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RESEARCH PAPER

# Effects of non-uniform root zone salinity on water use, $Na^+$ recirculation, and $Na^+$ and $H^+$ flux in cotton

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## Abstract

**A new split-root system was established through grafting to study cotton response to non-uniform salinity. Each root half was treated with either uniform (100/100 mM) or non-uniform NaCl concentrations (0/200 and 50/150 mM). In contrast to uniform control, non-uniform salinity treatment improved plant growth and water use, with more water absorbed from the non- and low salinity side. Non-uniform treatments decreased $Na^+$ concentrations in leaves. The [$Na^+$] in the '0' side roots of the 0/200 treatment was significantly higher than that in either side of the 0/0 control, but greatly decreased when the '0' side phloem was girdled, suggesting that the increased [$Na^+$] in the '0' side roots was possibly due to transportation of foliar $Na^+$ to roots through phloem. Plants under non-uniform salinity extruded more $Na^+$ from the root than those under uniform salinity. Root $Na^+$ efflux in the low salinity side was greatly enhanced by the higher salinity side. NaCl-induced $Na^+$ efflux and $H^+$ influx were inhibited by amiloride and sodium orthovanadate, suggesting that root $Na^+$ extrusion was probably due to active $Na^+/H^+$ antiport across the plasma membrane. Improved plant growth under non-uniform salinity was thus attributed to increased water use, reduced leaf $Na^+$ concentration, transport of excessive foliar $Na^+$ to the low salinity side, and enhanced $Na^+$ efflux from the low salinity root.**

**Key words:** Cotton, $Na^+/H^+$ antiporter, non-uniform salinity, split-root system, water use.

## Introduction

Soil salinity is one of the most widespread abiotic stresses and constitutes a stringent factor limiting plant productivity (Wang *et al.*, 2003). Plant response to salt stress can differ greatly depending on environmental factors in the soil. One of these factors is the distribution of salts in the root environment (Meiri and Plaut, 1985; Sonneveld and de Kreij, 1999). Soil salinity is often heterogeneous in saline fields, and thus it has attracted a number of studies on plant response to non-uniform salinity in the root zone (Meiri and Plaut, 1985; Dong *et al.*, 2008, 2010; Bazihizina *et al.*, 2009). Non-uniform salinity has been simulated with a plit-root system in the greenhouse or growth chamber, in which the root system was divided into two or more equal portions and each portion irrigated with varied concentrations of NaCl solution (Shani *et al.*, 1993; Messedi *et al.*, 2004; Lycoskoufis *et al.*, 2005; Bazihizina *et al.*, 2009; Dong *et al.*, 2010). Compared with uniform salinity, non-uniform salinity in the root zone increased the growth of tomato (Tabatabaie *et al.*, 2003), cucumber (Sonneveld and de Kreij, 1999), orange (Zekri and Parsons, 1990), and *Atriplex nummularia* (Bazihizina *et al.*, 2009). More recent studies showed that furrow seeding with plastic mulching could increase cotton growth and lint yield in saline fields because this practice resulted in non-uniform salinity in soil surface layers (Dong *et al.*, 2008, 2010). However, the underlying mechanism of growth improvement by non-uniform salinity is far from clear.

Soil salinity inhibits plant growth mainly due to osmotic stress and ion toxicity (Munns and Tester, 2008; Gorham *et al.*, 2009). High concentrations of salts in the soil make it

difficult for roots to absorb water, and high accumulation of salts in plant tissues results in ion toxicity (Munns and Tester, 2008). Plants can only grow under conditions of osmotic stress if they osmotically adjust. The most important means of osmotic adjustment is through the increased uptake of inorganic ions, both in halophytes (Flowers and Colmer, 2008; Hariadi *et al.*, 2011) and in glycophytes (Shabala and Lew, 2002). When roots were subjected to salinity, the water use of the whole plant decreased as NaCl concentrations in the medium increased (Shani *et al.*, 1993; Bazihizina *et al.*, 2009). However, when the plant roots were subjected to non-uniform salinity saline environments, the water use of the whole plant was higher than when roots were subject to high uniform salinity, and more water was absorbed from the low salinity side (Shani *et al.*, 1993; Bazihizina *et al.*, 2009).

Another specific feature of salt stress is $Na^+$ accumulation in plant tissues. Upon prolonged exposure of a plant to NaCl, $Na^+$ is translocated from the roots to the transpiring leaves where it can accumulate to toxic levels. Leaf $Na^+$ usually accumulates less under non-uniform than under uniform salinity (Shani *et al.*, 1993; Bazihizina *et al.*, 2009; Dong *et al.*, 2010). Many studies have suggested that $Na^+$ sequestration in vacuoles or recirculation from shoots to roots are two efficient ways to protect leaf cells from $Na^+$ injury (Apse *et al.*, 1999; Zhang and Blumwald, 2001; Munns, 2002; Berthomieu *et al.*, 2003). In the case of $Na^+$ recirculation, uptake from the xylem into pith cells of the stem must occur, followed by $Na^+$ efflux directed towards the phloem vessel. The pith cell which communicates between the xylem and phloem played a decisive role in the recirculation of $Na^+$ to the root through phloem in sweet pepper (Blom-Zandstra *et al.*, 1998). Berthomieu *et al.* (2003) confirmed that AtHKT1 is involved in $Na^+$ recirculation from shoots to roots, probably by mediating $Na^+$ loading into the phloem sap in shoots and unloading in roots.

Another means of sustaining $Na^+$ homeostasis in the cytosol is $Na^+$ extrusion to the apoplast or external environment (Blumwald *et al.*, 2000; Tester and Davenport, 2003; Zhu, 2003; Apse and Blumwald, 2007). The plasma membrane (PM) $Na^+/H^+$ antiporter, SOS1, is an important tolerance determinant, involved in the exclusion of sodium ions from cells (Shi et al., 2000, 2002; Martínez-Atienza *et al.*, 2007). This antiporter forms one component in a mechanism based on sensing of the salt stress that involves increases in cytosolic $[Ca^{2+}]$, protein interactions, and reversible phosphorylation, with SOS1 acting in concert with SOS2 and SOS3 (Halfter *et al.*, 2000; Quintero *et al.*, 2002; Guo *et al.*, 2004). Sodium chloride-induced activity of the PM $Na^+/H^+$ antiporter has been reported in many plant species such as tomato (Wilson and Shannon, 1995), *Arabidopsis* (Qiu et al., 2002, 2003), rice (Martínez-Atienza *et al.*, 2007), *Thellungiella* (Oh *et al.*, 2009), and *Populus* (Sun *et al.*, 2009). Overexpression of the $Na^+/H^+$ antiporter genes *AtSOS1* and *ThSOS1* decreased the accumulation of $Na^+$ in transgenic *Arabidopsis* under NaCl stress and increased the salt tolerance of *Arabidopsis* (Shi *et al.*, 2002; Oh *et al.*, 2009). Extrusion of $Na^+$ through these $Na^+/H^+$ antiporters is driven by an inwardly directed proton gradient created by $H^+$-ATPases (Blumwald *et al.*, 2000; Zhu, 2003). NaCl-induced $H^+$ pumping is therefore fundamental to $Na^+/H^+$ exchange and salinity tolerance (Ayala *et al.*, 1996; Vitart *et al.*, 2001; Chen *et al.*, 2007; Gévaudant *et al.*, 2007). Using the scanning ion-selective electrode technique, Sun *et al.* (2009) found that $Na^+/H^+$ exchange in root tissues and cells was inhibited by amiloride (an $Na^+/H^+$ antiporter inhibitor) and sodium orthovanadate (a PM $H^+$-ATPase inhibitor), and the $Na^+$ extrusion in stressed *Populus euphratica* roots was the result of an active $Na^+/H^+$ antiport across the PM with the involvement of PM $H^+$-ATPase.

