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# *Paxillus involutus* Strains MAJ and NAU Mediate $K^+/Na^+$ Homeostasis in Ectomycorrhizal *Populus* × *canescens* under Sodium Chloride Stress[1][C][W][OA]

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Salt-induced fluxes of $H^+$, $Na^+$, $K^+$, and $Ca^{2+}$ were investigated in ectomycorrhizal (EM) associations formed by *Paxillus involutus* (strains MAJ and NAU) with the salt-sensitive poplar hybrid *Populus* × *canescens*. A scanning ion-selective electrode technique was used to measure flux profiles in non-EM roots and axenically grown EM cultures of the two *P. involutus* isolates to identify whether the major alterations detected in EM roots were promoted by the fungal partner. EM plants exhibited a more pronounced ability to maintain $K^+/Na^+$ homeostasis under salt stress. The influx of $Na^+$ was reduced after short-term (50 mM NaCl, 24 h) and long-term (50 mM NaCl, 7 d) exposure to salt stress in mycorrhizal roots, especially in NAU associations. Flux data for *P. involutus* and susceptibility to $Na^+$-transport inhibitors indicated that fungal colonization contributed to active $Na^+$ extrusion and $H^+$ uptake in the salinized roots of *P.* × *canescens*. Moreover, EM plants retained the ability to reduce the salt-induced $K^+$ efflux, especially under long-term salinity. Our study suggests that *P. involutus* assists in maintaining $K^+$ homeostasis by delivering this nutrient to host plants and slowing the loss of $K^+$ under salt stress. EM *P.* × *canescens* plants exhibited an enhanced $Ca^{2+}$ uptake ability, whereas short-term and long-term treatments caused a marked $Ca^{2+}$ efflux from mycorrhizal roots, especially from NAU-colonized roots. We suggest that the release of additional $Ca^{2+}$ mediated $K^+/Na^+$ homeostasis in EM plants under salt stress.

Soil salinization is a serious factor restricting the expansion of agriculture and forestry around the world. Among the novel biotechnological tools that can enhance salt resistance, inoculation with ectomycorrhizal (EM) fungi has been suggested to be an important measure for enhancing the performance of and ensuring biomass production by woody species in saline environments (Luo et al., 2009). In general, EM fungi enhance the growth of host plants by increasing mineral nutrition and reducing the uptake of sodium ions ($Na^+$) under salt stress (Hall, 2002; Polle and Schützendübel, 2003; Smith and Read, 2008). For example, *Scleroderma bermudense* significantly increases phosphorus and $K^+$ levels but decreases $Na^+$ and $Cl^-$ concentrations in *Coccoloba uvifera* plants (Bandou et al., 2006). Similarly, *Hebeloma crustuliniforme* and *Laccaria bicolor* reduce tissue $Na^+$ and $Cl^-$ concentrations and alleviate salt injury in white spruce (*Picea glauca*), black spruce (*Picea mariana*), and jack pine (*Pinus banksiana*) seedlings (Muhsin and Zwiazek, 2002; Nguyen et al., 2006). In contrast to conifers, EM associations did not decrease tissue concentrations of $Na^+$ and $Cl^-$ in NaCl-treated trembling aspen (*Populus tremuloides*) and paper birch (*Betula papyrifera*; Yi et al., 2008), showing that the fungal effect on salt accumulation varies with EM fungus and host plant species.

*Paxillus involutus* strains MAJ and NAU have been identified as highly salt-tolerant fungi (Gafur et al., 2004; Langenfeld-Heyser et al., 2007; Zhang et al., 2008). Colonization with *P. involutus* strain MAJ reduces the buildup of $Na^+$ but enhances $K^+$

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accumulation in the leaves of a salt-sensitive hybrid poplar, *Populus* × *canescens* (Langenfeld-Heyser et al., 2007, Luo et al., 2011). The maintenance of a high $K^+/Na^+$ ratio is critical for salt tolerance in herbaceous plants (Shabala, 2000; Tester and Davenport, 2003; Chen et al., 2007; Shabala and Cuin, 2008) and woody species, including *Populus* species (Chen et al., 2001, 2002a, 2002b, 2003; Sun et al., 2009a, 2009b, 2010, 2012). At the cellular level, salinized plants avoid $Na^+$ toxicity in the cytosol by compartmentalizing $Na^+$ into the vacuole and excreting $Na^+$ into the external environment or the apoplast (Blumwald et al., 2000; Hasegawa et al., 2000; Zhu, 2001, 2003; Ottow et al., 2005; Apse and Blumwald, 2007; Munns and Tester, 2008; Chen and Polle, 2010). The driving force for $Na^+/H^+$ antiporters is provided by $H^+$-ATPases, which make an important contribution to the maintenance of low $Na^+$ levels in the cytosol (Chen and Polle, 2010). In addition, NaCl-induced $K^+$ deficiency in plants is regulated by depolarization-activated (DA) outward-rectifying $K^+$ channels (KORCs) and DA nonselective cation channels (NSCCs; Shabala et al., 2005, 2006a; Demidchik and Maathuis, 2007; Shabala and Cuin, 2008). Moreover, salinity is known to cause oxidative stress (Zhu, 2003; Demidchik et al., 2010), and a large proportion of NSCCs are reactive oxygen species activated (Demidchik et al., 2002, 2003). Roots of EM plants accumulate more, and leaves less, $Na^+$ than the respective tissues of non-EM plants, probably due to decreased xylem loading (Langenfeld-Heyser et al., 2007). EM roots increase the supply of $K^+$ under salt stress (Langenfeld-Heyser et al., 2007). However, how EM fungi assist plants by improving $Na^+$ and $K^+$ relationships after exposure to salinity is not yet clear.

It has been shown that different strains of *P. involutus* differ in their abilities to form typical mycorrhizal structures with *P.* × *canescens* roots (Gafur et al., 2004). While the fungal strain MAJ forms a typical hyphal mantle and Hartig net with roots of *P.* × *canescens*, NAU induces defense reactions, such as cell wall thickening, and is unable to intrude between the host cells (Gafur et al., 2004). Clarifying whether and how the incompatible fungal isolate affects the salt tolerance of host plants is necessary.

*P.* × *canescens* roots exhibit increased $Ca^{2+}$ enrichment during mycorrhizal symbiosis with *P. involutus* strain MAJ (Langenfeld-Heyser et al., 2007). Ramos et al. (2009) found that EM roots are more efficient than non-EM roots in taking up $Ca^{2+}$ from the external medium. Under NaCl stress, $Ca^{2+}$ regulates $K^+/Na^+$ homeostasis in a *salt-overly-sensitive3* mutant and wild-type Arabidopsis (*Arabidopsis thaliana*; Liu and Zhu, 1997). $Ca^{2+}$ has been suggested to restrict $Na^+$ uptake via voltage-independent-NSCCs (Demidchik and Tester, 2002; Tester and Davenport, 2003) and to restrain $K^+$ loss through DA-KORCs and DA-NSCCs (Shabala et al., 2006a; Sun et al., 2009b; Chen and Polle, 2010). Although $Ca^{2+}$ is well known to ameliorate salt stress, how $Ca^{2+}$ impacts $K^+/Na^+$ homeostasis via EM associations is unclear.

Using ion-selective vibrating microelectrodes, significant correlations between anion, $Ca^{2+}$, and $H^+$ fluxes were found on root surfaces, with increased fluxes after colonization of different hosts with either ectomycorrhizae or arbuscular mycorrhizae (Ramos et al., 2008, 2009). The goal of this study was to examine the role of ectomycorrhizae in ion homeostasis under salt stress. We used the ~~scanning ion-elective electrode technique (SIET)~~ to measure steady and transient profiles of ion fluxes ($Na^+$, $H^+$, $K^+$, and $Ca^{2+}$) in *P.* × *canescens-P. involutus* associations, non-EM roots, and fungal mycelia of the two *P. involutus* isolates MAJ and NAU. We also examined the effects of $Ca^{2+}$ on $K^+$ and $Na^+$ fluxes in *P.* × *canescens* roots; *P.* × *canescens* exhibited $Ca^{2+}$ enrichment upon colonization with the EM fungus *P. involutus*.

## RESULTS

### $Na^+$, $K^+$, and $Ca^{2+}$ Concentrations in Roots and Leaves

Scanning electron microscopy-energy dispersive x-ray spectrometry (SEM-EDX) was used to measure relative changes in the $Na^+$, $K^+$, and $Ca^{2+}$ concentrations in the cross-sections of roots and leaves. NaCl treatment (50 mM) for 1 week significantly increased $Na^+$ in the roots and leaves, with the exception of plants colonized with *P. involutus* strain NAU (Fig. 1). In contrast to $Na^+$, root and leaf $K^+$ concentrations decreased in salt-treated plants, especially nonmycorrhizal (NM) plants (Fig. 1). As a result, the $K^+/Na^+$ ratio in roots and leaves was markedly reduced by salt stress, with a more pronounced effect in NM plants compared with EM plants (Fig. 1). *P. involutus* mycorrhization increased $Ca^{2+}$ by 19% to 24% in the roots and by 53% to 72% in the leaves of nonstressed plants (Fig. 1). Salinized *P.* × *canescens* had a reduced $Ca^{2+}/Na^+$ ratio in the roots and leaves; however, the salt effect was less evident in NAU-mycorrhizal plants compared with NM and MAJ-mycorrhizal plants (Fig. 1).

### Steady and Transient Ion Fluxes in Roots and EM Fungus

#### *$Na^+$ Flux*

~~SIET~~ analyses showed that the pattern of $Na^+$ fluxes in EM roots differed from that of NM roots after short-term (ST; 24 h) and long-term (LT; 7 d) exposure to 50 mM NaCl (Fig. 2). In NM roots, ST stress caused a net $Na^+$ influx at the region 1,400 to 2,000 $\mu$m from the apex, whereas LT salinity resulted in a stable and constant influx along the whole measured distance from 50 to 2,000 $\mu$m (Fig. 2). Compared with NM roots, the salt-induced entry of $Na^+$ was less pronounced in MAJ- and NAU-mycorrhizal roots when considering both spatially resolved values along the scanned surface (Fig. 2) and mean values (Fig. 3A). A salt-induced efflux of $Na^+$ was detected in some regions along these mycorrhizal roots (Fig. 2).

