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REGULAR ARTICLE

ZxSOS1 is essential for long-distance transport and spatial distribution of Na^+ and K^+ in the xerophyte *Zygophyllum xanthoxylum*

Qing Ma · Yi-Xiao Li · Hui-Jun Yuan · Jing Hu · Li Wei · Ai-Ke Bao · Jin-Lin Zhang · Suo-Min Wang

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Abstract
Background and aims Two major adaptive strategies used by *Zygophyllum xanthoxylum*, a C_3 succulent xerophyte, against arid environments are absorbing a great quantity of Na^+ from low-salinity soil which is efficiently transported to the leaves, and maintaining the stability of K^+ concentration in those leaves. The plasma membrane Na^+/H^+ antiporter SOS1 has been suggested to be involved in Na^+ transport and correlated with K^+ nutrition in glycophytes. In this study, we investigated the function of the plasma membrane Na^+/H^+ antiporter ZxSOS1 in long-distance transport and spatial distribution of Na^+ and K^+ in the xerophyte *Z. xanthoxylum*.
Methods The responses of *ZxSOS1* to NaCl, KCl treatments and osmotic stress were investigated by semi-quantitative RT-PCR, then the role of ZxSOS1 in regulating plant growth and Na^+, K^+ transport and spatial distribution in *Z. xanthoxylum* was studied by using post-transcriptional gene silencing.
Results We found that *ZxSOS1* was preferentially expressed in roots and was induced and regulated by salt treatments and osmotic stress. Using post-transcriptional gene silencing, we found that *ZxSOS1*-silenced plants exhibited reduced growth rate compared to wild-type (WT) plants under both normal and saline conditions. *ZxSOS1*-silenced plants accumulated more Na^+ in their roots but less Na^+ in leaves and stems than WT under 50 mM NaCl. Furthermore, *ZxSOS1*-silenced plants had a lower net K^+ uptake rate than WT plants under both normal and saline conditions, and more interestingly, accumulated less K^+ in leaves under normal conditions than WT plants. *ZxSOS1*-silenced plants also showed a decreased concentration and spatial distribution of K^+ in leaves and roots than WT under 50 mM NaCl. In addition, *ZxSOS1*-silenced plants possessed an increased selective transport (ST) capacity for K^+ over Na^+ from root to stem while a decreased ST value from stem to leaf compared with WT plants when both were grown in 50 mM NaCl.
Conclusions These results demonstrate that ZxSOS1 is not only essential in long-distance transport and spatial distribution of Na^+ and even K^+, but also vital for regulating K^+ and Na^+ transport system and maintaining Na^+ and K^+ homeostasis in *Z. xanthoxylum*, thereby regulating its normal growth.

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Introduction

Drought and soil salinity are two prime abiotic stresses that contribute, worldwide, to desertification with a progressive reduction of vegetation and rapid soil erosion (Ben Hassine et al. 2010; Martínez et al. 2005). Crop yield losses due to drought and salinity are considerable since most crop plants are highly sensitive to these stresses (Rozema and Flowers 2008; Sambatti and Caylor 2007; Yamaguchi and Blumwald 2005). The xerophyte or halophyte species found in arid and saline regions, however, have developed various multiple protective mechanisms to enable them to grow successfully in these harsh environments (Ashraf 2010; Flowers and Colmer 2008). Through an understanding of the mechanisms leading to successful adaptation to drought and salt in these xerophytes and halophytes, it has already been possible to identify key genes able to alter metabolism and increase plant tolerance to drought and salt (Ashraf 2010; Chaves and Oliveira 2004).

Zygophyllum xanthoxylum, a perennial fodder shrub with high palatability and nutrient value that colonizes arid areas in China and Mongolia, is a C_3 succulent xerophyte with excellent adaptability to adverse arid environments (Liu et al. 1987, 1988; Ma et al. 2012; Pei et al. 2004). This species has a strong ability for sand-fixing, soil and water conservation in desert regions, and possesses high medicinal value (Wu et al. 2004; Zhou et al. 2006). Our previous studies showed that *Z. xanthoxylum* absorbed, from low-salinity soil, a great quantity of Na^+ that was transported to leaves under arid field conditions (Wang et al. 2004). Further investigations revealed that *Z. xanthoxylum* responded to salt with increased growth and, moreover, became more tolerant to drought in the presence of moderate salinity (Ma et al. 2010, 2012; Yue et al. 2012). NaCl at 50 mM mitigated deleterious impacts of drought on its growth by improving relative water content and increasing leaf turgor pressure due to a significant increase in the contribution of Na^+ to leaf osmotic potential and a significant drop in leaf water potential, and concomitantly, increasing chlorophyll concentrations and photosystem II activity resulting in an enhancement of overall plant photosynthetic activity (Ma et al. 2012). Indeed, for *Z. xanthoxylum* growing under 50 mM NaCl, about 95 % of accumulated Na^+ was found in the shoots, wherein 80 % was in the vacuoles of the succulent leaves, compartmentalized by the tonoplast Na^+/H^+ antiporter ZxNHX (Wu et al. 2011). This suggests that *Z. xanthoxylum* has a strong ability to transport vast quantities of Na^+ efficiently from root to leaf in order to reduce osmotic potential of cells and increase water uptake capacity by using Na^+ as an important osmoregulatory substance, thus maintaining plant growth when subjected to drought (Ma et al. 2012; Wu et al. 2011; Yue et al. 2012). However, mechanisms underlying such long-distance Na^+ transport in *Z. xanthoxylum* have not been explored.

Na^+ is transported from root to shoot via the transpiration stream in the xylem. As much as 10–15 % of the surface area of xylem vessels is in contact with xylem parenchyma cells (XPCs) (Apse and Blumwald 2007). This large surface area can unquestionably accommodate the large quantities of ions and water that must be passed from root to shoot (Apse and Blumwald 2007; Craig and Møller 2010; Tester and Davenport 2003). Numerous studies in recent years have been devoted to investigate Na^+ unloading from xylem vessels to XPCs in roots or the retrieval of Na^+ from xylem into phloem sap in shoots (Berthomieu et al. 2003; Davenport et al. 2007; Hauser and Horie 2010; Sunarpi et al. 2005). However, research on the molecular mechanism of Na^+ loading into the xylem from XPCs is limited. Several studies indicated that the plasma membrane Na^+/H^+ antiporter SOS1 might control long-distance Na^+ transport from root to shoot by loading Na^+ into xylem in Arabidopsis and tomato (Olías et al. 2009a, b; Shi et al. 2002).

SOS1 is the only Na^+ efflux protein at the plasma membrane of plants characterized so far (Xu et al. 2008), although recent evidence showed that there might be another transporter mediating Na^+ efflux under high affinity conditions (Schulze et al. 2012). Analysis by using a *SOS1* promoter:GUS fusion revealed preferential expression of SOS1 in epidermal cells at the root tip and in XPCs at the xylem/symplast boundary throughout the plant (Shi et al. 2002). As undifferentiated cells at the root tip lack vacuoles large enough for significant Na^+ compartmentalization, SOS1 might be vital to prevent Na^+ from accumulating in these cells by actively extruding Na^+ back to the soil solution (Gou et al. 2012; Martínez-Atienza et al. 2007; Pardo et al. 2006; Shi et al. 2000; Takahashi et al. 2009; Xu et al. 2008). However, direct *in*

planta demonstrations of the role of SOS1 in extruding Na^+ are actually very scarce (Kronzucker and Britto 2011) and multiple apparently contradictory evidences obscure the details of the undeniable role of SOS1 in plant salt tolerance (Ding and Zhu 1997; Oh et al. 2009; Yang et al. 2009). The preferential expression of SOS1 in XPCs suggested a role of this transporter in long-distance Na^+ transport, which has been confirmed by the fact that, under moderate salinity, an *atsos1* mutant accumulated less Na^+ in its shoots than wild-type (WT) plants, indicating that AtSOS1 might function in loading Na^+ into xylem for transport to the shoot and subsequent compartmentation under moderate salinity (Shi et al. 2002). Recently, researchers found that SlSOS1 is critical for the partitioning of Na^+ in plant organs and the ability of tomato (*Solanum lycopersicum*) to retain Na^+ in stems, thus preventing Na^+ from reaching the photosynthetic tissues under saline conditions (Olías et al. 2009a, b). However, Arabidopsis and tomato might not be the best choice to understand the pathway of Na^+ transport from root to leaf in plants because they have a weak ability of transporting Na^+ from root to shoot, and contrary to *Z. xanthoxylum*, limiting Na^+ accumulation in leaf is central to their salt tolerance (Essah et al. 2003; Estañ et al. 2005; Munns 2002; Oh et al. 2009).