In the present study, a split-root system established through grafting was used to study physiological response to non-uniform salinity in cotton, focusing on: (i) water uptake from both sides of the split root; (ii) $Na^+$ concentration in various plant tissues; (iii) the recirculation of $Na^+$ to and its extrusion from roots under salinity stress; and (iv) the mechanism of $Na^+$ extrusion from cotton roots under salinity stress.

## Materials and methods

### *Establishment of plant culture and split-root system*

A commercial Bt (*Bacillus thuringiensis*) transgenic cotton (*Gossypium hirsutum* L.), SCRC28 developed by the Cotton Research Center, Shandong Academy of Agricultural Sciences, Jinan, was used in the experiment. Acid-delinted seeds were sown at ~3 cm depth in plastic boxes (60 cm×45 cm×15 cm) containing sterilized wet sand. Boxes were placed in growth chambers with light/dark regimes of 16/8 h, light intensity of 400 μmol $m^{-2}$ $s^{-1}$ PAR, and temperature of 30±2 °C. At full emergence, seedlings were thinned to 100 plants per box.

When most seedlings reached the two true leaf stage, uniform seedlings were carefully removed from the sand and washed with water. Split-root systems were established through grafting with these seedlings (Fig. 1). Briefly, a '/' shaped incision was made with a blade on the hypocotyl 2 cm below the two cotyledons, leaving about one-third of the hypocotyl tissues intact. The top of the rootstock was cut to form a deep 'Δ' at the same position of the hypocotyl from another seedling. The 'Δ' section was then inserted into the '/' incision of the plant and closely wrapped with parafilm. Grafted seedlings were transferred to plastic pots containing aerated nutrient solution, sprayed with water, and immediately covered with plastic bags to prevent wilting. The nutrient solution was topped up with deionized water as required and renewed weekly. The solution consisted of (mM): 1.25 $Ca(NO_3)_2$, 1.25 $KNO_3$, 0.5 $MgSO_4$, 0.25 $NH_4H_2PO_4$, 0.05 EDTA-FeNa; and (μM): 10 $H_3BO_3$, 0.5 $ZnSO_4$, 0.1 $CuSO_4$, 0.5 $MnSO_4$, 0.0025 $(NH_4)_6Mo_7O_{24}$, and was adjusted to pH 6 with KOH. When a new leaf emerged from the grafted seedling at 2 weeks after grafting, the plastic bag and parafilm were removed. Grafted seedlings with two uniform split-root systems were transferred to a naturally lit greenhouse to grow under 28–32/20–24 °C and 60–70% relative humidity for 20 d. Nutrient solutions were renewed daily during the period of growth. Healthy seedlings with two uniform split-root systems (Fig. 1B, C) were selected for further experiments.

### *Non-uniform salinity and water use measurements*

Healthy seedlings with uniform split roots were selected and each root portion was put into one of two pots (15 cm in height and

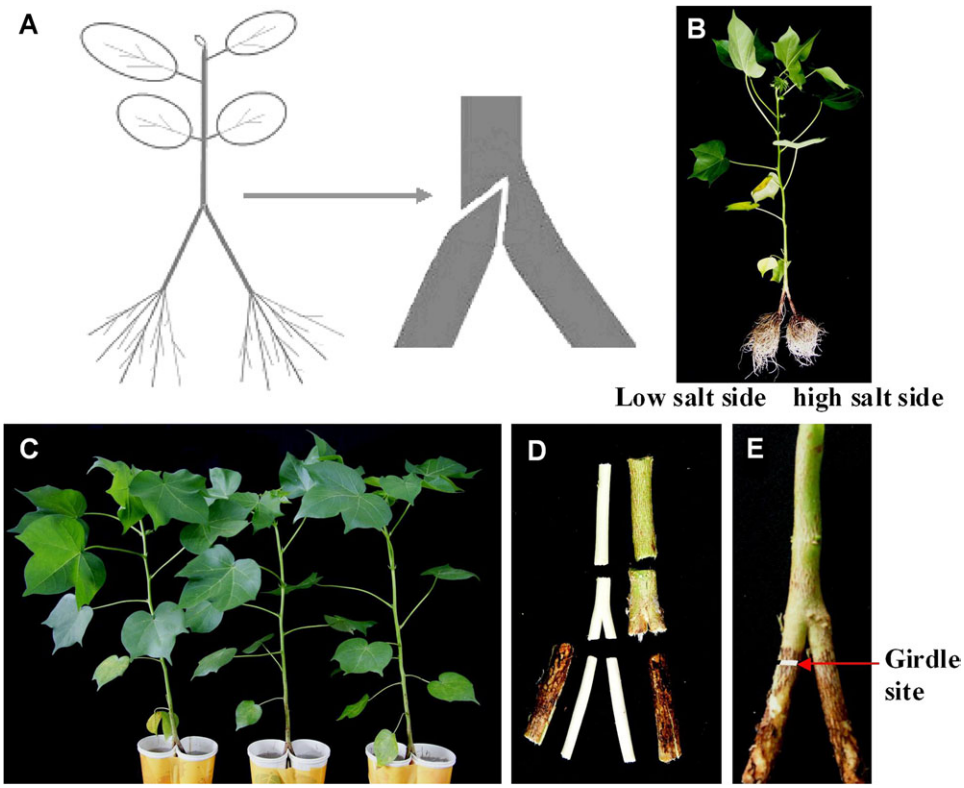


**Fig. 1.** Split-root system used in this study, showing the schematic diagram of the graft–split-root system (A); root system of cotton exposed to uniform NaCl concentrations (equal on both root sides, such as 0/0 mM and 100/100 mM NaCl treatment) or to non-uniform NaCl concentrations (different in the low and high salt sides, such as 0/200 mM and 50/150 mM NaCl treatment) for 1, 3, and 7 d (B); the grafted plants were uniform before treatment (C); the xylem and phloem of the two halves of the hypocotyl and stem were harvested separately after treatment (D); the girdled position of the grafted cotton is shown (E).

10 cm in diameter) held together by adhesive tape. Thus the two root portions of each seedling could be exposed to different NaCl concentrations at the same time (Fig. 1C). The two root portions under the same or different salt treatments were denoted as 0/0, 0/200, 50/150, and 100/100. In the non-uniform salinity treatments (0/200 mM and 50/150 mM NaCl), the low salt side was indicated as the 0 or 50 side, while the high salt side was indicated as the 200 or 150 side. It should be noted that either side of a uniform salinity treatment (0/0 or 100/100 mM NaCl) was considered as the low or high salt side (Fig. 1B).

To measure water use, two similar pots containing 1.0 liter of nutrient solutions were fitted into the potted split-root system and then the roots of the grafted plant were positioned in the split-root pot. The pots and nutrient solutions were measured and renewed in the morning (7:00–7:30 h) every day and then the water use was determined.

### *NaCl stress and girdling treatments*

The NaCl was increased in both compartments of all the split-root pots in increments of 12.5, 25, 37.5, and 50 mM every 12 h, until NaCl concentrations reached 50, 100, 150, and 200 mM, respectively. The nutrient solutions containing different NaCl concentrations were renewed in the morning (7:00–7:30 h) each day after reaching the final concentrations. Plants irrigated with non-saline nutrient solution served as the 0/0 control. Non-uniform salinity treatments such as 0/200 or 50/150 were established by irrigating one root portion with 0 mM or 50 mM NaCl, and the other part with 200 mM or 150 mM NaCl. A uniform salinity treatment (100/100) was achieved by irrigating both root portions with equal concentrations of NaCl solutions (100 mM NaCl).

