ZxAKT1 is essential for K⁺ uptake and K⁺/Na⁺ homeostasis in the succulent xerophyte *Zygophyllum xanthoxylum*

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SUMMARY

The inward-rectifying K⁺ channel AKT1 constitutes an important pathway for K⁺ acquisition in plant roots. In glycophytes, excessive accumulation of Na⁺ is accompanied by K⁺ deficiency under salt stress. However, in the succulent xerophyte *Zygophyllum xanthoxylum*, which exhibits excellent adaptability to adverse environments, K⁺ concentration remains at a relatively constant level despite increased levels of Na⁺ under salinity and drought conditions. In this study, the contribution of ZxAKT1 to maintaining K⁺ and Na⁺ homeostasis in *Z. xanthoxylum* was investigated. Expression of ZxAKT1 rescued the K⁺-uptake-defective phenotype of yeast strain CY162, suppressed the salt-sensitive phenotype of yeast strain G19, and complemented the low-K⁺-sensitive phenotype of Arabidopsis akt1 mutant, indicating that ZxAKT1 functions as an inward-rectifying K⁺ channel. ZxAKT1 was predominantly expressed in roots, and was induced under high concentrations of either KCl or NaCl. By using RNA interference technique, we found that ZxAKT1-silenced plants exhibited stunted growth compared to wild-type *Z. xanthoxylum*. Further experiments showed that ZxAKT1-silenced plants exhibited a significant decline in net uptake of K⁺ and Na⁺, resulting in decreased concentrations of K⁺ and Na⁺, as compared to wild-type *Z. xanthoxylum* grown under 50 mM NaCl. Compared with wild-type, the expression levels of genes encoding several transporters/channels related to K⁺/Na⁺ homeostasis, including ZxSKOR, ZxNHX, ZxSOS1 and ZxHKT1;1, were reduced in various tissues of a ZxAKT1-silenced line. These findings suggest that ZxAKT1 not only plays a crucial role in K⁺ uptake but also functions in modulating Na⁺ uptake and transport systems in *Z. xanthoxylum*, thereby affecting its normal growth.

Keywords: *Zygophyllum xanthoxylum*, ZxAKT1, K⁺ uptake, K⁺/Na⁺ homeostasis, salinity.

INTRODUCTION

Drought and salinity are two major abiotic stresses restricting crop growth and agricultural production worldwide (Tang *et al.*, 2015). These two hostile stresses frequently co-occur in agricultural ecosystems as the secondary salinization induced by irrigation becomes increasingly severe, especially in arid and semi-arid areas (Yan *et al.*, 2013; Tang *et al.*, 2015). The xerophytic or halophytic species widely distributed in arid and saline regions, however, have evolved various protective strategies to ensure their own survival and the establishment of their offspring under these harsh environments (Flowers and Colmer, 2008; Ashraf, 2010; Burrieya *et al.*, 2012; Ahmed *et al.*, 2013). One common mechanism is regulating K⁺, Na⁺ transport to re-establish cell osmotic and ionic homeostasis during stress (Blumwald *et al.*, 2000; Yuan *et al.*, 2015; Gu *et al.*, 2016; Shabalala *et al.*, 2016).

*Zygophyllum xanthoxylum*, a perennial shrub widely distributed in the desert areas of northwest China and Mongolia, is a salt-accumulating succulent xero-halophyte belonging to Zygophyllaceae with excellent resistance to drought and salinity (Ma *et al.*, 2012). This species plays a vital role in sand-fixing, water and soil conservation in...
desert areas (Ma et al., 2012). Our previous studies indicated that *Z. xanthoxylum* could absorb a large amount of Na⁺ even from low salt soils (Wang et al., 2004), and moderate concentrations of NaCl could stimulate the growth of *Z. xanthoxylum* under well irrigated conditions and, more interestingly, mitigate the deleterious impacts of drought that are closely linked to the high Na⁺ accumulation in leaves (Ma et al., 2012; Yue et al., 2012). In most glycophytes, a high external concentration of Na⁺ would disturb the ability to acquire K⁺ resulting from the competition between Na⁺ and K⁺ for the major binding sites of K⁺ channels or transporters, and, consequently, triggers the imbalance of cytoplasmic cations, thereby affecting many key metabolic processes in cells (Shabala et al., 2016). However, although the concentration of Na⁺ in leaves is tremendously increased under salt and drought conditions in *Z. xanthoxylum*, the concentration of K⁺ only exhibited a slight decrease under salt treatment and maintained unchanged under drought stress (Wu et al., 2011; Ma et al., 2012; Yue et al., 2012; Hu et al., 2016), suggesting that, besides absorbing a great quantity of Na⁺ which is efficiently accumulated in leaves, maintaining the stability of K⁺ concentration in those leaves is another important adaptive strategy for *Z. xanthoxylum* to survive in saline and arid environments. Therefore, an efficient K⁺ transport system at the plasma membrane of root cells is of crucial importance for maintaining K⁺ acquisition and homeostasis in *Z. xanthoxylum* under drought and salt conditions.

