

# Effect of NaCl on leaf H<sup>+</sup>-ATPase and the relevance to salt tolerance in two contrasting poplar species

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**Abstract** During a 30-day period of increasing salinity, we examined the effects of NaCl on leaf H<sup>+</sup>-ATPase and salinity tolerance in 1-year-old plants of *Populus euphratica* Oliv. (salt resistant) and *P. popularis* 35–44 (*P. popularis*) (salt sensitive). Electron probe X-ray microanalysis of leaf mesophyll revealed that *P. euphratica* had a higher ability to retain lower NaCl concentrations in the cytoplasm, as compared to *P. popularis*. The sustained activity of H<sup>+</sup> pumps (by cytochemical staining) in salinised *P. euphratica* suggests a role in energising salt transport through the plasma membrane (PM) and tonoplast. Salt-induced alterations of leaf respiration, ATP content and expression of PM H<sup>+</sup>-ATPase were compared between the two species. Results show that *P. euphratica* retained a constant respiratory rate, ATP production and protein

abundance of PM H<sup>+</sup>-ATPase (by Western blotting) in salt-stressed plants. *P. euphratica* was able to maintain a comparatively high capacity of ATP hydrolysis and H<sup>+</sup> pumping during prolonged salt exposure. By contrast, the activity and expression of PM H<sup>+</sup>-ATPase were markedly decreased in *P. popularis* leaves in response to salt stress. Furthermore, NaCl-stressed *P. popularis* plants showed a marked decline of respiration (70%) and ATP production (66%) on day 30. We conclude that the inability of *P. popularis* to transport salt to the apoplast and vacuole was partly due to the decreased activity of H<sup>+</sup> pumps. As a consequence, cytosolic ion concentrations were observed to be comparatively high for an extended period of time, so that cell metabolism, in particular respiration, was disrupted in *P. popularis* leaves.

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## Introduction

The ability to successfully sequester salt ions (Na<sup>+</sup> and Cl<sup>-</sup>) is considered to be a key determinant of plant salt tolerance. Compartmentalising Na<sup>+</sup> and Cl<sup>-</sup> into vacuoles and extruding these salts to the apoplast are two main strategies to retain low concentrations of Na<sup>+</sup> and Cl<sup>-</sup> in the cytoplasm (Hasegawa et al. 2000; Blumwald et al. 2000; Zhu 2001, 2003). Ottow et al. (2005a) showed a drastic accumulation of Na<sup>+</sup> in the apoplast relative to the protoplast in succulent *Populus euphratica* leaves. Similarly, we found that more Na<sup>+</sup> and Cl<sup>-</sup> ions accumulated in the cortical walls of *P. euphratica* roots, as compared to the salt-sensitive poplar species (Chen et al. 2002, 2003). It is

noteworthy that *P. euphratica* has a greater ability to sequester salt ions, especially  $\text{Cl}^-$  in root cortical vacuoles (Chen et al. 2002, 2003). The effective salt compartmentation in *P. euphratica* roots (including salt exclusion and vacuolar salt sequestration) is suggested to make a contribution to the restriction of ion loading into the root xylem during radial transport, and thus limits subsequent axial transport (Chen et al. 2002, 2003).

Salt compartmentation in vacuoles is partly dependent on active transport of salt ions across the tonoplast via  $\text{Na}^+/\text{H}^+$  antiporter systems (Blumwald and Poole 1985; Garbarino and Dupont 1988) or  $\text{H}^+$ -coupled  $\text{Cl}^-$  antiport (Schumaker and Sze 1987). It is suggested that the greater capacity for  $\text{Na}^+$  exclusion in *P. euphratica* is the result of an active  $\text{Na}^+$  extrusion via  $\text{Na}^+/\text{H}^+$  antiporters across the PM (Sun et al. 2009). *PeNhaD1* and *PeSOS1* likely function as PM  $\text{Na}^+/\text{H}^+$  antiporters in *P. euphratica* (Ottow et al. 2005b; Lü et al. 2007; Wu et al. 2007; Sun et al. 2009). The transcript levels of *PeNhaD1* and *PeSOS1* were maintained constant in *P. euphratica* under saline conditions (Ottow et al. 2005b; Wu et al. 2007) but declined in a salt-sensitive genotype, *P. canescens* (*PeNhaD1*, Ottow et al. 2005b; *PtSOS1*, Escalante-Pérez et al. 2009).  $\text{H}^+$ -ATPase pumps protons and maintains electrochemical  $\text{H}^+$  gradients, thus promoting the secondary active  $\text{Na}^+/\text{H}^+$  antiport through the plasma membrane (PM) and tonoplast (Blumwald et al. 2000; Zhu 2003). It can be inferred that the regulation  $\text{H}^+$ -ATPase activity represents an important cellular mechanism for salt tolerance.  $\text{H}^+$  pumping activity of  $\text{H}^+$ -ATPase in tonoplast vesicles, isolated from *P. euphratica* suspension cells, increased upon NaCl treatment (Ma et al. 2002). This is thought to be of benefit for energising the transport of salts into the vacuole. Shabala and Newman suggested the involvement of PM  $\text{H}^+$ -ATPase in  $\text{Na}^+/\text{H}^+$  antiport under NaCl stress (Shabala 2000; Shabala and Newman 2000).  $\text{Na}^+$  movement across PM vesicles of *P. tomentosa* callus is highly dependent on  $\text{H}^+$ -ATPase activity requiring ATP and  $\text{Mg}^{2+}$  (Yang et al. 2007a). Using the scanning ion-selective electrode technique (SIET), we found that the inhibitor of the PM  $\text{H}^+$ -ATPase, sodium orthovanadate, simultaneously decreased  $\text{Na}^+$  efflux and  $\text{H}^+$  influx along the root axis of long-term-stressed *P. euphratica* plants (Sun et al. 2009). Evidence by Western blotting proved that the expression of PM  $\text{H}^+$ -ATPase was up-regulated by NaCl stress in calluses and leaf tissues of *P. euphratica* (Yang et al. 2007b; Zhang et al. 2007). However, the localisation of  $\text{H}^+$ -ATPases in tissue cells of *P. euphratica* and their response to salinity were not investigated.

In this study, we used cytochemical technique to investigate the effects of NaCl stress on the activity of  $\text{H}^+$ -ATPase in a salt-tolerant species, *P. euphratica* and a salt-sensitive species, *P. popularis*. Western blotting was

employed to assess the protein expression of PM  $\text{H}^+$ -pumps during the period of salt treatment. The aim of this study is to assess the relevance of leaf  $\text{H}^+$ -ATPases for salt tolerance of poplar plants. Moreover, we want to examine the effects of increasing and prolonged salt stress on leaf respiration, since  $\text{H}^+$ -ATPase requires plenty of energy (ATP), delivered by mitochondrial respiration, to mediate salt exclusion from the cytosol.