For the girdling treatment, stems (hypocotyls) were girdled by cutting through the bark with a razor blade and removing the entire ring of bark including phloem without injuring the xylem (Wilson and Gartner, 2002; Dai and Dong, 2011). The completeness of total phloem removal was checked ~20 min after girdling. A proper girdle is characterized by the appearance of an all white, fibrous xylem ring (Fig. 1E). Any browning of the ring indicates an incomplete girdle and the remaining tissue must be cleaned out. In this study, a 3 mm width of bark on one side of the hypocotyl at 2 cm below the grafted position was removed with a blade to stop nutrient flux through the phloem (Fig. 1E). Girdling was performed on either side of a split-hypocotyl under 0/0 mM NaCl treatment, and on the 0/200-0 side in the non-uniform salinity treatment (0/200). The experiment was arranged into a completely randomized design with six replications.

### *Plant sampling*

Plants were sampled at 1, 3, and 7 d after treatments. To determine differences between sides, the two halves of the root system and hypocotyls were harvested separately and the stem above the two cotyledons was also sampled separately.

Cotton is a woody plant and the hypocotyl at the seedling stage mainly consists of the bark, cambium, xylem, and pith tissues (from the outer to the inner). The bark mainly includes the epidermis in the outmost part and the phloem tissues beneath the epidermis. Between the xylem and phloem is the cambium, which can be a few cell layers thick with the only function of renewing both the xylem and phloem tissues. As the cambium is very active in cotton seedlings, it forms a very clear boundary between the xylem and phloem, making the xylem and phloem tissues very easy to separate. In this study, tissues inside the cambium were

collectively referred to as xylem tissues (mainly xylem and pith) and those outside the cambium were considered as phloem tissues (mainly phloem and epidermis). The xylem and phloem were separated by hand immediately after harvesting (Fig. 1D). Briefly, a vertical incision was made on each stem (hypocotyls) sample with a double-sided blade. The incision was 2 mm deep and equivalent to the hypocotyl in length. The bark (xylem tissue) was then peeled from the xylem tissues with the help of the incision. Roots were washed three times with deionized water. For clarity, the root systems in the (0/0)-0, (0/200)-0, (50/150)-50, and (100/100)-100 sides were described as the low salt side and the other side as the high salt side (Fig. 1B). Similarly, the hypocotyls (including xylem and phloem) in the (0/0)-0, (0/200)-0, (50/150)-50, and (100/100)-100 sides were described as the low salt side and the other side as the high salt side; the portion above the two cotyledons was described as the stem. The hypocotyls in the low and high salt sides as well as the stem were each 4 cm long, and their cutting positions for the different treatments were similar (Fig. 1D).

### *Physiological measurements*

Plants were removed from pots by hand and all the organs were collected. Fresh weights were recorded. Dry weights were determined after oven drying for 48 h at 80 °C. Net photosynthetic and transpiration rates, and stomatal conductance of the third fully expanded young leaf from the end on the main stem were measured between 09:00 h and 11:00 h on cloudless days when ambient photosynthetic photon flux density exceeded 1500 μM $m^{-2}$ $s^{-1}$, using a LI-6400 portable photosynthesis system (Li-Cor, Lincoln, NE, USA). Osmotic potentials of the third fully expanded young leaf were determined with a freezing point osmometer (Fiske Associates, Needham Heights, MA, USA). All samples (the third main stem leaf, root, xylem, and phloem) were digested with $HNO_3$. Concentrations of $Na^+$ and $K^+$ were determined using an atomic absorption spectrophotometer (TAS-990, Beijing, China).

### *Measurements of net $Na^+$ and $H^+$ flux with NMT*

Net fluxes of $Na^+$ and $H^+$ were measured non-invasively using the Non-Invasive Micro-Test Technology (NMT) [NMT system BIO-IM; Younger USA, LLC.; Amherst, MA (Kühtreiber and Jaffe, 1990; Kochian *et al.*, 1992; Vincent *et al.*, 2005; Xu *et al.*, 2006; Sun *et al.*, 2009)]. The concentration gradients of $Na^+$ and $H^+$ were measured by moving the ion-selective microelectrode between two positions close to the plant material in a pre-set excursion (30 μm for excised roots in the present experiment). The NMT can measure ionic fluxes down to pM/c$M^{-2}$ $s^{-1}$ levels but must be measured slowly at ~5 s per point.

Pre-pulled and silanized glass micropipettes (2–4 μM aperture, XY-Na-04, XY-H-01; Xuyue Sci. & Tech. Co., Ltd) were first filled with a backfilling solution (Na, 100 mM NaCl; H, 40 mM $KH_2PO_4$; and 15 mM NaCl, pH 7.0) to a length of ~1 cm from the tip. Then the micropipettes were front filled with ~25 μM columns of selective liquid ion-exchange cocktails (LIXs: Na, Sigma 71178; H, Sigma 95293). An Ag/AgCl wire electrode holder (XY-ER-01; Xuyue Sci. and Tech. Co. Ltd.) was inserted in the back of the electrode to establish an 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: (i) $Na^+$, 0.5 mM and 5.0 mM (the $Na^+$ concentration was 0.9 mM in the measuring buffer for root samples); (ii) $H^+$, pH 5.5 and 6.5 (the pH of the measuring buffer was adjusted to 6.0 for root samples).

Only electrodes with Nernstian slopes ~58 mV per decade were used in the present study. Ion flux was calculated by Fick's law of diffusion:

$$J = -D(\mathrm{d}c/\mathrm{d}x)$$

where $J$ represents the ion flux in the $x$ direction, $\mathrm{d}c/\mathrm{d}x$ 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 IM-Flux software, part of the NMT system.

After exposure to the saline (1 d and 7 d) treatments, root segments with 2–3 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, and the flux rate decreased gradually to a steady level within 10 min. The measuring site was 5000 μM from the root apex, in which a vigorous flux of $Na^+$ or $H^+$ was usually observed. After equilibrating for 30 min, roots were transferred to the measuring chamber containing 10–15 ml of a fresh measuring solution. After the roots were immobilized on the bottom, ion flux measurements were started. The measured positions of roots could be visualized and defined under the NMT microscope because young roots were semi-transparent under light. Ions ($Na^+$ and $H^+$) were monitored in the following solutions: (i) $Na^+$, 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; (ii) $H^+$, 0.1 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5 mM NaCl, 0.2 mM $Na_2SO_4$, pH 6.0 adjusted with NaOH and HCl. Although buffered solutions could affect the actual magnitude of the $H^+$ flux (Arif *et al.*, 1995), the qualitative tendency of NaCl-induced $H^+$ flux was not altered.

The inhibitory effects of PM transport inhibitors on ion fluxes were examined in cotton roots. Roots were subjected to 500 μM sodium orthovanadate (a PM $H^+$-ATPase inhibitor) or 100 μM amiloride (a $Na^+/H^+$ antiporter inhibitor) for 30 min. Then measuring solutions containing sodium orthovanadate were removed slowly with a pipette and 10 ml of a 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 also scanned at location which was 5000 μM from the root apex.

Two-dimensional ionic fluxes were calculated using MageFlux developed by Yue Xu (http://xuyue.net/mageflux). Positive values in figures represent efflux.

### *Statistical analysis*

An analysis of variance was performed using PROC MIXED in the Statistical Analysis System (SAS, v. 9.1, SAS Institute, Cary, NC, USA). Treatment means were separated with Duncan's multiple range test at $P < 0.05$.