The mycelia of the two *P. involutus* strains, MAJ and NAU, exhibited a marked $Na^+$ efflux under ST and LT

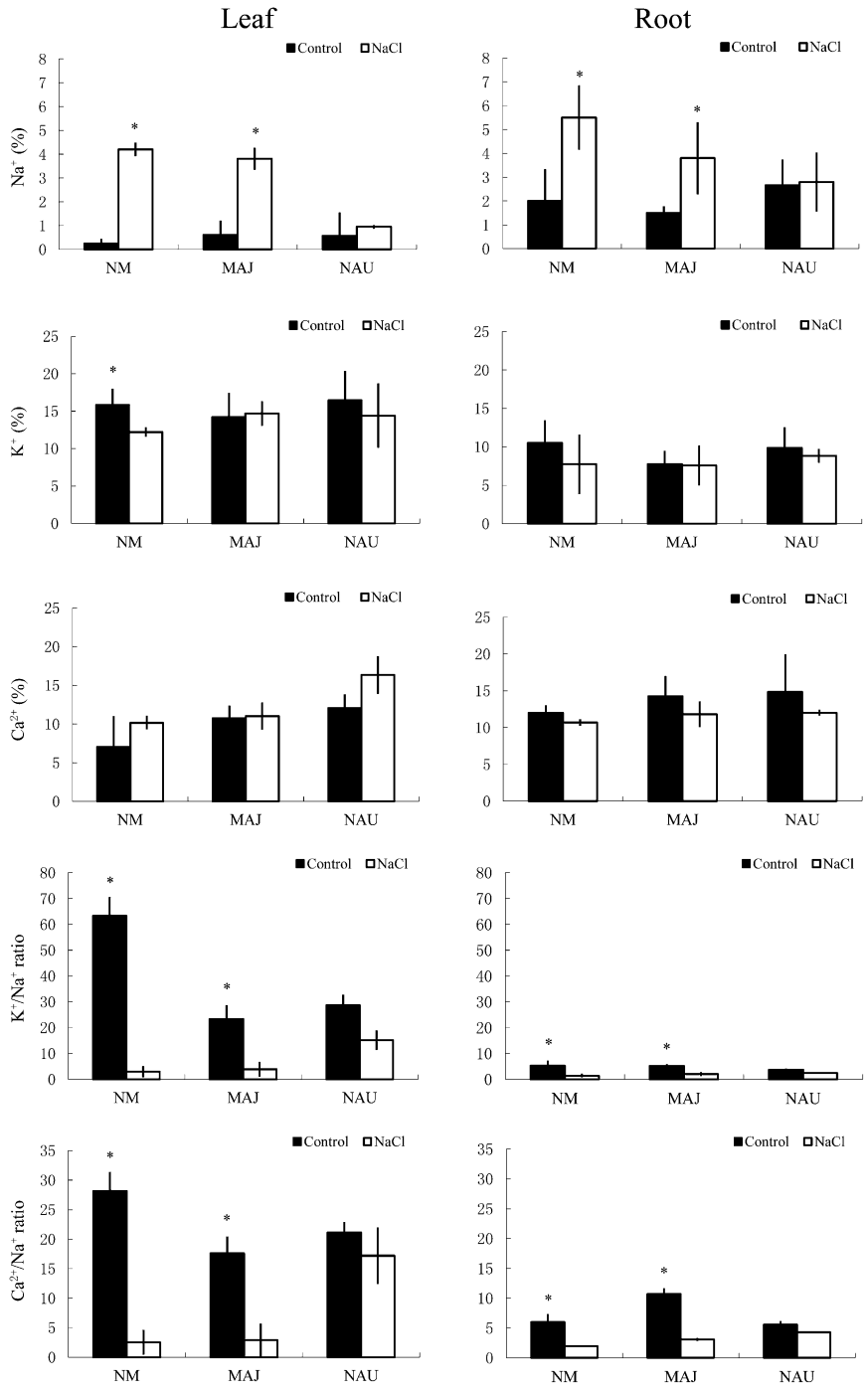


**Figure 1.** Effects of NaCl (50 mM, 1 week) on $Na^+$, $K^+$, $Ca^{2+}$, $K^+/Na^+$, and $Ca^{2+}/Na^+$ in the leaves and roots of mycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants. Amounts of $K^+$, $Na^+$, and $Ca^{2+}$ are expressed as atomic mass fraction (%). Each column is the mean of three to four individual plants, and error bars represent SE. Columns labeled with asterisks indicate significant differences at $P < 0.05$ between control and NaCl treatments.

stress (Fig. 3, B and C). The NaCl-induced $Na^+$ efflux was typically higher under conditions of high salinity (400 mM NaCl) compared with lower salinity (50, 100, and 200 mM NaCl; Fig. 3, B and C). However, the two *P. involutus* strains displayed different capacities to sustain the $Na^+$ efflux at high salt concentrations (400 mM NaCl). LT-treated NAU exhibited a flux rate similar to that of ST-treated hyphae, whereas a reduced $Na^+$ efflux was detected in MAJ under LT salinity (Fig. 3, B and C). Pharmacological experiments showed that the high-salt-induced $Na^+$ efflux in the two fungal strains was significantly reduced by the $Na^+/H^+$ antiporter inhibitor amiloride or the plasma membrane $H^+$-ATPase inhibitor sodium orthovanadate (Fig. 4A).

### *$H^+$ Flux*

SIET measurements of root apices revealed a net $H^+$ influx into NM roots in the absence of salt stress (Fig. 5; mean values are shown in Fig. 6A). In contrast, EM

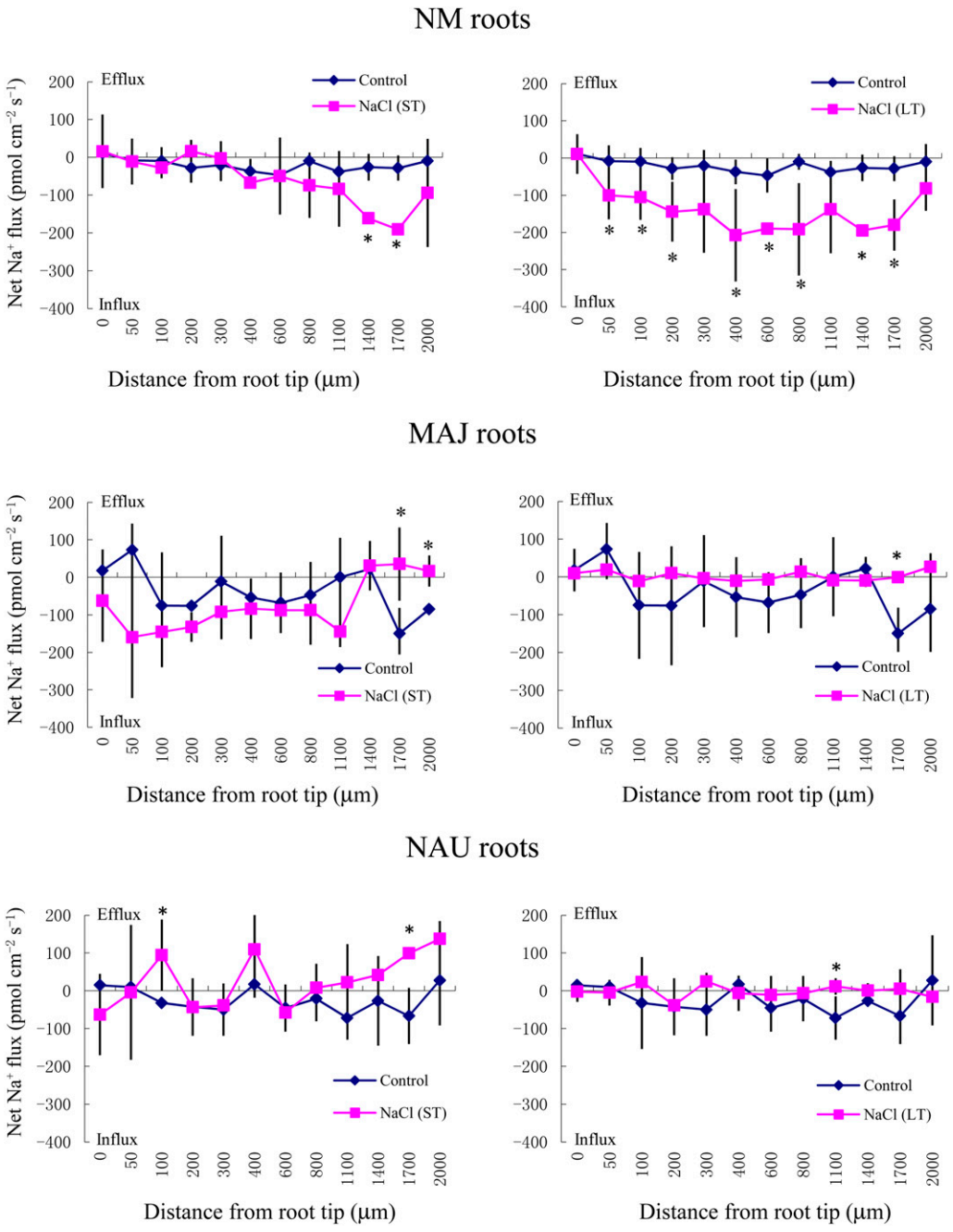


**Figure 2.** Effects of ST salinity (50 mM NaCl for 24 h) and LT salinity (50 mM NaCl for 7 d) on the net $Na^+$ flux in the mycorrhizal (MAJ and NAU) and NM roots of *P.* × *canescens* plants. Control roots were treated without NaCl. $Na^+$ fluxes were measured along root axes (0–2,000 $\mu$m from the apex) at intervals of 50 to 300 $\mu$m. Each point is the mean of five to six individual plants, and error bars represent SE. *$P < 0.05$ between treatments. [See online article for color version of this figure.]

roots were characterized by stable and constant $H^+$ effluxes along the measured regions (Figs. 5 and 6A). ST and LT salinity caused a typical shift of $H^+$ efflux toward an influx in EM roots (Figs. 5 and 6A). In the absence of fungal colonization, NaCl did not significantly change the $H^+$ flux profile along the root axis after ST or LT exposure, although the $H^+$ flux oscillated in the measured regions (Fig. 5).

MAJ and NAU mycelia exhibited a net $H^+$ efflux under control conditions similar to that of EM roots (Fig. 6, A–C). ST and LT salinity reduced the efflux of $H^+$ from strain MAJ (Fig. 6B). A similar trend was observed in the salinized hyphae of strain NAU, although $H^+$ fluxes in ST-treated NAU varied with salt concentration (Fig. 6C). The salinized fungus NAU exhibited a temporary influx (approximately 5–10 min)

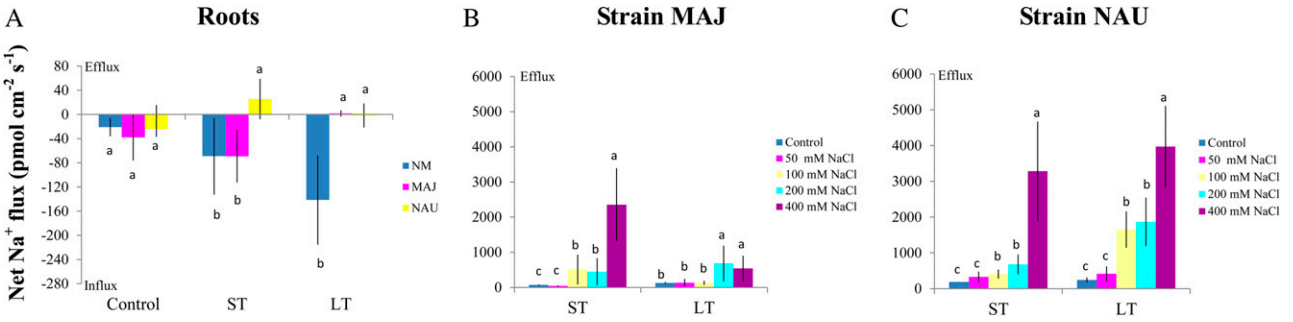


**Figure 3.** Effects of NaCl on steady $Na^+$ fluxes in *P.* × *canescens* roots (NM, MAJ, NAU) and *P. involutus* strains MAJ and NAU. A, Mycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants were subjected to ST salinity (50 mM NaCl for 24 h) and LT salinity (50 mM NaCl for 7 d), respectively. Control roots were treated without NaCl. For each plant, $Na^+$ fluxes were measured along root axes (0–2,000 $\mu$m from the apex at intervals of 50–300 $\mu$m), and mean values are given. Each column is the mean of five to six individual plants, and error bars represent SE. Columns labeled with different letters indicate significant differences at $P < 0.05$ between NM and EM roots. B and C, *P. involutus* isolates MAJ and NAU were subjected to ST salinity (50, 100, 200, or 400 mM NaCl for 24 h) and LT salinity (50, 100, 200, or 400 mM NaCl for 7 d), respectively. Control axenic mycelia were treated without NaCl. $Na^+$ fluxes of fungal hyphae were measured over a recording period of 30 to 40 min, and mean values are given. Each column is the mean of five to six axenic EM cultures (pelleted hyphae), and error bars represent SE. Columns labeled with different letters indicate significant differences at $P < 0.05$ between salinity levels. [See online article for color version of this figure.]

during the period of recording (30 min), especially under LT treatment, although the mean value indicated outward rectification (data not shown).