## Materials and methods

### Plant materials

The salt-tolerant poplar species *P. euphratica* Oliv. (*P. euphratica*, 1-year-old seedlings, obtained from the Xinjiang Uygur Autonomous Region of China) and salt-sensitive species *P. popularis* 35–44 (*P. popularis*, 1-year-old hardwood cuttings, obtained from the nursery of Beijing Forestry University, BFU) were used in this study. In April (2007, 2008), *P. euphratica* and *P. popularis* plants were planted in individual pots containing 10 L of loam soil and sand (soil:sand = 1:1, v/v) and placed in a greenhouse of BFU. The plants in pots were irrigated 2–3 times per week, depending on the evaporative demand and received 1 L of full-strength Hoagland's nutrient solution every 2 weeks. Plants were raised 3 months prior to the initiation of salt treatment (in July). Fifty to sixty uniform plants for each species, which were about 50–80 cm high, were selected for the following experiment.

### Salt treatment

Poplar plants were subjected to 30 days of increasing salinity, i.e. 1 L of 50, 100, 150, and 200 mM NaCl in full-strength Hoagland's nutrient solution was applied on day 1, day 8, day 15 and day 22, respectively. Control plants were kept well-watered and weekly fertilized with no addition of NaCl.

### Leaf harvests and measurements

At each sampling time, destructive harvest of leaves for biochemical analyses were carried out for three independent replicates (plants) per treatment. Western blotting of the plasma membrane (PM)  $\text{H}^+$ -ATPase was performed weekly. X-ray microanalysis and cytochemical staining of leaf cells were conducted on day 22 and 30. ATP content in leaves was measured at the end of experiment. Leaf respiratory rates were measured in non-destructive plants during the period of treatment (three individual plants were examined for each treatment).

## X-ray microanalysis

Energy-dispersive X-ray microanalysis was used to examine the salt distribution within cells. To stimulate the emission of characteristic X-rays from a specimen, a high-energy beam is focused into the sample being studied. The number and energy of the X-rays emitted from a specimen can be measured by an energy-dispersive spectrometer. In this study, leaves were sampled after 22 and 30 days of exposure to salt treatment for 3 independent replicates per treatment. Leaf sections, 2 × 2 mm, cut from upper mature leaves (leaf numbers 6–10 from shoot apex) were divided to two groups: one group for subcellular element examination and another for cytochemical staining of H<sup>+</sup>-ATPase (see below). Sample preparations for X-ray microanalysis were followed as Fritz (1989) with modifications. In brief, leaf sections were immediately placed in isopentane which had been cooled with liquid nitrogen. Thereafter, sections were vacuum freeze-dried at −106°C for 4 days. Following freeze-drying, the samples were allowed to equilibrate with room temperature for 24 h and then stored over silica gel until embedding in plastic. Freeze-dried samples (6–8 leaf sections sampled from each plant) were placed in vacuum-pressure chambers, infiltrated with diethyl ether for 24 h before infiltrating with plastic. The infiltration steps were 1:1 ether:plastic (v/v) for 24 h, 1:2 ether:plastic for 24 h, 1:3 ether:plastic for 24 h, and finally 100% plastic (24 h, ×2). Following infiltration, samples were transferred to gelatine capsules and polymerized at 60°C for at least 7 days. After polymerization, leaves (3 samples for each plant) were sectioned into 1-μm-thick slices on a dry glass knife with an ultramicrotome, mounted on copper grids (mesh 50), coated with carbon and stored over silica gel until analysis.

The slices were examined with a HITACHI-H800 transmission electron microscope assembled with an EDAX-9100 energy-dispersive X-ray analyzer. The accelerating voltage was 150 keV with the take-off angle of 25°. In the mesophyll cells, cell wall, vacuoles and cytoplasm were examined. For each compartment, 10–20 measurements were made at each section and the counting time was 60 s. The data were expressed as counts per second (CPS) of an element peak after subtraction of the background.

## Cytochemical analysis of H<sup>+</sup>-ATPase

For H<sup>+</sup>-ATPase activity analysis, sections were cut from the same leaves that were used for X-ray microanalysis. Pb(NO<sub>3</sub>)<sub>2</sub> staining was used to indicate ATPase activity as described by Malone et al. (1977) and Jian et al. (2000) with some modifications. The histochemical localization of ATPase activity is based on the reaction of Pb<sup>2+</sup> with the liberated free phosphate, which forms electron-dense

insoluble precipitates Pb<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>. The staining intensity indicates the hydrolysis activity of the ATPase. Leaf samples (12–16 sections for each plant) were fixed in a solution of 50 mM cacodylate buffer (pH 7.2), 4% (v/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde for 2 h. Thereafter, tissues were washed in 50 mM cacodylate buffer (pH 7.2) for 30 min (×4). Then the leaf samples (6–8 sections for each plant) were incubated for 3 h in a reaction mixture containing 50 mM Tris-maleate buffer (pH 7.2), 2 mM ATP, 5 mM MgSO<sub>4</sub> and 3 mM Pb(NO<sub>3</sub>)<sub>2</sub>. For the blank control, tissues (6–8 sections for each plant) were incubated in the reaction mixture without ATP addition. After rinsing with 50 mM Tris-maleate buffers (pH 7.2) (30 min, ×2) and 50 mM cacodylate buffer (pH 7.2) (30 min, ×4), the samples were fixed overnight in 50 mM cacodylate buffer (pH 7.2) containing 1% OsO<sub>4</sub> at room temperature. Finally, tissues were washed four times with redistilled water, dehydrated with acetone and embedded in Epon 812 resin. Embedded samples (three sections for each treatment) were sectioned into 0.02-μm-thick slices with an ultramicrotome and photographed with an electron microscope JEM 100 CX (JEOL, Tokyo, Japan).

## Western blotting of the PM H<sup>+</sup>-ATPase

### Protein extraction

Upper mature leaves (leaf numbers 6–10 from the shoot apex) were sampled weekly from control and salinised plants of the two species. Approximate 1 g tissue was homogenized in liquid nitrogen. This requires 8–10 leaves for *P. euphratica* and 1–2 leaves for *P. popularis*. At each sampling time, three individual plants were sampled for each treatment. The plant protease inhibitor, PVPP, was mixed with the ground tissue before the addition of 3 mL of pre-cooled extract containing 50 mM pH 7.5 Tris-HCl, 2% (w/v) SDS, 2 mM EDTA, 5 mM ascorbate, 14 mM β-mercaptoethanol, 1 mM PMSF, 1 μg L<sup>-1</sup> leupeptin, 1 μg L<sup>-1</sup> pepstatin, 1 μg L<sup>-1</sup> aprotinin and 0.15% (w/v) PVPP. The filtrate was centrifuged at 12,000g for 20 min at 4°C and supernatant was used for electrophoresis. The protein concentration of total extracts was determined with BSA as the standard according to Bradford (1976).

### SDS-PAGE

A 60-μL enzyme solution was mixed with 60 μL SDS-loading buffer containing 1 M Tris-HCl (pH 6.8), 25% (v/v) glycerol, 4% (w/v) SDS, 0.01% (w/v) bromocresol blue and 0.1% (v/v) β-mercaptoethanol. The mixture was incubated in thermostatic water bath at 100°C for 5 min, then centrifuged at 12,000g for 5 min at 4°C. The

supernatant was used for SDS-PAGE separation (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) (Laemmli 1970). A 50- $\mu$ g protein was loaded onto a discontinuous SDS-PAGE (4% stacking gel and 10% separating gel) and electrophoretic separation was performed with a mini electrophoresis system (Bio-Rad, Richmond California, USA). After the electrophoresis, one of the two identical gels was stained with Coomassie Brilliant Blue R-250 to check the protein of PM H<sup>+</sup>-ATPase. The stained gel was scanned by the Scanner UMAX Powerlook 2100XL.