## Results

### *Plant growth and photosynthetic parameters*

In Table 1, comparisons were made of plant performance with uniform treatments of 0/0 mM and 100/100 mM NaCl versus two non-uniform treatments, in which the average root-zone salinity was 100 mM NaCl, namely 50/100 mM and 0/200 mM NaCl. Plant growth, photosynthesis (Pn), transpiration (Tr), chlorophyll (Chl), and stomatal conductance (Cond) were significantly reduced under salinity stress regardless of salt distribution in both root portions (Table 1). Compared with the 0/0 mM NaCl control treatment, the 100/100 mM NaCl treatment (uniform salinity) decreased fresh weight (FW) by 24.3%, dry weight (DW) by 31.7%, Pn by 35.2%, Tr by 35.7%, Chl by 24.6%, and Cond by 57.6% at 7 d after stress. Treatment with 0/200 mM

**Table 1.** *Effects of non-uniform root zone salinity on cotton fresh weight (FW), dry weight (DW), net photosynthetic (Pn) and transpiration (Tr) rates, chlorophyll (Chl), and stomatal conductance (Cond) in the third main stem leaves from the end at 7 d after salinity stress*
Value of the NaCl concentration expressed in mM is given as x/y, which denotes the values in both root parts.

| NaCl (mM) | Shoot FW (g per plant) | Shoot DW (g per plant) | Pn ($\mu$mol $CO_2$ $m^{-2}$ $s^{-1}$) | Tr (mmol $m^{-2}$ $s^{-1}$) | Chl (mg $g^{-1}$ FW) | Cond (mol $m^{-2}$ $s^{-1}$) |
|---|---|---|---|---|---|---|
| 0/0 | 68.3 a | 12.09 a | 24.4 a | 3.39 a | 20.7 a | 0.405 a |
| 0/200 | 62.5 b | 10.54 b | 22.3 b | 3.25 a | 18.9 b | 0.348 b |
| 50/150 | 57.5 c | 9.32 c | 18.7 c | 2.91 b | 17.3 c | 0.282 c |
| 100/100 | 51.7 d | 8.26 d | 15.8 d | 2.18 c | 15.6 d | 0.176 d |

Means ($n$=6) within a column followed by the same letters are not significantly different at $P < 0.05$

NaCl (non-uniform salinity) decreased the FW, DW, Pn, Tr, Chl, and Cond by 8.5, 12.8, 8.6, 4.1, 8.7, and 16.1%, respectively, while the 50/150 mM NaCl (non-uniform salinity) treatment decreased them by 15.8, 22.9, 23.4, 14.2, 16.4, and 32%, respectively. However, compared with the uniform salinity control (100/100 mM NaCl), the non-uniform salinity treatments (0/200 mM and 50/150 mM NaCl) increased the FW by 20.9% and 11.2%, the DW by 27.6% and 12.8%, Pn by 41.1% and 18.4%, Tr by 49.1% and 33.5%, Chl by 21.2% and 10.9%, and Cond by 97.7% and 60.2% (Table 1). Between the non-uniform salinity treatments, the FW, DW, Pn, Tr, Chl, and Cond under the 0/200 mM NaCl treatment were higher than those under the 50/150 mM NaCl treatment (Table 1).

### *Water use*

Data for water uptake and use at 7 d after NaCl treatment are shown in Fig. 2. In comparison with the 0/0 control, whole plant water use decreased 19.2% and 48.3% in the non-uniform salinity (0/200 mM and 50/150 mM NaCl) treatments and 62.3% in the uniform salinity (100/100 mM NaCl) treatment (Fig. 2). The non-uniform salinity (0/200 mM and 50/150 mM NaCl) treatments significantly increased the whole plant water use by 114.1% and 37.1% compared with the uniform salinity control (Fig. 2). Under non-uniform salinity, 86.4% and 76.6% of the water was absorbed from the non- and lower salinity side of roots, and only 15.6% and 23.4% was absorbed from the higher salinity side (Fig. 2). Although plants absorbed more water from the '0' side and less from the '200' side in the 0/200 treatment than either side in the 0/0 treatment, the total water use for the whole plant in the 0/200 treatment was lower than that in the 0/0 treatment (Fig. 2).

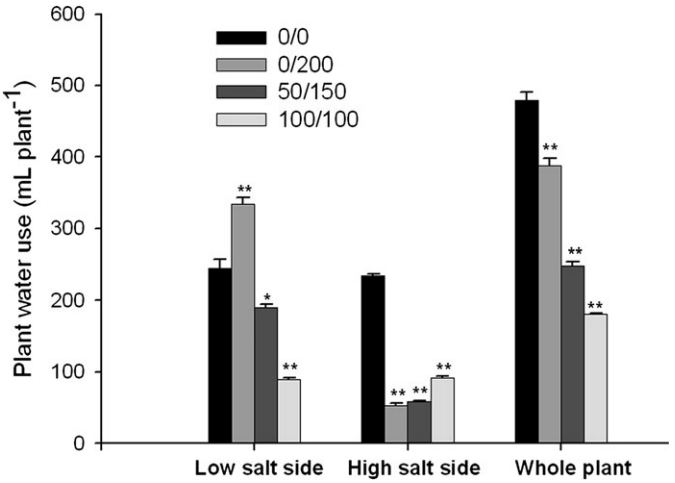


**Fig. 2.** Effects of non-uniform root zone salinity on whole plant water use 7 d after salinity stress (B). Values are means ±SE ($n$=6). * and ** show significant differences from the 0/0 control at $P < 0.05$ and $P < 0.01$.

### *Osmotic potential and tissue $Na^+$ and $K^+$ concentration*

The osmotic potential of the third main stem leaves was significantly reduced under salinity stress regardless of salt distribution in both root portions (Fig. 3). However, non-uniform salinity (0/200 mM and 50/150 mM NaCl) treatments significantly increased the osmotic potential by 30.4% and 12.3%, compared with uniform salinity (100/100 mM NaCl). The osmotic potential of the 0/200 mM NaCl treatment was significantly higher than that of the 50/150 mM NaCl treatment.

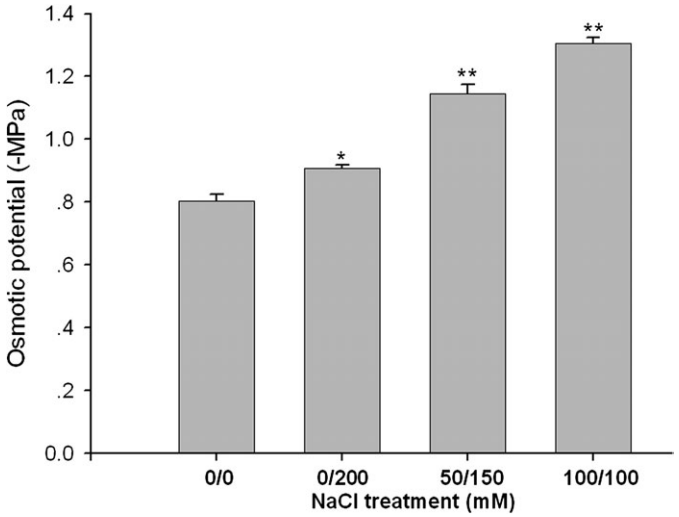


**Fig. 3.** Effects of non-uniform root zone salinity on osmotic potential in the third main stem leaves at 7 d after salinity stress. Values are means ±SE ($n$=6). * and ** show significant differences from the 0/0 control at $P < 0.05$ and $P < 0.01$.