The salt-induced transient $H^+$ kinetics in roots and fungal mycelia were also examined. In the absence of salt stress, $H^+$ efflux was detected in EM roots instead of $H^+$ influx in NM roots (Fig. 6D). Salt shock (50 mM NaCl) caused a pronounced shift in the $H^+$ efflux toward an influx in EM roots (Fig. 6D), but no significant changes in the $H^+$ kinetics of NM roots were observed during the recording period (Fig. 6D). The responses of the transient $H^+$ kinetics to salt shock were compared between the two *P. involutus* strains. After exposure to NaCl (50 to 400 mM), hyphae exhibited an instantaneous decrease in the $H^+$ efflux, which then remained constant during the period of recording (40 min; Fig. 6, E and F). Compared with strain NAU, the shock-induced reduction in $H^+$ efflux was more pronounced in strain MAJ over the concentration range of 50 to 400 mM NaCl (Fig. 6, E and F).

### *$K^+$ Flux*

Although the $K^+$ flux varied along the root axis (Fig. 7), ST and LT salt treatments resulted in an overall net $K^+$ efflux from NM roots (Fig. 8A). Mycorrhization of *P.* × *canescens* roots with *P. involutus* reduced the $K^+$ efflux under ST and LT treatments, with the exception of ST-stressed MAJ-mycorrhizal roots (Figs. 7 and 8A). ST and LT treatment caused a net $K^+$ efflux from the mycelia of both NAU and MAJ, but the effect was more pronounced in MAJ, especially under ST salinity (Fig. 8, B and C). Tetraethylammonium chloride (TEA), a $K^+$ channel blocker, significantly decreased the salt-induced $K^+$ efflux from the hyphae of the two strains (Fig. 4B).

In salinized *P.* × *canescens* roots, the transient $K^+$ kinetics in response to salt shock followed a trend similar to the steady-state measurements (Fig. 8, A and D). Salt shock caused an evident $K^+$ efflux in NM roots, but the flux rate was lower in MAJ- and NAU-mycorrhizal roots (Fig. 8D). In the hyphae of the two strains, the rate of $K^+$ efflux was reduced after the addition of NaCl (50–400 mM; Fig. 8, E and F). An instantaneous increase in the $K^+$ influx was detected in MAJ and NAU after the onset of salt shock (Fig. 8, E and F).

### *$Ca^{2+}$ Flux*

Steady-state flux measurements showed that ST and LT treatment accelerated $Ca^{2+}$ efflux along mycorrhizal roots, especially in NAU-mycorrhizal plants (Fig. 9; mean values are shown in Fig. 10A). In the absence of salt stress, the hyphae of the two strains exhibited $Ca^{2+}$ influx, with a higher flux rate in MAJ than in NAU (Fig. 10, B and C). ST-treated MAJ maintained the $Ca^{2+}$ influx, but the flux rate decreased with increasing NaCl concentrations (Fig. 10B). In strain NAU, ST salinity reduced the influx under low-salt conditions (50 and 100 mM NaCl) and shifted toward an efflux at high salinity (200 and 400 mM NaCl; Fig. 10C). Under LT stress, the pattern of $Ca^{2+}$ flux was similar in the two strains. LT salinity reduced the influx or reversed the rectification toward an efflux (Fig. 10, B and C).

When subjected to salt shock, EM roots exhibited a transient increase in $Ca^{2+}$ efflux, but no corresponding changes were observed in NM roots (Fig. 10D). The shock-induced $Ca^{2+}$ efflux was more pronounced in MAJ-mycorrhizal roots than NAU-colonized roots (Fig. 10D). $LaCl_3$, an inhibitor of $Ca^{2+}$-permeable channels, did not markedly restrict the high rate of $Ca^{2+}$ efflux from salt-shocked MAJ and NAU roots

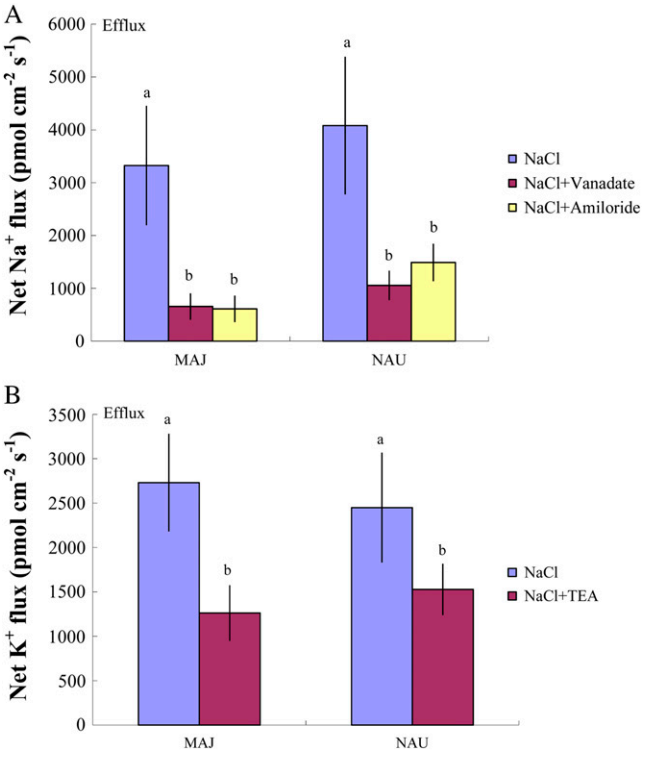


**Figure 4.** Effects of pharmacological agents on net $Na^+$ and $K^+$ fluxes in ST-treated *P. involutus* isolates MAJ and NAU. A, ST-treated (400 mM NaCl, 24 h) axenic mycelia were pretreated with 500 $\mu$M sodium orthovanadate or 50 $\mu$M amiloride for 30 min prior to measuring $Na^+$ flux. Measuring solutions containing sodium orthovanadate were removed slowly with a pipette, and 10 mL of fresh solution was then slowly added to the measuring chamber. B, ST-treated (400 mM NaCl, 24 h) axenic mycelia were pretreated with 50 $\mu$M TEA for 30 min prior to measuring $K^+$ flux. Each column is the mean of five to six axenic EM cultures (pelleted hyphae), and error bars represent SE. $Na^+$ and $K^+$ fluxes of fungal hyphae were measured over a recording period of 30 to 40 min, and mean values are given. Columns labeled with different letters indicate significant differences at $P < 0.05$ between treatments. [See online article for color version of this figure.]

(Supplemental Fig. S1). In the absence of salt stress, the mycelia of the two strains exhibited a stable and steady influx of $Ca^{2+}$, typically with higher flux rates in MAJ than in NAU (Fig. 10, E and F). NAU hyphae exhibited a $Ca^{2+}$ efflux immediately after the addition of NaCl (50–400 mM; Fig. 10F). Similarly, salt shock reduced the $Ca^{2+}$ influx in strain MAJ, although a transient increase in $Ca^{2+}$ influx was observed in the presence of 400 mM NaCl (Fig. 10E).

### Effects of $Ca^{2+}$ on Salt-Induced $K^+$ and $Na^+$ Fluxes in *P. × canescens* Roots

The effect of $Ca^{2+}$ on $K^+$ and $Na^+$ fluxes was examined in salinized roots. The aim was to investigate whether $Ca^{2+}$ released from salt-treated mycorrhizal associations benefits root cells in the control of $K^+/Na^+$ homeostasis. $Ca^{2+}$ application (10 mM) markedly limited the salt-induced $K^+$ efflux and enhanced the apparent $Na^+$ efflux in *P. × canescens* roots (Fig. 11). More profound effects were found in the meristematic zone ($K^+$ and $Na^+$) and elongation region ($Na^+$) than in other parts of the root.

## DISCUSSION

### EM Fungal Colonization Ameliorated $K^+/Na^+$ Homeostasis under Salt Stress

Colonization of the salt-sensitive *P. × canescens* with the EM fungus *P. involutus* (strain MAJ) was previously found to improve growth, prime for increased stress tolerance, and increase nutrition under salt stress (Langenfeld-Heyser et al., 2007; Luo et al., 2009, 2011). Similar findings were observed in this study. NM plants abscised old leaves from the lower shoots, and upper leaves displayed salt damage after 7 d of NaCl treatment (50 mM; Supplemental Fig. S2D). However, the salt injury was alleviated by EM colonization (Supplemental Fig. S2, D–F). Salinized *P. × canescens* exhibited an enhanced capacity to maintain $K^+/Na^+$ homeostasis in the presence of fungal colonization (strains MAJ and NAU). The maintenance of $K^+/Na^+$ homeostasis is crucial for *P. × canescens* to tolerate saline conditions (Chen et al., 2001, 2002a, 2002b, 2003; Sun et al., 2009a, 2009b, 2010; Chen and Polle, 2010). SEM-EDX microanalysis data indicate that the high $K^+/Na^+$ ratio in mycorrhizal plants was the result of less $Na^+$ accumulation and $K^+$ reduction under NaCl stress than in NM plants (Fig. 1). This finding is in accordance with our previous finding that MAJ-mycorrhizal *P. × canescens* had diminished leaf $Na^+$ but increased the $K^+$ supply under salt stress (Langenfeld-Heyser et al., 2007; Luo et al., 2009). Unexpectedly, we found that NAU-colonized plants exhibited a higher ability to maintain $K^+/Na^+$ homeostasis than MAJ, although NAU does form a Hartig net with *P. × canescens* and ensheathes the root tip with a loose mycelial network (Supplemental Fig. S2; Gafur et al., 2004). Thus, the formation of a physical barrier by the mantel formed by MAJ is likely not involved in modifying salt uptake. The increased capacity for ion balance control in the salt-sensitive host plant, which was promoted by fungal colonization, was probably due to the modulation of transport systems.

