

Cotton (*Gossypium hirsutum* L.) genotypes with contrasting K^+/Na^+ ion homeostasis: implications for salinity tolerance

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Abstract Cotton (*Gossypium hirsutum* L.) possesses notable inter- and intraspecific salt tolerance variation, but the mechanisms underlying this variation are, more or less, unclear. To explore salt stress responses, Na^+/K^+ transport and the transcriptional profiles of salinity tolerance candidate genes (e.g., *GhSOS1*, *GhNHX1*, and *GhAKT1*) were evaluated in four cotton cultivars (differing in salt sensitivities) using electrophysiological and qRT-PCR assays. Physiology and lipid peroxidation assays revealed the following pattern of salt tolerance, in decreasing order: CZ91 > CCRI44 > CCRI49 > Z571. Salinity caused ion imbalances in plants, but ion homeostasis was more pronounced in Z571, which accumulated more Na^+ and less K^+ in leaves. In contrast, salt-tolerant cultivars, especially CZ91, retained less Na^+ , but more K^+ under salt stress. The non-invasive micro-test technique revealed that Z571 exhibited a higher capacity to transport Na^+ from roots to shoots, but a lower capacity to extrude Na^+ and retain K^+ in leaves under salinity. Meanwhile, NaCl-induced changes in Na^+ and K^+ ion homeostasis were less pronounced in salt-tolerant cultivars, increasing leaf K^+/Na^+ ratios under salinity. Additionally, NaCl increased the *GhSOS1* transcript abundance in hypocotyls, but this increase was more

pronounced in salt-sensitive Z571. On the other hand, NaCl significantly enhanced the transcript abundances of *GhSOS1* in leaves and/or *GhAKT1* in hypocotyls of salt-tolerant cultivars. NaCl also increased the *GhNHX1* transcript abundance, but there was no strong correlation between *GhNHX1* expression level and salt resistance. The present findings provided an explanation for salt tolerance variation in cotton, illuminating the transcriptional regulation of K^+/Na^+ transport and the maintenance of the K^+/Na^+ ratio as underlying attributes.

Keywords Sodium exclusion · Sodium sequestration · Potassium retention · Non-invasive micro-test technique · Ion fluxes

Introduction

Salinity is one of the major environmental factors that severely limits productivity and yield in many areas worldwide (Wang et al. 2001). At present, about 7% of the total land area and 20% of the irrigated arable land are affected by salinity (Parihar et al. 2015). Farmland salinity has become a severe agricultural problem, partly due to unreasonable irrigation and fertilization. As such, enhancing crop salt tolerance could be a putative way to meet the challenge. Salinity tolerance is a complex trait regulated by various mechanisms; hence, a deeper understanding of various protective physiological mechanisms is essential for developing salt-tolerant cultivars (Flowers 2004).

Plants can survive under saline conditions and evolve to better tolerate saline conditions through special strategies utilizing physiological, biochemical, and molecular mechanisms (Munns and Tester 2008; Singh et al. 2015). These

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special strategies include ion homeostasis, photosynthesis adjustment, antioxidant enzyme induction, hormone overproduction, and combinations of these factors (Parida and Das 2005). Maintaining an optimal cytoplasmic K^+/Na^+ ratio is an important component of plant salinity tolerance (Wang et al. 2016).

Plants maintain an optimal cytosolic K^+/Na^+ balance via different strategies for the avoidance of excessive Na^+ accumulation by increasing efflux and/or increasing vacuolar sequestration of Na^+ (Tester and Davenport 2003). The key salt tolerance gene *salt overly sensitive-1* (*SOS1*) encodes a plasma membrane (PM) Na^+/H^+ antiporter that can extrude excess Na^+ from the cytosol (Shi et al. 2002). Overexpression of *SOS1* can significantly improve salt tolerance by enhancing Na^+ exclusion from the cytosol to extracellular spaces (Ma et al. 2014; Qiu et al. 2002). Transporting Na^+ from the cytoplasm into the vacuole by *NHX1*, a vacuolar membrane-bound Na^+/H^+ antiporter, is another effective method of minimizing Na^+ concentrations in the cytoplasm (Munns and Tester 2008). Previous studies have shown that the overexpression of *NHX1* in transgenic plants can increase salt tolerance (Banjara et al. 2012; Chen et al. 2008). Maintaining cytosolic K^+/Na^+ homeostasis in tissues can be achieved not only through stronger Na^+ exclusion and/or compartmentalization, but also by improved K^+ retention (Wu et al. 2011). In plants, K^+ acquisition is mainly mediated by K^+ transporters and ion channels (Martinez-Cordero et al. 2005; Wang and Wu 2013). Among them, the inward-rectifying potassium channel *AKT1* is considered the major route for K^+ uptake, and it plays a critical role in maintaining the K^+/Na^+ balance in salt-stressed plants (Hirsch et al. 1998; Lebaudy et al. 2007). Some reports have also shown that *AKT1* transcription levels are up-regulated along with an increase in K^+ uptake capacity under salt stress, ultimately contributing to the physiological adaptation to salt stress in plants (Boscari et al. 2009; Chen et al. 2013; Duan et al. 2015).