Numerous earlier studies found that K⁺ uptake exhibited bi-phasic kinetics in response to increasing external K⁺ concentrations with a high- or a low-affinity component in plants (Epstein et al., 1963; Maathuis and Sanders, 1994). In the model plant *Arabidopsis thaliana*, the high-affinity K⁺ transporter HAK5 and inward-rectifier K⁺ channel AKT1 (*Arabidopsis* K⁺ transporter 1) constitute important pathways for K⁺ acquisition in root cells (Maathuis et al., 1997; Wang and Wu, 2013). It has been shown that AtHAK5 only operates as a high-affinity K⁺ uptake system at external concentrations <10 μM, while AKT1 operates as a low-affinity K⁺ uptake system and plays a crucial role in K⁺ uptake at external concentrations >500 μM. When concentrations of external K⁺ are between 10 and 200 μM, both AtHAK5 and AKT1 contribute to K⁺ acquisition (Gierth et al., 2005; Rubio et al., 2008). Given that the typical K⁺ concentration in natural soils varies from 0.1 to 1 mM and potash fertilization is a common practice in many crop producing areas (Maathuis, 2009), it is likely that AKT1 plays a more important role in K⁺ acquisition in natural environments.

*AKT1* is expressed preferentially in roots hairs, epidermis, cortex and endodermis of mature roots, where cell types specialized in K⁺ uptake (Lagarde et al., 1996; Fuchs et al., 2005; Li et al., 2014). In Arabidopsis and rice, loss of function of *AKT1* led to a significant reduction of K⁺ uptake and made the plants hypersensitive to K⁺ deficiency (Hirsch et al., 1998; Spalding et al., 1999; Xu et al., 2006; Li et al., 2014). Besides mediating K⁺ uptake, AKT1 was also correlated with salt tolerance in plants. Golldack et al. (2003) found that *OsAKT1* transcript levels disappeared from the exodermis and endodermis in roots of relatively salt resistant (sodium-excluding) rice varieties whereas it was expressed in salt-sensitive (sodium-accumulating) variety under 150 mM NaCl. Interestingly, the expression pattern of *OsAKT1* in different varieties did not respond to external K⁺ concentrations (Golldack et al., 2003). These observations suggest that Na⁺ accumulation in the whole plant, to some extent, is related to the expression of *OsAKT1* under salt stress (Golldack et al., 2003). Furthermore, Wang et al. (2007) found that tetraethylammonium (TEA⁺), Cs⁺ and Ba²⁺, which inhibit the activity of AKT1 (Maathuis et al., 1997), significantly reduced ²²Na⁺ influx under higher NaCl concentration in the halophyte *Suaeda maritima*. These results indicate that AKT1 not only mediates K⁺ uptake in roots, but is also related to Na⁺ homeostasis under salt conditions. However, current knowledge about this channel in xerophyte species, especially, its roles in modulating K⁺ and Na⁺ accumulation and homeostasis remains unknown.

In this study, the *ZxAKT1* gene was isolated from the xerophyte *Z. xanthoxylum* and shown to be encoding an inward-rectifying K⁺ channel. The expression patterns of ZxAKT1 in response to KCl and NaCl were then investigated. By using RNA interference, we also evaluated the role of ZxAKT1 in K⁺, Na⁺ uptake and accumulation in *Z. xanthoxylum*. The transcript levels of *ZxSKOR*, *ZxNHX*, *ZxSOS1* and *ZxHKT1;1* in ZxAKT1-silenced lines and wild-type (WT) were also studied. Our results indicate that ZxAKT1 modulates K⁺ and Na⁺ transport and thus plays an important role in maintaining K⁺, Na⁺ homeostasis in *Z. xanthoxylum*.