### Ectomycorrhizae Enhance $Na^+$ Extrusion

The reduction in shoot $Na^+$ uptake has been suggested to be an important resistance mechanism in EM plants growing in salinized soil (Muhsin and Zwiazek, 2002; Nguyen et al., 2006). Leaf $Na^+$ in mycorrhizal *P. × canescens* was 6% (MAJ) and 76% (NAU) lower than in NM plants after 1 week of exposure to 50 mM NaCl (Fig. 1), which is in agreement with our previous findings (Langenfeld-Heyser et al., 2007). Similarly, the EM fungi *H. crustuliniforme* and *L. bicolor* have been

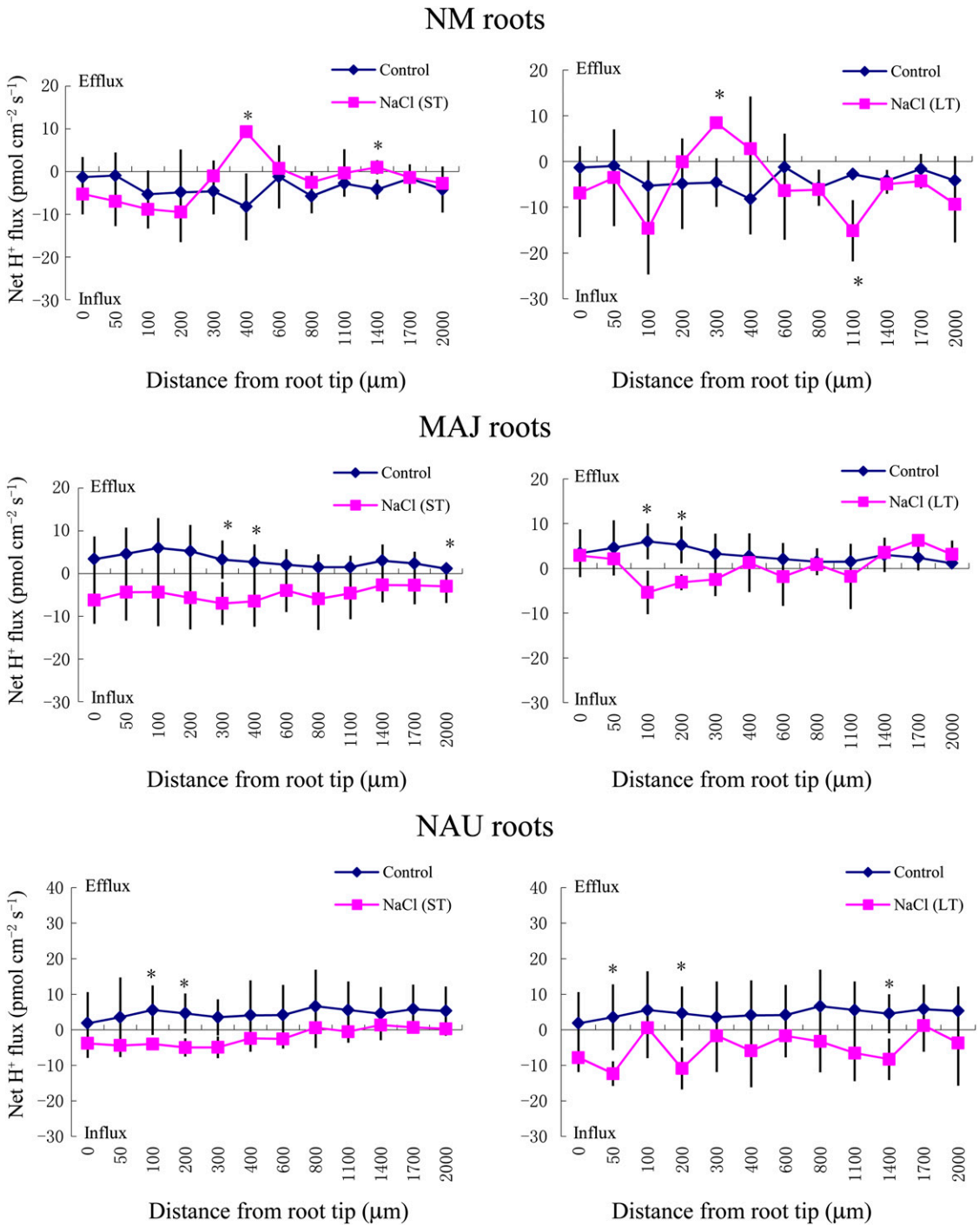


**Figure 5.** Effects of ST salinity (50 mM NaCl for 24 h) and LT salinity (50 mM NaCl for 7 d) on the net $H^+$ flux in roots of mycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants. Control roots were treated without NaCl. $H^+$ flux was measured along root axes (0–2,000 $\mu$m from the apex) at intervals of 50 to 300 $\mu$m. Each point is the mean of five to six individual plants, and error bars represent SE. $^*P < 0.05$ between treatments. [See online article for color version of this figure.]

shown to reduce tissue $Na^+$ concentrations and alleviate salt injury in several conifers (Muhsin and Zwiazek, 2002; Nguyen et al., 2006). The diminished buildup of salt in shoots is likely the result of salt uptake and transport restriction in roots (Chen et al., 2002a, 2003). Our SIET data show that the salt-induced entry of $Na^+$ was markedly impeded in EM plants, especially in NAU-colonized roots (Figs. 2 and 3). The same trend was shown in fungal hyphae, which exhibited a steady $Na^+$ efflux under ST and LT stress.

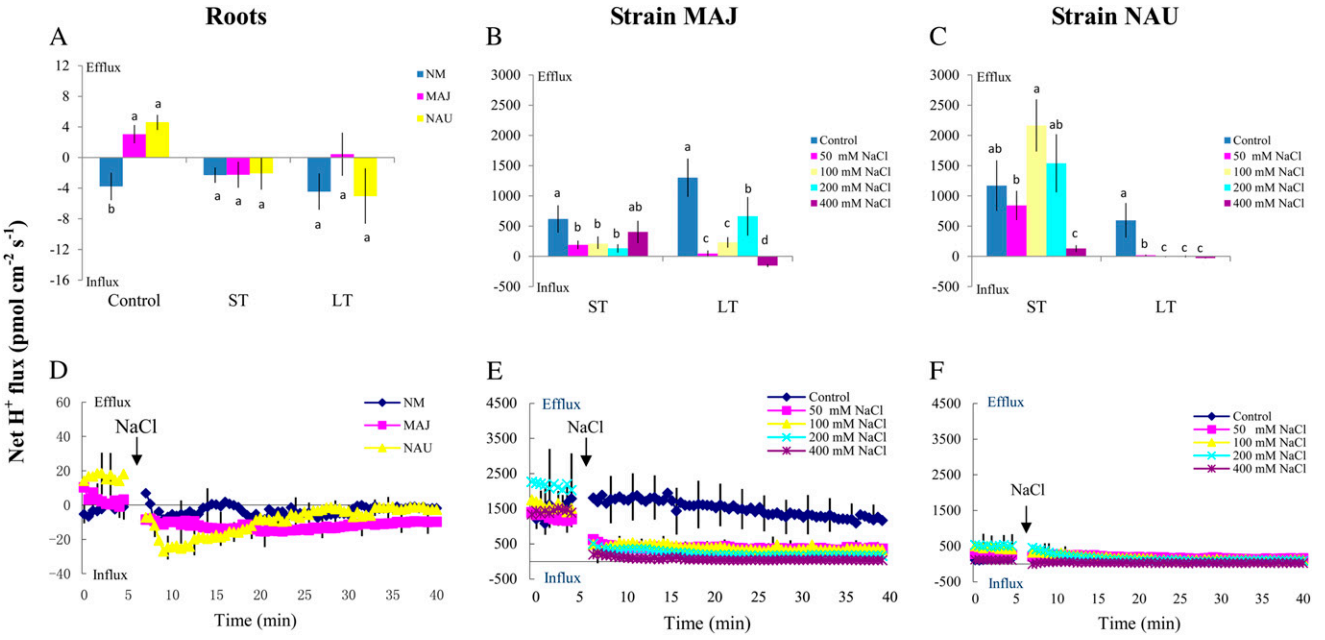


**Figure 6.** Effects of NaCl on steady and transient $H^+$ fluxes in *P.* × *canescens* roots (NM, MAJ, NAU) and *P. involutus* strains MAJ and NAU. A, Mycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants were subjected to ST salinity (50 mM NaCl for 24 h) and LT salinity (50 mM NaCl for 7 d), respectively. Control roots were treated without NaCl. For each plant, $H^+$ fluxes were measured along root axes (0–2,000 $\mu$m from the apex at intervals of 50–300 $\mu$m), and mean values are given. Each column is the mean of five to six individual plants, and error bars represent SE. Columns labeled with different letters indicate significant differences at $P < 0.05$ between NM and EM roots. B and C, *P. involutus* isolates MAJ and NAU were subjected to ST salinity (50, 100, 200, or 400 mM NaCl for 24 h) and LT salinity (50, 100, 200, or 400 mM NaCl for 7 d), respectively. Control axenic mycelia were treated without NaCl. $H^+$ fluxes of fungal hyphae were measured over a recording period of 30 to 40 min, and mean values are given. Each column is the mean of five to six axenic EM cultures (pelleted hyphae), and error bars represent SE. Columns labeled with different letters indicate significant differences at $P < 0.05$ between salinity levels. D, Mycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants were subjected to salt shock with 50 mM NaCl. $H^+$ kinetics were recorded at the apex (the measuring site was approximately 500 $\mu$m from the root tip) after the required amount of 200 mM NaCl stock was introduced into the measuring chamber. E and F, *P. involutus* isolates MAJ and NAU were subjected to salt shock with 50 to 400 mM NaCl. $H^+$ kinetics of axenic mycelia were recorded after the required amount of 200 to 800 mM NaCl stock was introduced into the measuring chamber. Before the salt shock, steady $H^+$ fluxes were monitored for approximately 5 min. In D to F, each point is the mean of four individual plants or axenic EM cultures (pelleted hyphae), and error bars represent SE. [See online article for color version of this figure.]

As amiloride, a $Na^+/H^+$ antiporter inhibitor, reduced $Na^+$ efflux in the salt-treated mycelia of both strains (Fig. 4), our data suggest that *P. involutus* contributed to active $Na^+$ extrusion in the salinized roots of *P.* × *canescens*.

Notably, the salt-enhanced $Na^+$ efflux was always associated with an $H^+$ influx into EM roots (Figs. 2, 3, 5, and 6), whereas EM roots exhibited a net $H^+$ efflux in the absence of salt stress (Figs. 5 and 6). The $H^+$ efflux in EM roots apparently resulted from the root-ensheathing fungus, as MAJ and NAU hyphae exhibited an evident $H^+$ efflux (Fig. 6). Our data agree with the findings of Ramos et al. (2009), who reported significant $H^+$ efflux from *Eucalyptus globulus* roots (the apex, meristematic, and elongation zones) colonized with *Pisolithus* species. The mycorrhiza-stimulated $H^+$ efflux is due to the activity of plasma membrane $H^+$-ATPase in the EM associations. In the roots of *Pinus sylvestris-Laccaria laccata*, Lei and Dexheimer (1988) showed the localization of ATPase activity along the Hartig net hyphal plasma membranes and the plasma membranes of contiguous living cortical cells. Arbuscular mycorrhizal symbioses induced the expression of two genes (*pma2* and *pma4*) responsible for de novo $H^+$-ATPase activity in the periarbuscular membrane of invaded cells (Gianinazzi-Pearson et al., 2000). Some host plasma membrane $H^+$-ATPase isoforms show high activity in arbuscular mycorrhizal associations (Ramos et al., 2005; Rosewarne et al., 2007). In addition, three plasma membrane $H^+$-ATPase genes (LHA1, LHA2, and LHA4) were found to be regulated by arbuscular mycorrhiza in tomato (*Solanum lycopersicum*) plants (Ferrol et al., 2002). The molecular analysis of ATPases in EM roots appears to be missing. In our study, salt exposure reversed the rectification of $H^+$ from efflux to influx in the EM roots of *P.* × *canescens* (Figs. 5 and 6). In accordance, the $H^+$ efflux from hyphae was diminished by various salt treatments (salt