#### Western blot analysis

Western blotting was conducted according to the method described by Pan (2005). In brief, immediately after electrophoresis, the polypeptides were electrophoretically transferred to a 0.45- $\mu$ m nitrocellulose membrane (Amersham Life Science) using a Transblot DYCZ-40D apparatus (Liuyi, Beijing, China). Transfer buffer contained 15.6 mM Tris, 120 mM Gly, and 20% (v/v) methanol. The constant flow was 100 mA at 4°C for 6 h during electrophoretic transfer. After this, the membrane was washed three times in TBS (Tris-buffered saline: 10 mM Tris-HCl and 150 mM NaCl, pH 7.5), blocked overnight with 2% (w/v) bovine serum albumin (BSA) and 0.05% (v/v) Tween 20. The protein-transferred nitrocellulose membrane was incubated with monoclonal antibodies against Arabidopsis PM H<sup>+</sup>-ATPase (AHA3) that was previously diluted 10,000-fold with blocking medium containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2% (w/v) BSA and 0.05% (v/v) Tween 20. The incubation system was gently shaken for 3 h at room temperature. After rinsing three times with TBS-T<sub>1</sub> [10 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% (v/v) Tween 20], the membrane was incubated for 1 h at room temperature with a 1:800 (v/v) diluted secondary antibody [alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (H + L), Bio-Rad] in TBS-T<sub>2</sub> containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% (v/v) Tween 20 and 1% (w/v) BSA. Then the membrane was washed three times with TBS-T<sub>2</sub>. Finally, the alkaline phosphatase reaction was developed in dark by 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP-NBT) alkaline phosphatase substrate (B-5655, Sigma-Aldrich). The coloration reaction appeared after about 10 min and was terminated by redistilled water. The images of PM H<sup>+</sup>-ATPases immuno-reactive bands of the membrane were captured using the Gel Imaging System (Bio-Rad). The image density of each band relative to that of the control was estimated using the software Gel-pro Analyzer (version 4.0, Media Cybernetics, Inc. Bethesda, MD, USA).

#### Leaf ATP content

##### ATP extraction

Leaves in upper shoot (leaf numbers 6–10 from the shoot apex) were sampled from control and NaCl-treated plants at the end of experiment. Three independent replicates were harvested for each treatment. Samples were immediately frozen in liquid nitrogen and stored at –80°C freezer for ATP content assay. ATP extraction was based on the method of Wang and Gu (1986) but with some modifications. Approximate 0.5 g leaves were immersed into 10 mL of heat buffer solution (20 mM pH 7.8 Tris buffer solution, 100°C) and incubated for 10 min at 100°C. Subsequently, the samples were cooled down on ice and the supernatant was used for ATP measurement. The average recovery ratio of leaf ATP was ca 92%. We found that the recovery ratio of *P. euphratica* (89–91%) was lower than *P. popularis* (93–95%).

##### ATP assay

The luciferin/luciferase method to measure the ATP concentration was applied according to Wang (1985). A 0.2 mL of ATP extract was mixed with 0.8 mL luciferin/luciferase solution (Shanghai Institute of Plant Physiology, Chinese Academy of Sciences) in the test tubule and then placed in the reaction darkroom of spectrophotometer. Luminescence was measured with the F-4500 FL Spectrophotometer (Hitachi, Japan) at 25°C and the luminiferous peak was recorded (relative light units, RLU). Fluorescence measurement conditions were as follows: 120 s of time scan, 0 nm excitation wavelength and 525 nm emission wavelength (excitation and emission slit was 2.5 nm). Concentrations of ATP in leaves were calculated according to the ATP standard curve.

##### ATP standard curve

To establish the ATP standard curve, a series of ATP concentrations were prepared (in luciferase buffer solution):  $5 \times 10^{-12}$ ,  $5 \times 10^{-11}$ ,  $5 \times 10^{-10}$  and  $5 \times 10^{-9}$  M. A 0.2 mL of ATP standard solution was sucked into the test tubule and reacted with 0.8 mL luciferin/luciferase. For the blank background, 0.2 mL of luciferase buffer, instead of ATP solution, was put into the tubule and the maximum RLU was recorded. In our study, the ATP versus RLU curve yielded a regression coefficient of 0.99. (There are two key points for ATP measurements: (1) luciferase-luciferin solution and ATP standard solutions should be prepared just before being used, and (2) luciferin/luciferase solution should be placed in thermostatic

water bath of 25°C to retain its activity during the measuring time.)

Leaf respiratory rate

The portable infra-red gas analysis system (CIRAS-2 portable IRGA system, PP-systems, Amesbury, MA, USA) was adopted for the assay of leaf respiratory rate according to Turcsányi et al. (2000). The CO<sub>2</sub> exchange of upper mature leaves (leaf numbers 6–10 from shoot apex, dark adapted) was determined at regular time during the period of 30 days of salt treatment. Three individual plants per treatment were examined at each measuring time (three leaves were measured for each plant) and the temperature in the greenhouse was 25–29°C. Leaves were dark adapted for 1 h prior to the gas exchange measurement. Leaf respiratory rate was examined at 29–31°C and the measured area in leaf cuvette was 1.7 cm<sup>2</sup>.

Data analysis

The experimental data of X-ray microanalysis, ATP content and leaf respiratory rate were subjected to ANOVA and significant differences between means were determined by Duncan’s multiple-range test. Unless

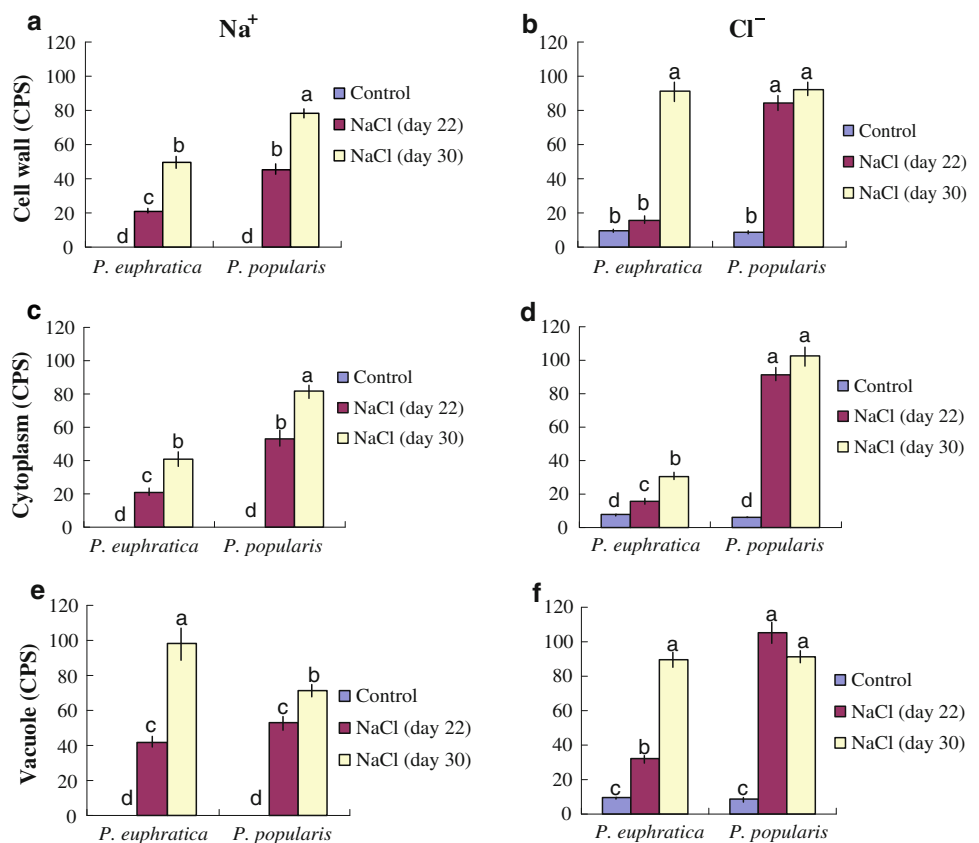
otherwise stated, differences were considered statistically significant when *P* < 0.05. Representative images illustrating the activity and protein content of leaf ATPase were given.

