NaCl-Induced Alternations of Cellular and Tissue Ion Fluxes in Roots of Salt-Resistant and Salt-Sensitive Poplar Species[1][C][W][OA]

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Using the scanning ion-selective electrode technique, fluxes of H^+, Na^+, and Cl^- were investigated in roots and derived protoplasts of salt-tolerant *Populus euphratica* and salt-sensitive *Populus popularis* 35-44 (*P. popularis*). Compared to *P. popularis*, *P. euphratica* roots exhibited a higher capacity to extrude Na^+ after a short-term exposure to 50 mM NaCl (24 h) and a long term in a saline environment of 100 mM NaCl (15 d). Root protoplasts, isolated from the long-term-stressed *P. euphratica* roots, had an enhanced Na^+ efflux and a correspondingly increased H^+ influx, especially at an acidic pH of 5.5. However, the NaCl-induced Na^+/H^+ exchange in root tissues and cells was inhibited by amiloride (a Na^+/H^+ antiporter inhibitor) or sodium orthovanadate (a plasma membrane H^+-ATPase inhibitor). These results indicate that the Na^+ extrusion in stressed *P. euphratica* roots is the result of an active Na^+/H^+ antiport across the plasma membrane. In comparison, the Na^+/H^+ antiport system in salt-stressed *P. popularis* roots was insufficient to exclude Na^+ at both the tissue and cellular levels. Moreover, salt-treated *P. euphratica* roots retained a higher capacity for Cl^- exclusion than *P. popularis*, especially during a long term in high salinity. The pattern of NaCl-induced fluxes of H^+, Na^+, and Cl^- differs from that caused by isomotic mannitol in *P. euphratica* roots, suggesting that NaCl-induced alternations of root ion fluxes are mainly the result of ion-specific effects.

Soil salinity causes increasingly agricultural and environmental problems on a worldwide scale, especially in arid areas. When plant roots are subjected to saline environments with high NaCl content, external Na^+ and Cl^- establish a large electrochemical gradient favoring the passive entry of salt ions through a variety of cation and anion channels and/or transporters in the plasma membrane (PM; Blumwald et al., 2000; Hasegawa et al., 2000; White and Broadley, 2001; Roberts, 2006; Demidchik and Maathuis, 2007). The entry and accumulation of toxic ions lead to disruption of ion homeostasis and finally cause secondary stress, e.g. oxidative bursts (Zhu, 2001, 2003). Accordingly, the maintenance of low salt concentration in the cytosol is of great importance for salt adaptation of plants (Greenway and Munns, 1980; Munns and Tester, 2008).

Active Na^+ extrusion to the apoplast or external environment is essential for sustaining Na^+ homeostasis in the cytosol (Blumwald et al., 2000; Tester and Davenport, 2003; Zhu, 2003; Apse and Blumwald, 2007). PM Na^+/H^+ antiporters have been widely considered to play a crucial role in active Na^+ extrusion under saline conditions (Shi et al., 2000, 2002; Qiu et al., 2002; Martínez-Atienza et al., 2007). NaCl-induced activity of PM Na^+/H^+ antiporter has been reported in crop species, tomato (*Solanum lycopersicum*;

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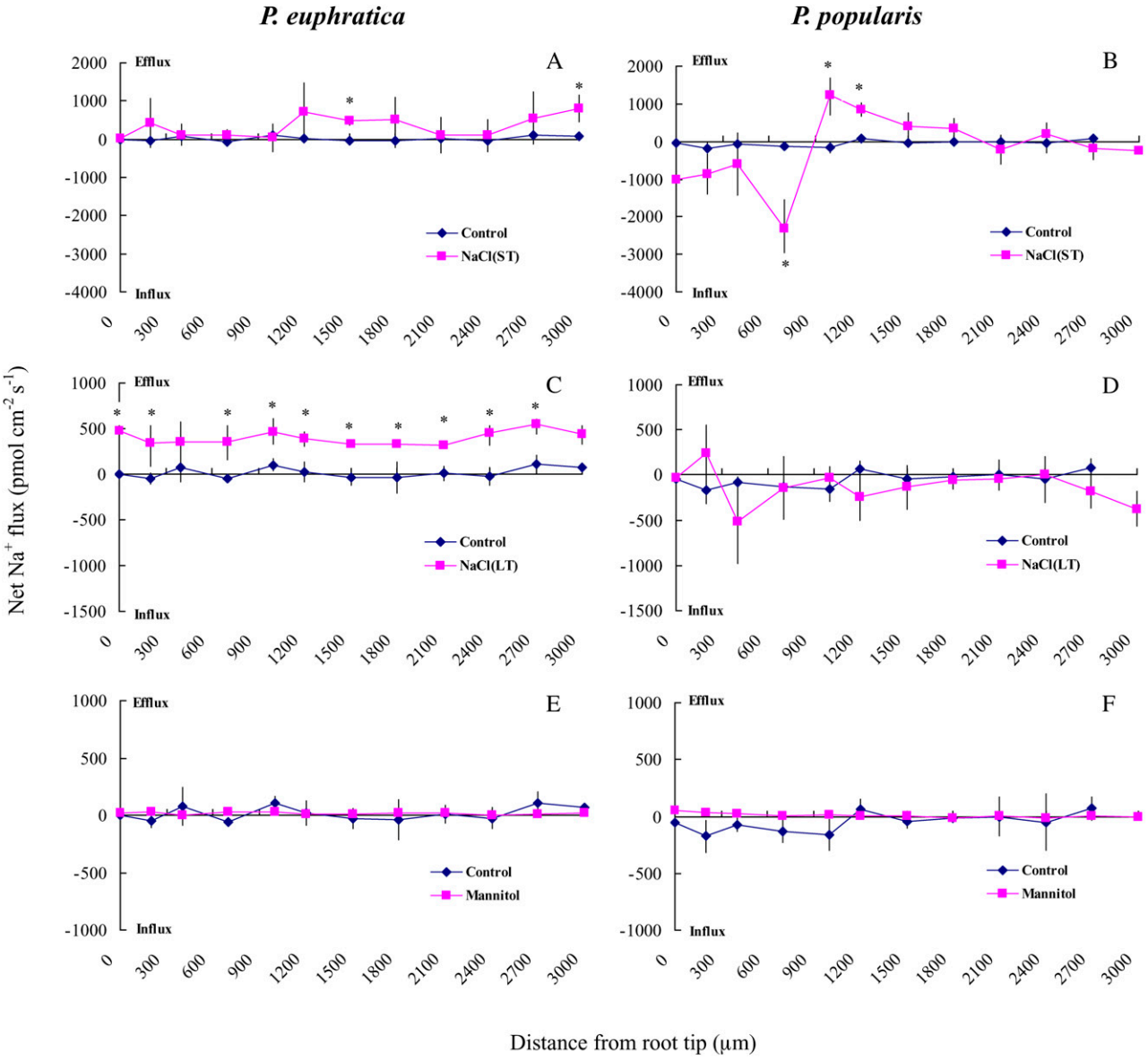

Figure 1. Effects of ST salinity (50 mM NaCl for 24 h; A and B), LT salinity (100 mM NaCl for 15 d; C and D), or hyperosmotic stress (85 mM mannitol, for 24 h; E and F) on net Na^+ fluxes in roots of *P. euphratica* and *P. popularis*. Control roots were treated without NaCl or mannitol. Na^+ fluxes were measured along root axes (0–3,000 μm from the apex) at intervals of 50 to 300 μm. Each point is the mean of five to six individual plants and bars represent the standard error of the mean. An asterisk denotes a significant difference at $P < 0.05$ between treatments. [See online article for color version of this figure.]

Wilson and Shannon, 1995), Arabidopsis (*Arabidopsis thaliana*; Qiu et al., 2002, 2003), and rice (*Oryza sativa*; Martínez-Atienza et al., 2007). Furthermore, overexpression of the Na^+/H^+ antiporter gene *AtSOS1* decreases the accumulation of Na^+ in transgenic Arabidopsis under NaCl stress (Shi et al., 2003). These PM Na^+/H^+ antiporters depend on electrochemical H^+ gradients, which are generated by PM H^+-ATPase (Blumwald et al., 2000; Zhu, 2003). Using an ion-selective microelectrode, Shabala and a colleague suggested the involvement of PM H^+-ATPase in the Na^+/H^+ antiport according to H^+ kinetics on salt shock (Shabala, 2000; Shabala and Newman, 2000). Therefore, the NaCl-induced H^+ pumping is fundamental to Na^+/H^+ exchange and salinity tolerance (Ayala et al., 1996; Vitart et al., 2001; Chen et al., 2007; Gévaudant et al., 2007). However, the active Na^+/H^+ antiport across PM and the contribution to salt exclusion have been rarely investigated in tree species.