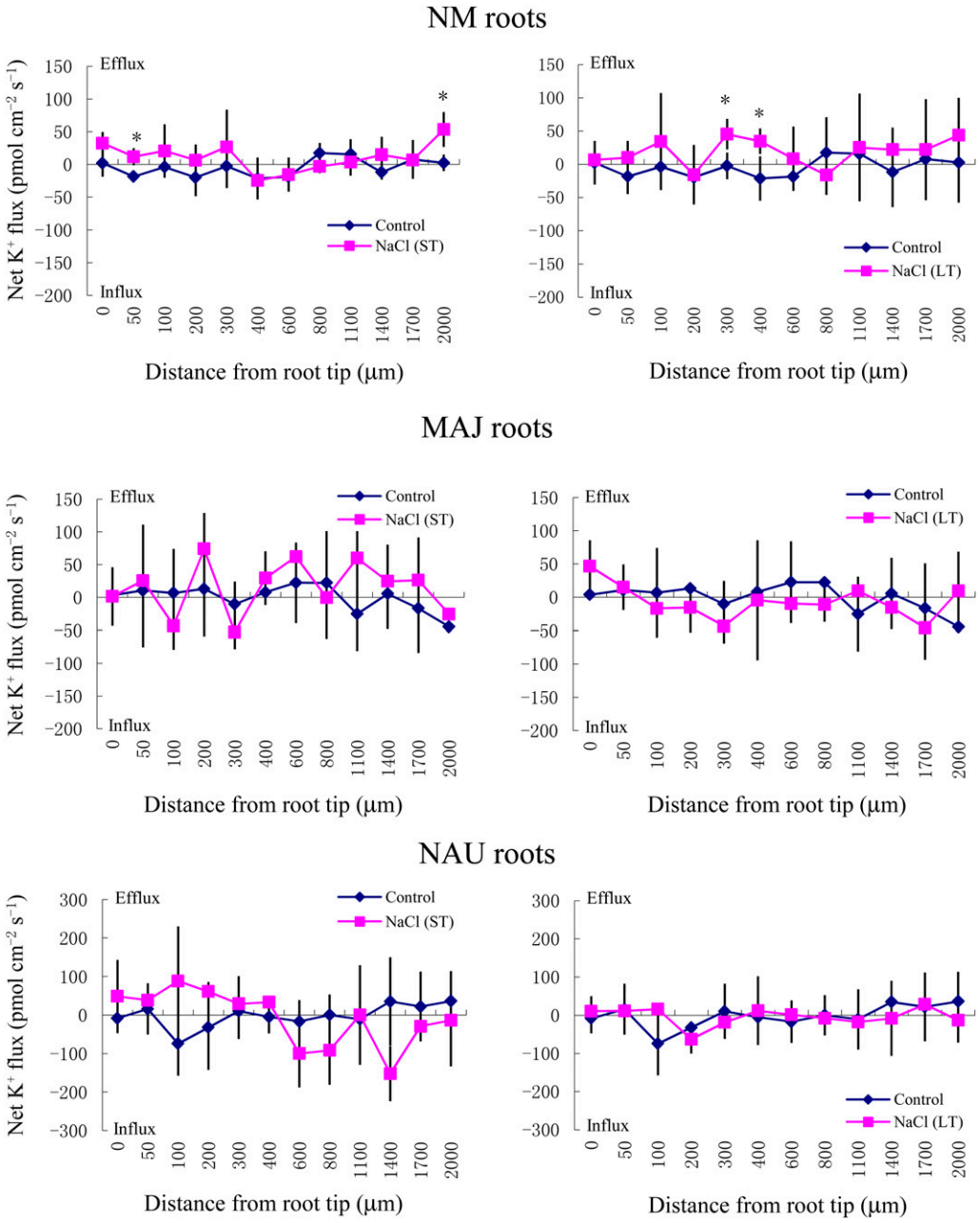


**Figure 7.** Effects of ST salinity (50 mM NaCl for 24 h) and LT salinity (50 mM NaCl for 7 d) on net $K^+$ flux in roots of mycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants. Control roots were treated without NaCl. The $K^+$ flux was measured along root axes (0–2,000 $\mu$m from the apex) at intervals of 50 to 300 $\mu$m. Each point is the mean of five to six individual plants, and error bars represent SE. *$P < 0.05$ between treatments. [See online article for color version of this figure.]

shock, ST, and LT treatments, with a few exceptions) or shifted to a net influx during the period of recording. In the presence of an inhibitor of the plasma membrane proton pump, sodium orthovanadate, the salt-enhanced $Na^+$ efflux was reduced in the two strains (Fig. 4). Taken together, these data suggest that colonization with *P. involutus* stimulates the $H^+$-ATPase activity in *P.* × *canescens*-ectomycorrhizae associations, which pumps protons to promote the secondary active $Na^+/H^+$ antiport at the plasma membrane (Blumwald et al., 2000; Zhu, 2003). We have noticed that under salt exposure, the influx of $H^+$ was not equivalent to the efflux of $Na^+$. The flux inconsistency of $Na^+$ and $H^+$ is mainly due to the superimposition of two effects: salt-induced $H^+$ excretion, as salinity stress is usually associated with increased $H^+$ efflux, and at the same time, higher SALT-OVERLY-SENSITIVE1 $Na^+/H^+$ exchanger activity of EM plants, which leads to an accelerated $H^+$ uptake. As such, the net $H^+$ flux will not be changed.

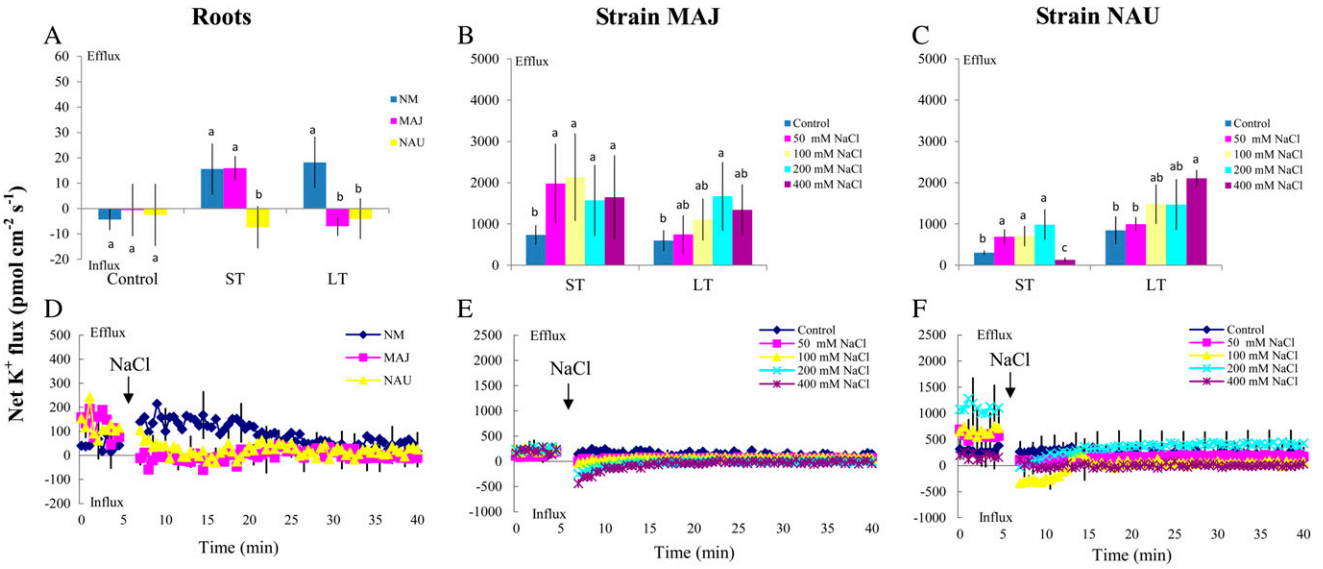


**Figure 8.** Effects of NaCl on steady and transient $K^+$ fluxes in *P.* × *canescens* roots (NM, MAJ, NAU) and *P. involutus* strains MAJ and NAU. A, Mycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants were subjected to ST salinity (50 mM NaCl for 24 h) and LT salinity (50 mM NaCl for 7 d), respectively. Control roots were treated without NaCl. For each plant, $K^+$ fluxes were measured along root axes (0–2,000 $\mu$m from the apex at intervals of 50–300 $\mu$m), and mean values are given. Each column is the mean of five to six individual plants, and error bars represent SE. Columns labeled with different letters indicate significant differences at $P < 0.05$ between NM and EM roots. B and C, *P. involutus* isolates MAJ and NAU were subjected to ST salinity (50, 100, 200, or 400 mM NaCl for 24 h) and LT salinity (50, 100, 200, or 400 mM NaCl for 7 d), respectively. Control axenic mycelia were treated without NaCl. $K^+$ fluxes of fungal hyphae were measured over a recording period of 30 to 40 min, and mean values are given. Each column is the mean of five to six axenic EM cultures (pelleted hyphae), and error bars represent SE. Columns labeled with different letters indicate significant differences at $P < 0.05$ between salinity levels. D, Mycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants were subjected to salt shock with 50 mM NaCl. $K^+$ kinetics were recorded at the apex (the measuring site was approximately 500 $\mu$m from the root tip) after the required amount of 200 mM NaCl stock was introduced into the measuring chamber. E and F, *P. involutus* isolates MAJ and NAU were subjected to salt shock with 50 to 400 mM NaCl. $K^+$ kinetics of axenic mycelia were recorded after the required amount of 200 to 800 mM NaCl stock was introduced into the measuring chamber. Before the salt shock, steady $K^+$ fluxes were monitored for approximately 5 min. In D to F, each point is the mean of four individual plants or axenic EM cultures (pelleted hyphae), and error bars represent SE. [See online article for color version of this figure.]

For $Na^+$ flux measurements, special attention needs to be paid to $Na^+$ microelectrodes because of the nonideal selectivity of the commercially available $Na^+$ liquid ion exchanger (LIX; Chen et al., 2005). $Na^+$ electrodes produced signals when we calibrated $Na^+$ LIX in a range of $K^+$ or $Ca^{2+}$ standards, in accordance with the finding by Chen et al. (2005). To reduce interfering effects of $K^+$ on $Na^+$ flux, $K^+$ was omitted from the bathing medium (Cuin et al., 2011). In our study, $Ca^{2+}$ and $K^+$ concentrations in the measuring solution were set to low concentrations, 0.1 and 0.5 mM, respectively. To estimate the interference of $K^+$ and $Ca^{2+}$ on $Na^+$ flux in plant materials, we measured root $Na^+$ flux in control and salinized *P.* × *canescens* plants (50 mM NaCl for 24 h) in the presence and absence of interfering ions ($K^+$ and $Ca^{2+}$). We found that 0.5 mM $K^+$ and 0.1 mM $Ca^{2+}$ had no significant effects on root $Na^+$ flux in no-salt controls (data not shown). However, in salt-treated roots, the absence of $K^+$ and $Ca^{2+}$ in the measuring buffer resulted in higher signals but did not change the tendency of root flux (data not shown). We found that the detected $Na^+$ signals in salinized roots were unstable and fluctuated greatly during the period of recording. This is presumably the plant response to nutrient deficiency in the root medium. In our study, the presence of $K^+$ and $Ca^{2+}$ may not affect the accuracy of our conclusions relating to $Na^+$ fluxes in mycorrhizal roots and axenic EM cultures. The experimental evidence and explanations are briefly listed here. (1) $Na^+$ electrodes exhibited much higher selectivity for $Na^+$ relative to $K^+$ and $Ca^{2+}$ in the presence of both $Na^+$ and interfering ions (Supplemental Table S1). Moreover, the released $Na^+$ from salinized mycorrhizal roots and hyphae would increase the ratio of $Na^+$ to $K^+$ and $Ca^{2+}$ near the electrode, thus increasing the selectivity of the $Na^+$ electrode during the period of recording. (2) Treatment with amiloride (an inhibitor of the $Na^+/H^+$ antiporter) significantly decreased the $Na^+$ efflux in axenic mycelia (Fig. 4), suggesting that the detected signals were largely carried by $Na^+$ across the plasma membrane. (3) The different trends of $Na^+$, $K^+$, and $Ca^{2+}$ fluxes suggest that the selectivity of $Na^+$ LIX was sufficient for $Na^+/K^+$ and $Na^+/Ca^{2+}$ discrimination. Salinized axenic mycelia exhibited an outward