Results

Sodium and chloride compartmentation in mesophyll cells

Under no-salt conditions, Na<sup>+</sup> was not detected in mesophyll compartments of both species (Fig. 1a, c, e). The fraction of Na<sup>+</sup> in the measured cell compartments increased with the duration of NaCl stress in the two poplars, but a more pronounced effect was usually observed in *P. popularis* (Fig. 1a, c, e). Na<sup>+</sup> concentrations in the cell wall, cytoplasm and vacuole were typically lower in *P. euphratica* than in *P. popularis* after 22 days of salt treatment (Fig. 1a, c, e). The same trend was observed on day 30 with the exception of vacuole, in which more Na<sup>+</sup> ions were accumulated in *P. euphratica*, as compared to *P. popularis* (Fig. 1a, c, e). It is worth noting that NaCl-treated *P. euphratica* showed a greater capacity to control Na<sup>+</sup> in the cytosol, i.e. Na<sup>+</sup> fraction in the cytoplasm was

**Fig. 1** Effects of NaCl on Na<sup>+</sup> and Cl<sup>-</sup> concentrations (CPS counts per seconds) in leaf mesophyll compartments of *P. euphratica* (salt tolerant) and *P. popularis* (salt sensitive). Leaves in upper shoot (leaf number 6–10 from shoot apex) were sampled and vacuum freeze-dried, infiltrated in ether and polymerised with plastic. Samples were cut into 1-µm-thick sections for X-ray microanalysis. Probe measurements were made on cell wall, cytoplasm and vacuole in mesophyll cells. Ten to 20 measurements were taken from each leaf section. Each column is the mean of three plants and bars represent the standard error of the mean. Columns labelled with different letters are significantly different at *P* < 0.05



lower than its concentration in the wall and vacuole over the observation periods (Fig. 1a, c, e).

The  $\text{Cl}^-$  compartmentation in mesophyll cells resembled a trend similar to  $\text{Na}^+$  during the period of salt stress (Fig. 1b, d, f). On day 22, salt-induced increase of  $\text{Cl}^-$  in all measured cell compartments remained lower in *P. euphratica* than in *P. popularis* (Fig. 1b, d, f). However,  $\text{Cl}^-$  in the wall and vacuole of *P. euphratica* markedly increased in the following 8 days, reaching a level similar to that of *P. popularis* on day 30 (Fig. 1b, f). Compared with *P. popularis*, salinised *P. euphratica* was effective to retain a lower  $\text{Cl}^-$  in the cytoplasm (Fig. 1d).

## $\text{H}^+$ -ATPase activity

$\text{Pb}(\text{NO}_3)_2$  staining for  $\text{H}^+$ -ATPase is based on the reaction product of  $\text{Pb}^{2+}$  with liberated free phosphate and the staining intensity represents the hydrolysis activity of the  $\text{H}^+$ -ATPase. As shown in Fig. 2a, b, i, j, staining was absent when the substrate, ATP, was not added to incubating solution.  $\text{Pb}(\text{NO}_3)_2$  staining was seen in control cells of the two species, but there were marked differences in the response to salinity (Fig. 2c–h, k–p). On day 22, the activity of  $\text{H}^+$ -ATPase in plasma membrane (PM) and tonoplast increased evidently in salinised

**Fig. 2** Representative TEM images showing cytochemical analysis of  $\text{H}^+$ -ATPase activity in leaf cells of *P. euphratica* (salt tolerant) and *P. popularis* (salt sensitive). For  $\text{H}^+$ -ATPase activity analysis, sections were cut from the same leaves that were used for X-ray microanalysis. The staining intensity of  $\text{Pb}(\text{NO}_3)_2$  represents the hydrolysis activity of the  $\text{H}^+$ -ATPase. Blank control (a, b, i, j) (ATP was absent in the reaction solution); Control (c, d, k, l); NaCl (day 22) (e, f, m, n), NaCl (day 30) (g, h, o, p). V vacuole, N nucleus, C chloroplast, T tonoplast, PM plasma membrane, Cyt cytosol. Arrows indicate the reaction product of  $\text{Pb}^{2+}$  with liberated free phosphate