Cotton (*Gossypium hirsutum* L.) is a widely grown fiber crop, and about 4 million ha are sown in China annually, accounting for 20% of the world's cotton production (Dai and Dong et al. 2014). Cotton has been reported to be moderately salt tolerant, but it has shown limited growth and yield in high salinity conditions (Leidi and Saiz 1997). Moreover, a large body of data regarding mechanisms of salt tolerance in cotton is now available (Kong et al. 2012, 2016a; Li et al. 2013; Wang et al. 2016). Plant salinity tolerance is a complex physiological trait exhibiting polygenic inheritance; variation in salt sensitivity occurs not only among species, as even genotypes belonging to a species perform differently under saline conditions (Shabala and Munns 2012). At the same time,

however, little information is available on the modulation of key candidate genes that regulate ion homeostasis in cotton genotypes with various salinity tolerance. Thus, it is very important to identify the specific components of protective mechanisms that underlie salt tolerance in cotton.

To further address the above questions, salt tolerance variation in cotton cultivars was comparatively evaluated. This study used atomic absorption spectroscopy to assess the effects of salt stress on the transport and compartmentalization of K^+ and Na^+ in seedlings of different cotton cultivars. The non-invasive micro-test technique (NMT) was also employed to study the kinetics of stress-induced net Na^+ and K^+ fluxes and these changes were linked to alterations in the relative abundances of *GhSOS1*, *GhNHX1*, and *GhAKT1* transcripts. We attempted to characterize and identify the salinity tolerance mechanisms among cultivars varying in K^+ and Na^+ homeostasis, which is likely linked to the expression of K^+ and Na^+ transport system under salinity.

Materials and methods

Plant materials and treatment

Four cotton cultivars [Z571 (salt sensitive), CCRI49 (moderately salt tolerant), CCRI44 (moderately salt tolerant), and CZ91 (superior line of Xinluzhong 61, salt tolerant)] were obtained from the Cotton Research Institute at the Chinese Academy of Agricultural Sciences (CCRI), Anyang, Henan, People's Republic of China. All seeds were surface sterilized in 0.1% (w/v) $HgCl_2$ solution for 10 min and subsequently rinsed several times with tap water to remove traces of $HgCl_2$. The seeds were sown in 10-cm diameter by 10-cm height pots filled with 1000 g of sandy clay soil (sterilized in an autoclave for 20 min). Then, the pots were placed in a growth chamber with a photosynthetic photon flux density, average humidity, and light/dark regime of $500 \mu mol m^{-2} s^{-1}$, ~80%, and 14/10 h at 28/20 °C, respectively. At the two-leaf stage (20 days after sowing), seedlings exhibiting uniform growth were randomly selected for salt treatment. The NaCl solution was added to the soil to reach a final concentration of 0.3% (w/w) and control plants were supplied with water. The completely randomized design with three replicates (i.e., pots) was used in all experiments. All experiments were separately repeated two times. To maintain soil moisture at field capacity, plants received 100 mL of distilled water every 2 days and 100 mL of Hoagland nutrient solution every 5 days. After 9 days under NaCl stress, physiological and biochemical parameters were assessed.

Measurement of net photosynthetic rate and chlorophyll content

Net photosynthetic rate (P_n) of the third fully expanded leaf was determined using a portable photosynthesis open system (Li-6400XT, Li-Cor Inc., USA) between 9:00 and 11:00 am at 28 °C, 60% relative humidity, 400 $\mu\text{mol mol}^{-1} \text{CO}_2$, and 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ quantum flux. Then, these leaves from each plant were detached for the determination of total chlorophyll content. Chlorophyll was extracted from 100 mg of fresh leaf tissues in 5 mL of 80% acetone. The absorbance of the extracts was measured using a spectrophotometer at two wavelengths (645 and 663 nm). Chlorophyll content was calculated according to the method described by Lichtenthaler (1987).

Measurement of relative electric conductivity and malondialdehyde content

After chlorophyll assessment, relative electric conductivity (REC) and malondialdehyde (MDA) content of the third fully expanded leaf were measured. Electrolyte leakage was measured as described by Nayyar et al. (2005) using young segments (0.8-cm diameter) of leaf. Samples were placed in closed vials containing 10 mL of deionized water and incubated at 30 °C on a rotary shaker for 3 h, and the initial electrical conductivity (L_1) of the solution was determined. Samples were then boiled for 30 min at 100 °C and the final electrical conductivity (L_2) was obtained after equilibration at 25 °C. REC was expressed by the formula: $\text{REC} (\%) = (L_1/L_2) \times 100$. The lipid peroxidation level was determined by assessing the MDA content using the thiobarbituric acid (TCA) test as described by Shi et al. (2010). For MDA extraction, fresh leaf material (0.2 g) was ground in 10% TCA. The mixture was centrifuged at 10,000 $\times g$ for 20 min, and 2 mL of the supernatant was mixed with 2 mL of 0.6% thiobarbituric acid (TAB). The samples were heated in a boiling water bath for 10 min and then centrifuged at 10,000 $\times g$ for 5 min, and the absorbance was monitored at 600, 532, and 450 nm, respectively. The MDA content was calculated as follows: $C (\mu\text{mol L}^{-1}) = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$.