The $Na^+$ and $K^+$ concentrations in leaves, roots, xylem, and phloem of the stems and hypocotyls were measured at 1, 3, and 7 d after treatment (Figs 4, 5). As $Na^+$ and $K^+$ concentrations were not significantly different between two hypocotyls as well as roots of plants under uniform salinity (0/0 mM and 100/100 mM NaCl), the data presented are pooled averages (Figs 4, 5). The $Na^+$ concentration in leaves, roots, xylem, and phloem of the stems increased with increasing NaCl treatment time and concentrations (Fig. 4). Compared with uniform salinity (100/100 mM NaCl), non-uniform salinity (0/200 mM and 50/150 mM NaCl) decreased the $Na^+$ concentration in the leaves (Fig. 4A), xylem, and phloem (stem) (Fig. 4F, I). The $Na^+$ concentration in the root, hypocotyls xylem and phloem on the '0' side in the 0/200 treatment was similar to that on either side in 0/0 at 1 d and 3 d after treatment (Fig. 4B, D, G). However, the $Na^+$ concentration on the '0' side increased by 91.7, 50.9, and 43.4 %, respectively, compared with either side in the 0/0 treatment at 7 d after NaCl treatment (Fig. 4B, D, G). Under NaCl treatments, the $Na^+$ concentration in the xylem and phloem of the high salt side was much higher than that in the stem (Fig. 4E, F, H, I). The $Na^+$ concentration in the phloem was ~1.2-fold higher than in the xylem under 0/0 mM NaCl treatment and ~1.6-fold higher under NaCl treatments (data not shown).

The $K^+$ concentration in leaves, roots, xylem and phloem of the stems decreased with increasing NaCl treatment time and concentrations in the medium (Fig. 5). However, non-uniform (0/200 mM and 50/150 mM NaCl) salinity increased the $K^+$ concentration in leaves (Fig. 5A) as well as in the xylem and phloem of stems (Fig. 5F, I). The patterns of the $K^+/Na^+$ ratio were similar to that of $K^+$ under NaCl treatment, and non-uniform salinity also significantly increased the $K^+/Na^+$ ratio in the leaf and stem relative to the uniform salinity control (data not shown).

### *Girdling effects on $Na^+$ in split-root plants*

Girdling was performed 2 cm below the grafted position on either side of the split-hypocotyl under 0/0 mM NaCl treatment and on the 0/200-0 side in the non-uniform salinity treatment (0/200). Without salinity stress (0/0 mM NaCl), the $Na^+$ concentration in leaves and roots of the girdled plants was similar to that of the non-girdled plants (Fig. 6). The concentration of $Na^+$ in roots on the '200' side of 0/200 treatment also did not differ from non-girdled plants (Fig. 6B). However, girdling increased leaf $Na^+$ by 15.6%, and decreased root $Na^+$ concentration in the '0' side of 0/200 by 43.7% at 7 d after NaCl treatment (Fig. 6).

### *Root $Na^+$ and $H^+$ fluxes in response to salt stress and PM transport inhibitors*

There was a net $Na^+$ influx in cotton root under 0/0 mM NaCl treatment, but the net $Na^+$ flux was reversed to an

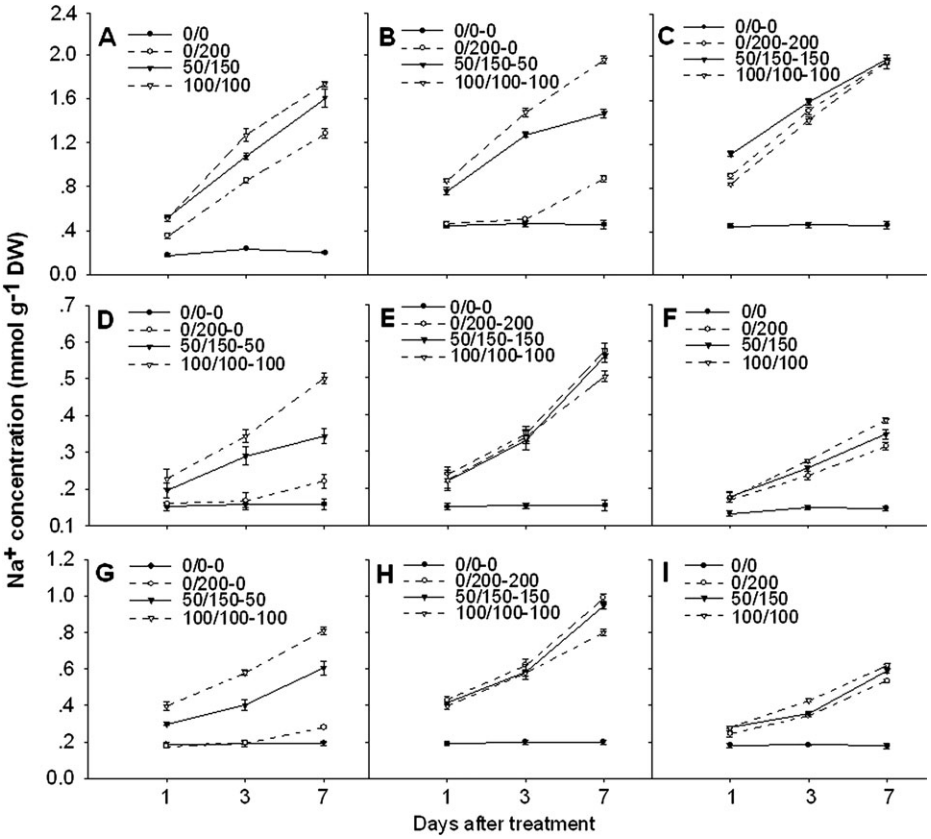


**Fig. 4.** Concentration of $Na^+$ in the third main stem leaves (A); roots (B), xylem (D), and phloem (G) of the low salt side; roots (C), xylem (E), and phloem (H) of the high salt side, and xylem (F) and phloem (I) of the stem at 1, 3, and 7 d after 0/0, 0/200, 50/150, and 100/100 mM NaCl treatment. Values are means ±SE ($n$=6).