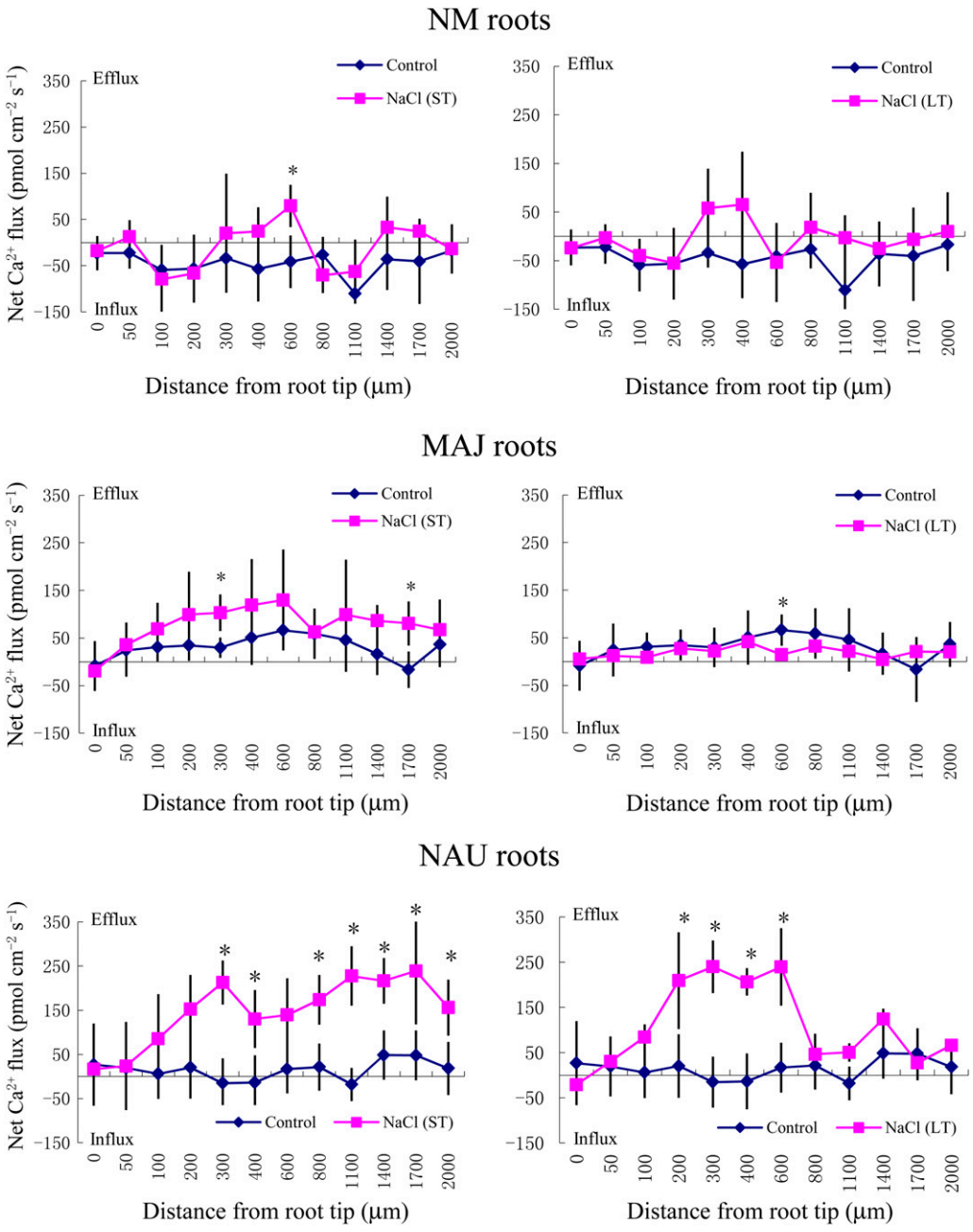


**Figure 9.** Effects of ST salinity (50 mM NaCl for 24 h) and LT salinity (50 mM NaCl for 7 d) on the net $Ca^{2+}$ flux in the roots of mycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants. Control roots were treated without NaCl. The $Ca^{2+}$ flux was measured along root axes (0–2,000 $\mu$m from the apex) at intervals of 50 to 300 $\mu$m. Each point is the mean of five to six individual plants, and error bars represent SE. *$P < 0.05$ between treatments. [See online article for color version of this figure.]

rectification of $Na^+$ and $K^+$, but with different patterns with increasing NaCl concentrations (Figs. 3 and 8). Salt treatment caused an evident $Na^+$ efflux in EM cultures, while the detected $Ca^{2+}$ efflux was low and an inward rectification was usually seen in salinized mycelia (Figs. 3 and 10). Salinity induced an evident $Ca^{2+}$ efflux in mycorrhizal roots, whereas there was no equivalent $Na^+$ flux corresponding to the $Ca^{2+}$ efflux in these roots (Figs. 2, 3, 9, and 10).

### EM Ameliorates $K^+$ Homeostasis

In our study, $K^+$ concentrations in the roots and leaves were reduced by salt treatment to a lesser extent in mycorrhizal plants than in NM plants. This finding agrees with the results of Langenfeld-Heyser et al. (2007), who found that MAJ-mycorrhizal plants contained higher leaf $K^+$ levels than non-EM plants. Our studies show that *P. involutus* fungi assist host plants

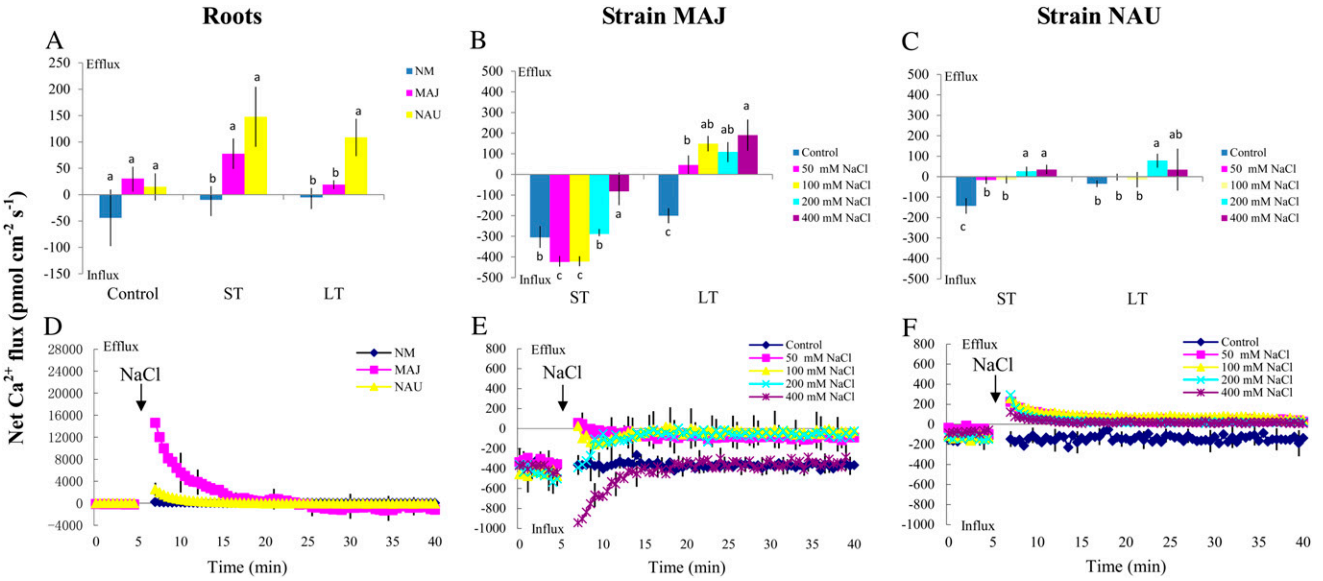


**Figure 10.** Effects of NaCl on steady and transient $Ca^{2+}$ fluxes in *P.* × *canescens* roots (NM, MAJ, NAU) and *P. involutus* strains MAJ and NAU. A, Mycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants were subjected to ST salinity (50 mM NaCl for 24 h) and LT salinity (50 mM NaCl for 7 d), respectively. Control roots were treated without NaCl. For each plant, $Ca^{2+}$ fluxes were measured along root axes (0–2,000 $\mu$m from the apex at intervals of 50–300 $\mu$m), and mean values are given. Each column is the mean of five to six individual plants, and error bars represent SE. Columns labeled with different letters indicate significant differences at $P < 0.05$ between NM and EM roots. B and C, *P. involutus* isolates MAJ and NAU were subjected to ST salinity (50, 100, 200, or 400 mM NaCl for 24 h) and LT salinity (50, 100, 200, or 400 mM NaCl for 7 d), respectively. Control axenic mycelia were treated without NaCl. $Ca^{2+}$ fluxes of fungal hyphae were measured over a recording period of 30 to 40 min, and mean values are given. Each column is the mean of five to six axenic EM cultures (pelleted hyphae), and error bars represent SE. Columns labeled with different letters indicate significant differences at $P < 0.05$ between salinity levels. D, Mycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants were subjected to salt shock with 50 mM NaCl. $Ca^{2+}$ kinetics were recorded at the apex (the measuring site was approximately 500 $\mu$m from the root tip) after the required amount of 200 mM NaCl stock was introduced into the measuring chamber. E and F, *P. involutus* isolates MAJ and NAU were subjected to salt shock with 50 to 400 mM NaCl. $Ca^{2+}$ kinetics of axenic mycelia were recorded after the required amount of 200 to 800 mM NaCl stock was introduced into the measuring chamber. Before the salt shock, steady $Ca^{2+}$ fluxes were monitored for approximately 5 min. In D to F, each point is the mean of four individual plants or axenic EM cultures (pelleted hyphae), and error bars represent SE. [See online article for color version of this figure.]

in the maintenance of $K^+$ homeostasis by delivering the nutrient to the plant and slowing the loss of $K^+$ under NaCl stress. Zhang et al. (2008) reported that *P. involutus* mycelium, especially strain MAJ, increased the uptake of $K^+$ after exposure to salt treatment. Salt shock caused an instantaneous influx of $K^+$ into the fungal mycelium; however, ST- and LT-stressed hyphae exhibited a $K^+$ efflux (Fig. 8). Our data indicate that prolonged NaCl treatment resulted in $K^+$ loss from *P. involutus*. Currently, we cannot conclude that $K^+$ efflux is mediated by cation channels, although the $K^+$ efflux caused by NaCl (400 mM) was significantly reduced by the $K^+$ channel blocker (TEA) in the two tested strains (Fig. 4). The high rate of $K^+$ efflux detected in the control and salinized mycelia (ST and LT treatments) relates, to some extent, to the concentrations of the $K^+$ sources (Fig. 8). The enriched $K^+$ in EM hyphae is thought to be transferred to the host during the period of salt treatment, which caused a lesser reduction of $K^+$ in the roots and leaves of EM plants (Fig. 1). Rygiewicz and Bledsoe (1984) reported that external hyphae in EM symbiosis have a high capacity to take up $K^+$ and deliver the nutrient to the host plant. In addition to stimulating $K^+$ uptake, *P. involutus* colonization benefits the salinized hosts by slowing down the rate of $K^+$ loss. Steady and transient flux data for *P.* × *canescens* roots indicate that the salt-induced $K^+$ efflux was decreased by *P. involutus* colonization (Figs. 7 and 8). The reduced rate of $K^+$ efflux in ST- and LT-treated EM roots is partly the result of a delayed $K^+$ loss from inner host cells. In the fungus-ensheathed roots, $K^+$ in the compatible (MAJ) or incompatible (NAU) hyphae was replaced by $Na^+$ before $K^+$ exchange with the root cells of the host was expected. Moreover, the released $Ca^{2+}$ ions that are replaced by $Na^+$ can assist plants in maintaining $K^+$ homeostasis under salt stress.