Munns and Tester (2008) claimed that Cl^- toxicity is more important than Na^+ toxicity in some woody species, e.g. citrus. Similarly, we have noticed that the inability to restrict Cl^- uptake contributes to the NaCl-induced salt damage in salt-sensitive poplar (*Populus*

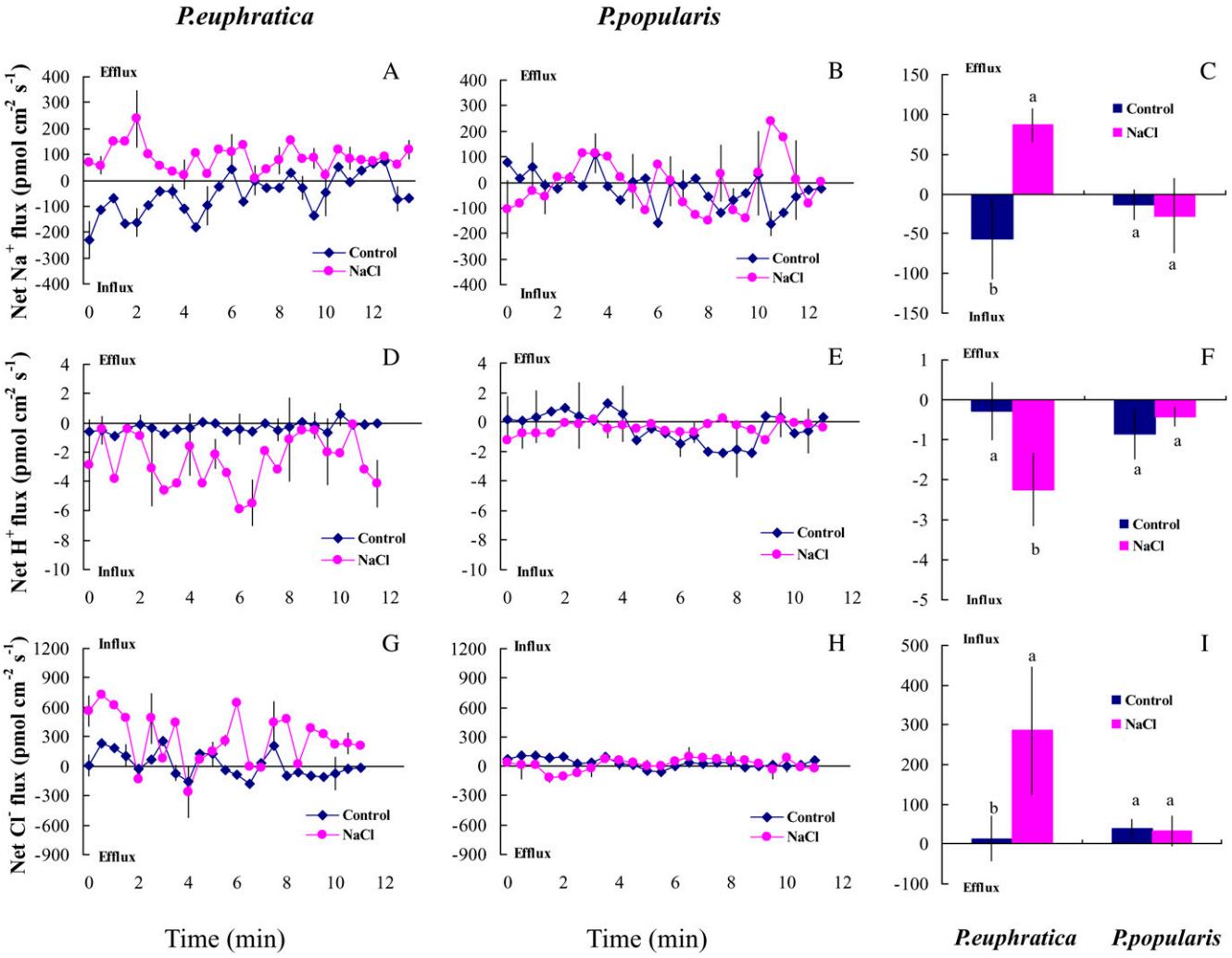

Figure 2. Net fluxes of Na^+ (A and B), H^+ (D and E), and Cl^- (G and H) in protoplasts isolated from control and LT-stressed roots of *P. euphratica* and *P. popularis*. A continuous flux recording of 10 to 12 min was conducted for each protoplast in corresponding measuring solutions (pH 6.0). Each point represents the mean of five to six individual protoplasts and bars represent the standard error of the mean. The mean fluxes of Na^+, H^+, and Cl^- within the measuring periods are shown (C, F, and I). Columns labeled with different letters (a and b) are significantly different at $P < 0.05$. [See online article for color version of this figure.]

spp.) species, in addition to toxicity of excess Na^+ (Chen et al., 2001, 2002, 2003). The differences in Cl^- tolerance exhibited by plants are usually related to the ability to restrict Cl^- transport to the aerial part (Greenway and Munns, 1980; White and Broadley, 2001). Excluding Cl^- from the xylem seems to be an effective mechanism for lotus to cope with the interactive effect of salt and water logging (Teakle et al., 2007). An influx of Cl^-, immediately after addition of NaCl, was observed in bean (*Vicia faba*) mesophyll tissue (Shabala, 2000). The Cl^- flux response to salt shock is helpful to reveal the rapid adjustments of plants to salinity. However, Cl^- fluxes in salt-adapted roots, which are necessary to clarify plant adaptations to long durations of salinity, have not been examined.

Populus euphratica has been widely considered as a model plant to elucidate physiological and molecular mechanisms of salt tolerance in woody species (Chen et al., 2001, 2002, 2003; Gu et al., 2004; Ottow et al., 2005a, 2005b; Junghans et al., 2006; Wang et al., 2007, 2008; Wu et al., 2007; Zhang et al., 2007). Comparative studies have shown that salt-stressed *P. euphratica* seedlings accumulate less Na^+ and Cl^- in root and shoot tissues than salt-sensitive poplar species (Chen et al., 2001, 2002). It is suggested that the greater capacity to exclude NaCl in *P. euphratica* is likely the result of salt uptake and transport restriction in roots (Chen et al., 2002, 2003). However, this needs further investigations, e.g. by electrophysiology, to clarify.

In this study, we used a noninvasive ion flux technique to measure the tissue and cellular fluxes of H^+, Na^+, and Cl^- in roots of the salt-tolerant *P. euphratica* and salt-sensitive *P. popularis* 35-44. The aim was to compare the NaCl-induced alternations of ion fluxes in poplar species differing in salt tolerance. Furthermore, we examined the effects of pH, salt shock, and