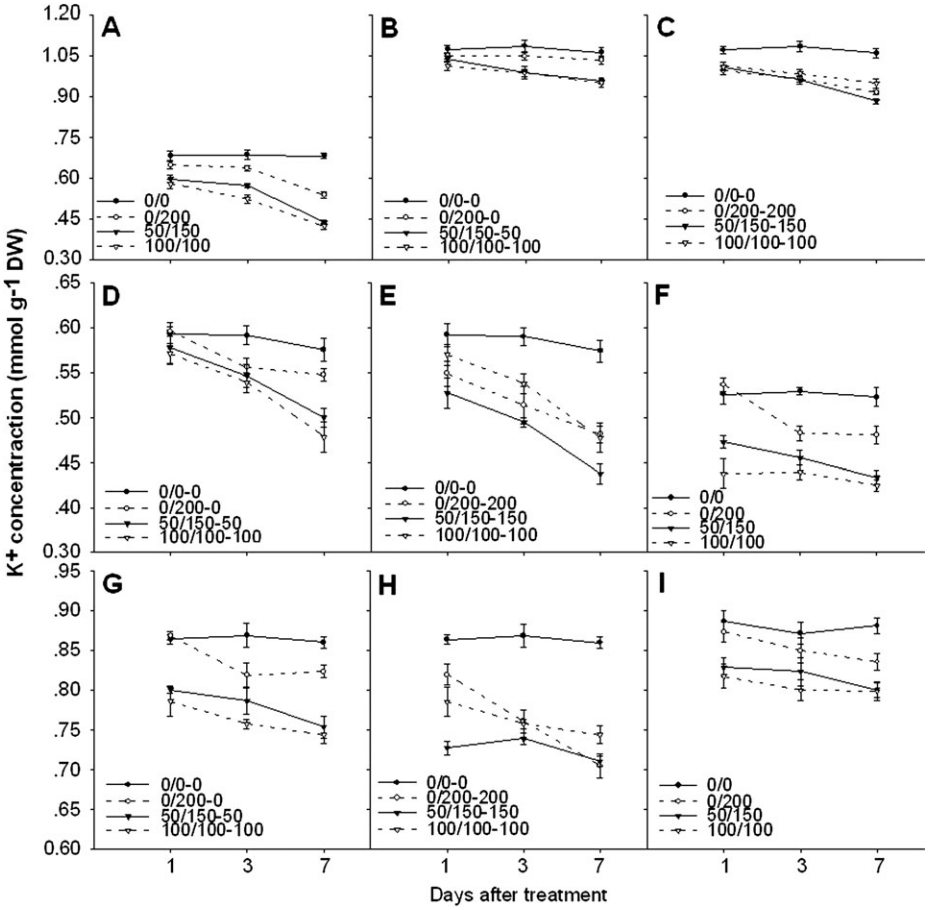


**Fig. 5.** The $K^+$ concentration in the third main stem leaves (A); roots (B), xylem, (D), and phloem (G) of the low salt side; roots (C), xylem, (E), and phloem (H) of the high salt side, and xylem (F) and phloem (I) of the stem at 1, 3, and 7 d after 0/0, 0/200, 50/150, and 100/100 mM NaCl treatment. Values are means ±SE ($n$=6).

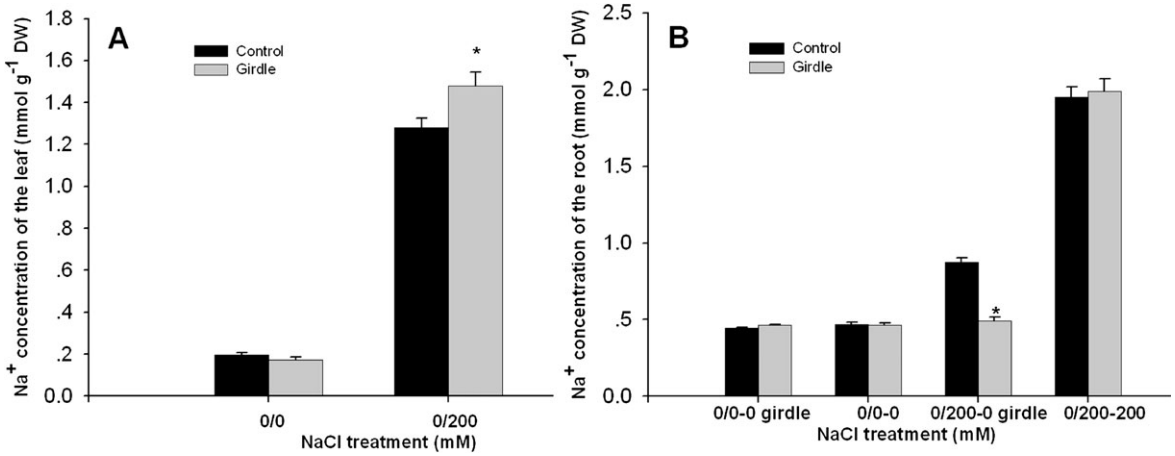


**Fig. 6.** Comparison of the $Na^+$ concentration in the third main stem leaves (A) and roots (B) in girdled and non-girdled plants at 7 d after 0/0 mM and 0/200 mM NaCl treatment. Values are means ±SE ($n$=6). * denotes significant differences at $P < 0.05$.

efflux when treated with NaCl and the efflux increased as the NaCl concentration increased (Fig. 7). Interestingly, the $Na^+$ influx in the '0' side of the 0/200 mM NaCl treatment was reversed to an efflux compared with the 0/0 mM NaCl treatment control, possibly due to the NaCl treatment on the 0/200-200 side (Fig. 7; Supplementary Fig. S1 available at *JXB* online). The net $Na^+$ efflux in the '150' side of 50/150 mM NaCl treatment was similar to either side in the 150/150 mM NaCl treatment, but the '50' side of the 50/150 mM NaCl treatment was higher than either side in the 50/50 mM NaCl treatment at 1 d and 7 d after treatments (Fig. 7). A net $H^+$ efflux in cotton root was also observed

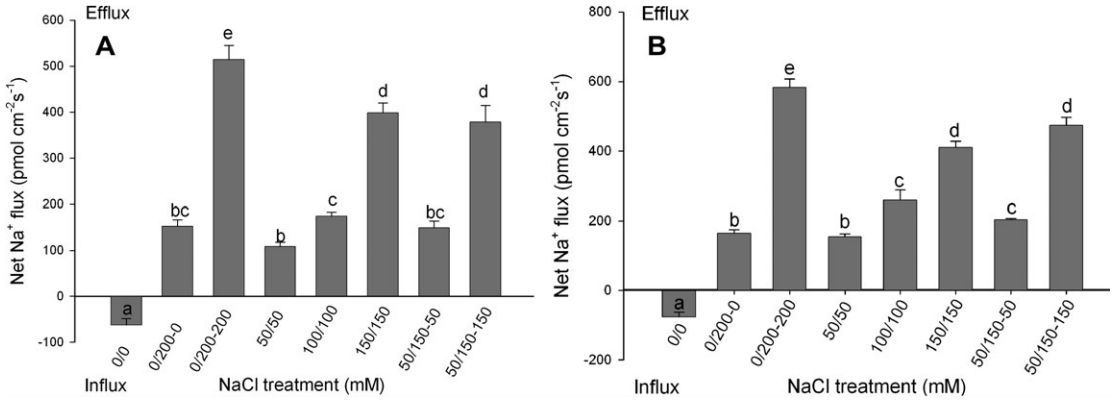


**Fig. 7.** Effects of non-uniform (0/200 mM and 50/150 mM NaCl) and uniform (0/0, 50/50, 100/100, and 150/150 mM NaCl) root zone salinity on net $Na^+$ fluxes in roots of cotton at 1 d (A) and 7 d (B) after treatment. The data are main fluxes of $Na^+$ within the measuring periods (15 min). Values are means ±SE ($n$=6). Bars with different letters (a, b, and c) differ significantly at $P < 0.05$.

under 0/0 mM NaCl treatment, but the net $H^+$ flux was reversed to influx when treated with NaCl while the net $H^+$ influx increased as the NaCl concentration rose in the medium at 1 d after treatment (Fig. 8B). The $H^+$ flux on the '0' side root of the 0/200 mM NaCl treatment was reversed to influx compared with the 0/0 mM NaCl treatment control, which may have been induced by the NaCl treatment in the 0/200-200 side (Fig. 8B).

Amiloride (100 μM), an inhibitor of the $Na^+/H^+$ antiporter, and sodium orthovanadate (500 μM), an inhibitor of PM $H^+$-ATPase, significantly reduced the NaCl-induced $Na^+$ efflux and $H^+$ influx in roots, although the reduction in $Na^+$ efflux was greater than that in $H^+$ influx (Fig. 8; Supplementary Fig. S2 at *JXB* online). The $Na^+$ efflux and $H^+$ influx in the '0' side of 0/200 were also reduced by amiloride and orthovanadate (Fig. 8). Under 0/0 mM NaCl treatment, amiloride had no effect on $Na^+$ and $H^+$ flux. Orthovanadate did not affect $Na^+$ flux but significantly reduced $H^+$ efflux under 0/0 mM NaCl treatment (Fig. 8).