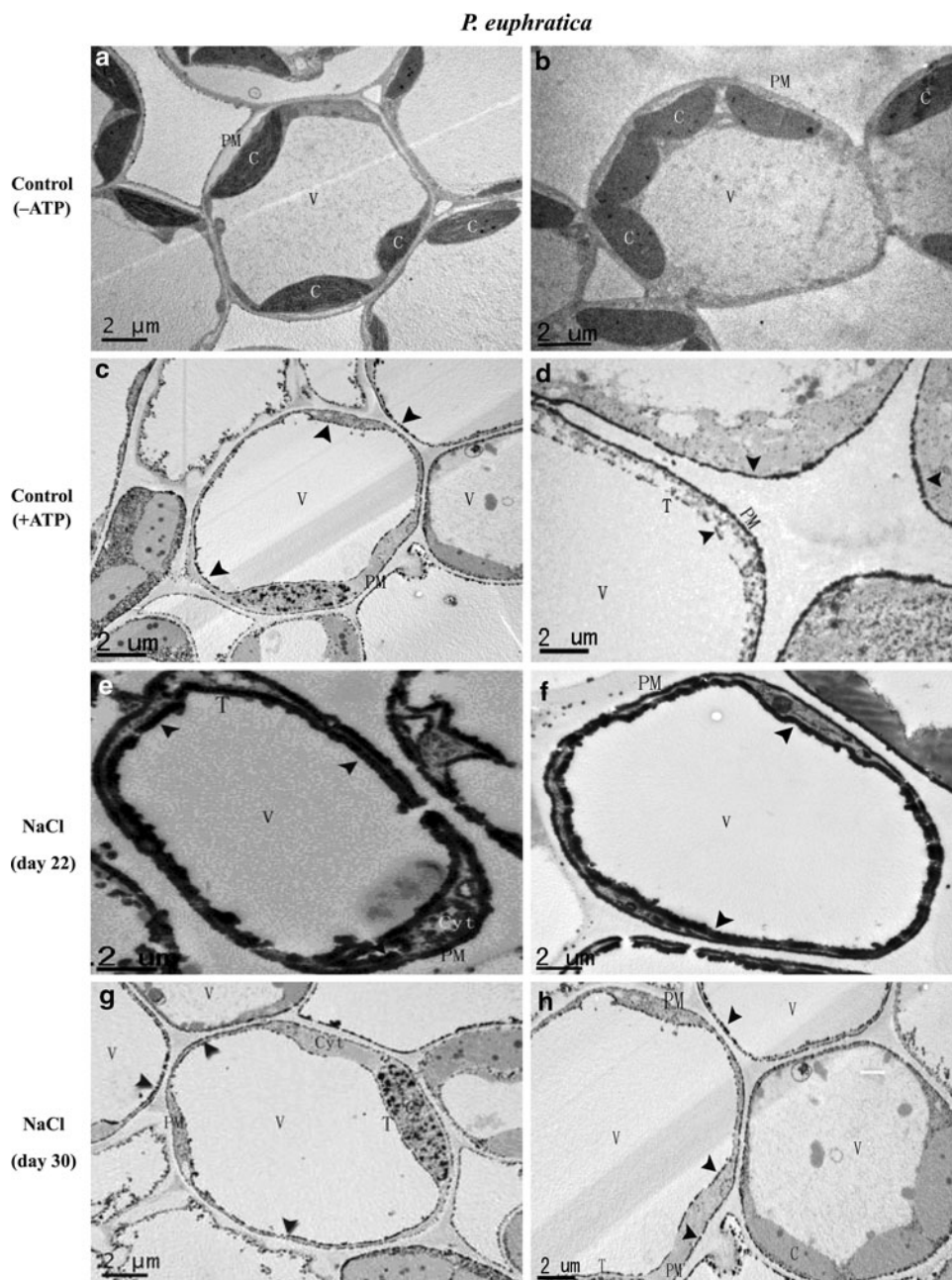
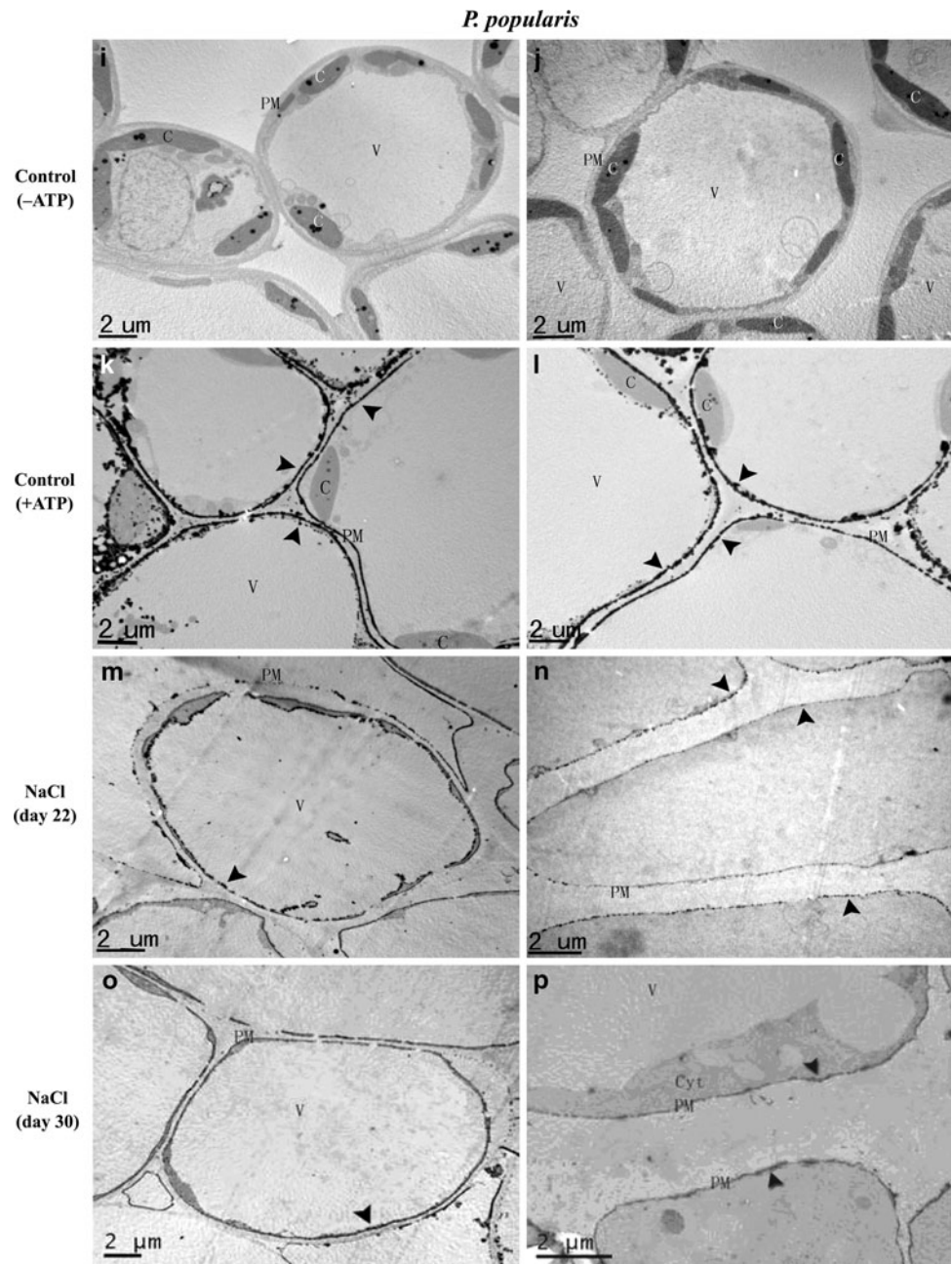


Fig. 2 continued

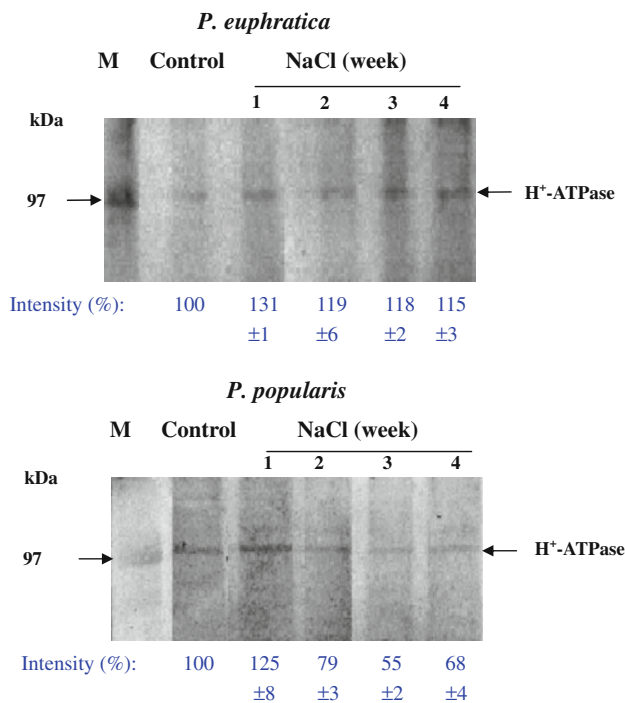


*P. euphratica*, whereas there were no corresponding changes in *P. popularis* (Fig. 2e, f, m, n). The same trend was observed on day 30, although *P. euphratica* displayed a less intense staining as compared to day 22 (Fig. 2g, h, o, p).