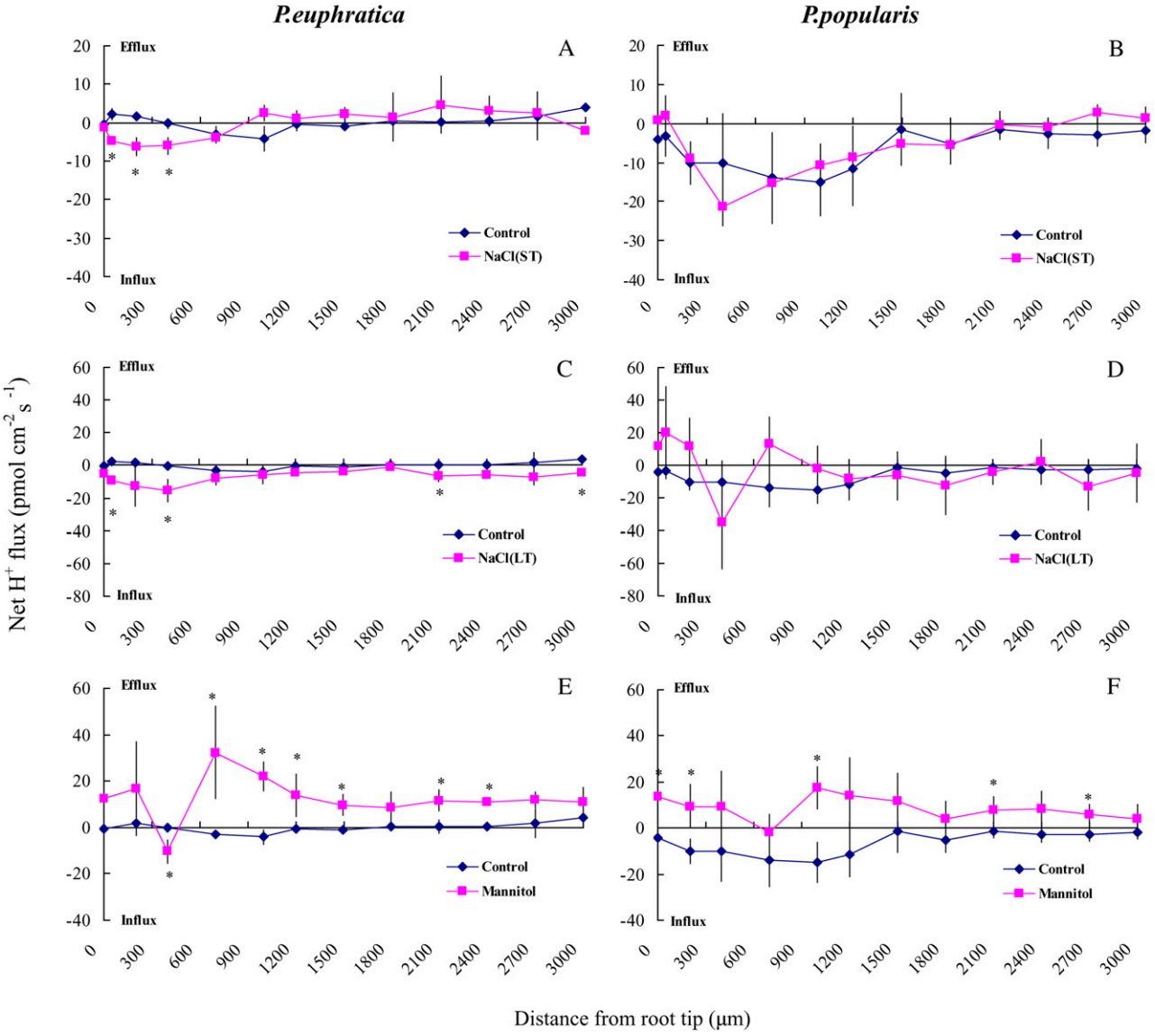

Figure 3. Effects of ST salinity (A and B), LT salinity (C and D), or hyperosmotic stress (E and F) on net H^+ fluxes in roots of *P. euphratica* and *P. popularis*. Control roots were treated without NaCl or mannitol. H^+ fluxes were measured along root axes (0–3,000 μm from the apex) at intervals of 50 to 300 μm. Each point is the mean of seven to eight individual plants and bars represent the standard error of the mean. An asterisk denotes a significant difference at $P < 0.05$ between treatments. [See online article for color version of this figure.]

PM transport inhibitors on Na^+ and H^+ fluxes in root-derived protoplasts of the salt-tolerant species, *P. euphratica*, which exhibited an evident Na^+ exclusion under saline conditions.

RESULTS

Tissue and Cellular Ion Fluxes in Response to Salt and Osmotic Stress in Two Poplar Species

Na^+ Fluxes

The scanning ion-selective electrode technique (SIET) data show that the pattern of Na^+ fluxes in roots of *P. euphratica* differs from that in *P. popularis* after a short-term (ST) exposure to 50 mM NaCl (24 h) and a long-term (LT) exposure to higher salinity, i.e. 100 mM NaCl (15 d; LT). The ST stress caused a net Na^+ efflux, ranging from 20 to 800 pmol cm^{-2} s^{-1} in the measured regions of *P. euphratica* roots (0–3,000 μm from the apex), but Na^+ fluxes in *P. popularis* roots varied within the apical zone and elongation region (Fig. 1, A and B). NaCl induced a net Na^+ influx (600–2,300 pmol cm^{-2} s^{-1}) at the region of 0 to 1,000 μm (apical zone, meristem plus associated root cap), but a pronounced shift toward an efflux (300–1,200 pmol cm^{-2} s^{-1}) was seen at the region of 1,100 to 1,800 μm

from the apex (root elongation region; Fig. 1B). After being subjected to the LT stress, *P. euphratica* roots exhibited a stable and constant efflux with a mean value of 400 pmol cm^{-2} s^{-1} (Fig. 1C). However, a net Na^+ influx was usually detected along *P. popularis* roots, although there were several exceptions (Fig. 1D).

An isoosmotic stress caused by 85 mM mannitol (24 h, osmotic potential of 85 mM mannitol is equal to 50 mM NaCl) did not change the Na^+ flux profile along the root axis in either species (Fig. 1, E and F).

Cellular fluxes of Na^+ were recorded in protoplasts isolated from control and LT-stressed roots of the two species. Control *P. euphratica* protoplasts showed an inward rectification with a mean value of 60 pmol cm^{-2} s^{-1} (Fig. 2, A and C). LT-stressed *P. euphratica* cells exhibited an enhanced Na^+ efflux, although the flux rates oscillated during the period of recording (10–12 min; Fig. 2, A and C). In contrast to *P. euphratica*, salinity did not significantly change the rectification and flux rate of Na^+ in root protoplasts of *P. popularis* during the recording periods (Fig. 2, B and C).

H^+ Fluxes

NaCl induced a marked H^+ influx at the apical zone (50–400 μm) in ST-stressed *P. euphratica* and the effect was even more pronounced in LT-stressed roots, in which a net H^+ influx was also observed at the elongation region in addition to the apex (Fig. 3, A and C). In contrast to *P. euphratica*, the pattern of H^+ flux in *P. popularis* roots did not significantly change, regardless of NaCl concentrations and exposure durations (Fig. 3, B and D).

In general, the hyperosmotic stress (85 mM mannitol, 24 h) caused a drastic H^+ efflux along the root axis in both species (Fig. 3, E and F).

At the cellular level, a NaCl-induced H^+ influx was found in *P. euphratica* protoplasts; its mean flux rate increased 7-fold (Fig. 2, D and F). However, H^+ flux in LT-stressed *P. popularis* cells did not significantly differ from the control (Fig. 2, E and F).

As shown in Figure 4, transient H^+ kinetics response to salt shock was compared in protoplasts, isolated from LT-stressed roots of the two species. After exposure to the 50 mM NaCl, H^+ fluxes in protoplasts exhibited a two-phase response, with an instantaneous increase of efflux, 7 to 10 pmol cm^{-2} s^{-1}, followed by a continuous drift toward larger influxes (Fig. 4A). However, salt-shocked *P. euphratica* cells exhibited a typically greater influx than *P. popularis* over the period of recording (Fig. 4B).

Cl^- Fluxes

There were marked differences in Cl^- fluxes upon salinity between the ST- and LT-stressed plants in the two species. LT-stressed *P. euphratica* roots exhibited a drastic Cl^- efflux in the region of 400 to 1,200 μm, while there were no corresponding changes in ST-stressed roots (Fig. 5, A and C). In contrast to *P. euphratica*, NaCl-induced Cl^- efflux was seen along the root axes of ST-stressed *P. popularis* (with the exception of the root tip) but absent in LT-stressed roots (Fig. 5, B and D).

The hyperosmotic stress (85 mM mannitol, 24 h) caused a drastic Cl^- efflux along the root axis in *P. popularis* (Fig. 5F). However, mannitol treatment did not cause a net Cl^- efflux along *P. euphratica* roots; instead, an enhanced Cl^- influx was seen in the 700 to 1,200 μm region (Fig. 5E).

Compared to control protoplasts, an accelerated Cl^- influx was observed in LT-stressed *P. euphratica* cells, where the mean flux rate increased to 290 pmol cm^{-2} s^{-1} (Fig. 2, G and I). However, Cl^- fluxes in salt-stressed *P. popularis* cells did not significantly differ from controls (Fig. 2, H and I).

Effects of pH, Salt Shock, and PM Transport Inhibitors on Na^+ and H^+ Fluxes in *P. euphratica*

We used root protoplasts of LT-stressed *P. euphratica*, which exhibited a clear Na^+/H^+ exchange, to charac-

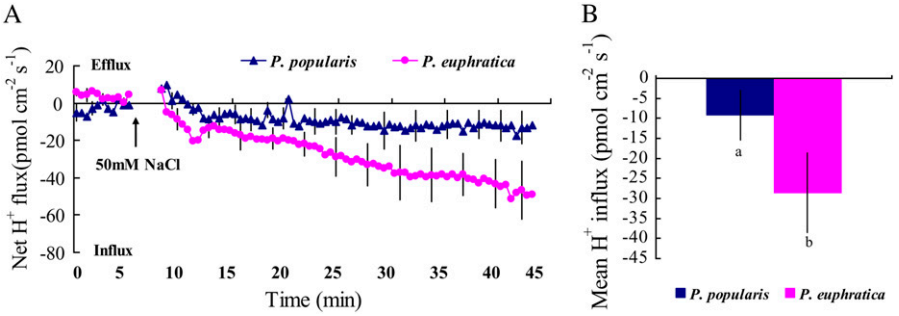

Figure 4. Transient H^+ kinetics of salt shock (50 mM NaCl) in protoplasts isolated from LT-stressed roots of *P. euphratica* and *P. popularis*. A, H^+ kinetics recorded after the required amount of 1.0 M NaCl stock solution was added to the chamber. Prior to the salt shock, steady H^+ fluxes of cells were examined for approximately 5 min. Each point represents the mean of five to six protoplasts and bars represent the standard error of the mean. B, The mean rate of H^+ flux in protoplasts of the two poplar species during the period of salt shock. Columns labeled with different letters (a and b) are significantly different at $P < 0.05$ between the two species. [See online article for color version of this figure.]