Besides accumulating Na^+, maintaining the stability of K^+ concentration in leaves is another important strategy for *Z. xanthoxylum* to adapt to saline and arid environments (Ma et al. 2012; Wu et al. 2011). It has been reported that SOS1 might also be involved in K^+ nutrition in plants (Kronzucker and Britto 2011; Pardo et al. 2006). The *atsos1* mutant was defective in high affinity K^+ uptake (Wu et al. 1996), K^+ content was significantly lower than in WT under salt stress (Zhu et al. 1998), and K^+ efflux from *atsos1* root was greater than that seen in WT (Shabala et al. 2005). Qi and Spalding (2004) revealed that SOS1 was necessary for protecting K^+ uptake mediated by AKT1 and the elevated cytoplasmic Na^+ levels resulting from loss of SOS1 function impaired K^+ uptake ability in root cells and compromised K^+ nutrition under salt stress. However, very little is known about the function of SOS1 in the xerophyte species, especially, its roles in long-distance transport and partition of Na^+ and K^+ among plant organs.

We have identified a cDNA fragment highly homologous to *AtSOS1* by transcriptomic analysis of salt and drought-treated *Z. xanthoxylum*, then we cloned and characterized the full-length cDNA which was designated as *ZxSOS1* (registered at GenBank under accession number GU177864) (Ma Q and Wang SM, unpublished data). In this study, we investigated its expression patterns in plants exposed to salt or drought; and using posttranscriptional gene silencing, we evaluated the role of ZxSOS1 in Na^+, K^+ transport and partition among plant organs. The results indicate that ZxSOS1 controls long-distance transport and spatial distribution of Na^+ and K^+ and plays an important role in maintaining Na^+, K^+ homeostasis in *Z. xanthoxylum*.

Materials and methods

Semi-quantitative RT-PCR analysis of *ZxSOS1* responding to different treatments

Seeds of *Zygophyllum xanthoxylum* were collected from wild plants in Alxa League (39°05′N, 105°34′E; elevation 1360 m) in the Inner-Mongolia Autonomous Region of China. The mean annual rainfall and temperature of the area are 60–150 mm and 8 °C, respectively, and the mean annual wind velocity 3.4 to 4.7 m s^{-1}. The concentrations of available N, P and K in soil were 2.1, 0.08 and 4.7 μ mol g^{-1} dry soil, respectively (Ma et al. 2012). After removal of the bracts, seeds were surface sterilized for 1 min in 75 % ethanol (v/v) and rinsed 3 times with distilled water, soaked in distilled water for 1 day and then germinated at 25 °C in the dark for 2 days. Uniform seedlings were transplanted to plugged hole in plastic containers (5 cm×5 cm×5 cm; 1 seedling/container) filled with sand and irrigated with modified ½ strength Hoagland nutrient solution containing 2 mM KNO_3, 0.5 mM $NH_4H_2PO_4$, 0.25 mM $MgSO_4{\cdot}7H_2O$, 0.1 mM $Ca(NO_3)_2{\cdot}4H_2O$, 50 μM Fe-citrate, 92 μM H_3BO_3, 18 μM $MnCl_2{\cdot}4H_2O$, 1.6 μM $ZnSO_4{\cdot}7H_2O$, 0.6 μM $CuSO_4{\cdot}5H_2O$ and 0.7 μM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$. Solutions were renewed every 3 days. Seedlings were grown in a greenhouse where the temperature was 28 °C/23 °C (day/night), the daily photoperiod was 16/8 h (light/dark; the flux density was approximately 800 μ mol m^{-2} s^{-1}) and relative humidity was about 65 %.

Three-week-old plants were used for the following treatments. (i) Plants were treated with modified ½ strength Hoagland nutrient solutions supplemented with additional 0, 5, 25, 50, 100 and 150 mM NaCl for 48 h. (ii) Plants were treated with modified ½ strength Hoagland nutrient solutions supplemented with additional 50 mM NaCl and harvested at 0, 3, 6, 12, 24, 48, 72, 96, 120 and 144 h after treatments. (iii) Plants were treated with modified ½

strength Hoagland nutrient solutions deprived of KNO_3 for 3 days, where 2 mM KNO_3 was substituted by 1 mM NH_4NO_3. Then different concentrations of K^+ treatments were supplemented by adding 0, 0.1, 0.5, 1, 5 and 10 mM KCl for 48 h. (iv) Plants were treated with modified ½ strength Hoagland nutrient solutions supplemented with additional mannitol where the osmotic potential was −0.5 MPa for 24 h. Treatment solutions were changed every day to maintain a constant ion concentration or osmotic potential.

Total RNA was extracted with a Trizol Kit (Sangon Biotech Co., Ltd, Shanghai, China) following the manufacturer's instructions. RNA samples were quantified by absorbance at 260 nm and the purity was assessed by the 260/280 nm ratio and on a 1.0 % (w/v) agarose gel stained with ethidium bromide (EtBr). First strand cDNA was synthesized from 4 μg of total RNA with MMLV-reverse transcriptase (Sangon Biotech Co., Ltd, Shanghai, China). Semi-quantitative RT-PCR was performed with the primer pairs P1 and P2 (Supplementary Table S1), which yielded a RT-PCR product of 533 bp. The PCR was performed as follows: 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 53 °C for 45 s and 72 °C for 50 s; and a final extension at 72 °C for 10 min. *ACTIN* was used as the internal control in the semi-quantitative RT-PCR. The specific primers of *ACTIN* that amplified a 598 bp fragment are A1 and A2 (Supplementary Table S1), designed according to the cDNA sequence of *ACTIN* from *Z. xanthoxylum* (GenBank accession no. EU019550). The PCR for *ACTIN* was performed as follows: 94 °C for 2 min; 25 cycles of 94 °C for 30 s, 53 °C for 40 s, 72 °C for 50 s; and a final extension at 72 °C for 10 min. PCR products were separated on 1.2 % (w/v) agarose gels containing EtBr and visualized by AlphaImager (ProteinSimple Inc., Santa Clara, CA, USA) for subsequent analysis. The ratios of the quantity of mRNA for *ZxSOS1* to that for *ACTIN* were calculated and results reflect the relative expression level. Experiments were repeated at least three times.

RNA interference silencing of *ZxSOS1*

Stable gene silencing via agrobacterium-mediated transformation was done using a pHANNIBAL vector (Wesley et al. 2001) designed for producing a hairpin RNA construct of *ZxSOS1*. A 627 bp PCR fragment encoding 209 amino acids located in the long hydrophilic tail in the C-terminal end (amino acids from 899 to 1107) was obtained using primers P3 (Supplementary Table S1, Xba I and Xho I restriction sites underlined) and P4 (Supplementary Table S1, Hind III and Kpn I restriction sites underlined). The whole Not I cassette bearing the RNA interference (RNAi) construct was subcloned into the corresponding site of the binary vector pART27, under the control of the CAMV35S promoter, which was introduced into *Agrobacterium tumefaciens* strain GV3101 cells and used for plant transformation of *Z. xanthoxylum.*

The transformation was performed using a procedure modified from that described by Li et al. (1997) and Weeks et al. (2008). Briefly, seeds of *Z. xanthoxylum* were germinated as described above and then seedlings were transplanted into plastic pots (5 cm×5 cm×5 cm; 2 seedling/container) filled with vermiculite irrigated with modified (see above) ½ strength Hoagland nutrient solution and grown in the greenhouse as described above. After the cotyledons had expanded and the apical node emerged, a cotyledon and the apical node were excised and the wound covered with cotton wool. Then an *Agrobacterium* suspension - obtained after incubation in 100 ml Luria-Bertani (LB) liquid medium (pH 7.0) containing 50 μ g ml^{-1} kanamycin overnight at 28 °C under constant rotation at 200 rpm and resuspension in the appropriate volume of LB liquid medium to an OD_{600} of 0.6–0.7 - was injected into the cotton wool using a syringe under low light conditions. For wild type (WT) control, the *Agrobacterium* suspension was replaced by water. After 3 h, seedlings were putted in the dark for 3 days. Thereafter, seedlings were transferred into and grown in the greenhouse and irrigated with modified ½ strength Hoagland nutrient solution as described above. Five days later, a new apical node emerged from the wound region and formed new shoots 4 weeks later. Individual plants of WT and each RNAi-transgenic line were obtained by propagation from stem cuttings: stem sections with two nodes were excised using a sharp razor blade and the base placed into moist vermiculite under a photoperiod of 16/8 h (light/dark) at 25±2 °C and 90 ±5 % relative humidity in a growth chamber and irrigated with modified ½ strength Hoagland nutrient solution. After about 10 days when the adventitious roots formed, seedlings were taken out from the growth chamber and grown in the greenhouse for 2 weeks.

