additional mechanism possibly involved in the response of *P. euphratica* to salt may be inferred from the observed reduction of the cell size after a salt pulse, suggesting an osmotically induced reduction in surface area in response to the decrease of extracellular water activity, possibly via an endocytic internalization of the cell boundary as already reported in other plant species and cell models (Kubitscheck and others 2000; Bahaji and others 2003). The *P. euphratica* suspension-cultured cells can be adapted to high salinity and might also provide a good experimental system for investigation of these hypotheses.

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