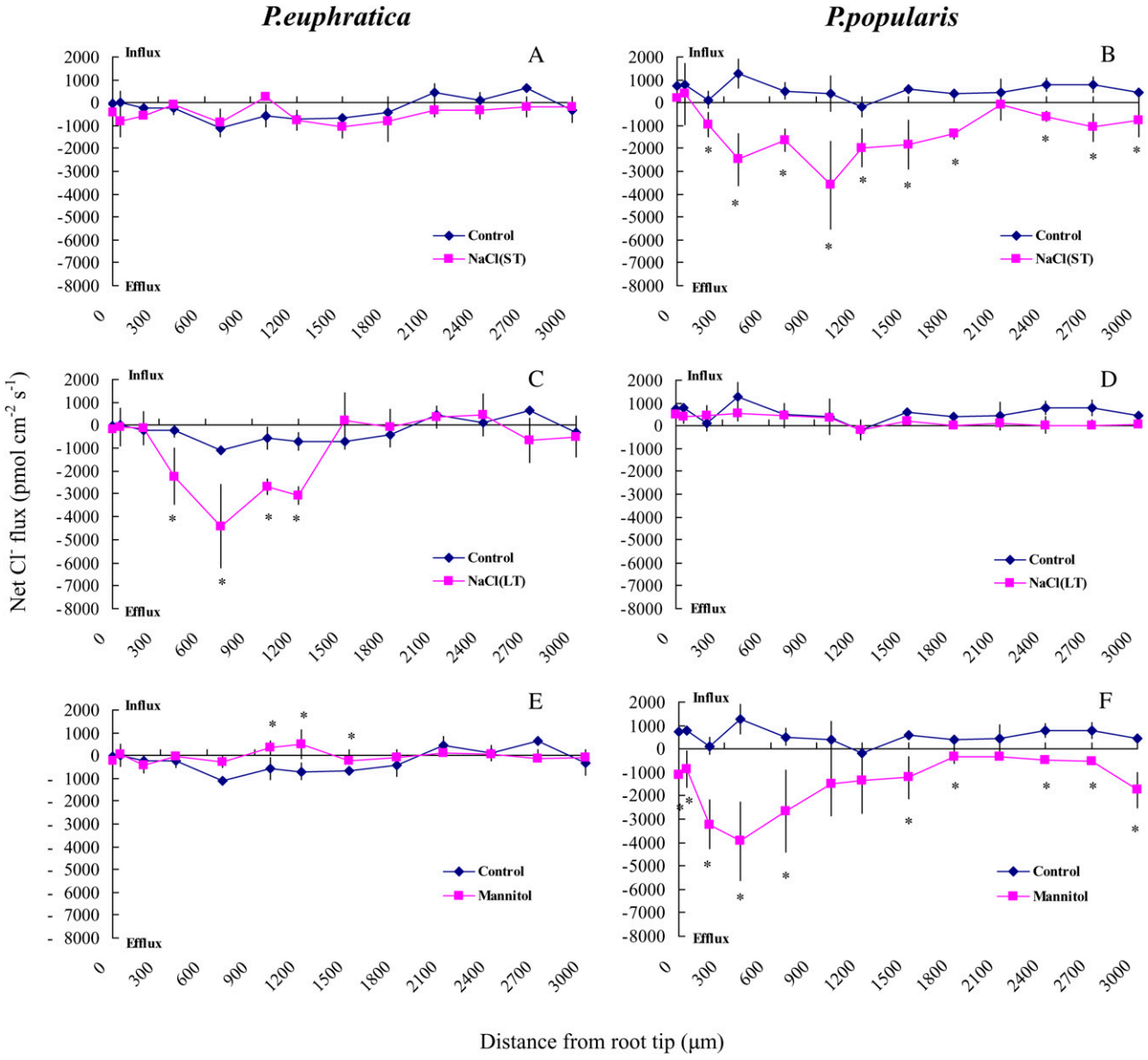

Figure 5. Effects of ST salinity (A and B), LT salinity (C and D), or hyperosmotic stress (E and F) on net Cl^- fluxes in roots of *P. euphratica* and *P. popularis*. Control roots were treated without NaCl or mannitol. Cl^- fluxes were measured along root axes (0–3,000 μm from the apex) at intervals of 50 to 300 μm. Each point is the mean of five to six individual plants and bars represent the standard error of the mean. An asterisk denotes a significant difference at $P < 0.05$ between treatments. [See online article for color version of this figure.]

terize the effects of pH, salt shock, and PM transport inhibitors on cellular Na^+ and H^+ fluxes.

External pH

Cellular fluxes of Na^+ and H^+ depend on pH values in measuring solutions and an acidic environment was found to accelerate Na^+ efflux (Fig. 6A) and H^+ influx (Fig. 6B). More pronounced effects were observed at pH 5.5, compared to pH 6.0 and a neutral pH (Fig. 6).

PM Transport Inhibitors

Amiloride (50 μM), the specific inhibitor of Na^+/H^+ antiporter, or sodium orthovanadate (500 μM), the specific inhibitor of PM H^+-ATPase, significantly reduced the NaCl-induced Na^+ efflux and H^+ influx along the roots (Fig. 7, A–D). A more pronounced inhibitory effect was observed in individual cells. Amiloride reversed the rectifications of Na^+ (efflux → influx) and H^+ (influx → efflux), irrespective of external pH values, 5.5 or 6.0 (Fig. 7, E and F). Similarly,

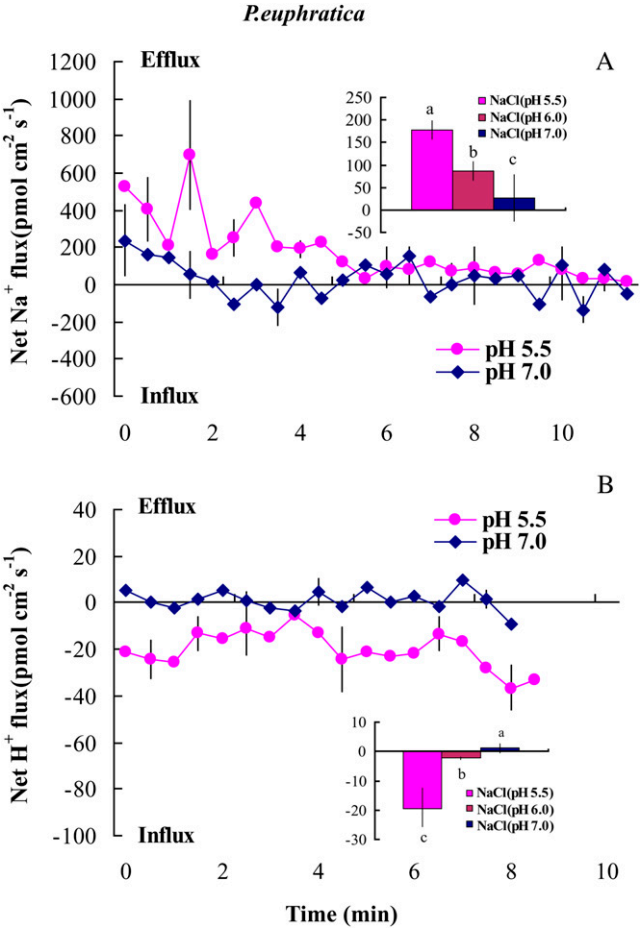

Figure 6. Effects of pH on net Na^+ and H^+ fluxes in protoplasts isolated from LT-stressed *P. euphratica* roots. Ion flux measurements were carried out in measuring buffers with pH at 5.5, 6.0, and 7.0. Each point represents the mean of five to six protoplasts and bars represent the standard error of the mean. Inserted sections show the mean flux rates of Na^+ and H^+ within the measuring period of 8 to 10 min. Columns labeled with different letters (a, b, and c) denote significant difference at $P < 0.05$. [See online article for color version of this figure.]

sodium orthovanadate markedly reduced the H^+ influx and caused a drastic shift of Na^+ efflux toward an influx (Fig. 7, E and F).

NaCl Shock

As shown in Figure 8A, NaCl shock (50 mM) induced a net H^+ influx in protoplasts isolated from LT-stressed *P. euphratica* roots and the influx increased with an increase in the length of exposure. However, PM transport inhibitors amiloride or sodium orthovanadate significantly inhibited the H^+ influx, although salt shock caused a biphasic pattern of H^+ flux in amiloride-pretreated protoplasts: with an instantaneous increase of efflux, followed by a continuous drift toward influx (Fig. 8, A–C).