Analysis of *ZxSOS1*-silenced plants

The transformed plants were screened by a PCR assay using pHANNIBAL-specific primers and DNA obtained

from the leaf in order to detect the presence of the RNAi construct. Positive plants were selected to study *ZxSOS1* expression level by semi-quantitative RT-PCR performed with the primer pairs P1 and P2 (Supplementary Table S1) as described above and four *ZxSOS1*-silenced lines (L3, L5, L9 and L13, obtained from cuttings as described above) with different reduced expression level of *ZxSOS1* were chosen for further analysis.

For physiological analysis, plants were treated with modified ½ strength Hoagland nutrient solutions supplemented with or without additional 50 mM NaCl. After 7 days, roots were washed twice for 8 min in ice-cold 20 mM $CaCl_2$ to exchange cell wall-bound Na^+, leaves and stems were rinsed in deionised water to remove surface salts (Wang et al. 2007), and then tissue fresh weights were determined. Then tissues were immediately incubated in an oven at 80 °C. After 72 h, tissue dry weights were measured. The relative increase in dry weight induced by salt was calculated by the formula: $[DW_{(50)}-DW_{(C)}]/DW_{(C)}\times 100$ % (Cohen et al. 1980), where $DW_{(50)}$ and $DW_{(C)}$ were the dry weight of whole plant under NaCl and control treatments, respectively. Na^+ and K^+ were extracted from dried plant tissues in 100 mM acetic acid at 90 °C for 2 h. Ion analysis was performed using a flame spectrophotometer (2655–00, Cole-Parmer Instrument Co., Vernon Hills, IL, USA). Selective transport (ST) capacity by different parts of plant for K^+ over Na^+ were estimated according to the following equation as described by Wang et al. (2002): $ST_{(A/B)}$ = (Na^+/K^+ in part A)/(Na^+/K^+ in part B). The higher ST value indicates the stronger net capacity of selection for transport of K^+ over Na^+ from part A to part B (Wang et al. 2002). Net uptake rate (NUR) of Na^+ and K^+ in whole plants was calculated according to the following equation (Wang et al. 2009): NUR = [Δ whole plant Na^+ (or K^+) content between salt-treated plant and BT plant]/root fresh weight (RFW)/Δ time, where BT means before treatments. The relative distribution of Na^+ (or K^+) in different tissues were estimated by the formula: Na^+ (or K^+) relative distribution (%) = Na^+ (or K^+) content in each tissue/Na^+ (or K^+) content in the whole plant.

Data analysis

Results of growth, ion concentrations and gene expression level are presented as means with standard deviation and data analysis was performed by one-way analysis of variance (ANOVA) using SPSS statistical software (Ver.13.0, SPSS Inc., Chicago, IL, USA). Duncan's multiple range test was used to detect a difference between means at a significance level of $P<0.05$ ($n\geq 3$).

Results

The expression of *ZxSOS1* was induced by NaCl treatments and osmotic stress

To investigate the tissue-specific expression of *ZxSOS1* under saline condition, plants were subjected to 50 mM NaCl for 48 h. In the absence of NaCl, the expression level of *ZxSOS1* was very low in all tissues, but was higher in root than that in leaf and stem tissue (Fig. 1). The addition of 50 mM NaCl significantly increased the transcript of *ZxSOS1* in all tissues, and the level in roots was 93 % and 3-fold higher than in stems and leaves, respectively (Fig. 1).

The mRNA level of *ZxSOS1* was assayed in plants treated with different concentrations of NaCl and KCl. The expression level of *ZxSOS1* in root tissue significantly increased with the increase of external NaCl concentrations and peaked in 100 mM NaCl, which was 6.7-fold higher than that under control condition (0 mM NaCl) (Supplementary Fig. S1). However, different concentrations of KCl had no effect on the expression of *ZxSOS1* in any tissues (Supplementary Fig. S2).

To further determine the kinetics for salt-induced activation of *ZxSOS1* in all tissues, semi-quantitative RT-PCR analysis of its transcript level was performed in plants exposed to 50 mM NaCl over a 144 h period. The expression level of *ZxSOS1* in all tissues increased with the increase of treatment time reaching their peak values at 48 and 72 h in roots and stems, respectively, and at 120 h in leaves, and then remained constant (Fig. 2). Moreover, the expression level in root was always remarkable higher than that in stem and leaf tissue (Fig. 2).

We also investigated the tissue-specific expression of *ZxSOS1* under osmotic stress. As shown in Fig. 3, −0.5 MPa osmotic stress significantly increased the transcript level of *ZxSOS1* in all tissues, but the level in root was 1.2- and 5.5-fold higher than in leaf and stem, respectively.

Taken together, these results suggested that *ZxSOS1* was preferentially expressed in roots, and the transcript

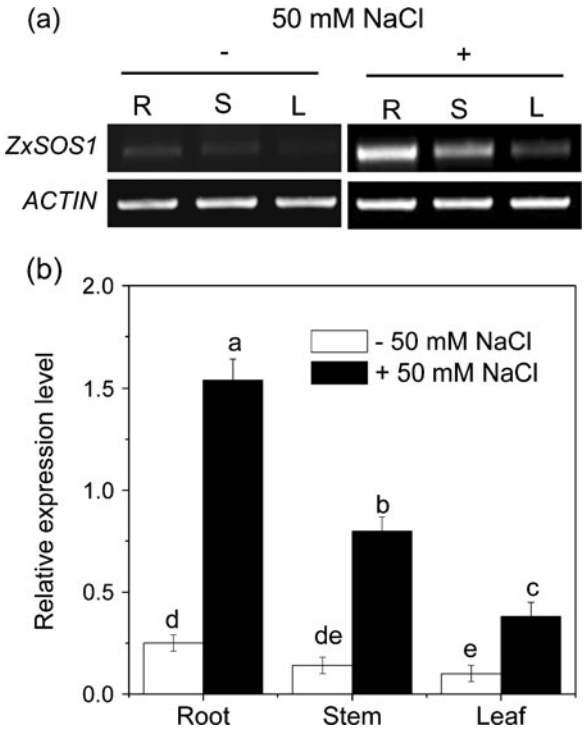

Fig. 1 Tissue-specific expression of *ZxSOS1* in *Z. xanthoxylum* under salt treatment. **a** Semi-quantitative RT-PCR analysis of *ZxSOS1* mRNA in root, stem, and leaf tissue of 3-week-old plants treated without (−) or with (+) 50 mM NaCl for 48 h. *ACTIN* was used as an internal control. Experiments were repeated at least three times (with similar results). **b** The relative expression level of *ZxSOS1* (related to *ACTIN*) in various tissues. Values are means ± SD (n=3) and *bars* indicate SD. Columns with different *letters* indicate significant differences at P<0.05 (Duncan test)

was induced and regulated by salt treatments and osmotic stress.