Measurement of Na^+ and K^+ fluxes

Net ion fluxes (Na^+ and K^+) in hypocotyl and leaf mesophyll tissues were measured using NMT (NMT-YG-100, Younger, USA) at Xuyue Sci. & Tech. Co. (Beijing, China). The principle behind this method and the instrument used were described previously (Lei et al. 2014; Wu et al. 2015). The hypocotyl and leaf samples ($\sim 5 \times 5$ mm) were cut from near the cotyledon (Fig. 1a) and the middle part of the third leaf (Fig. 1b), exposing leaf mesophyll tissue. Cut

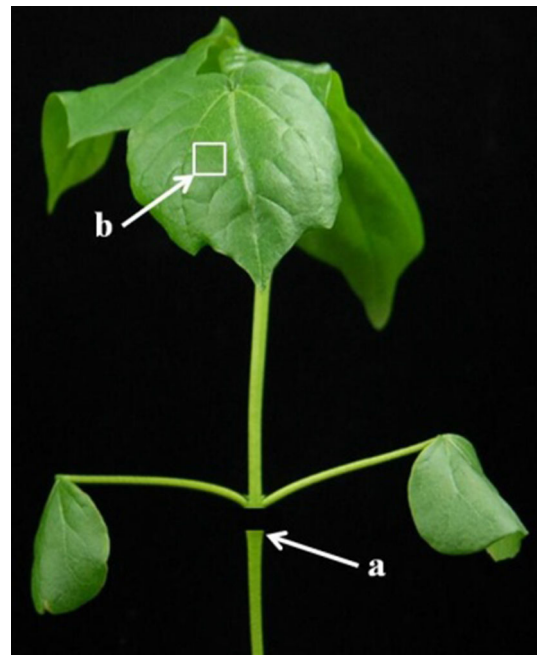


Fig. 1 The cutting and measuring points for net Na^+ and K^+ fluxes in the hypocotyls (a) and leaf mesophyll (b) tissues of the different cotton cultivars using the non-invasive micro-test technique (NMT)

samples were immediately fixed on the bottom of a measuring dish, incubated in the measuring solution, and allowed to equilibrate for 30 min. The measuring solution consisted of 0.1 mM KCl, 0.1 mM CaCl_2 , 0.1 mM MgCl_2 , 0.5 mM NaCl, 0.3 mM MES, and 0.2 mM Na_2SO_4 (pH 6.0, maintained using Tris or HCl). The measuring site was 300 μm from the edge of the samples, into which a vigorous flux of Na^+ and K^+ ions was measured, having infiltrated the tissues from the fresh measuring solution. The steady-state ion fluxes were continuously recorded for 20–25 min, and the ion flux estimates were calculated using JCal V3.0 (Xuyue Sci. and Tech., China).

Measurement of Na^+ and K^+ content

After salt treatment, roots were soaked in deionized water for 10 min, and shoots were washed with distilled water to remove surface salts. From each plant, the roots, stems, and leaves were harvested and dried at 80 °C for 72 h. Each sample (0.1 g) was digested in 1.0 mol L^{-1} HCl solution for 24 h at 30 °C, and then shaken for 60 min. The Na^+ and K^+ contents were determined using an atomic absorption spectrophotometer (SpectAA-50/55, Varian, Australia).

RNA isolation and quantitative real-time PCR analysis

Under salinity stress, changes of related-gene expression are a relatively quick response (Horie et al. 2012). So, cotton

seedlings grown as described earlier were subjected to NaCl stress for time periods of 3, 6, 9, 12, 18, 24, and 48 h. Then, the sampled tissues (hypocotyls near the cotyledon and juvenile leaves) were crushed in liquid nitrogen using a precooled mortar and pestle. Total RNA from the different cotton seedling tissues was extracted using an RNAPrep Pure Plant Kit (Tiangen, China) and purified with RNase-free DNase I (Tiangen). A first-strand cDNA fragment was synthesized from total RNA samples using Superscript II reverse transcriptase (Invitrogen, USA). Then, quantitative real-time PCR assays were performed on the 7500 Real-time PCR System (Applied Biosystems, USA) using the reverse-transcribed cDNAs extracted from the different tissues. Gene-specific primers were obtained based on primer sequences for *GhSOS1*, *GhNHX1*, and *GhAKT1* acquired from NCBI, and *UBQ7* was chosen as the reference gene for standardizing RT-PCR experiments (Table 1). According to the manufacturer's protocol, a melting curve analysis was carried out to determine the specificity of amplification. Relative quantization of gene expression was calculated based on the comparative threshold cycle method using *UBQ7* as an internal control and normalized to the gene expression values.

Statistical analysis

The experiment was repeated two times with results showing similar trends and there was no interaction among repetitions of the experiments; hence, the pooled data were presented. All data were statistically analyzed using SPSS 17.0 (SPSS Inc., USA). The differences between treatments were analyzed using analysis of variance (ANOVA) followed by Duncan's multiple range test, and significance was determined at $P < 0.05$.

Results

Effect of salinity on leaf photosynthesis

Non-significant differences in the chlorophyll content and P_n of the different cotton cultivars were observed under the control conditions (Fig. 2). As shown in Fig. 2, salinity resulted in a significant reduction in chlorophyll content and P_n in all cultivars. However, more pronounced effects

were observed in the salt-sensitive cultivar Z571, as the chlorophyll content and P_n decreased by 55.9 and 65.4%, respectively. The cultivars ranked according to the values of these parameters in seedling leaves, in descending order, were CZ91, CCRI44, CCRI49, and (last) Z571.

Effect of salinity on relative electric conductivity and malondialdehyde content

Compared with controls, the REC of leaves increased under salinity (Fig. 3a). Less pronounced effects were observed in leaves of CZ91, as its REC increased by only 118.2%. However, in leaves of Z571, CCRI49, and CCRI44, this parameter increased by 232.3, 192.8, and 151.8%, respectively. Additionally, salinity significantly increased the MDA content in cotton leaves (Fig. 3b). The descending order of cultivars according to seedling leaf increases in MDA was Z571, CCRI49, CCRI44, and (last) CZ91, as the MDA contents increased by 258.4, 185.6, 174.4, and 59.3%, respectively.