DISCUSSION

The leaves of the salt-sensitive species, *P. popularis*, exhibited symptoms of salt damage after 15 d of salt stress (100 mM NaCl), whereas there was no visible injury in leaves of *P. euphratica* during the period of our experiment. This agrees with the result of Gu et al. (2004), who found that *P. euphratica* could tolerate an even higher concentration of NaCl, 450 mM, under hydroponic conditions. Leaf injury in *P. popularis* likely resulted from an oxidative burst, induced by an excessive accumulation of toxic salts in cell compartments, e.g. in chloroplasts (Wang et al., 2007, 2008). We have suggested that the greater capacity to exclude NaCl in *P. euphratica* is the result of salt uptake and transport restriction in roots (Chen et al., 2002, 2003). Using SIET in this study, we conclude that active salt extrusion in *P. euphratica* roots contributes to ionic homeostasis under saline conditions.

Na^+/H^+ Antiport across the PM under NaCl Stress

ST and LT salinity caused a net Na^+ efflux in *P. euphratica* roots, although the more pronounced effect was observed in LT-stressed plants (Fig. 1, A and C). Similarly, a net Na^+ efflux was obtained in bean leaf mesophyll after NaCl addition (Shabala, 2000). Salt-stressed *P. euphratica* roots exhibited a net H^+ influx in the apical zone (ST) or along the root axis (LT; Fig. 3, A and C). NaCl-induced H^+ influx was also seen in the root apex of a wild-type Arabidopsis (Shabala et al., 2005). In accord with root tissues, LT-stressed *P. euphratica* cells exhibited an evident net Na^+ efflux (Fig. 2, A and C) and a corresponding influx of H^+ (Fig. 2, D and F). In this study, the increase in H^+ influx corresponding to the Na^+ efflux suggests that the Na^+ extrusion in salt-stressed *P. euphratica* roots is mainly the result of an active Na^+/H^+ antiport across the PM. The experimental evidence is briefly listed below. (1) Pharmacological evidence at tissue and cellular levels. The PM transport inhibitors, amiloride (inhibitor of the Na^+/H^+ antiporter) and sodium orthovanadate (inhibitor of the PM H^+-ATPase) simultaneously decreased Na^+ efflux and H^+ influx along the root axis of LT-stressed plants (Fig. 7, A–D). At cellular levels, the rectification of Na^+ flux was correspondingly reversed when H^+ influx was restricted by the inhibitors, regardless of pH values (5.5 or 6.0) in the measuring buffers (Fig. 7, E and F). (2) Experimental results from multiple cycles of salt stress. In this study, LT-stressed cells were shocked with 50 mM NaCl to resalinize these cells to confirm that the enhanced H^+ influx was the result of NaCl stress. Protoplast isolation and purification were carried out in enzymatic solutions without NaCl addition and thus, the salt stress was alleviated during the period of protoplast preparation (approximately 7 h). Noteworthy is that the enhanced H^+ influx was refound when these cells were resalinized with 50 mM NaCl (Fig. 8). Moreover, the NaCl-induced H^+ influx in these restressed cells was inhibited by

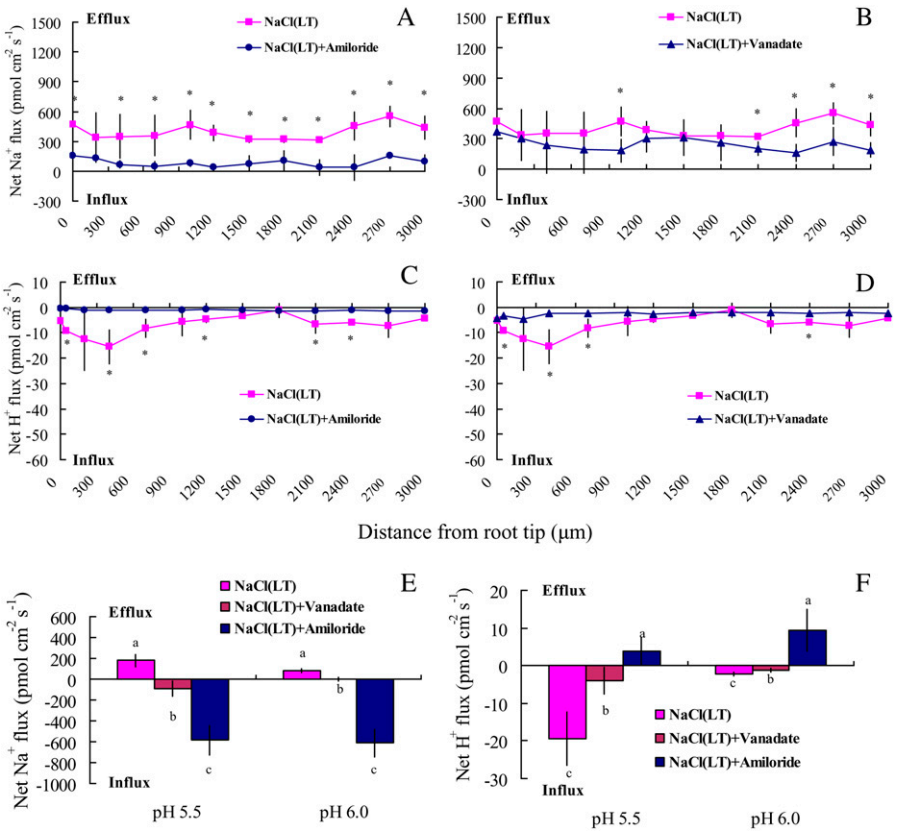

Figure 7. Effects of sodium orthovanadate (500 μM) and amiloride (50 μM) on net Na^+ and H^+ fluxes in LT-stressed roots (A–D) and derived protoplasts (E and F) of *P. euphratica*. Roots and protoplasts were pretreated with 500 μM sodium orthovanadate or 50 μM amiloride for 30 min prior to flux measurements. A to D, Na^+ and H^+ fluxes were measured along root axes (0–3,000 μm from the apex) at intervals of 50 to 300 μm (pH value in the measuring solutions was 6.0). Each point is the mean of five to six individual roots and bars represent the standard error of the mean. An asterisk denotes a significant difference at $P < 0.05$ between treatments. E and F, Cellular fluxes of Na^+ and H^+ were recorded in the measuring solutions with pH values at 5.5 and 6.0 (the continuous recording period was 10 min). Each column is the mean of five to six individual protoplasts and bars represent the standard error of the mean. Columns labeled with different letters (a, b, and c) are significantly different at $P < 0.05$. [See online article for color version of this figure.]

inhibitors of the Na^+/H^+ antiporter and PM H^+-ATPase, suggesting a contribution of H^+ influx to Na^+ extrusion in *P. euphratica* cells (Fig. 8).

PeNhaD1 and PeSOS1 likely function as Na^+/H^+ antiporters in the PM of *P. euphratica*. The transcriptional level of *PeNhaD1* was maintained at the control levels in *P. euphratica* but declined in the salt-sensitive poplar after 12-h salt stress (Ottow et al., 2005a). Yeast complementation experiments have shown that the mutant strain ANT3 (*Saccharomyces cerevisiae*, Δena1-4::HIS3 Δnha1::LEU2), which lacks the PM Na^+/H^+ antiporter gene *ScNHA1*, resumes the capacity for salt exclusion after *PeNhaD1* transformation (Lü et al., 2007). Furthermore, *PeNhaD1* overexpression in salt-sensitive poplar species enhanced salt exclusion in transgenic plants (Chen, 2007). An acidic environment, e.g. pH 5.5 or 6.0, promoted Na^+/H^+ exchange in *P. euphratica* protoplasts, compared to a neutral pH (Fig. 6). This agrees with Ottow et al. (2005a), who have confirmed that PeNhaD1 is strictly pH dependent and only functions under acidic conditions of a pH 5.5 (the tested pH values were 5.5, 7.0, and 8.0). The gene *PeSOS1*, encoding the putative PM Na^+/H^+ antiporter of *P. euphratica*, has been recently characterized by Wu et al. (2007). PeSOS1 levels in *P. euphratica* leaves were found to increase after NaCl treatment (Wu et al., 2007). Given these results, we conclude that Na^+ extrusion in salt-stressed *P. euphratica* cells is correlated with increasing activity of PM Na^+/H^+ antiporters.

PM H^+-ATPase pumps protons and maintains electrochemical H^+ gradients, thus promoting the secondary active Na^+/H^+ antiport at the PM (Blumwald et al., 2000; Zhu, 2003). Shabala and a colleague suggested the involvement of PM H^+-ATPase in Na^+/H^+ antiport (Shabala, 2000; Shabala and Newman, 2000). In this study, pharmacological experiments at tissue and cellular levels showed that the Na^+/H^+ exchange in LT-stressed *P. euphratica* was associated with the PM H^+-ATPase (Fig. 7). Evidence by western blotting also proved that the expression of PM H^+-ATPase was up-regulated by NaCl stress in calluses and leaves of *P. euphratica* (Yang et al., 2007; Zhang et al., 2007).