ZxSOS1 silencing induced a significant inhibition on the growth of *Z. xanthoxylum*

In order to investigate further the function of ZxSOS1 *in planta*, the gene was silenced by RNAi. Fifteen transgenic lines harbouring the RNAi construct were identified (data not shown). Since the transcript of *ZxSOS1* was preferentially expressed in roots and significantly induced by 50 mM NaCl (Fig. 1), the expression levels of *ZxSOS1* in root tissue of each transgenic line treated with 50 mM NaCl for 48 h were determined by semi-quantitative RT-PCR. Out of the 15 positive lines, four *ZxSOS1*-silenced lines (L3, L5, L9 and L13) with different reduced expression level of *ZxSOS1* were chosen for further analysis. Results showed that the silencing levels of *ZxSOS1* in L3, L5, L9 and L13 were 44 %, 39 %, 80 % and 45 %, respectively (Fig. 4a, b), indicating that the expression of *ZxSOS1* was, to various extents, reduced by RNAi in each transgenic line. The expression level of *ZxSOS1* in root, stem and leaf tissue of L9, which had the highest silencing efficiency of *ZxSOS1*, were further analyzed after plants were treated with 50 mM NaCl for 0, 6 and 48 h, respectively. As expected, the expression of *ZxSOS1* was induced by NaCl in all tissues in L9 and WT, and expression levels in all tissues were significantly lower in L9 than that in WT (Fig. 4c, d).

Phenotypically, *ZxSOS1*-silenced plants differed significantly from WT under salt treatment and even control condition (Fig. 5a). Under control condition, the silencing of *ZxSOS1* triggered a significant decrease in fresh weight, dry weight, shoot height and root length (Fig. 5b, c, d, e). In L9 especially, the above parameters were significantly decreased by 70 %, 71 %, 50 % and 30 %, respectively, in comparison to that of WT (Fig. 5b, c, d, e).

The salt treatment, 50 mM NaCl for 7 day, significantly increased fresh weight, dry weight and shoot height not only in WT but also in all the *ZxSOS1*-silenced plants compared to plants grown under control condition (Fig. 5b, c, d, e). However, the promotive effect of NaCl on the growth of WT was greater than that of *ZxSOS1-silenced* lines: the relative increase in dry weight was only 17 % for L9, whereas 45 % for WT (Table 1). These results indicated that *ZxSOS1* silencing significantly inhibited the growth of *Z. xanthoxylum* under both normal and salt conditions.

ZxSOS1 silencing altered Na^+ and K^+ uptake, their concentrations and partitioning in plant tissues

Under control condition, no significant difference in net Na^+ uptake rate was observed between WT and *ZxSOS1*-silenced plants, but net K^+ uptake rate in L9 was significantly lower by 17 % than that in WT (Table 2). The addition of 50 mM NaCl significantly increased net Na^+ uptake rate but decreased net K^+ uptake rate in both *ZxSOS1*-silenced plants and WT (Table 2). Under 50 mM NaCl, net Na^+ uptake rate was significantly higher by 21 % while net K^+ uptake rate was significantly lower by 56 % in L9 compared to that in WT (Table 2).

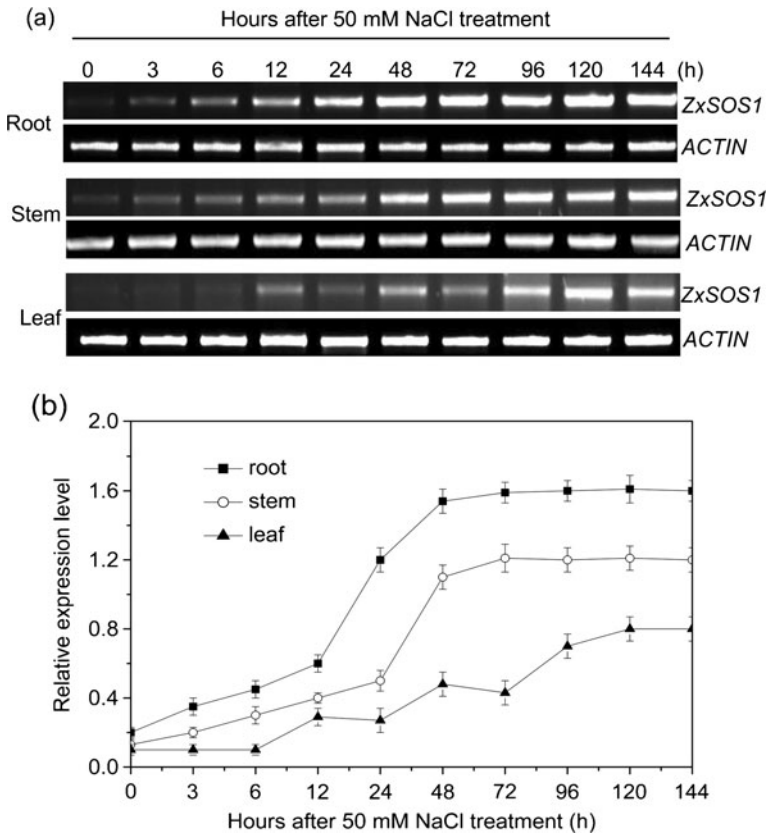

Fig. 2 Time courses of *ZxSOS1* expression in root, stem, and leaf of *Z. xanthoxylum* in 50 mM NaCl. **a** Semi-quantitative RT-PCR analysis of *ZxSOS1* mRNA in 3-week-old plants treated with 50 mM NaCl over a 144 h period. *ACTIN* was used as an internal control. Experiments were repeated at least three times (with similar results). **b** The relative expression level of *ZxSOS1* (related to *ACTIN*) under 50 mM NaCl over a 144 h period. Values are means ± SD (n=3) and *bars* indicate SD

Under control condition, Na^+ concentrations in different tissues were the same between *ZxSOS1*-silenced and WT, whereas the K^+ concentration in leaf tissue was significantly lower by 17 % in both L5 and L9, compared to that in WT (Fig. 6). Compared with the control, the addition of 50 mM NaCl significantly increased Na^+ concentrations but decreased K^+ concentrations in all tissues in both *ZxSOS1*-silenced and WT plants (Fig. 6). In 50 mM NaCl, L9 accumulated more Na^+ in its roots (by 19 %) but less Na^+ in leaves and stems (by 15 % and 23 %, respectively) than WT (Fig. 6a, b, c). However, K^+ concentrations in leaves and roots in L9 were significantly reduced compared to that in WT while that in the stems remained unchanged (Fig. 6). In 50 mM NaCl, the selective transport capacity for K^+ over Na^+ (ST value) from root to stem was significantly higher by 33 % in L5 and 83 % in L9 while the ST value from stem to leaf was significantly lower by 25 % and 35 % in L5 and L9, respectively, when compared to WT plants (Fig. 7a, b).

Under control condition, the relative distribution of K^+ in leaves was significantly lower in L5 and L9 than in WT plants, although there was no difference between these lines and the WT in the proportion of K^+ in the stems and roots (Table 3). In 50 mM NaCl, the relative distributions of K^+ in leaves and roots were significantly lower whereas that in stem was significantly higher in L9 compared to that in WT (Table 3); however, L9 had more Na^+ in its roots but less in stems and leaves than WT (Table 4). Taken together, these data indicate that ZxSOS1 is not only involved in transport and spatial distribution of Na^+, but also regulates that of K^+ in *Z. xanthoxylum*.