## Discussion

The split-root system has been used to study plant response to heterogeneous soil conditions such as partial root drying (Lawlor, 1973; Sobeih *et al.*, 2004), unequal salt distribution (Shani *et al.*, 1993; Messedi *et al.*, 2004; Lycoskoufis *et al.*, 2005; Bazihizina *et al.*, 2009), and heterogeneous nutrient distribution (Drew and Saker, 1978; Arredondo and Johnson, 1999; Paterson *et al.*, 2006). The conventional split-root system in cotton is established by dividing the lateral roots into two equal parts after cutting of the main root of a seedling (Dong *et al.*, 2010); however, in this study, we established an entirely new split-root system by grafting cotton seedlings (Fig. 1). In contrast to the conventional split-root, the main roots of the new system remained intact, which may provide a better and more convenient system for studying cotton response to unequal salt distribution in the root zone. The new system was suitable for plant growth in nutrient solution and for a girdling experiment because there was some distance between the root and position of the graft, so the two root systems could be fully immerged in the nutrient solution (Fig. 1). In the new system, it was found that plant growth, leaf photosynthesis, transpiration, chlorophyll concentration, and stomatal conductance were significantly inhibited by salinity stress regardless of salt distribution in both root portions (Table 1). The decreased shoot dry mass was correlated with decreased photosynthesis, which in turn was correlated with decreased transpiration, stomatal conductance, and leaf chlorophyll concentration. These correlations suggest that the decreases in plant growth were caused by reduced photosynthesis (Supplementary Fig. S3 at *JXB* online). However, compared with uniform salinity (100/100 mM NaCl), non-uniform salinity (0/200 mM and 50/150 mM NaCl) increased plant growth, photosynthesis, transpiration, and stomatal conductance as previously reported for conventional split-root systems (Papadopoulos *et al.*, 1985; Bazihizina *et al.*, 2009; Dong *et al.*, 2008, 2010).

### *Water uptake and use*

The presence of salt in the medium imposes both ionic and osmotic stresses on plants (Tester and Davenport, 2003), as well as a disturbance of mineral nutrient uptake by roots (Silberbush and Ben-Asher, 2001). In some plant species, one portion of the root system can compensate for the dwindling supply of water to the other portion. Well-supplied parts of apple root systems (West, 1978) or tomato (Papadopoulos *et al.*, 1985) compensated for the dwindling supply of water by salinized roots. However, reduced water use for a whole plant was recorded in *A. nummularia* where the root system was split and exposed to non-uniform salinity, because the low salinity side of roots could not fully compensate for the reduced uptake by the high salinity side (Bazihizina *et al.*, 2009). In the present study, total water use decreased under salinity stress regardless of salt distribution in both root portions. However, compared with uniform salinity (100/100 mM NaCl), non-uniform salinity (0/200 mM and 50/150 mM NaCl) significantly increased whole plant water use (Fig. 2). Most of the water (76.6–86.4%) was taken up from the low salinity side, which

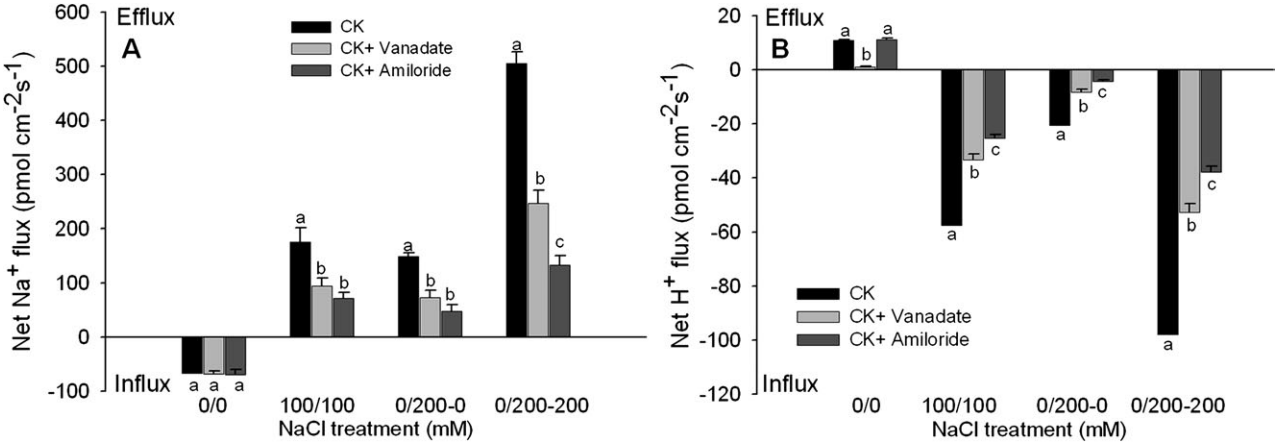


**Fig. 8.** Effects of sodium orthovanadate (500 μM) and amiloride (100 μM) on net $Na^+$ (A) and $H^+$ (B) fluxes in cotton roots at 1 d after 0/0, 0/200, and 100/100 mM NaCl treatment. CK denotes the test solution without inhibitor. The data are main fluxes of $Na^+$ and $H^+$ within the measuring periods (15 min). Values are means ±SE ($n$=6). Bars with different letters (a, b, and c) differ significantly at $P < 0.05$.

seemed to compensate partially for the reduced uptake from the high salinity side (Fig. 2).

Leaf osmotic potentials were also decreased under salt stress, but were greatly increased under non-uniform salinity compared with those under uniform salinity (Fig. 3). The results are in agreement with a previous report on *A. nummularia* (Bazihizina *et al.*, 2009). Osmotic adjustment can be achieved by means of accumulation of inorganic ions (Shabala and Lew, 2002; Flowers and Colmer, 2008; Hariadi *et al.*, 2011); therefore, the decreased osmotic potential under salt stress might be due to increased $Na^+$ concentration in the leaf (Fig. 4A).

### *$Na^+$ recirculation from shoot to root*

Since $Na^+$ accumulates in the leaf blade after being deposited in the transpiration stream, the leaf blade was more prone to $Na^+$ toxicity than roots under salinity (Munns, 2002). It is easy to understand that less accumulation of salts in leaves was very important for protecting plants against salinity (Khan *et al.*, 2004; Ali *et al.*, 2009). The present data showed that non-uniform salinity decreased leaf $Na^+$ concentration relative to uniform salinity (Fig. 4A). Such a reduction in leaf $Na^+$ under non-uniform salinity was also reported in the halophyte, *A. nummularia* (Bazihizina *et al.*, 2009). It was supposed that $Na^+$ recirculation from shoots to roots involving AtHKT1 might be an important mechanism for preventing deleterious $Na^+$ build up in the leaf blade (Berthomieu *et al.*, 2003). Recirculation of $Na^+$ from shoots (including leaves) to roots by the phloem sap limits $Na^+$ accumulation in leaves (Munns 2002). This assumption was supported by Blom-Zandstra *et al.* (1998) who found that the $Na^+$ concentration in the xylem sap of sweet pepper decreased towards the apex and $^{22}Na^+$ entered the unlabelled root part and leaked into the medium. Blom-Zandstra *et al.* (1998) suggested that the phloem participates in $Na^+$ recirculation from shoot to root. It was also found that the $Na^+$ concentration in the xylem and phloem decreased towards the apex (Fig. 4E, F, H, I), and the concentration in the '0' side of roots in the 0/200 mM NaCl treatment was much higher than that in either side of the root in the 0/0 mM NaCl treatment control at 7 d after treatment (Fig. 4B). However, girdling of the hypocotyl increased leaf $Na^+$ concentration by 15.6%, and significantly decreased root $Na^+$ concentration in the '0' side of the 0/200 treatment at 7 d after NaCl treatment (Fig. 6). The decreased root $Na^+$ concentration in the '0' side and the increased $Na^+$ concentration in the leaf under the 0/200 treatment due to inhibited $Na^+$ recirculation by girdling suggested that $Na^+$ recirculated to roots of cotton mainly through the phloem. Interestingly, the $Na^+$ concentration in the phloem was ~1.2-fold higher than in the xylem under 0/0 mM NaCl treatment, but ~1.6-fold higher under NaCl stress, further suggesting that the phloem was involved in recirculation of toxic ions in cotton (data not shown). There may be some homologues of AtHKT1 in cotton which transport xylem $Na^+$ to phloem sap under salt stress.