In contrast to *P. euphratica*, *P. popularis* roots did not exhibit a marked Na^+ efflux along the root axis in LT-stressed plants, although a net Na^+ efflux was observed in the elongation region of ST-stressed roots (Fig. 1, B and D). These results are consistent with our previous findings that *P. popularis* plants have a lower capacity to exclude Na^+ under LT saline conditions (Chen et al., 2002). Compared with controls, NaCl did not induce a marked H^+ influx in either ST- or LT-stressed roots (Fig. 3, B and D). The same trends of Na^+ and H^+ fluxes were seen in the protoplasts isolated from LT-stressed *P. popularis* roots (Fig. 2, B, C, E, and F). These results suggest that the PM Na^+/H^+ antiport system in this species is insufficient to exclude the entry of Na^+, which presumably occurs through voltage-independent nonselective cation channels

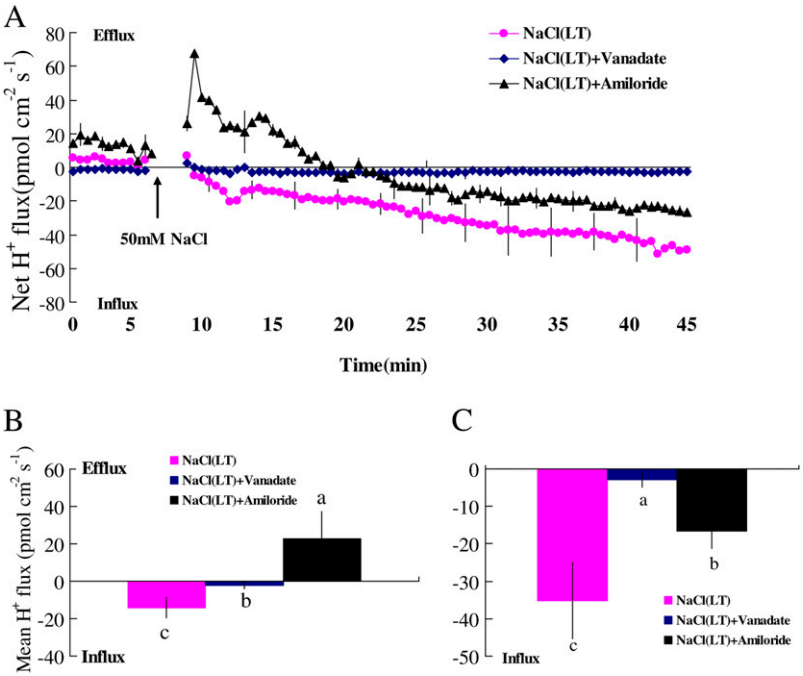

Figure 8. Effects of sodium orthovanadate (500 μM) and amiloride (50 μM) on salt shock-induced transient H^+ kinetics in protoplasts isolated from LT-stressed roots of *P. euphratica*. Protoplasts were pretreated with 500 μM sodium orthovanadate or 50 μM amiloride for 30 min prior to fluxes measurements. A, H^+ kinetics recorded after the required amount of 1.0 M NaCl stock solution was added to the chamber. Prior to the salt shock, steady H^+ fluxes were measured for approximately 5 min in protoplasts pretreated with or without inhibitors. Each point represents the mean of five to six protoplasts and bars represent the standard error of the mean. B and C show the mean flux rate of H^+ at 0 to 10 min and 10 to 35 min after the onset of salt shock. Columns labeled with different letters (a, b, and c) denote significant difference at $P < 0.05$. [See online article for color version of this figure.]

(Maathuis and Sanders, 2001; Demidchik and Tester, 2002; Demidchik et al., 2002; White and Davenport, 2002; Tester and Davenport, 2003; Maathuis, 2006; Shabala et al., 2006). When provided with a salt shock, LT-stressed *P. popularis* cells exhibited a significant lower H^+ influx, compared with *P. euphratica* (Fig. 4). This suggests that the inability to extrude Na^+ from root tissues and cells likely results from its lower H^+ pumping activity of H^+-ATPase. An electron microscopic-cytochemical study showed that PM H^+-ATPase was down-regulated in *P. popularis* after exposure to NaCl stress (L. Deng, X. Ma, J. Li, and S. Chen, unpublished data).

Cl^- Fluxes under NaCl Stress

Excessive accumulations of Cl^- contribute to salt damage in woody species (Chen et al., 2001, 2002, 2003; Munns and Tester, 2008). *P. euphratica* exhibits a net Cl^- efflux in LT-stressed roots (Fig. 5C). Similarly, NaCl-induced Cl^- efflux was observed in Arabidopsis (Lorenzen et al., 2004). However, protoplasts isolated from LT-stressed *P. euphratica* roots did not show a corresponding Cl^- efflux; instead, an influx of Cl^- was usually registered (Fig. 2, G and I). Therefore, Cl^- efflux in stressed roots is presumably, at least in part, the result of ion release from Cl^- sources. We found a marked Cl^- compartmentation in cell walls and vacuoles of salt-stressed *P. euphratica* roots and Cl^- concentrations were much higher than those of Na^+ (Chen et al., 2002, 2003). The flux rates of Cl^- in roots may reveal the concentration of Cl^- sources. Salt-induced Cl^- efflux in *P. popularis* was only seen in ST-stressed plants, but not observed in LT-stressed ones (Fig. 5, B and D), implying that Cl^- was less retained in roots after a longer duration of stress. The Cl^- had presumably been transported to the leaves via the transpiration stream, since *P. popularis* roots are unable to block efficiently the salt radical translocation through both the symplastic and apoplastic pathways (Chen et al., 2002).

Osmotic and Ionic Effects on Ion Fluxes

Fluxes of H^+ and Na^+ upon salt stress (50 mM NaCl, 24 h) differ from the response to hyperosmotic stress (85 mM mannitol, 24 h). In the two tested species, NaCl-induced Na^+ fluxes were not seen in the isotonic mannitol treatment (Fig. 1, A, B, E, and F). Hyperosmotic stress accelerated the H^+ efflux along the root axes in the two poplar species (Fig. 3, E and F), but the iso-NaCl induced an H^+ influx at the root apex of *P. euphratica* or had no effects on *P. popularis* (Fig. 3, A and B). Our results indicate that ionic responses of roots to a hyperosmotic treatment were highly stress specific (Shabala, 2000). However, there were marked differences between the two species in Cl^- flux given the mannitol treatment. Hyperosmotic stress caused a drastic Cl^- efflux in roots of *P. popularis* but not in *P. euphratica* (Fig. 5, E and F). The Cl^- efflux in osmotically stressed *P. popularis* was absent when the isotonic mannitol was introduced to the measuring solution (Supplemental Fig. S1), suggesting that the Cl^- efflux enhanced by hyperosmotic stress is likely mediated by stretch-activated anion channels. ST-stressed *P. popularis* roots showed a trend similar to mannitol treatment (Fig. 5, B and F), indicating that stretch-activated anion channels contributed to salt-induced Cl^- efflux

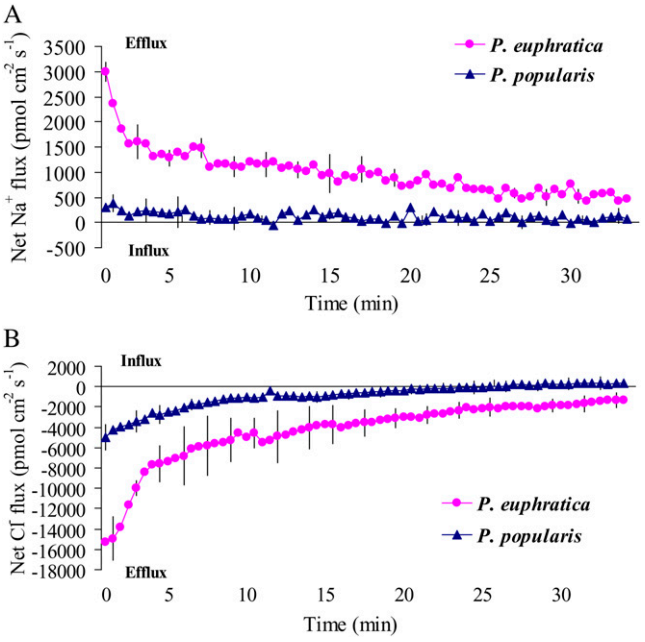

Figure 9. Fluxes of Na^+ (A) and Cl^- (B) in excised roots of LT-stressed *P. euphratica* and *P. popularis*. Na^+ and Cl^- fluxes were measured immediately after roots were sampled and the measuring site was 500 μm from the root apex, in which a vigorous flux of Na^+ or Cl^- was usually observed. Each point is the mean of five to six individual plants and bars represent the standard error of the mean. [See online article for color version of this figure.]