Discussion

Z. xanthoxylum is valuable for characterizing long-distance Na^+ transport *in planta*

To date, the possible molecular mechanisms of long-distance Na^+ transport from root to shoot in higher plants has only been reported in *A. thaliana*, *Eutrema salsugineum* (formerly *Thellungiella salsuginea*) and

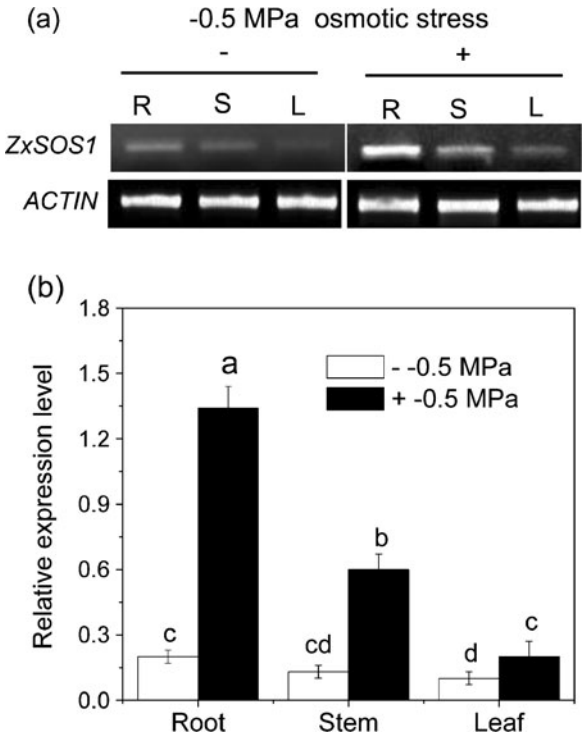

Fig. 3 Tissue-specific expression of *ZxSOS1* in *Z. xanthoxylum* under osmotic stress. **a** Semi-quantitative RT-PCR analysis of *ZxSOS1* mRNA in root, stem, and leaf tissue of 3-week-old plants treated without (−) or with (+) ½ strength Hoagland nutrient solutions supplemented with additional mannitol where the osmotic potential was −0.5 MPa for 24 h. *ACTIN* was used as an internal control. Experiments were repeated at least three times (with similar results). **b** The relative expression level of *ZxSOS1* (related to *ACTIN*) in various tissues. Values are means ± SD (n=3) and *bars* indicate SD. Columns with different *letters* indicate significant differences at P<0.05 (Duncan test)

tomato (*S. lycopersicum*) (Amtmann 2009; Oh et al. 2009; Olías et al. 2009a, b; Shi et al. 2002). However, these plants have a weak ability of transporting Na^+ from root to shoot (Essah et al. 2003; Estañ et al. 2005; Munns 2002; Oh et al. 2009). Furthermore, *A. thaliana* and *E. salsugineum* have very short stems, which do not allow a precise dissection of the relative content of Na^+ in stem versus leaf (Olías et al. 2009b). In this sense, these plants might not be the best choice to understand the pathway of Na^+ transport from root to leaf in plants.

Z. xanthoxylum is a good alternative for studying long-distance Na^+ transport because of its physiological and anatomical structure. *Z. xanthoxylum* has clear a well-defined stem and can absorb a great quantity of Na^+ from low-salinity soil and transport Na^+ to its leaves under arid field conditions (Wang et al. 2004). Our previous studies discovered that adding 5 to 100 mM NaCl significantly increased the growth of *Z. xanthoxylum*, and the optimal growth was obtained at 50 mM NaCl (Ma et al. 2012; Yue et al. 2012). For *Z. xanthoxylum* growing under 50 mM NaCl, 94 % of the Na^+ that is accumulated is in shoot (81 % in leaf and 13 % in stem, Table 4), and there is evidence that little or no Na^+ was recirculated from shoot to root or secreted onto leaf surfaces (Liu et al. 1988; Wang et al. 2004), suggesting that virtually all the Na^+ that enters root is efficiently transported to the leaves. Moreover, *Z. xanthoxylum* has succulent leaves with enlarged cells in which the vacuoles occupy most of the cell volume (Liu et al. 1987; Wang et al. 2004). Further investigations revealed that Na^+ is sequestered in vacuoles in these large cells mediated by the tonoplast Na^+/H^+ antiporter ZxNHX (Wu et al. 2011). This allows *Z. xanthoxylum* plants to use Na^+ as a beneficial element for osmotic adjustment, and as a result, enhance photosynthesis and improve water status (Ma et al. 2012; Yue et al. 2012). In contrast to *Z. xanthoxylum*, *A. thaliana*, *E. salsugineum* and *S. lycopersicum* cannot accumulate such high Na^+ concentrations in leaves, except when those leaves are dying or dead (Wang et al. 2007; Taleisnik and Grunberg 1994). Therefore, *Z. xanthoxylum* is a valuable plant material for characterizing the pathway of Na^+ transport from root to leaf.

Expression pattern of *ZxSOS1* indicates a critical role of SOS1 for different plants to adapt to varied environments

In Arabidopsis, the expression of *AtSOS1* was up-regulated by NaCl stress, and *AtSOS1* mRNA was more abundant in roots than shoots (Shi et al. 2000). A similar expression pattern was observed in *OsSOS1* (Martinez-Atienza et al. 2007), *TaSOS1* (Xu et al. 2008), *ThSOS1* (Oh et al. 2009), *PtSOS1* (Guo et al. 2012) and *ZxSOS1* (Figs. 2 and 3). However, in a salt-secreting plant *Mesembryanthemum crystallinum*, the transcript level of *McSOS1* is up-regulated in leaves but not in roots under NaCl treatment. This suggests that McSOS1 functions in the maintenance of a low cytosolic Na^+ concentration in the leaves of *M. crystallinum*, especially in photosynthetic cells (Cosentino et al. 2010), rather than, as AtSOS1, extruding Na^+ from root to soil or loading Na^+ into xylem (Shi et al. 2002). Unlike many other salt stress-responsive genes, the up-regulated expression of *AtSOS1* expression is very specific to NaCl and does not occur under other abiotic treatments such as ABA

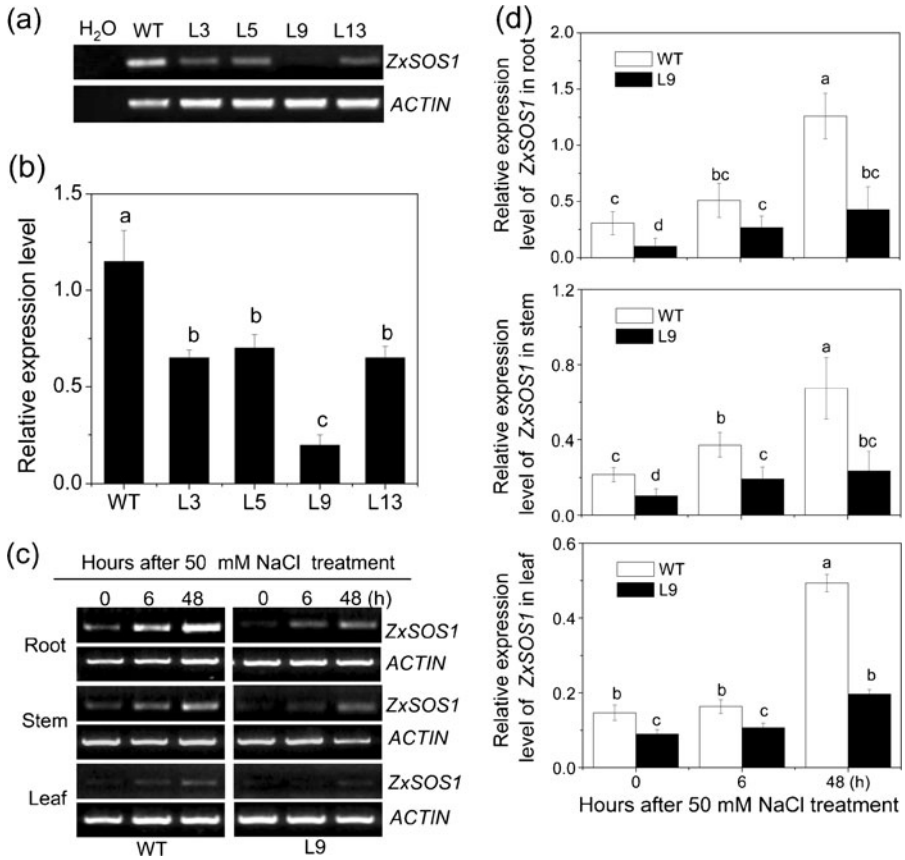

Fig. 4 Analysis by RT-PCR of *ZxSOS1* expression in transgenic *ZxSOS1*-silenced lines (L3, L5, L9 and L13). **a** Semi-quantitative RT-PCR analysis of *ZxSOS1* mRNA in root tissue of wild type (WT) and transgenic *ZxSOS1*-silenced lines (L3, L5, L9 and L13) treated with 50 mM NaCl for 48 h. H_2O was used as a template for the negative control of RT-PCR reaction. **b** The relative expression level of *ZxSOS1* (related to *ACTIN*) in root tissue of WT and transgenic *ZxSOS1*-silenced lines (L3, L5, L9 and L13). **c** Semi-quantitative RT-PCR analysis of *ZxSOS1* mRNA in root, stem and leaf of WT and L9 treated with 50 mM NaCl for 0, 6 and 48 h, respectively. **d** The relative expression level of *ZxSOS1* (related to *ACTIN*) in root, stem and leaf tissue of WT and L9 treated with 50 mM NaCl for 0, 6 and 48 h, respectively. *ACTIN* was used as an internal control. Experiments were repeated at least three times (with similar results). Values are means ± SD (n=3) and *bars* indicate SD. Columns with different *letters* indicate significant differences at P<0.05 (Duncan test)