Potassium is an essential mineral nutrient in every organism and the most abundant cation in the cytosol. Maintenance of a high cytosolic $K^+/Na^+$ ratio is a key feature of plant salt tolerance (Maathuis and Amtmann, 1999). In the present study, salinity induced more $Na^+$ and less $K^+$ accumulation and then a lower $K^+/Na^+$ ratio in cotton regardless of salt distribution in both root portions (Figs 4, 5). However, under non-uniform salinity, the $K^+$ concentration and $K^+/Na^+$ ratio were much higher than those under uniform salinity control. Increased $K^+/Na^+$ in leaves under non-uniform salinity was therefore probably due to enhanced accumulation of $K^+$ and decreased $Na^+$ due to recirculation.

### *$Na^+$ and $H^+$ flux*

Non-invasive micro-test technology revealed an $Na^+$ influx into roots under 0/0 mM NaCl treatment, whereas a net $Na^+$ efflux in cotton roots was observed under NaCl stress (Fig. 7). Such a net $Na^+$ efflux was also observed in bean

leaf mesophyll (Shabala, 2000), *P. euphratica* (Sun *et al.*, 2009), and wheat (Cuin *et al.*, 2011) roots after NaCl treatment. The $H^+$ could efflux from roots under 0/0 mM NaCl treatment, but the $H^+$ flux was reversed to an influx when treated with NaCl (Fig. 8B). An NaCl-induced $H^+$ influx was also noticed in the root apex of wild-type *Arabidopsis* (Shabala *et al.*, 2005) and salt-tolerant *P. euphratica* (Sun *et al.*, 2009). Under salt stress conditions, the $H^+$ influx increased with increases in $Na^+$ efflux and also decreased when $Na^+$ efflux was decreased by the inhibitors. Regression analysis showed that the net $Na^+$ efflux was positively correlated with net $H^+$ influx ($R^2$=0.9146, $n$=36) (Supplementary Fig. S4 at *JXB* online). In accordance with a previous study of *P. euphratica* (Sun *et al.*, 2009), the increase in $H^+$ influx corresponding to the $Na^+$ efflux suggests that the $Na^+$ extrusion in salt-stressed cotton roots was mainly attributed to an active $Na^+/H^+$ antiport across the PM, although the cotton PM $Na^+/H^+$ antiport gene has not been cloned. Cuin *et al.* (2011) found that amiloride (an inhibitor of the $Na^+/H^+$ antiporter) and sodium orthovanadate (an inhibitor of the PM $H^+$-ATPase) reduced wheat root $Na^+$ efflux under NaCl stress. The present data also showed that the PM transport inhibitors amiloride and sodium orthovanadate simultaneously decreased $Na^+$ efflux and $H^+$ influx of salt-stressed cotton roots although the reduction in $Na^+$ efflux was greater than that of $H^+$ influx (Fig. 8; Supplementary Fig. S2 at *JXB* online). This is possibly because amiloride could also block some non-selective cation channels and orthovanadate could inhibit other P-type pumps (Marunaka, 1996; Møller *et al.*, 1996). PM $Na^+/H^+$ antiporters extrude $Na^+$ out of the cell depending on electrochemical $H^+$ gradients generated by PM $H^+$-ATPase. The 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). These results further suggested the involvement of $Na^+/H^+$ antiport across the PM in $Na^+$ extrusion after NaCl stress.

The *SOS1* transcript levels and activity of the PM $Na^+/H^+$ antiporter were increased in many plant species, such as tomato (Wilson and Shannon, 1995), *Arabidopsis* (Qiu *et al.*, 2002, 2003), rice (Martínez-Atienza *et al.*, 2007), *Thellungiella* (Oh *et al.*, 2009), and *Populus* (Wu *et al.*, 2007; Sun *et al.*, 2009) under NaCl stress. In this study, the net $Na^+$ efflux and $H^+$ influx simultaneously increased with increasing NaCl concentrations in the medium at 1 d and 7 d after treatments (Figs 7, 8). The $Na^+$ flux in the '0' side was reversed to an efflux due to 200 mM NaCl in the 200 side of the 0/200 treatment (Fig. 7). The change might be attributed to the increased transcript level of *SOS1* and the activity of the PM $Na^+/H^+$ antiporter. Increased activity of the PM $Na^+/H^+$ antiporter in the 0/200-0 side may be induced by some endogenous signalling molecules (such as abscisic acid, $H_2O_2$, and NO) (Desikan *et al.*, 2004; Zhang *et al.*, 2007; Chen *et al.*, 2010) originating from the high salinity side (200 mM NaCl), except for $Na^+$, because the $Na^+$ concentration in the '0' side of the 0/200 treatment was similar to that of either side in the 0/0 mM NaCl treatment at 1 d and 3 d after stress (Fig. 4B). As observed for the 0/200 mM NaCl treatment, the $Na^+$ efflux in the '50' side of the 50/150 mM NaCl treatment also increased due to induction by the high salinity side (Fig. 7; Supplementary Fig. S1 at *JXB* online). The net $Na^+$ efflux in the '150' side of the 50/150 mM NaCl treatment was similar to that on either side in the 150/150 mM NaCl treatment (Fig. 7). Under non-uniform conditions, the $Na^+$ efflux from the low salinity side was induced by the high salinity side; therefore, the $Na^+$ the efflux ability of the whole plant was higher than that of their uniform control. The signalling molecules and their underlying mechanisms for $Na^+$ efflux in the low salinity side need further study.

In conclusion, the results showed that non-uniform salinity improved plant growth, leaf photosynthesis, and transpiration relative to uniform salinity in cotton. Such an improvement was attributed to increased water use, decreased $Na^+$ accumulation, and increased $K^+$ and $K^+/Na^+$ ratio in leaves. Recirculation of $Na^+$ from the shoot to the low salinity side of roots through the phloem is an important mechanism for reducing $Na^+$ accumulation in leaves. Enhanced $Na^+$ efflux from the low salinity root side induced by the high salinity root side might also play an important role in decreasing foliar $Na^+$ accumulation. The $Na^+$ extrusion in salt-stressed cotton roots is mainly attributed to an active $Na^+/H^+$ antiport across the PM.

## Supplementary data

Supplementary data are available at *JXB* online.

**Figure S1.** $Na^+$ flux in cotton roots 1 d after 100/100, 0/200, and 50/150 mM NaCl treatment.

**Figure S2.** $Na^+$ (A) and $H^+$ (B) fluxes in cotton roots 1 d after 100/100 mM NaCl, 100/100-100 mM NaCl with amiloride (100 μM), and 100/100 mM NaCl with orthovanadate (500 μM) treatment.

**Figure S3.** Correlation between net photosynthetic rate (Pn) and dry weight (DW) (A), Pn and transpiration (Tr) rates (B), Pn and stomatal conductance (Cond) (C), and Pn and chlorophyll (Chl) concentrations (D) in the third main stem leaves 7 d after salinity stress ($n$=12).

**Figure S4.** Correlation between net $Na^+$ and $H^+$ fluxes in cotton roots 1 d after 0/0, 0/200, and 100/100 mM NaCl with and without sodium orthovanadate (500 μM) and amiloride (100 μM) treatment ($n$=36).

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