Effect of salinity on Na⁺ and K⁺ ion partitioning

As Table 2 shows, salinity produced a significant increase in tissue Na⁺ concentration, with the highest increase observed in the susceptible cultivar Z571. Among the most sampled tissues, Na⁺ concentration was lowest in CZ91, especially in CZ91 leaves. Contrasting results were found for K⁺ concentrations in stems and leaves of the four tested cultivars, which were significantly reduced in response to salt stress (Table 2). Among the cultivars, CZ91, CCRI44, and CCRI49 showed comparatively mild decreases (2.3, 14.7, and 23.1%, respectively) in leaf K⁺ compared to Z571 (36.8%), while the first three cultivars showed higher decreases (24.1, 25.2, and 12.7%, respectively) in stem K⁺ concentration than Z571 (5.7%) under salt stress. In contrast to leaves and stems, K⁺ concentration of root was markedly increased by NaCl treatment, and more pronounced effects were observed in Z571 and CCRI49. Irrespective of the treatment effect, the ratio of K⁺/Na⁺ was also found to be considerably reduced across all tissues (Table 2). The descending order of cultivars with respect to their K⁺/Na⁺ ratios in leaves was CZ91, CCRI44, CCRI49, and, last, Z571, while no differences among the cultivars were found in roots and stems.

Table 1 Gene primer sequences used for the quantitative real-time PCR analysis

Gene name/gene ID	Forward primer (5'–3')	Reverse primer (5'–3')
<i>GhUBQ7</i> /DQ116441	GAAGGCATTCCACCTGACCAAC	CTTGACCTTCTTCTTCTTGCTTG
<i>GhSOS1</i> /KM986873	CCCTTCCTTCTAGTGTCCGC	AAGCCCAACGTACTIONCATG
<i>GhNHX1</i> /AF515632.2	CCAGCATGCTCTCAGACCAA	GGAACGAAGGGCACAAAACC
<i>GhAKT1</i> /KF294166	CAATTGCCCTCTCGCTACT	ATGCTCGATCGGATGGCTTT

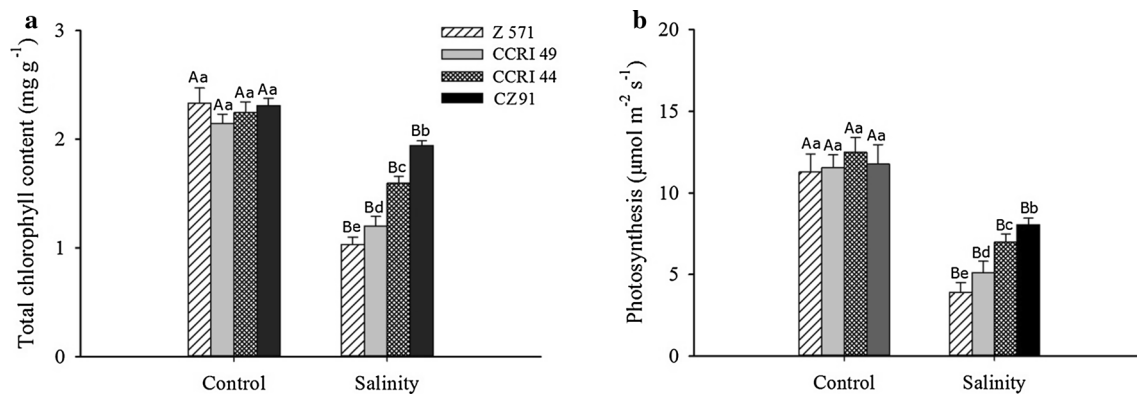


Fig. 2 Changes in chlorophyll content (a) and photosynthetic rate (b) in the leaves of the four cotton cultivars after treatment with NaCl for 9 days. Different capital letters denote significant differences

between treatments in each cultivar; different lowercase letters denote significant differences between cultivars ($P < 0.05$)

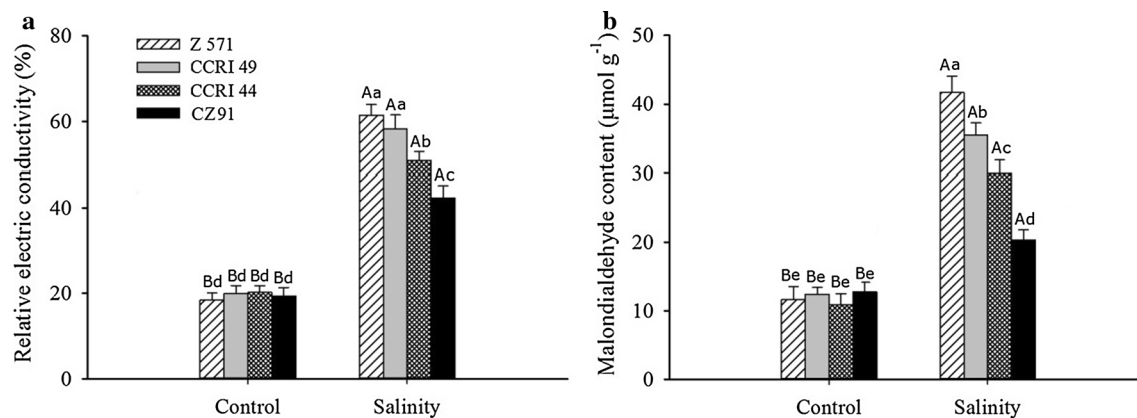


Fig. 3 Changes in relative electric conductivity (a) and malondialdehyde content (b) in the leaves of the four cotton cultivars after treatment with NaCl for 9 days. Different capital letters denote

significant differences between treatments in each cultivar; different lowercase letters denote significant differences between cultivars ($P < 0.05$)