or cold (Shi et al. 2000; Zhu 2000). However, recent evidence showed that nitric oxide could increase the expression of *SOS1* in the leaves of a mangrove, *Avicennia marina*, under high salinity, and therefore enhance salt secretion from salt glands in leaves (Chen et al. 2010). In the present study, the expression of *ZxSOS1* was not only induced by NaCl treatments (Figs. 1 and 2), but also up-regulated by osmotic stress, especially in root tissue (Fig. 3), indicating that *ZxSOS1* is important for *Z. xanthoxylum* responding to arid environments. For these reasons, we speculate that SOS1 plays a critical role in different plants growing under their specific environments, not simply restricted to episodes of saline conditions.

ZxSOS1 is involved in spatial distribution of Na^+ and K^+, thus regulating Na^+, K^+ homeostasis in *Z. xanthoxylum*

In glycophytes, both passive and active models have been vigorously advocated for the transport mechanisms involved in Na^+ loading into xylem (Munns and Tester 2008; Wegner and Raschke 1994). However, thermodynamically, passive Na^+ loading into xylem would happen only if the membrane potential values of XPCs were less negative than −17 mV (Shabala and Mackay 2011). This seems not to be the case in halophytes since some authors reported highly negative values (e.g. −130 to −140 mV) for halophyte XPCs

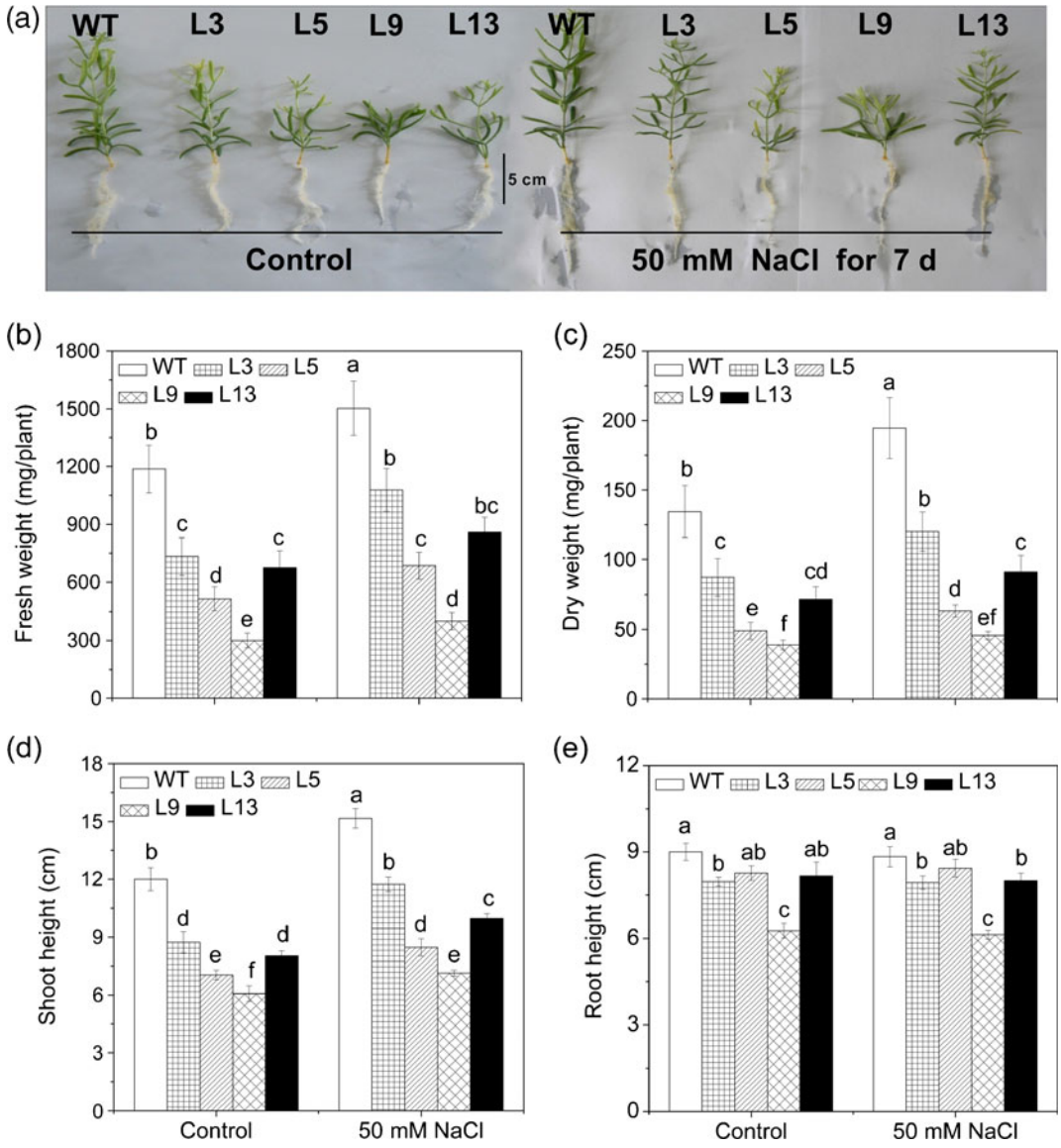

Fig. 5 The growth status (**a**), fresh weight (**b**), dry weight (**c**), shoot height (**d**) and root length (**e**) of WT and *ZxSOS1*-silenced lines (L3, L5, L9 and L13) under control (no additional NaCl) and 50 mM NaCl for 7 days. The scale bar in (**a**) equals 5 cm. Values in (**b**), (**c**), (**d**) and (**e**) are means ± SD (n=4) and *bars* indicate SD. Columns with different *letters* indicate significant differences at P<0.05 (Duncan test)

(Anderson et al. 1977). This leaves active Na^+ loading operated by SOS1 as the only option in halophytes (Adolf et al. 2013; Sun et al. 2009a; Shabala and Mackay 2011) and plants like *Z. xanthoxylum* which possesses a feature typical for salt-accumulating halophytes (Janz and Polle 2012; Ma et al. 2012).

Under moderate salinity, the Arabidopsis mutant, *atsos1* accumulated less Na^+ in its shoots than the WT, indicating that SOS1 might function in loading Na^+ into xylem for controlling delivery to the shoot and storage in leaf mesophyll cells under moderate salt stress in Arabidopsis (Shi et al. 2002). Olías et al. (2009a, b) cloned *SlSOS1* from tomato, an important crop plant with clear differentiation of stem and leaf, and found SlSOS1 in tomato controlled the partitioning of Na^+ in plant organs. In the present study, we also found that ZxSOS1 was involved in long-distance transport and spatial distribution of Na^+ among plant organs in *Z. xanthoxylum*, presumably by acting at xylem loading (Shi et al. 2002). This view is supported by the facts that

Table 1 Relative increase in dry weight (%) of WT and *ZxSOS1*-silenced lines (L3, L5, L9 and L13) under 50 mM NaCl for 7 days. Values are means ± SD (n=4) and bars indicate SD. Columns with different letters indicate significant differences at P<0.05 (Duncan test)

Lines	WT	L3	L5	L9	L13
Relative increase in dry weight	45±12 a	38±13 a	29±10 ab	17±7 b	27±11 ab

Na^+ concentration and its proportion in leaf and stem decreased significantly, whereas it significantly increased in root tissue in L9 compared to WT (Fig. 6; Table 4). However, unlike *Z. xanthoxylum*, transgenic tomato with reduced expression of *SlSOS1* possessed higher accumulation of Na^+ in leaves and roots, but lower concentration in stems compared to WT (Olías et al. 2009a), which suggests that, although SOS1 controls long-distance transport and spatial distribution of Na^+, it might plays different roles in the response of *Z. xanthoxylum* and tomato to salt. As most glycophytes, tomato plants limit salt accumulation in the photosynthetic leaf tissue by retaining Na^+ in stems (Guerrier 1996; Taleisnik and Grunberg 1994), and SlSOS1 is critical for this process (Olías et al. 2009a). By contrast, *Z. xanthoxylum* responds to salt with increased growth by transporting a great quantity of Na^+ into and accumulating it in leaf tissue, a feature typical for halophytes (Flowers et al. 1977; Janz and Polle 2012); moreover the plants become more tolerant to drought in the presence of moderate salinity (50 mM NaCl) (Ma et al. 2012; Yue et al. 2012).