#### Western blot analysis of PM H<sup>+</sup>-ATPase

Using the antibody against Arabidopsis PM H<sup>+</sup>-ATPase, the content of the expressed protein was examined weekly during the period of salt treatment. There are species

differences in the response to salinity between the two poplars although a low protein abundance (i.e. the immuno-reactive bands) was found on the membrane (Fig. 3). The expression of PM H<sup>+</sup>-ATPase in *P. popularis* leaves increased on the first week of stress, but markedly declined in the following 3 weeks (Fig. 3). Unlike *P. popularis*, PM H<sup>+</sup>-ATPase content in *P. euphratica* increased upon the salt stress and remained at a constant level over the observation periods, although the level of the expressed protein in control plants was lower than *P. popularis* (Fig. 3).



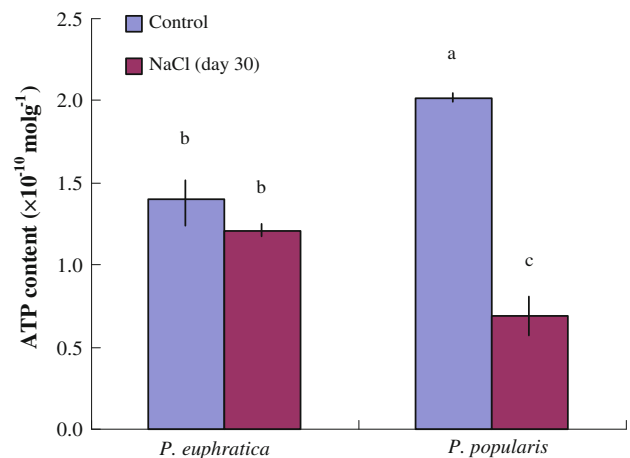
**Fig. 3** Representative images showing Western blotting of plasma membrane  $H^+$ -ATPase in leaves of *P. euphratica* (salt tolerant) and *P. popularis* (salt sensitive). *M* standard markers for molecular mass, *Control* control plants, lane 1 NaCl (1 week), lane 2 NaCl (2 weeks), lane 3 NaCl (3 weeks), lane 4 NaCl (4 weeks). A total of 50  $\mu$ g of protein was loaded per lane. After SDS-PAGE and Western blotting, immunodecoration was performed with monoclonal primary antibody against Arabidopsis PM  $H^+$ -ATPase (AHA3) and with secondary antibody [alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (H + L)]. The image density of each band was expressed as % of the control and each value ( $\pm$ SD) is the mean of three individual plants

#### ATP content of leaves

As shown in Fig. 4, control plants of *P. popularis* typically have a higher ATP content than *P. euphratica*. Salinity reduced ATP content in leaves of the two species, but the inhibitory effect of NaCl on ATP level was more pronounced in the salt-sensitive species, *P. popularis* (Fig. 4). Until the end of the experiment, ATP content decreased by 66% in stressed *P. popularis* but the decline was 14% in *P. euphratica* (Fig. 4).

#### Leaf respiratory rate

The respiratory rate ( $R_n$ ) in *P. popularis* leaves tends to decrease with increasing duration of salt stress and reached the minimum on day 30, at which time a 70% decline was found (Fig. 5). In contrast to *P. popularis*,  $R_n$  in NaCl-treated *P. euphratica* leaves markedly increased after 7 days of salt treatment, and remained 25–35% higher than control plants during the following days of treatment (Fig. 5).



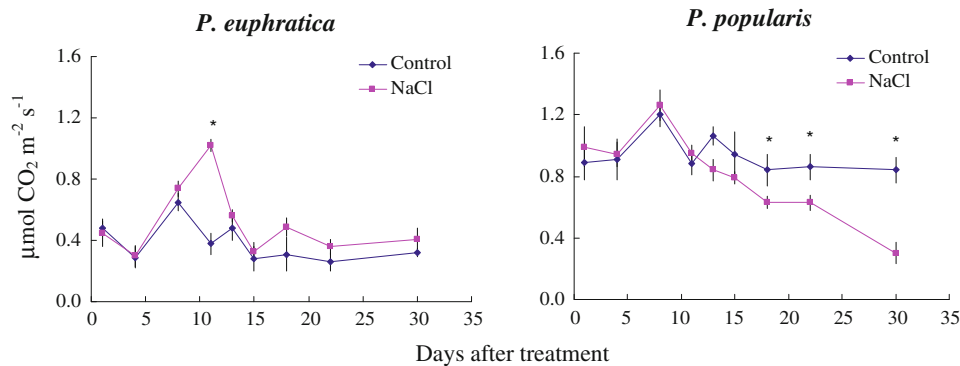
**Fig. 4** Effects of NaCl on ATP content in leaves of *P. euphratica* (salt tolerant) and *P. popularis* (salt sensitive). Leaves in upper shoot (leaf number 6–10 from shoot apex) were sampled from control and salt-stressed plants. Each column is the mean of three plants and bars represent the standard error of the mean. Columns labelled with different letters are significantly different at  $P < 0.05$

## Discussion

### Leaf NaCl compartmentation and salinity tolerance

X-ray microanalysis shows that the salt-resistant species *P. euphratica* generally exhibits lower  $Na^+$  and  $Cl^-$  concentrations in all subcellular compartments as compared to the salt-sensitive *P. popularis*, after 22 days of salt treatment (Fig. 1). This implies that salt uptake into the roots, or subsequent transport to the leaves was controlled more efficiently in the salt-tolerant species (Chen et al. 2002, 2003). Moreover, long-term stressed *P. euphratica* had a higher ability to retain a low concentration of  $Na^+$  and  $Cl^-$  in the cytoplasm, which may result from the vacuolar salt compartmentation and salt extrusion (Fig. 1). Vacuolar fractions of  $Na^+$  and  $Cl^-$  in *P. euphratica* markedly increased coincident with the increasing salinity (Fig. 1). It is also evident that the rate of salt uptake into the vacuole (and the apoplast) between day 22 and 30 was higher in *P. euphratica* as compared to *P. popularis* (Fig. 1). In the latter species, full storage capacity of salt ions in the apoplast and vacuole was apparently achieved already on day 22, whereas *P. euphratica* continued to exclude salt ions from the cytosol beyond this time point (Fig. 1). It is noteworthy that the rise of  $Na^+$  and  $Cl^-$  in *P. euphratica* was lower in the cytoplasm than in the cell wall after an extended period of salt treatment (Fig. 1). Similarly, Ottow et al. (2005a) found that a large amount of  $Na^+$  accumulated in the leaf apoplast of *P. euphratica*. The preferential accumulation of  $Na^+$  and  $Cl^-$  in cell wall relative to the cytoplasm implies the active extrusion of salts from the cytosol, in addition to ion sequestration in the vacuole. This





**Fig. 5** Effects of NaCl on respiratory rates (Rn) in leaves of *P. euphratica* (salt tolerant) and *P. popularis* (salt sensitive). Gas exchange measurements were conducted on leaves in upper shoot (leaf number 6–10 from shoot apex). Each point is the mean of three plants

(three leaves were examined for each plant) and bars represent the standard error of the mean. An asterisk denotes a significant difference at  $P < 0.05$  between control and NaCl treatments

pattern of salt compartmentation suggests a reduction of the toxic effects of salts in the cytoplasm, thus limiting the over production of ROS and avoiding secondary oxidative stress in leaf cells of *P. euphratica* (Wang et al. 2007, 2008).