Table 2 K^+ and Na^+ concentrations and K^+/Na^+ ratios of the four cotton cultivars after treatment with NaCl for 9 days

Variables	Cultivars	Root		Stem		Leaf	
		Control	Salt	Control	Salt	Control	Salt
Na^+ ($mg\ g^{-1}$)	Z571	2.72 Bc	10.80 Aa	3.30 Bc	15.40 Aa	2.15 Bd	18.80 Aa
	CCRI49	2.68 Bc	8.80 Ab	3.70 Bc	11.90 Ab	2.00 Bd	15.20 Ab
	CCRI44	2.53 Bc	9.20 Aab	3.50 Bc	11.10 Ab	1.89 Bd	14.10 Ab
	CZ91	2.60 Bc	8.40 Ab	4.00 Bc	10.10 Ab	2.10 Bd	9.80 Ac
K^+ ($mg\ g^{-1}$)	Z571	17.31 Bd	24.61 Aa	21.83 Aa	20.59 Aab	16.85 Aa	10.60 Bd
	CCRI49	18.29 Bcd	23.94 Aa	21.50 Aa	18.76 Bbc	16.65 Aa	12.81 Bc
	CCRI44	18.65 Bcd	20.43 Ab	22.06 Aa	16.50 Bcd	17.35 Aa	14.80 Bb
	CZ91	17.40 Bd	19.25 Abc	20.69 Aab	15.71 Bd	17.10 Aa	16.70 Aa
K^+/Na^+	Z571	6.37 Aa	2.28 Bb	6.61 Aa	1.34 Bb	7.84 Ab	0.56 Bf
	CCRI49	6.82 Aa	2.85 Bb	5.81 Aa	1.58 Bb	8.33 Aa	0.84 Be
	CCRI44	7.37 Aa	2.22 Bb	6.30 Aa	1.49 Bb	9.18 Aa	1.02 Bd
	CZ91	6.69 Aa	2.19 Bb	5.17 Aa	1.56 Bb	8.14 Aab	1.70 Bc

Different capital letters denote significant differences between treatments in each cultivar; different lowercase letters denote significant differences between cultivars ($P < 0.05$)

Effect of salinity on Na⁺ and K⁺ ion fluxes

To understand how salinity influenced the transport and compartmentalization of Na⁺ and K⁺ in cotton seedlings, Na⁺ and K⁺ fluxes of the hypocotyls and leaves were assessed. Under control conditions, no significant differences in Na⁺ and K⁺ efflux rates were found among cotton cultivars (Fig. 4). As shown in Fig. 4a, b, salt stress induced a marked Na⁺ efflux in hypocotyls and leaves. Upon salinity exposure, the descending order of cultivars in net Na⁺ efflux of hypocotyls was Z571, CCRI44, CCRI49, and last, CZ91. However, the net Na⁺ efflux of leaves exhibited the reverse trend, and a more pronounced effect was observed in CZ91 and CCRI49 (5747 and 4851 pmol cm⁻² s⁻¹, respectively) compared with Z571 and CCRI44 (4062 and 3654 pmol cm⁻² s⁻¹, respectively). Salinity markedly restrained the net K⁺ efflux in hypocotyls; a more pronounced effect was recorded in CCRI49 (with a steady-state value of 1962 pmol cm⁻² s⁻¹), and a less pronounced effect was observed in CCRI44 (with a steady state value of 3553 pmol cm⁻² s⁻¹; Fig. 4c). In contrast to hypocotyls, the net K⁺ effluxes in leaves were markedly increased by salinity (Fig. 4d). Under

salinity, the net K⁺ effluxes in leaves of Z571 and CCRI44 (1505 and 1117 pmol cm⁻² s⁻¹, respectively) were significantly higher than those in leaves of CCRI49 and CZ91 (782 and 700 pmol cm⁻² s⁻¹, respectively).

Effect of salinity on *SOS1*, *NHX1*, and *AKT1* gene expression

To determine whether Na⁺ and K⁺ transport-related genes were up-regulated in hypocotyls and leaves under salinity, the expression patterns of *GhSOS1*, *GhNHX1*, and *GhAKT1* were analyzed by qRT-PCR at 3, 6, 9, 12, 18, 24, and 48 h after salt stress (HAS). The expression of *GhSOS1* in the different cultivars increased gradually during the initial salt exposure and then gradually decreased (Fig. 5a, b). Compared with CCRI49 and CZ91, the *GhSOS1* expression level in the hypocotyls of Z571 and CCRI44 plants was considerably higher at 12–18 HAS (Fig. 5a). In contrast, the *GhSOS1* expression levels in leaves of CZ91 were significantly up-regulated at 12 HAS, and less pronounced effects were observed in leaves of CCRI44 (Fig. 5b). Similar patterns were also observed for *GhNHX1* expression levels under salinity (Fig. 5c, d). However, *GhNHX1*