### Mediation of $Ca^{2+}$ in $K^+/Na^+$ Homeostasis in EM Plants

In our study, EM *P.* × *canescens* plants exhibited an enhanced $Ca^{2+}$ uptake ability in the absence of salinity stress (Fig. 1). The $Ca^{2+}$ enrichment was caused by

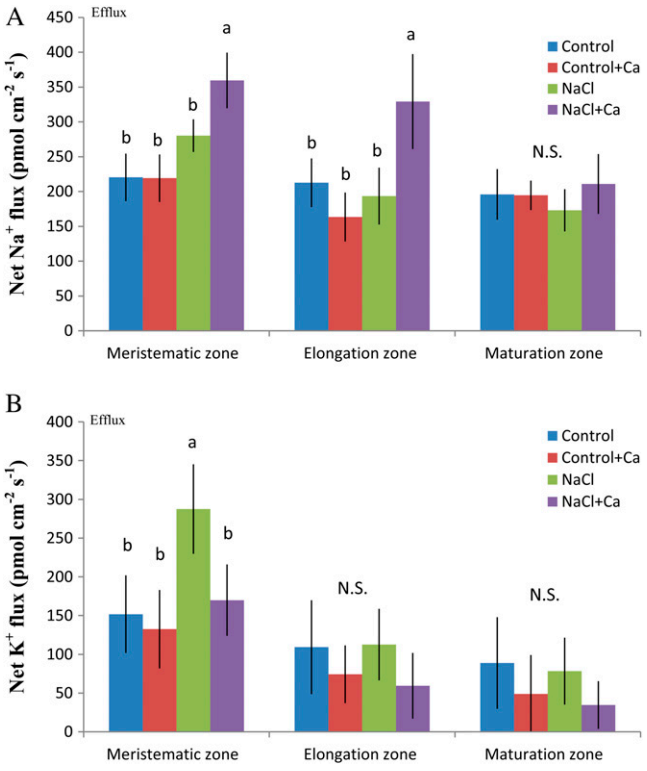


**Figure 11.** Effects of NaCl and $Ca^{2+}$ supplementation on $Na^+$ (A) and $K^+$ (B) flux at the meristematic, elongation, and maturation zones of *P.* × *canescens* roots. Plants were subjected to 1 week of NaCl stress (50 mM) supplemented with or without 10 mM $CaCl_2$. Control plants were well fertilized but treated without additional NaCl. Roots were sampled at 24 h and 7 d, and mean flux values are given. Steady fluxes at the meristematic, elongation, and maturation regions were measured along the root axes at intervals of 30 to 50 $\mu$m. Each column is the mean of six to eight individual plants, and error bars represent SE (for each plant, the average fluxes at the meristematic, elongation, and maturation zones were calibrated from measuring points in the measured region). Columns labeled with different letters indicate significant differences at $P < 0.05$ between treatments. N.S., No significant difference. [See online article for color version of this figure.]

colonization with *P. involutus*, because the mycelium also displayed a stable $Ca^{2+}$ influx under these conditions (Fig. 10). Similarly, the EM roots of *E. globulus* colonized by *Pisolithus* species exhibit a high uptake capacity for $Ca^{2+}$ from the external medium (Ramos et al., 2009). Transient and steady flux measurements of mycorrhizal roots revealed a significant $Ca^{2+}$ efflux under salt stress, suggesting that the $Ca^{2+}$ accumulated in mycorrhizal roots could be replaced by $Na^+$ (Figs. 9 and 10). The salt-induced $Ca^{2+}$ flux may originate from the cell wall (Arif et al., 1995; Shabala and Newman, 2000); $LaCl_3$, an inhibitor of $Ca^{2+}$-permeable channels, did not markedly restrict the high rate of $Ca^{2+}$ efflux from salt-shocked MAJ and NAU roots (Supplemental Fig. S1). A large amount of cell wall $Ca^{2+}$ has been shown to be exchangeable in mycorrhizal roots (Peterson and Enstone, 1996; Kuhn et al., 2000; Bücking et al., 2002). The increase in leaf $Ca^{2+}$ as a consequence of NaCl treatment indicates that the root-derived element could be transported to the shoots in addition to being released from the root surface. Thus, freed $Ca^{2+}$ is able to assist plants in ameliorating the $K^+/Na^+$ ratio under salt stress. $Ca^{2+}$ inhibits DA-KORCs and DA-NSCCs to reduce $K^+$ efflux in Arabidopsis under saline conditions (Shabala et al., 2006a; Sun et al., 2009b). Exogenous $Ca^{2+}$ improved the $K^+/Na^+$ balance by inhibiting $K^+$ efflux and increasing the apparent $Na^+$ efflux in *P.* × *canescens* roots (Fig. 11). These results are consistent with our previous findings in NM roots of another salt-sensitive poplar, *Populus popularis* (Sun et al., 2009b). Therefore, we conclude that the increased availability of free $Ca^{2+}$, which was released by $Ca^{2+}/Na^+$ exchange from EM roots, favored the establishment of $K^+/Na^+$ homeostasis in *P.* × *canescens* under salt treatment.

Differences in $K^+/Na^+$ maintenance between MAJ- and NAU-colonized roots are clear (Fig. 1), which might have resulted from differences in the release of exchangeable $Ca^{2+}$ from mycorrhizal associations. MAJ-mycorrhizal roots lost a large amount of $Ca^{2+}$ after being subjected to salt shock (Fig. 10), whereas NAU-colonized roots exhibited a long-sustained release of free $Ca^{2+}$ during ST and LT salt treatments (Figs. 9 and 10). Consequently, the $Ca^{2+}$ ions released from the mycorrhizal associations would benefit inner root cells controlling $K^+/Na^+$ homeostasis in terms of the effects of $Ca^{2+}$ on $K^+$ and $Na^+$ fluxes in *P.* × *canescens* roots. We noticed that MAJ-mycorrhizal roots displayed a significant $Ca^{2+}$ efflux under salt stress; however, a net $Ca^{2+}$ influx was usually recorded in the fungal mycelium of this strain after exposure to salt shock and ST treatment (Figs. 9 and 10). Therefore, the salt-induced $Ca^{2+}$ efflux that we detected from MAJ-mycorrhizal roots likely came from inner host cells, at least in part, as a result of $Na^+/Ca^{2+}$ exchange. Compared with NAU, the inner root cells might have been more accessible to $Na^+$ because MAJ forms a typical Hartig net with the roots of *P.* × *canescens*, whereas NAU exhibits cell wall thickening (Supplemental Fig. S2; Gafur et al., 2004).

## MATERIALS AND METHODS

### Plant and Fungal Cultures for EM Colonization

The EM fungi used in this study were the *Paxillus involutus* isolates MAJ and NAU. Plants, fungal cultures, and the technique for synthesizing ectomycorrhizae followed the procedures described by Gafur et al. (2004). In brief, the two isolates were grown on 2% modified Melin Norkrans (MMN) agar medium [the medium contains the following components in g $L^{-1}$: 0.5 $KH_2PO_4$, 0.25 $(NH_4)_2SO_4$, 0.15 $MgSO_4 \cdot 7H_2O$, 0.05 $CaCl_2 \cdot 2H_2O$, 0.025 NaCl, 0.01 $FeCl_3 \cdot 6H_2O$, 0.0001 thiamine-HCl, 10 Glc, and 3 malt extract, pH 5.0; Gafur et al., 2004]. After fungal inoculation, the petri dishes (diameter, 90 mm) were sealed with a strip of Parafilm and kept in permanent darkness at 23°C.

In this study, we used a petri dish culture system for the colonization of *Populus* × *canescens* with the *P. involutus* strains MAJ and NAU (Gafur et al., 2004). In brief, regenerated plantlets of *P.* × *canescens* were grown for 2 to 3 weeks on Murashige and Skoog rooting medium. The fungi were pregrown on the agar culture medium for 1 week prior to colonization. Vigorous plantlets with sufficient roots were used for ectomycorrhization. Rooted plantlets from

sterile culture were carefully freed from agar particles. Rooted plantlets were placed on the MMN agar medium in the presence or absence of EM mycelium. During the period of incubation, the room temperature was 24°C ± 1°C with a light period of 14 h (6:00 AM to 8:00 PM). Photosynthetic active radiation of 200 $\mu$mol $m^{-2}$ $s^{-1}$ was supplied by cool-white fluorescent lamps. Root-fungus associations were formed during 1 month after colonization. All plantlets exhibited well-developed shoots and roots (Supplemental Fig. S2, A–C). Some leaves at lower shoots became dry at the tips (Supplemental Fig. S2, A–C). Samples of EM and NM root tips for anatomical investigations were embedded, stained, and photographed as described previously (Gafur et al., 2004). Anatomical analyses of mycorrhizal roots showed that the hyphae of strain MAJ penetrated into the cell walls of the cortex, whereas NAU hyphae were only detected on the outer epidermal cell walls (Supplemental Fig. S2, G–I). Uniform mycorrhizal and NM plants were used for acclimation and salt treatment.

## Liquid Culture of Fungi

Liquid culture of *P. involutus* was grown at the College of Biological Sciences and Technology, Beijing Forestry University. Strains MAJ and NAU were obtained from the Büsgen Institute of Forest Botany and Tree Physiology, Göttingen University. For liquid culture, agar was absent and the medium was buffered with citrate (Ott et al., 2002). Mycelium from the agar plate was homogenized, transferred into 150 mL of liquid medium in flasks, and incubated on a rotary shaker in darkness (150 rpm, 23°C; Langenfeld-Heyser et al., 2007). *P. involutus* in submerged culture grew in the form of compact spherical masses of mycelium (pellets). For salt treatment, sterile filtered NaCl solutions were added to achieve final concentrations of 0, 50, 100, 200, and 400 mM. After ST (24 h) or LT (7 d) treatment, axenic cultures of MAJ and NAU were used for steady flux measurements of $Na^+$, $H^+$, $K^+$, and $Ca^{2+}$.