(Falke et al., 1988; Cosgrove and Hedrich, 1991; Teodoro et al., 1998; Qi et al., 2004). Cl^- efflux in *P. euphratica* roots was not significantly enhanced at a hyperosmotic stress, irrespective of measuring solutions supplemented with or without mannitol (Fig. 5E; Supplemental Fig. S1), suggesting that NaCl-induced Cl^- efflux in *P. euphratica* roots accounted for the specific salt effects.

CONCLUSION

In conclusion, SIET data show that *P. euphratica* roots have a higher capacity to exclude Na^+ in ST and LT treatments. Steady fluxes of H^+ and Na^+ in protoplasts of LT-stressed *P. euphratica* roots indicate that the active Na^+/H^+ antiport across a PM contributes to Na^+ extrusion, especially under acidic conditions. Inhibition of Na^+/H^+ antiporter by amiloride (a Na^+/H^+ antiporter inhibitor) or sodium orthovanadate (a PM H^+-ATPase inhibitor) at tissue and cellular levels verified the involvement of Na^+/H^+ antiporters and a H^+ pump (PM H^+-ATPase) in Na^+ extrusion. In comparison, the Na^+/H^+ antiport system in *P. popularis* roots is insufficient to exclude Na^+ at tissue and cellular levels, leading to perturbations of ion homeostasis over long periods of salinity. Moreover, salt-treated *P. euphratica* roots retain a higher capacity for Cl^- exclusion than *P. popularis*, especially under LT salinity. The greater capacity to exclude NaCl in *P. euphratica* roots may restrict the root-to-shoot salt transport and the subsequent accumulation in aerial tissues. Noteworthy, the pattern of root H^+, Na^+, and Cl^- flux in NaCl-stressed *P. euphratica* differs from the response to hyperosmotic stress, suggesting that NaCl-induced alternations of root ion fluxes in this species are mainly the result of ion-specific effects.

MATERIALS AND METHODS

Plant Materials

In April (2007, 2008), 1-year-old seedlings of *Populus euphratica*, obtained from the Xinjiang Uygur Autonomous Region of China, and hardwood cuttings of *Populus popularis* 35-44 (*P. popularis*), from the nursery of Beijing Forestry University, were planted in individual pots (10 L) containing loam soil and placed in a greenhouse at Beijing Forestry University. Potted plants were well irrigated according to evaporation demand and watered with 1 L full-strength Hoagland nutrient solution every 2 weeks. Plants were raised 3 months prior to the beginning of hydroponic culture. In mid-July, uniform plants were washed free of soil and transferred to individual porcelain pots containing 2 L one-quarter strength Hoagland nutrient solution. Plants were continuously aerated by passing air through the solution. The temperature in the greenhouse was 20°C to 25°C with a 16-h photoperiod (7 AM–11 PM), 150 μmol m^{-2} s^{-1} of photosynthetically active radiation. Nutrient solutions were renewed every 48 h and plants were raised for 15 to 20 d under hydroponic conditions prior to salt and hyperosmotic treatments.

Treatments

Plants of the two species were subjected to a ST 50 mM NaCl solution for 24 h and a LT salinity of 100 mM NaCl, for 15 d. The required amounts of NaCl were added to the Hoagland nutrient solution. After 15 d of salt treatment (100 mM NaCl), *P. popularis* exhibited visible necrosis in old leaves and chlorosis generally occurred at the margin of upper leaves. However, no salt injury was seen in *P. euphratica* during the period of salt stress. The number of newly built roots in *P. popularis* was decreased by salinity but NaCl did not reduce root growth in *P. euphratica*. In a 24-h hyperosmotic treatment, plants of the two species were exposed to 85 mM mannitol, instead of iso-NaCl (50 mM). Control plants were not treated with NaCl or mannitol. Young roots with apices of 2 to 3 cm were sampled from the control and from stressed plants of the two species and used for H^+, Na^+, and Cl^- flux measurements.

Isolation of Root Protoplasts

Protoplasts were isolated from control and LT-stressed plants of *P. euphratica* and *P. popularis*. The procedures for protoplasts isolation were followed, as described in Shabala et al. (1998) and Demidchik and Tester (2002). A brief description is given below. Root tips (2–3 cm) were chopped into 0.5 mm in length and incubated in 4 mL of a basic solution (10 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM MES, 0.1% bovine serum albumin [w/v], and 330 mM mannitol, pH 5.7 was adjusted with Tris) supplemented with the following enzymes (in w/v): 1.5% cellulase Onozuka R-10 (Yakult Honsha, lot no. 216013), 1% Cellylysin (CalBiochem, lot no. B70487), and 0.1% pectolyase Y-23 (Yakult Honsha, lot no. Y-007). Segments of roots were gently shaken at 60 rpm in the enzyme solution at 28°C for 6 h. Then protoplasts were filtered through a nylon mesh with 50-μm-diameter pores. Undigested tissues were placed in a small beaker containing 3 mL of a holding solution (5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Suc, 10 mM Glc, 2 mM MES, pH 5.7 was adjusted with Tris). Osmotic potentials of basic and holding solutions, 290 to 300 mOsM kg^{-1}, were adjusted by 330 mM mannitol with a vapor pressure osmometer (Wescor 5520). By gentle agitation with a glass rod, additional protoplasts were released from the undigested tissues and collected by filtering through the nylon mesh. Thereafter, a duplicate dilution method was adopted to minimize mechanical damage caused by the enzyme solution. The collected filtrate was centrifuged at 300*g* for 5 min to remove the enzyme solution. Then protoplasts were resuspended and washed with 2 to 3 mL of a holding solution (×2).

Finally, protoplast suspensions (1 mL) were diluted with a 2-mL holding solution and used for ion flux measurements, including the steady-state measurements (Chen et al., 2005) and transient kinetics (Shabala, 2000).

Measurements of Net Na^+, H^+, and Cl^- Fluxes with SIET

Net fluxes of Na^+, H^+, and Cl^- were measured noninvasively using SIET (the SIET system BIO-001A; Younger USA Sci. & Tech. Corp.; Applicable Electronics Inc.; and ScienceWares Inc.; Kühtreiber and Jaffe, 1990; Kochian et al., 1992; Zonia et al., 2002; Vincent et al., 2005; Xu et al., 2006). The concentration gradients of the target ions were measured by moving the ion-selective microelectrode between two positions close to the plant material in a preset excursion (30 μm for excised roots and 10 μm for protoplasts in our experiment) at a programmable frequency in the range of 0.3 to 0.5 Hz. The SIET can measure ionic fluxes down to picomolar levels but must be measured slowly at approximately 1 to 2 s per point. This is mainly due to the mechanical disturbance of the gradient by the electrode movement, although the time constant of the liquid ion exchanger (LIX) electrodes is also a factor. The ionic gradient is accurately measured due to the following reasons: (1) while conducting the experiment, the electrode stays in one position for a certain time (from 0.8 to a few seconds) to let the gradient recover before it takes a reading of that position; (2) the time the electrode spends in one position is also needed for the electrode to get stabilized to give a reliable measurement. In fact, the time spent on electrode stabilization is longer than the time for gradient recovery, which is one of the key factors determining the efficiency of the electrode (Kunkel et al., 2006). The electrode is stepped from one position to another in a predefined sampling routine while also being scanned with the three-dimensional microstepper motor manipulator (CMC-4).

Prepulled and silanized glass micropipettes (2–4 μm aperture, XYPG120-2; Xuyue Sci. and Tech. Co., Ltd.) were first filled with a backfilling solution (Na: 100 mM NaCl; Cl: 100 mM KCl; H: 40 mM KH_2PO_4 and 15 mM NaCl, pH 7.0) to a length of approximately 1 cm from the tip. Then the micropipettes were front filled with approximately 15-μm columns of selective liquid ion-exchange cocktails (LIXs; Na: Fluka 71178; H: Fluka 95293; Cl: Fluka 24902). An Ag/AgCl wire electrode holder (XYEH01-1; Xuyue Sci. and Tech. Co., Ltd.) was inserted in the back of the electrode to make electrical contact with the electrolyte solution. DRIREF-2 (World Precision Instruments) was used as the reference electrode. Ion-selective electrodes of the following target ions were calibrated prior to flux measurements: (1) Na^+: 0.9 mM, 2.0 mM, 5.0 mM (Na^+ concentration was usually 0.9 mM in the measuring buffer for root and cell samples); (2) H^+: pH 5.5, 6.0, 6.5 (pH of the measuring buffer was usually adjusted to 6.0 for root and cell samples; pH tests of protoplasts were carried out at pH 5.5, 6.0, and 7.0); (3) Cl^-: 0.25 mM, 0.5 mM, 2.0 mM (Cl^- concentration was usually 0.5 mM in the measuring buffer for root and cell samples).

Only electrodes with Nernstian slopes >50 mV/decade (−50 mV/decade for Cl^- electrodes) were used in our study. Ion flux was calculated by Fick's law of diffusion:

$$J = -D(dc/dx)$$

where J represents the ion flux in the x direction, dc/dx is the ion concentration gradient, and D is the ion diffusion constant in a particular medium. Data and image acquisition, preliminary processing, control of the three-dimensional electrode positioner, and stepper-motor-controlled fine focus of the microscope stage were performed with ASET software, part of the SIET system.