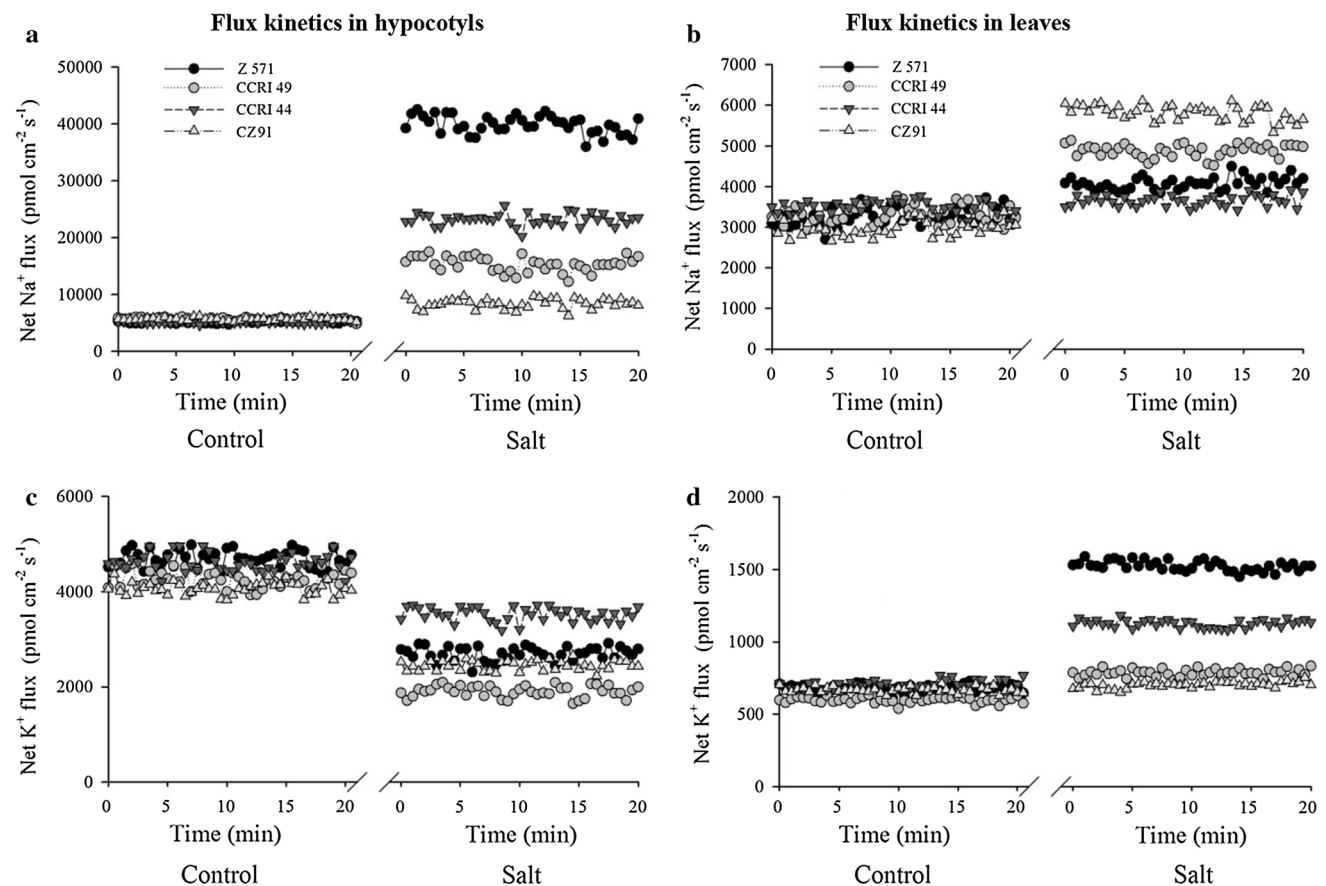


Fig. 4 Changes in Na⁺ (a, b) and K⁺ (c, d) flux kinetics in the hypocotyls (a, c) and leaves (b, d) of the four cotton cultivars after NaCl for 9 days