Leaf respiration markedly decreased in *P. popularis*, but not in *P. euphratica*, after 2 weeks of salt treatment (Fig. 5). The greater decline of leaf respiration in the salt-sensitive species may result from the excessive salt accumulation in the cytoplasm (Fig. 1). The stable respiration in *P. euphratica* was favourable for salt-stressed cells to provide a constant ATP supply (Fig. 4), which benefits  $H^+$ -ATPase to energise salt transport through the plasma and vacuolar membranes.

#### Leaf $H^+$ -ATPase and salinity tolerance

Maintenance of the  $H^+$ -pumping activity is crucial for plant cells to adapt to saline conditions. An Arabidopsis mutant, disrupted in the  $H^+$ -ATPase gene *AHA4*, was found to increase sensitivity to salt stress (Vitart et al. 2001). In tobacco, expressing a constitutively activated  $H^+$ -ATPase enhanced the salt tolerance during the germination and vegetative growth (Gévaudant et al. 2007). The greater capacity of *P. euphratica* for salt compartmentation is presumably associated with the activity of  $H^+$  pumps. Our cytochemical data show that  $H^+$ -ATPase activity did not decline in response to NaCl in both the PM and tonoplast of *P. euphratica* cells (Fig. 2), indicating that  $H^+$ -pumps could maintain the electrochemical  $H^+$  gradients and promote the active  $Na^+/H^+$  antiport under NaCl stress (Blumwald et al. 2000; Zhu 2003). Vacuolar  $H^+$ -ATPase appears to compartmentalise toxic ions into the vacuoles via  $Na^+/H^+$  antiporter systems (Blumwald and Poole 1985; Garbarino and Dupont 1988) or  $H^+$ -coupled  $Cl^-$  antiport (Schumaker and Sze 1987). For salt extrusion at the

PM,  $H^+$ -ATPase has been claimed to be involved in PM  $Na^+/H^+$  antiport by means of a non-invasive ion flux technique [microelectrode ion flux estimation (MIFE)] (Shabala 2000; Shabala and Newman 2000). Our pharmacological experiments at tissue and cellular levels showed that the  $Na^+/H^+$  exchange in long-term-stressed *P. euphratica* was associated with the PM  $H^+$ -ATPase (Sun et al. 2009). Therefore, the  $Na^+$  extrusion through  $Na^+/H^+$  antiporters in *P. euphratica*, e.g. PeNhaD1 (Ottow et al. 2005b), PeSOS (Wu et al. 2007), is thought to be activated by the  $H^+$  gradients generated by the PM  $H^+$ -ATPase (Blumwald et al. 2000; Kerkeb et al. 2001; Zhu 2003; Yamaguchi and Blumwald 2005). Accordingly, *P. popularis* was unable to sequester  $Na^+$  and  $Cl^-$  into vacuoles or to extrude these salts to the apoplast, presumably due to the lower  $H^+$  pumping activity.

Western blot analysis shows that the protein abundance of PM  $H^+$ -ATPase in *P. euphratica* was not decreased after exposure to a longer-term salt stress (Fig. 3). This is consistent with cytochemical analysis of  $H^+$ -ATPase (Fig. 2), indicating that the sustained activity of  $H^+$  pump is partly the result of protein abundance. Similarly, the expression of PM  $H^+$ -ATPase was found to be up-regulated by NaCl stress in calluses and leaves of *P. euphratica* (Yang et al. 2007b; Zhang et al. 2007). The abundance of  $H^+$ -ATPase protein is presumably associated with the level of gene expression. Using Affymetrix poplar gene chips, microarray data show that *P. euphratica* retains a typically higher transcript abundance of the genes encoding  $H^+$ -ATPases, as compared to *P. popularis* (Ding et al. 2010). Accordingly, the two salt-tolerant Italian *P. alba* clones, 14P11 and 2AS11, up-regulated *V-ATPase* genes at intermediate salinity (14 days) (Beritognolo et al. 2007). In the present study, the immuno-reactive band of the membrane was not clear, which was mainly due to (1) the lower abundance of  $H^+$ -ATPase in the PM, and/or (2) the lower

affinity of *P. euphratica* H<sup>+</sup>-ATPase with the antibody AHA3 (Arabidopsis PM H<sup>+</sup>-ATPase), which originated from a herbaceous species.

We conclude that *P. euphratica* was able to retain the activity of H<sup>+</sup>-ATPase during the period of salt exposure. As a consequence, cytosolic ion concentration remained comparatively low (for a longer time), so that cell metabolism was not disrupted; in particular respiration (as observed for *P. popularis*). This enables *P. euphratica* cells to provide plenty of energy (ATP), delivered by mitochondrial respiration for H<sup>+</sup> pumps to continuously exclude salt from the cytosol.