Experimental Protocols

Root Tissues

After exposure to the saline (ST and LT) and hyperosmotic treatments, root segments with 2 to 3 cm apices were sampled for ion flux measurements. To decrease the effect of salt release on flux recording (preloaded Na^+ and Cl^- would diffuse from the surface of NaCl-stressed roots in a buffer with lower Na^+ and Cl^- concentrations), roots were rinsed with redistilled water and immediately incubated in the measuring solution to equilibrate for 30 min (as shown in Fig. 9, a rapid efflux of Na^+ and Cl^- took place after the roots were immediately sampled); then the flux rate decreased gradually and reached a steady level within 10 min, although *P. euphratica* exhibited a typical higher efflux rate than *P. popularis*. The measuring site was 500 μm from the root apex, in which a vigorous flux of Na^+ or Cl^- was usually observed. Afterward, roots were transferred to the measuring chamber containing 10 to 15 mL of a fresh measuring solution. After the roots were immobilized on the bottom, ion flux measurements were started from the apex and went along the root axis until 3,000 μm (ion flux rates in the region of 0–3,000 μm are significantly larger than mature zones; data not shown). The measured positions of roots could be visualized and defined under the SIET microscope because young roots were semitransparent under light. Generally, control roots have an approximately 0.5- to 1.0-mm apex length and an 8- to 10-mm-long elongation zone. For LT-stressed *P. popularis* roots, the position and length of the root apex were similar to those of the controls but the full length of the elongation zone was reduced to 4–7 mm. Nevertheless, this did not affect the flux measurement in the elongation region since the SIET recordings were performed at a root length of 3 mm from the apex. For *P. euphratica*, there was no morphological difference between control and stressed roots. Na^+, H^+, and Cl^- were monitored in the following solutions, respectively: (1) Na^+: 0.1 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5 mM NaCl, 0.3 mM MES, 0.2 mM Na_2SO_4, pH 6.0, adjusted with choline and HCl; (2) H^+: 0.1 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5 mM NaCl, 0.3 mM MES, 0.2 mM Na_2SO_4, pH 6.0 adjusted with NaOH and HCl; (3) Cl^-: 0.05 mM KCl, 0.05 mM $CaCl_2$, 0.05 mM $MgCl_2$, 0.25 mM NaCl, 0.2 mM Na_2SO_4, pH 6.0, adjusted with choline and H_3PO_3 (MES was absent in the Cl^- measuring solution because it had negative effects on the selectivity of Cl^- LIX; Messerli et al., 2004).

Although buffered solutions may affect the actual magnitude of the H^+ flux (Arif et al., 1995), the qualitative tendency of NaCl- or mannitol-induced H^+ flux is not altered.

The inhibitory effects of PM transport inhibitors on ion fluxes were examined in *P. euphratica*. LT-stressed *P. euphratica* roots were subjected to 500 μM sodium orthovanadate (a PM H^+-ATPase inhibitor) or 50 μM amiloride (a Na^+/H^+ antiporter inhibitor) for 30 min. Then measuring solutions containing sodium orthovanadate were removed slowly with a pipette and a 10-mL fresh solution was slowly added to the measuring chamber. Measuring solutions containing amiloride were not replaced because amiloride had no obvious effect on the Nernstian slopes of Na^+ and H^+ electrodes. Fluxes of Na^+ and H^+ were scanned along the root axes (0–3,000 μm) at intervals of 50 to 300 μm.

Protoplasts

Prior to cellular ion flux recording, protoplasts were first fixed on the bottom of the measuring chamber to reduce the mobility caused by moving the electrode during measurements. Glass slips treated with a poly-L-Lys (Sigma) solution (Mazea et al., 1975) were used to affix individual protoplasts. Briefly, 20- × 20-mm coverslips were placed in a potassium bichromate washer for several hours and rinsed with redistilled water, then dried at 160°C for 2 to 3 h in an oven. After that, the coverslips were treated with poly-L-Lys solutions at concentrations of 0.002% (w/v). Slips were finally stored in a sealed container for air drying at room temperature. A 100 to 200 μL of protoplast suspensions was put in the middle of the poly-L-Lys-pretreated coverslips in the measuring chamber. When protoplasts had been settled on the surface (requiring several minutes), a 10-mL measuring solution of Na^+, H^+, or Cl^- was slowly added to the measuring chamber. The ionic compositions and pH of the measuring solutions were the same as those used for tissue flux measurements but for two changes: (1) The concentration of MES was decreased from 0.3 to 0.05 mM (Na^+, H^+), and (2) 330 mM mannitol was introduced to the measuring solutions (Na^+, H^+, and Cl^-). The steady-state flux measurements were started and continued for 10 to 12 min. The effects of salt shock, pH, and PM transporter inhibitors on ion fluxes were examined, which are briefly described as follows. (1) Salt shock: Transient H^+ flux kinetics in response to NaCl (50 mM) were measured in protoplasts isolated from LT-stressed root tips of the two species. A steady-state H^+ flux was recorded (5–6 min) prior to the salt shock. Then NaCl stock (1 M) was slowly added to the measuring solution until the final NaCl concentration in the buffer reached 50 mM. After that, the H^+ flux recording was restarted and continued for a further 40 to 50 min. The data measured during the first 2 to 3 min was discarded due to the diffusion effects of stock addition (Shabala, 2000; in our study, blank measurements, i.e. without protoplast addition, were carried out to exclude the disturbance of NaCl addition on H^+ flux measurements). (2) pH: Protoplasts of LT-stressed *P. euphratica* roots were used in our experiment and pH values of measuring solutions were adjusted to 5.5, 6.0, and 7.0, respectively. Steady-state Na^+ and H^+ fluxes were recorded and the continuous recording period was 8 to 10 min. (3) Inhibitor treatments: LT-stressed *P. euphratica* protoplasts were subjected to sodium orthovanadate (500 μM) or amiloride (50

μM) for 30 min in the measuring solutions. Prior to the flux recording, the measuring solutions containing sodium orthovanadate were replaced with a 10-mL fresh solution, but measuring solutions with amiloride were not renewed (amiloride had no clear effect on the Nernstian slopes of the Na^+ and H^+ electrodes). Then the steady-state Na^+ and H^+ fluxes were measured in protoplasts pretreated with or without inhibitors at pH 5.5 and 6.0. Moreover, the H^+ kinetics of the salt shock (50 mM NaCl) were recorded in these cells as described above.

Data Analysis

Three-dimensional ionic fluxes were calculated using MageFlux developed by Yue Xu (http://www.youngerusa.com/mageflux or http://xuyue.net/mageflux). Positive values in figures represent cation efflux or anion influx and vice versa.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Effects of hyperosmotic stress (85 mM mannitol, 24 h) on net Cl^- fluxes in roots of *P. euphratica* and *P. popularis*.

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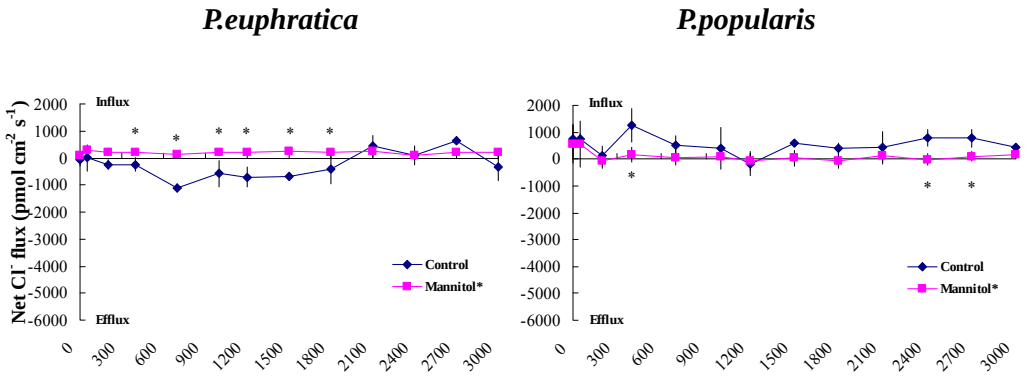

Supplemental Figure 1. Effects of hyperosmotic stress (85 mM mannitol, 24 h) on net Cl^- fluxes in roots of *P. euphratica* and *P. popularis*. Mannitol*, roots were pretreated with mannitol for 24 h and ion fluxes were recorded in the measuring buffer supplemented with 85 mM mannitol. Control roots were pretreated without mannitol. Cl^- fluxes were measured along root axis (0-3000 µm from the apex) at intervals of 50 to 300 µm. Each point is the mean of 5 to 6 individual plants and bars represent the standard error of the mean. An asterisk denotes a significant difference at $p < 0.05$ between treatments.