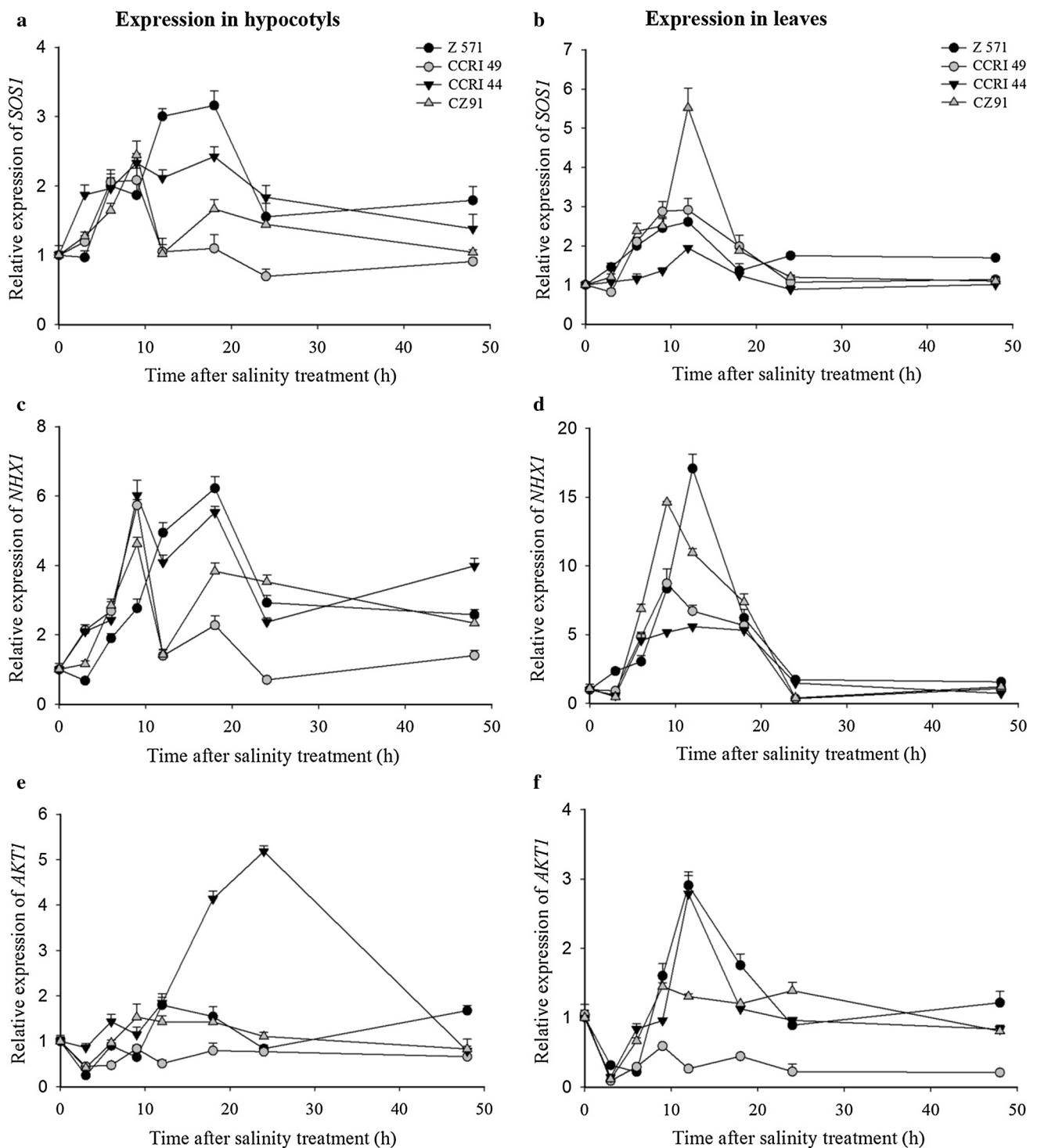


Fig. 5 Changes in *SOS1* (a, b), *NHX1* (c, d), and *AKT1* (e, f) expression levels in the hypocotyls (a, c, e) and leaves (b, d, f) of the four cotton cultivars as monitored by RT-PCR at 0, 3, 6, 9, 12, 18, 24 and 48 h after salinity treatment

expression levels in hypocotyls of CCRI44 were significantly higher after exposure to salinity, while the expression level in leaves was lower than that in other cultivars. Irrespective of the treatment effect, Z571 exhibited higher *GhNHX1* expression levels at 12–18 HAS in hypocotyls

and at 12 HAS in leaves. *GhNHX1* expression in leaves of CZ91 was also significantly up-regulated at 9 HAS. Salinity decreased the *GhAKT1* expression level in CCRI49, especially the expression level in leaves (Fig. 5e, f). Unlike the *GhAKT1* expression pattern in CCRI49, those

of Z571, CZ91, and CCRI44 decreased shortly after treatment (3–6 h) and then increased gradually after exposure to salinity, followed by a gradual decrease. Among cultivars, the expression level of *GhAKT1* was higher in CCRI44 at 18–24 HAS in hypocotyls and at 12 HAS in leaves. *GhAKT1* expression in leaves of Z571 was also significantly up-regulated at 12–18 HAS.

Discussion

Like other cultivated plant species, cotton is relatively tolerant to salinity and possesses a certain degree of variability in salinity tolerance at species level (Dai et al. 2014; Li et al. 2013; Wang et al. 2016). In the present study, distinct differences in salt tolerance among the four cultivars were found. The physiological function assays (Fig. 2) suggested that Z571 was the most sensitive to salinity, exhibiting elevated damage characterized by higher MDA accumulation and REC (Fig. 3). The descending order of salt tolerance among cultivars was CZ91, CCRI44, CCRI49, and last, Z571. To overcome salt-induced limitations, salt-tolerant plants have employed special adaptive mechanisms to cope with salt toxicity, i.e., enhanced Na^+ exclusion from shoot tissue, Na^+ tissue tolerance, and K^+ retention in leaves (Shabala et al. 2015; Wu et al. 2015).

Under salinity, Na^+ exerts toxic effects on physiological metabolism and plant growth. Thus, maintaining a low Na^+ content is a key factor for plant adaptation to high salinity conditions (Munns and Tester 2008; Kong et al. 2016a). In our study, the four cotton cultivars showed a significant increase in Na^+ content under salinity, which is a common response to ionic imbalance in plants under salinity (Chen et al. 2008; Pitann et al. 2013). However, the salt-susceptible cultivar Z571 had the highest Na^+ concentration in its tissues under stress, while less pronounced effects of salinity were observed in cultivar CZ91. These results indicated that Z571 exhibited poor regulation of ion transport, which was supported by increased Na^+ efflux in hypocotyls with decreased Na^+ efflux in leaves under salinity (Fig. 4a, b). These findings are in accordance with previous results showing that salt-tolerant plants often maintain lower Na^+ contents by regulating Na^+ ion homeostasis (Dai et al. 2014; Wang et al. 2016).