## Plant Acclimation and Salt Treatments

Prior to salt treatment, mycorrhizal and NM plants were carefully removed from MMN agar medium, planted in individual pots containing fine sand, and grown in a greenhouse at the College of Biological Sciences and Technology, Beijing Forestry University. The room temperature was 24°C ± 1°C with a light period of 14 h (6:00 AM to 8:00 PM), and photosynthetic active radiation of 200 $\mu$mol $m^{-2}$ $s^{-1}$ was supplied by cool-white fluorescent lamps. Mycorrhizal and NM plantlets were exposed to 50 mM NaCl for ST (24 h) or LT (7 d) treatments. The required NaCl concentrations were added to the LN (Matzner et al., 1982) nutrient solution (Langenfeld-Heyser et al., 2007). Control plantlets received LN solution without NaCl. On days 1 and 7, roots with apices of 1 to 2 cm were sampled from mycorrhizal and NM plants and used for steady-state measurements of the net $Na^+$, $H^+$, $K^+$, and $Ca^{2+}$ fluxes. At the final harvest, leaf segments (2–3 mm long and 1–2 mm wide) and root segments with 1.0-cm apices were sampled from mycorrhizal and NM plantlets, freeze dried, and used for microanalysis by SEM-EDX.

## $CaCl_2$ Treatment

Plantlets of *P.* × *canescens* were multiplied by micropropagation as described by Leplé et al. (1992). Rooted plantlets were cultivated in hydroponic LN nutrient solutions with low nitrogen supply (Gafur et al., 2004). To acclimate the plants to ambient conditions, plantlets were covered with plastic bags and illuminated with low light (50 $\mu$mol $m^{-2}$ $s^{-1}$) supplied by cool-white fluorescent lamps. The plastic bags were gradually opened over 1 week. Acclimated plants were subjected to NaCl (50 mM) or NaCl (50 mM) plus $CaCl_2$ (10 mM). The required amounts of NaCl and $CaCl_2$ were added to the nutrient solution. Control plants were treated in the same manner without the addition of NaCl. Plants were continuously aerated by passing air to hydroponic LN nutrient solution, which was regularly renewed. Steady fluxes of $K^+$ and $Na^+$ in meristematic, elongation, and maturation zones were measured after 24 h and 7 d of treatment.

## Inhibitor Treatments

ST-treated (400 mM NaCl) *P. involutus* isolates MAJ and NAU were subjected to sodium orthovanadate (500 $\mu$M), amiloride (50 $\mu$M), or TEA (50 $\mu$M) for 30 min in the measuring solutions. Prior to recording $Na^+$ flux, the measuring solutions containing sodium orthovanadate were replaced with 10 mL of fresh solution, but the measuring solution with amiloride and TEA was not renewed (amiloride and TEA had no clear effect on the Nernstian slopes of $Na^+$ and $K^+$ electrodes; Sun et al., 2009a, 2009b). Steady-state fluxes of $Na^+$ (orthovanadate or amiloride treatment) and $K^+$ (TEA treatment) were measured in axenic mycelia (pelleted hyphae) pretreated with or without inhibitors at pH 6.0.

## Steady-State Measurements of Net $Na^+$, $H^+$, $K^+$, and $Ca^{2+}$ Fluxes

The net fluxes of $Na^+$, $H^+$, $K^+$, and $Ca^{2+}$ in roots and mycelia were measured using a ~~noninvasive SIET~~ (BIO-001A; ~~Younger USA Science and Technology;~~ Xu et al., 2006; Sun et al., 2009a, 2009b). The concentrations of the ions and concentration gradients were measured by moving the ion-selective microelectrode between two positions close to the materials in a preset excursion (30 $\mu$m) at a programmable frequency in the range of 0.3 to 0.5 Hz.

Ion-selective microelectrodes for the target ions were calibrated prior to flux measurements: (1) $Na^+$, 0.1, 0.5, 1.0 mM ($Na^+$ concentration was usually 0.1 mM in the measuring buffer for roots and axenic mycelia); (2) $H^+$, pH 4.0, 5.0, 6.0 (pH was 5.0 in the measuring buffer); (3) $K^+$, 0.1, 0.5, 1.0 mM ($K^+$ was 0.5 mM in the measuring buffer); and (4) $Ca^{2+}$, 0.1, 0.5, 1.0 mM ($Ca^{2+}$ was 0.1 mM in the measuring buffer). All electrodes used for steady and transient recordings were usually corrected two to three times by calibrations during the experiments. The ion flux rate was calculated using Fick's law of diffusion:

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

where $J$ is the ion flux in the $x$ direction, $dc$ represents the ion concentration difference, $dx$ is the microelectrode movement between two positions, $dc/dx$ is the ion concentration gradient, and $D$ represents the ion diffusion coefficient in a particular medium.

### *Flux Oscillations*

Rhythmic (ultradian) flux oscillations are ubiquitous in the measured plant species (Souda et al., 1990; Toko et al., 1990; Shabala et al., 1997, 2003, 2006b; Shabala and Knowles, 2002). $H^+$ flux oscillations have been widely reported in a variety of plant species. In our study, oscillations in the $H^+$ flux in *P.* × *canescens* roots were not as noticeable as in herbaceous species. The $H^+$ oscillations were more like fluctuations, and oscillations in the $Na^+$, $K^+$, and $Ca^{2+}$ fluxes displayed similar trends as $H^+$ (Supplemental Fig. S3). This finding is presumably due to a lower growth rate of woody roots compared with crop species. Toko et al. (1990) found that roots exhibit no oscillations when they have a slow growth speed. Salinity caused a significant decrease in the period of $H^+$ flux oscillations (Shabala, 2003). Our data show that the oscillatory periods of measured ions ($H^+$, $Na^+$, $K^+$, and $Ca^{2+}$) in *P.* × *canescens* roots are usually in the range of several minutes. In our scanning studies, fluxes were recorded for 8 to 10 min at each point, which is long enough to ensure the absence of oscillations.

### *Ion Selectivity of $Na^+$ Electrodes*

We found that $Na^+$ microelectrodes were not able to record $Na^+$ flux in a measuring buffer containing high $Na^+$ due to the low signal/noise ratio of $Na^+$ LIX (Fluka 71178; Sun et al., 2009a). $Na^+$-selective microelectrodes were also found to be unsuitable for screening $Na^+$ fluxes, because of the nonideal selectivity of the commercially available $Na^+$ LIX (Chen et al., 2005). $K^+$ was omitted from the bathing medium to reduce the interfering effects of $K^+$ on $Na^+$ flux (Cuin et al., 2011). To determine the interfering effects of $K^+$ and $Ca^{2+}$ on $Na^+$-selective electrodes, $Na^+$-selective microelectrodes were calibrated in $Na^+$ solution (0.1, 0.5, 1.0 mM) in the presence or absence of $K^+$ (0.1, 0.5, 1.0 mM) and $Ca^{2+}$ (0.1, 0.5, 1.0 mM). The calibration characteristics (Nernst slope) of the $Na^+$ electrode were not altered by the interfering $K^+$ ion (concentration range of 0.1–1.0 mM; Supplemental Table S1). In the presence of $Ca^{2+}$, the Nernst slope of the $Na^+$ electrodes was reduced up to 24% at 1.0 mM $Ca^{2+}$ (Supplemental Table S1). To reduce the interfering effects of $Ca^{2+}$ on $Na^+$ electrodes, the $Ca^{2+}$ concentration in the measuring solution was set to 0.1 mM. The Nernst slope and intercept of the $Na^+$ electrodes in the measuring solution (0.5 mM KCl, 0.1 mM NaCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$) were 50.0031 ±

1.9249 and 73.1593 ± 0.6543, similar to the values in the absence of $K^+$ and $Ca^{2+}$ (Nernst slope, 55.7648 ± 1.9751; Nernst intercept, 75.9074 ± 1.8814).

## Experimental Protocols for SIET Measurements

Roots sampled from mycorrhizal and NM plants and mycelial hyphae collected from the liquid culture medium were rinsed with distilled water and incubated in the basic measuring solution (0.5 mM KCl, 0.1 mM NaCl, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$) to equilibrate for 30 min. To record the $Na^+$ flux in mycelial hyphae, the axenic EM cultures (pelleted form) were equilibrated for 60 min to reach a stable flux rate (Supplemental Fig. S4). The roots or hyphae were transferred to petri dishes containing 10 mL of fresh measuring solution. Prior to recording the flux, root and fungal samples were immobilized on the bottom. The $Na^+$, $H^+$, $K^+$, and $Ca^{2+}$ fluxes in the roots were measured along the root apex (0–2,000 $\mu$m from the tip) at intervals of 50 to 300 $\mu$m. Ion fluxes in the mycelium were measured over a recording period of 30 to 40 min. Real-time flux measurements of NM roots, EM roots, and axenic mycelia (pelleted hyphae) are shown in Supplemental Figure S5.

## Transient Flux Kinetics

Mycorrhizal and NM roots were sampled from nonsalinized plants. After equilibration to the basic measuring solution, the steady-state fluxes of $H^+$, $K^+$, and $Ca^{2+}$ in the apical region (500 $\mu$m from the root apex) were recorded (5–6 min) prior to salt shock. NaCl (200 mM) was slowly added to the measuring solution until the final NaCl concentration in the buffer reached 50 mM. Ion flux recording was continued for 30 to 40 min. The effect of lanthanum chloride (200 $\mu$M) on salt shock-induced transient $Ca^{2+}$ kinetics was examined in the roots of mycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants.

Fungal mycelia were exposed to 0, 50, 100, 200, or 400 mM NaCl to induce salt shock. $H^+$, $K^+$, and $Ca^{2+}$ fluxes were monitored over a continuous recording period of 30 to 40 min. For transient flux kinetics, the data measured during the first 2 to 3 min were discarded due to the diffusion effects of stock addition.

## X-Ray Microanalysis

The samples from control and stressed plantlets were rapidly frozen in liquid nitrogen and vacuum freeze dried at −100°C for 7 d. The freeze-dried samples were gold coated in a high-vacuum sputter coater and analyzed using a Hitachi S-3400N scanning electron microscope equipped with an energy-dispersive x-ray spectrometer (EX-250; Horiba). Probe measurements of roots and leaves were made with a broad electron beam covering the whole cross-section. The relative amounts of $K^+$, $Na^+$, and $Ca^{2+}$ were expressed as atomic mass fractions (%).

## Data Analysis

Three-dimensional ionic fluxes were calculated using MageFlux, developed by Xu Yue (http://xuyue.net/mageflux). All mean data were subjected to ANOVA. Significant differences between means were determined by Duncan's multiple range test. Unless otherwise stated, differences were considered significant at $P < 0.05$.

## Supplemental Data

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

**Supplemental Figure S1.** Effects of lanthanum chloride (200 $\mu$M) on salt shock-induced transient $Ca^{2+}$ kinetics in the roots of mycorrhizal (MAJ and NAU) and NM *P.* × *canescens* plants.

**Supplemental Figure S2.** EM colonization of *P.* × *canescens* and performance of mycorrhizal (MAJ and NAU) and NM plants under salt stress.

**Supplemental Figure S3.** Fluctuations and oscillations in the net $H^+$ flux in the elongation zone of *P.* × *canescens* roots.

**Supplemental Figure S4.** Kinetics of the net $Na^+$ efflux in axenic mycelia of *P. involutus* strains MAJ and NAU after being exposed to 400 mM NaCl for 24 h.

**Supplemental Figure S5.** Representative images showing real-time flux measurements of NM roots, EM roots, and axenically grown EM cultures.

**Supplemental Table S1.** Interfering effects of $K^+$ and $Ca^{2+}$ on $Na^+$-selective electrodes.

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