Although some early studies have suggested that SOS1 is a specific transporter for Na^+ and unable to transport K^+ (Qiu et al. 2002; Quintero et al. 2002; Shi et al. 2002), numerous publications have demonstrated that the activity of SOS1 is correlated with K^+ nutrition, mainly focused on K^+ uptake (Kronzucker and Britto 2011; Pardo et al. 2006; Shabala et al. 2005; Wu et al. 1996, Zhu et al. 1998). In the present study, we also found that ZxSOS1 could regulate K^+ uptake in *Z. xanthoxylum* (Table 2). It has been reported that SOS1 was necessary for protecting the K^+ uptake mediated by inward-rectifying channel AKT1 on which growth depends and that the elevated cytoplasmic Na^+ levels resulting from loss of SOS1 function impaired K^+ uptake ability in root cells and compromised K^+ nutrition under salt stress (Qi and Spalding 2004). Furthermore, *atsos1* plants were impaired in high-affinity K^+ uptake, independent of saline conditions (Ding and Zhu 1997). Such a protective effect of SOS1 on root K^+ uptake might exist in *Z. xanthoxylum* under salt treatment and normal condition, since net uptake rate of K^+ was significantly decreased in L9 under both conditions in comparison to that in WT (Table 2).

Furthermore, we surprisingly found that ZxSOS1 could regulate long-distance transport and spatial distribution of K^+ in *Z. xanthoxylum*. Under normal condition, K^+ concentration and the proportion of K^+ in leaves were significantly reduced in L9 compared to that in WT (Fig. 6; Table 3). Recently, Bassil et al. (2011) and Barragán et al. (2012) revealed that tonoplast $Na^+(K^+)/H^+$ antiporters NHX1 and NHX2 could mediate active K^+ uptake into vacuoles and thus maintain K^+ accumulation in leaf in Arabidopsis. Whether ZxSOS1 might regulate gene expression or activities of these antiporters to maintain K^+ accumulation in leaves in *Z. xanthoxylum* under normal condition needs to be further investigated. Under salt treatment, the disruption of ZxSOS1 by RNAi probably reduced extrusion of Na^+ back to the rhizosphere mediated by ZxSOS1, since net Na^+ uptake rate was significantly higher in L9 compared to that in WT (Table 2). It has been reported that even in

Table 2 Net uptake rate (nmol $gRFW^{-1}$ min^{-1}) of Na^+ and K^+ in whole plants of WT and *ZxSOS1*-silenced lines (L3, L5, L9 and L13) under control condition (no additional NaCl) or 50 mM NaCl for 7 days. Values are means ± SD (n=4) and bars indicate SD. Columns with different letters indicate significant differences at P<0.05 (Duncan test)

	Net Na^+ uptake rate		Net K^+ uptake rate	
	Control	50 mM NaCl	Control	50 mM NaCl
WT	0.2±0.1 a	135±12 b	6.5±0.3 a	−5.1±1.4 a
L3	0.2±0.2 a	124±16 b	6.1±0.4 ab	−5.3±2.3 ab
L5	0.2±0.1 a	134±27 ab	6.0±0.3 ab	−7.1±1.3 ab
L9	0.2±0.1 a	163±21 a	5.5±0.4 b	−8.0±1.4 b
L13	0.2±0.2 a	142±12 ab	6.1±0.5 ab	−6.7±1.7 ab

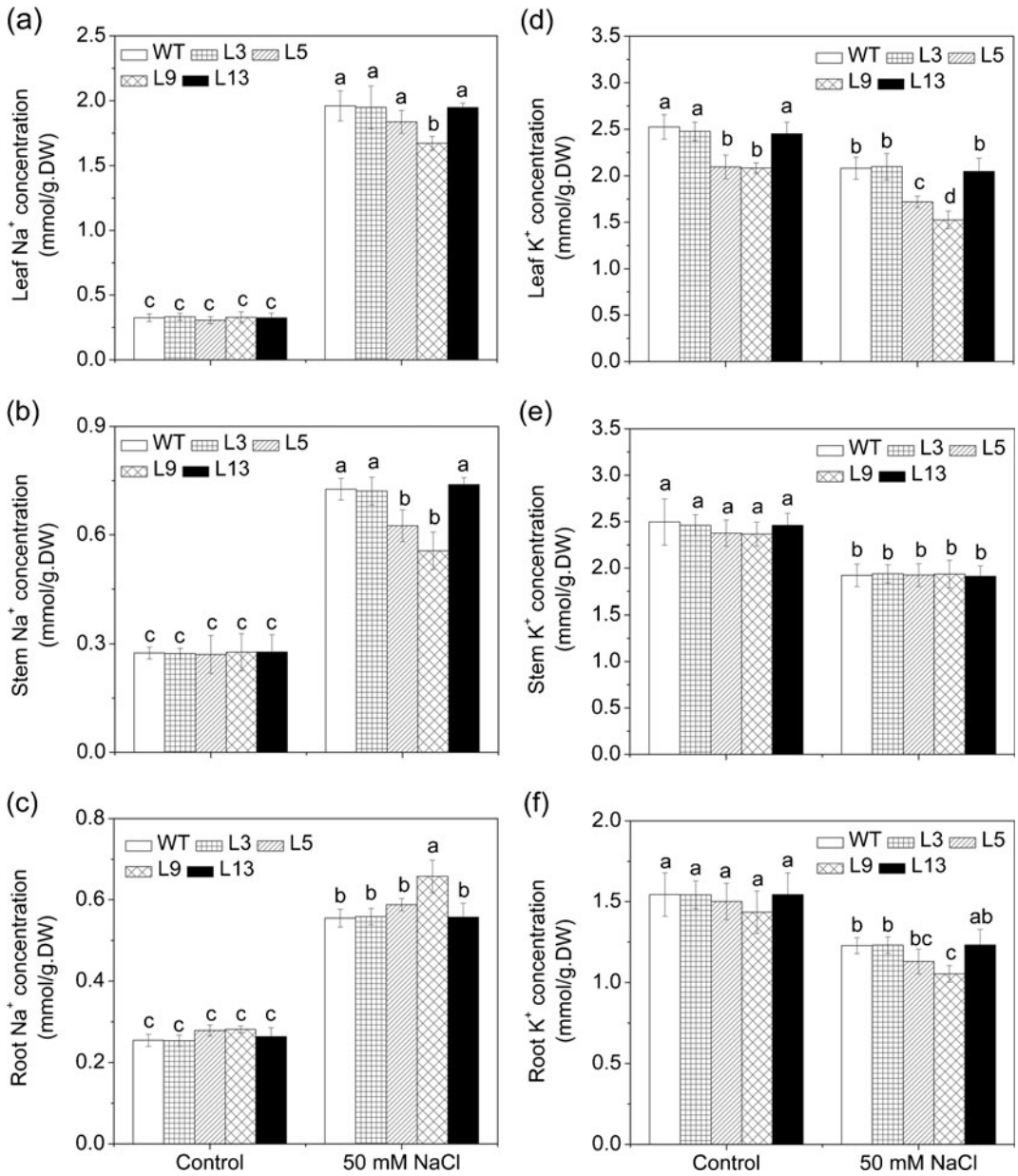

Fig. 6 Na^+ (**a**, **b**, **c**) and K^+ (**d**, **e**, **f**) concentrations in the leaf, stem and root tissue of WT and *ZxSOS1*-silenced lines (L3, L5, L9 and L13) under control (no additional NaCl) and 50 mM NaCl for 7 days. Values are means ± SD (n=4) and *bars* indicate SD. Columns with different *letters* indicate significant differences at $P<0.05$ (Duncan test)

halophytes, undifferentiated cells at the root tip are sensitive to the rapid accumulation of Na^+ (Hajibagheri et al. 1985), and plants need to secrete certain amounts of Na^+ back to the rhizosphere to protect cells of root meristematic region and elongation zone (Oh et al. 2009). Reduced Na^+ extrusion would increase Na^+ entry into root XPCs via radial transport and induce a sequential depolarization of root XPCs membrane, and then result in an efflux of K^+ from XPCs to the xylem (Shabala et al. 2010; Wegner et al. 2011), probably via membrane depolarization-activated K^+ outward-rectifying channel SKOR (Britto and Kronzucker 2008; Gaymard et al. 1998; Wegner and Raschke 1994; Wegner and De Boer 1997) or non-selective cation channels