SOS1, an Na^+/H^+ antiporter, is not only crucial for Na^+ exclusion, but also to be involved in long-distance Na^+ transport from roots to shoots under salt stress (Ma et al. 2014; Munns and Tester 2008; Shi et al. 2002). Kong et al. (2016b) found that the enhancement of salt tolerance by foliar sodium nitroprusside (SNP) application to cotton can be ascribed to the altered Na^+ levels via feedback regulation of *GhSOS1* expression. In our study, the addition of NaCl significantly increased the expression of *SOS1*

(Fig. 5a, b), consistent with previous studies (Ma et al. 2014; Qiu et al. 2002). The *GhSOS1* expression level in Z571 was higher in hypocotyls, but lower in leaves than the expression levels in other cultivars, suggesting that salinity induced more Na^+ transport from roots to shoots, but less Na^+ export from leaves to the phloem (Ma et al. 2014). However, salt-tolerant cultivars possessed a more efficient salt-sensing system that was able to translate changes in saline condition into both transcriptional (Fig. 5a, b) and functional changes (Fig. 4a, b). Thus, it appears that this salt-inducible ability to control *GhSOS1* expression partly contributed to the differential salinity tolerance of the cultivars. It has to be noted that the moderately salt-tolerant cultivar CCRI44 also exhibited a higher induction of Na^+ transport to its shoots, but lower Na^+ export from the leaves (Fig. 4a, b). Thus, multiple adaptive strategies can confer salinity stress tolerance in cotton.

Previous reports on various species have suggested that NHX1 plays a key role in Na^+ sequestration into vacuoles, thus minimizing cytoplasmic Na^+ concentrations (Chen et al. 2008; Munns and Tester 2008; Pitann et al. 2013). The expression of *NHX1* was remarkably induced by salinity (Kong et al. 2016b; Zhang et al. 2016), which is consistent with our findings (Fig. 5c, d). Therefore, the transcriptional activation of *GhNHX1* can decrease Na^+ accumulation in the cytoplasm via the enhancement of Na^+ vacuolar sequestration capabilities. Several studies have also shown that the relative transcription of *NHX1* was significantly higher in salt-tolerant genotypes than in salt-sensitive ones after high salinity implementation (Pitann et al. 2013; Saqib et al. 2005). However, there was no strong correlation between *GhNHX1* expression levels and salt tolerance in our study and, thus, Na^+ sequestration into vacuoles by GhNHX1 might play an important, but non-essential role in the underlying genetic variability of salinity tolerance in cotton.

In general, high Na^+ levels can compete with K^+ for uptake into cells, leading to a decrease in K^+ content, thus impairing metabolic activity and often triggering programmed cell death (Munns and Tester 2008; Shabala et al. 2015). In the present study, salinity lowered K^+ concentrations in shoots, but increased K^+ concentrations in roots (Table 2), suggesting that salt inhibited K^+ transport from roots to shoots. This result was confirmed by measuring K^+ efflux of the hypocotyls and leaves under salt stress (Fig. 4c, d). Efficient maintenance of K^+ homeostasis has always been considered as one of the most, if not the most prominent feature of halophytes (Munns and Tester 2008; Shabala et al. 2015). Our result is in close agreement with previous findings, as the K^+ retention capacity in leaves was, in descending order, CZ91, CCRI44, CCRI49, and Z571, matching the descending order of salt tolerance (Table 2). These results suggested that maintaining K^+

homeostasis to provide an optimal cytosolic K^+/Na^+ ratio could be an essential mechanism underlying salinity tolerance in cotton.

Some studies have indicated that AKT1 is a very likely key factor in controlling the selective absorption capacity of K^+ over Na^+ under salinity (Ardie et al. 2010; Boscari et al. 2009; Chen et al. 2013). *AKT1* transcripts have been reported to be regulated by external Na^+ concentrations; however, somewhat contradictory results have been reported (Ardie et al. 2010; Duan et al. 2015; Wang et al. 2015). These differences may be attributed to variation in plant species, tissues, and duration of exposure to salinity (Ardie et al. 2010; Munns and Tester 2008). In our study, *GhAKT1* expression patterns were not similar in response to salinity (Fig. 5e, f). There was also a significant correlation between hypocotyl *GhAKT1* expression levels (Fig. 5e) and K^+ export flux values (Fig. 4c) under salt stress, indicating that *GhAKT1* is very likely the key factor in maintaining the selective absorption capacity for K^+ from roots to shoots under salinity. Interestingly, however, a significant negative correlation between leaf *GhAKT1* expression levels (Fig. 5f) and K^+ export flux values (Fig. 4d) was observed in response to saline treatment. This inconsistent result could be attributed to depolarized plasma membranes caused by the massive entry of external Na^+ , resulting in a K^+ loss mediated by AKT1 (Chérel et al. 2014; Wu et al. 2015). However, the specific details of the mechanism underlying this pattern remain obscure and must be examined in follow-up studies.

Conclusion

Salt tolerance in cotton is conferred by at least three complementary physiological mechanisms: (1) Na^+ transport from roots to shoots, (2) Na^+ export from the leaves, and (3) K^+ retention ability resulting from salt stress. These traits may be determined by the efficient transcriptional regulation of key transport genes, i.e., *GhSOS1* and *GhAKT1*. The above mechanisms appear to be coordinated, thus enabling a more optimal K^+/Na^+ ratio, which is a key mechanism conferring salinity tolerance in cotton. Salinity tolerance in plants is a complex polygenic trait, involving other key genes and mechanisms, and these factors should be addressed in future experiments.

Author contribution statement GY and QH conceived and designed the experiments. NW, HQ and WQ performed the experiments and analyzed the data. JS, QX and HZ contributed reagents and materials. NW, GY and QH wrote the article. All the authors read and approved the final manuscript.

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