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## References

- Beritognolo I, Piazzai M, Benucci S, Kuzminsky E, Sabatti M, Mugnozza GS, Muleo R (2007) Functional characterisation of three Italian *Populus alba* L. genotypes under salinity stress. *Trees Struct Funct* 21:465–477
- Blumwald E, Poole RJ (1985) Na<sup>+</sup>/H<sup>+</sup> antiporter in isolated tonoplast vesicles form storage tissue of *Beta vulgaris*. *Plant Physiol* 78:163–167
- Blumwald E, Aharon GS, Apse MP (2000) Sodium transport in plant cells. *Biochim Biophys Acta* 1465:140–151
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 72:248–254
- Chen SL, Li JK, Fritz E, Wang SS, Hüttermann A (2002) Sodium and chloride distribution in roots and transport in three poplar genotypes under increasing NaCl stress. *For Ecol Manag* 168:217–230
- Chen S, Li J, Wang S, Hüttermann A, Altman A (2003) Effects of NaCl on shoot growth, transpiration, ion compartmentation and transport in regenerated plants of *Populus euphratica* and *Populus tomentosa*. *Can J For Res* 33:967–975
- Ding M, Hou P, Shen X, Wang M, Deng S, Sun J, Xiao F, Wang R, Zhou X, Lu C, Zhang D, Zheng X, Hu Z, Chen S (2010) Salt-induced expression of genes related to Na<sup>+</sup>/K<sup>+</sup> and ROS homeostasis in leaves of salt-resistant and salt-sensitive poplar species. *Plant Mol Biol*. doi:10.1007/s11103-010-9612-9
- Escalante-Pérez M, Lautner S, Nehls U, Selle A, Teuber M, Schnitzler JP, Teichmann T, Fayyaz P, Hartung W, Polle A, Fromm J, Hedrich R, Ache P (2009) Salt stress affects xylem differentiation of grey poplar (*Populus × canescens*). *Planta* 229:299–309
- Fritz E (1989) X-ray microanalysis of diffusible elements in plant cells after freeze-drying, pressure infiltration with ether and embedding in plastic. *Scanning Microsc* 3:517–526
- Garbarino J, Dupont FM (1988) NaCl induces a Na<sup>+</sup>/H<sup>+</sup> antiporter in tonoplast vesicles from barley roots. *Plant Physiol* 86:231–236
- Gévaudant F, Duby G, von Stedingk E, Zhao R, Morsomme P, Boutry M (2007) Expression of a constitutively activated plasma membrane H<sup>+</sup>-ATPase. *Plant Physiol* 144:1763–1776
- Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. *Ann Rev Plant Physiol Plant Mol Biol* 51:463–499
- Jian L-C, Sun L-H, Li J-H, Wang H, Sun D-L, Li PH (2000) Ca<sup>2+</sup>-homeostasis differs between plant species with different cold-tolerance at 4°C chilling. *Acta Bot Sin* 42:358–366
- Kerkeb L, Donaire JP, Venema K, Rodriguez-Rosales MP (2001) Tolerance to NaCl induces changes in plasma membrane lipid composition, fluidity and H<sup>+</sup>-ATPase of tomato calli. *Physiol Plant* 113:217–224
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
- Lü PP, Hu J, Chen SL, Shen X, Yin WL, Chen YH, Sun YR, Hu ZM (2007) Function of the putative Na<sup>+</sup>/H<sup>+</sup> antiporter gene *PeNhaD1* from salt-resistant *Populus euphratica* Oliv. *J Plant Physiol Mol Biol* 33:173–178 (in Chinese with English abstract)
- Ma T, Liu Q, Li Z, Zhang X (2002) Tonoplast H<sup>+</sup>-ATPase in response to salt stress in *Populus euphratica* cell suspensions. *Plant Sci* 163:499–505
- Malone CP, Burke JJ, Hanson JB (1977) Histochemical evidence for the occurrence of oligomycin-sensitive plasmalemma ATPase in corn roots. *Plant Physiol* 60:916–922
- Ottow EA, Polle A, Brosché M, Kangasjärvi J, Dibrov P, Zörb C, Teichmann T (2005a) Molecular characterization of *PeNhaD1*: the first member of the NhaD Na<sup>+</sup>/H<sup>+</sup> antiporter family of plant origin. *Plant Mol Biol* 58:73–86
- Ottow EA, Brinker M, Teichmann T, Fritz E, Kaiser W, Brosché M, Kangasjärvi J, Jiang X, Polle A (2005b) *Populus euphratica* displays apoplastic sodium accumulation, osmotic adjustment by decreases in calcium and soluble carbohydrates, and develops leaf succulence under salt stress. *Plant Physiol* 139:1762–1772
- Pan W (2005) β-Catenin regulates myogenesis by relieving I-mfa-mediated suppression of myogenic regulatory factors in P19 cells. *PNAS* 102:17378–17383
- Schumaker KS, Sze H (1987) Decrease of pH gradients in tonoplast vesicles by NO<sub>3</sub><sup>-</sup> and Cl<sup>-</sup>: evidence for H<sup>+</sup>-coupled anion transport. *Plant Physiol* 83:490–496
- Shabala S (2000) Ionic and osmotic components of salt stress specifically modulate net ion fluxes from bean leaf mesophyll. *Plant Cell Environ* 23:825–837
- Shabala S, Newman IA (2000) Salinity effects on the activity of plasma membrane H<sup>+</sup> and Ca<sup>2+</sup> transporters in bean leaf mesophyll: masking role of the cell wall. *Ann Bot* 85:681–686
- Sun J, Chen S, Dai S, Wang R, Li N, Shen X, Zhou X, Lu C, Zheng X, Hu Z, Zhang Z, Song J, Xu X (2009) NaCl-induced alternations of cellular and tissue ion fluxes in roots of salt-resistant and salt-sensitive poplar species. *Plant Physiol* 149:1141–1153

- Turcsányi E, Lyons T, Plöchl M, Barnes J (2000) Does ascorbate in the mesophyll cell walls form the first line of defence against ozone? Testing the concept using broad bean (*Vicia faba* L). *J Exp Bot* 51:901–910
- Vitart V, Baxter I, Doerner P, Harper JF (2001) Evidence for a role in growth and salt resistance of a plasma membrane  $H^+$ -ATPase in the root endodermis. *Plant J* 27:191–201
- Wang W (1985) Determination of ATP by bio-luminescence. *Experiment Manual of Plant Physiol*, pp 115–117 (in Chinese)
- Wang W, Gu J (1986) Comparison of the methods for extracting ATP from plant leaves. *Plant Physiol Commun* 5:54–55 (in Chinese with English abstract)
- Wang R, Chen S, Deng L, Fritz E, Hüttermann A, Polle A (2007) Leaf photosynthesis, fluorescence response to salinity and the relevance to chloroplast salt compartmentation and anti-oxidative stress in two poplars. *Trees Struct Funct* 21:581–591
- Wang R, Chen S, Zhou X, Shen X, Deng L, Zhu H, Shao J, Shi Y, Dai S, Fritz E, Hüttermann A, Polle A (2008) Ionic homeostasis and reactive oxygen species control in leaves and xylem sap of two poplars subjected to NaCl stress. *Tree Physiol* 28:947–957
- Wu Y, Ding N, Zhao X, Zhao M, Chang Z, Liu J, Zhang L (2007) Molecular characterization of PeSOS1: the putative  $Na^+/H^+$  antiporter of *Populus euphratica*. *Plant Mol Biol* 65:1–11
- Yamaguchi T, Blumwald E (2005) Developing salt-tolerant crop plants: challenges and opportunities. *Trends Plant Sci* 10:615–620
- Yang YQ, Hu L, Chen X, Ottow EA, Polle A, Jiang X (2007a) A novel method to quantify  $H^+$ -ATPase-dependent  $Na^+$  transport across plasma membrane vesicles. *Biochim Biophys Acta* 1768:2078–2088
- Yang YL, Zhang F, Zhao M, An L, Zhang L, Chen N (2007b) Properties of plasma membrane  $H^+$ -ATPase in salt-treated *Populus euphratica* callus. *Plant Cell Rep* 26:229–235
- Zhang F, Wang Y, Yang YL, Wu H, Wang D, Liu JQ (2007) Involvement of hydrogen peroxide and nitric oxide in salt resistance in the calluses from *Populus euphratica*. *Plant Cell Environ* 30:775–785
- Zhu J-K (2001) Plant salt tolerance. *Trends Plant Sci* 6:66–71
- Zhu J (2003) Regulation of ion homeostasis under salt stress. *Curr Opin Plant Biol* 6:1–5