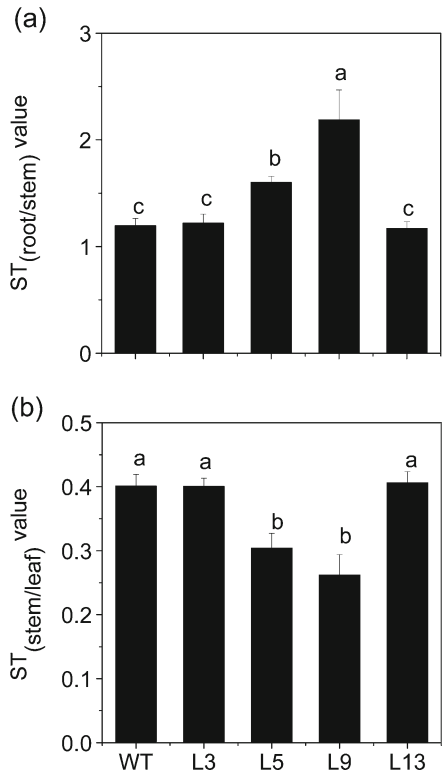

Fig. 7 The selective transport capacity for K^+ over Na^+ (ST value) from root to stem (**a**) and that from stem to leaf (**b**) in WT and *ZxSOS1*-silenced lines (L3, L5, L9 and L13) grown in 50 mM NaCl for 7 days. Values are means ± SD (n=4) and *bars* indicate SD. Columns with different *letters* indicate significant differences at P<0.05 (Duncan test)

(NSCCs) (Shabala and Cuin 2008; Sun et al. 2009b). Such an efflux would consequently trigger a decrease in K^+ concentration and the proportion of K^+ in root tissue while increasing the proportion of K^+ in stems under salt treatment (Fig. 6, Table 3).

Moreover, besides the suppression of K^+ accumulation in leaf tissue as discussed above, the disruption of ZxSOS1 in leaves might also inhibit K^+ loading into mesophyll cells from the xylem, thus decrease the selective transport capacity for K^+ over Na^+ from stem to leaf (Fig. 7b), which is consistent with the fact that *ZxSOS1-RNAi* plants accumulated less K^+ in leaves but more in stems (Fig. 6; Table 3). In leaves, K^+ channels AKT2 and KAT2 have been proposed to be likely candidates to account for K^+ loading into mesophyll cells from xylem (Lacombe et al. 2000; Lebaudy et al. 2007). Therefore, it is intriguing to investigate whether ZxSOS1 can regulate the function of these K^+ channels and so affect K^+ transport from stem to leaf in *Z. xanthoxylum*.

Taken together, we suggest that ZxSOS1 is involved in the spatial distribution of Na^+ and K^+ and thus regulating Na^+, K^+ homeostasis in *Z. xanthoxylum* - probably by regulating the function of Na^+, K^+ transporters/channels, but the detailed molecular bases need to be further examined.

Table 4 Na^+ relative distribution (%) in different parts of WT and *ZxSOS1*-silenced lines (L3, L5, L9 and L13) under 50 mM NaCl for 7 days. Values are means ± SD (n=4) and bars indicate SD. Columns with different letters indicate significant differences at P<0.05 (Duncan test)

	Leaf	Stem	Root
WT	80.6±1.1 a	13.0±1.0 a	6.4±1.1 b
L3	79.6±1.9 a	12.5±0.9 a	7.9±1.1 b
L5	77.6±1.5 ab	10.2±0.3 b	12.2±1.8 a
L9	74.9±1.7 b	10.1±0.7 b	15.0±1.7 a
L13	79.7±1.6 a	12.9±0.8 a	6.4±2.3 b

ZxSOS1 might be essential for normal growth and development of *Z. xanthoxylum*

Previous studies showed that SOS1 is not essential for normal plant growth and development, but critical for

Table 3 K^+ relative distribution (%) in different parts of WT and *ZxSOS1*-silenced lines (L3, L5, L9 and L13) under control condition (no additional NaCl) or 50 mM NaCl for 7 days. Values are means ± SD (n=4) and bars indicate SD. Columns with different letters indicate significant differences at P<0.05 (Duncan test)

	Control			50 mM NaCl		
	Leaf	Stem	Root	Leaf	Stem	Root
WT	73.1±2.7 a	18.1±2.9 a	8.8±1.2 a	73.2±1.6 a	18.2±2.4 b	8.6±1.0 a
L3	72.9±2.9 a	19.5±2.5 a	7.6±1.4 a	70.2±2.0 a	22.9±1.9 b	6.9±1.1 ab
L5	65.7±3.1 b	24.7±4.8 a	9.6±2.1 a	65.1±2.1 b	27.2±2.5 a	7.7±1.0 ab
L9	65.5±3.2 b	25.3±4.8 a	9.2±2.3 a	64.7±2.0 b	28.8±2.3 a	6.5±1.0 b
L13	72.1±4.6 ab	19.3±3.3 a	8.6±1.9 a	73.2±2.2 a	18.8±2.0 b	8.0±1.1 ab

the salt tolerance of glycophytes (Olías et al. 2009a; Shi et al. 2002; Wu et al. 1996). Here, our results firstly showed that silencing of *ZxSOS1* had strong negative effects on plant growth under salt treatment (Fig. 5), which was partly caused by the significantly reduced accumulation of K^+ and especially Na^+ that has positive effect on the growth of *Z. xanthoxylum* in leaf after the expression of *ZxSOS1* was reduced by RNAi (Fig. 6; Tables 3 and 4). These data provide evidences for expanding the role of SOS1 in regulating normal plant growth and development. It is worth noting that *ZxSOS1*-silencing also carried ill effects on plant growth even under non-stress conditions (Fig. 5). While all SOS1-silenced lines showed significant growth reduction compared with WT (Fig. 5; Table 1), only one line (L9, which had the highest silencing efficiency of *ZxSOS1*) showed a significant difference in the concentration and relative distribution of Na^+ in leaf tissue (Fig. 6; Table 4). This indicates that, besides regulating spatial distribution of Na^+ and K^+, other functional processes that modulate normal plant growth and development were possibly affected by silencing of *ZxSOS1* in *Z. xanthoxylum*. In fact, several cellular functions of AtSOS1 have been suggested, including regulating Ca^{2+} and H^+ homeostasis (Guo et al. 2009; Oh et al. 2010; Shabala et al. 2005), vacuolar morphology and membrane trafficking (Oh et al. 2010) and, possibly, signal transduction (Chung et al. 2008). Recent work has indicated that a large number of changes in the expression of genes encoding pH, membrane trafficking-related proteins, aquaporins, and ion transporters were triggered by *atsos1* mutation, even in the absence of salt stress (Oh et al., 2010). These may be other reasons why ZxSOS1 could be involved in regulating growth and development of *Z. xanthoxylum*.

In conclusion, our results demonstrate that ZxSOS1 is not only essential in long-distance transport and spatial distribution of Na^+ and K^+, but also has a more complicated function in regulating the K^+ and Na^+ transport system and maintaining Na^+, K^+ homeostasis in *Z. xanthoxylum*, thereby regulating its normal growth. Further studies are worthwhile to uncover the detailed molecular bases of the role(s) of ZxSOS1.

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