RESULTS

Isolation and characterization of *ZxAKT1*

A DNA fragment of 404 bp was amplified from *Z. xanthoxylum* cDNA using degenerate primers P1 and P2 (Table S1) by RT-PCR. After performing 5' and 3'RACE, a 3021 bp full-length cDNA was obtained, which contained a 5'-untranslated region (UTR) of 163 bp nucleotides, a predicted open reading frame of 2607 bp nucleotides encoding a protein of 869 amino acids, and a 3'-UTR of 251 bp nucleotides. Phylogenetic analysis revealed that this protein was highly homologous to inward-rectifying K⁺ channel AKT1, rather than KAT1, AKT2, AtKC1 and SKOR, from Arabidopsis and other plant species (Figure S1). It shared common features such as six transmembrane segments (S1–S6), a pore domain (P) carrying the hallmark GYG/E motif of highly K⁺ selective channels, a putative cyclic nucleotide-binding domain (cNBD), and an ankyrin domain
(A1–A5) (Figure S2), as previously reported for AKT1 proteins from other plants (Sentenac et al., 1992; Gambale and Uozumi, 2006). These results suggested that the obtained cDNA encodes an AKT1-like inward-rectifying K+ channel. We therefore designated this gene as ZxAKT1.

The K+ transport activity of ZxAKT1 was tested in yeast mutant strain CY162, which is defective in K+ uptake as a result of deletions of two K+ transporters TRK1 and TRK2 (Anderson et al., 1992). AKT1 from Arabidopsis (AtAKT1) was used as a positive control. Growth assays showed that under high K+ conditions (100 mM), all tested yeast strains showed similar growth (Figure 1a), while on the low K+ (0, 0.1 or 1 mM) medium, the growth of CY162 transformed with empty vector was significantly depressed, and both ZxAKT1 and AtAKT1 could rescue this growth defect (Figure 1a).

To determine if ZxAKT1 is involved in Na+ uptake, we expressed ZxAKT1, AtAKT1 and AtHKT1;1 (used as a positive control for Na+ uptake as it endows yeast cells with increased Na+ sensitivity by mediating Na+ uptake; Uozumi et al., 2000) in the yeast mutant strain G19, which exhibits higher sensitivity to Na+ due to disruptions in Na+-extruding ATPases ENA1 to ENA4 (Quintero et al., 1996). All the yeast cells grew well on the control medium (0 mM additional Na+) (Figure 1b). As expected, with increases of external Na+ concentrations (10–100 mM), the expression of AtHKT1;1 led to salt-hypersensitivity on G19 when compared with control cells (transformation of empty vector) (Figure 1b), while G19 cells that expressed ZxAKT1 and AtAKT1 showed increased growth compared with the control cells (Figure 1b). These results suggest that ZxAKT1 could not function directly as a Na+ transporter in yeast.

Figure 1. Functional characterization of ZxAKT1 in yeast and Arabidopsis.
(a) ZxAKT1 complemented the K+-uptake-deficient yeast mutant strain CY162 on AP medium containing different K+ concentrations (0, 0.2, 1 or 100 mM). CY162 expressing AtAKT1 and empty vector were used as positive and negative controls, respectively.
(b) Expression of ZxAKT1 in yeast mutant strain G19. Yeast cells were plated on AP medium containing 1 mM K+ and various concentrations of Na+ (0, 10, 30, 50, 70 or 100 mM). G19 expressing AtHKT1;1 and empty vector were used as positive and negative controls, respectively.
(c) RT-PCR analysis of transgenic akt1 lines overexpressing ZxAKT1 (CZ1-4) and AtAKT1 (CA1-4).
(d–f) Phenotype comparison of wild-type Arabidopsis (WS), akt1 mutant, and transgenic lines (akt1/ZxAKT1 and akt1/AtAKT1) grown on MS medium or low K+ medium (0.5 and 0.1 mM K+) for 10 days.
(g, h) Primary root length and seedling fresh weight of different plant materials grown on MS or low K+ medium for 10 days. Values are means ± SD (n = 5) and bars indicate standard deviation (SD). Columns with different letters indicate significant differences at P < 0.05 (Duncan’s test).
(i, j) K+ concentrations in shoots and roots of different plant materials. K+ concentrations were determined after the plants were grown on MS and low K+ medium for 10 days. [Colour figure can be viewed at wileyonlinelibrary.com].

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To further examine the possible function of ZxAKT1 in planta, the coding sequence of ZxAKT1 and AtAKT1 was transformed into an Arabidopsis akt1 mutant [ecotype Wassilewskija (WS)], and two transgenic lines (CZ2 and CA1, representing akt1/ZxAKT1 and akt1/AtAKT1, respectively) were selected for phenotypic tests (Figure 1). The akt1 mutant showed a low K⁺-sensitive phenotype and a significant reduction in K⁺ content when grown on low K⁺ (0.1 or 0.5 mM) medium (Figure 1d–j). The low K⁺-sensitive phenotype of the akt1 mutant was rescued in these two transgenic lines, which displayed a similar phenotype as wild-type (WT) plants under low K⁺ conditions (Figure 1d–f). K⁺ concentrations in shoots and roots of WT and the two transgenic lines were significantly higher than that of the akt1 mutants on MS medium or at 0.5 mM K⁺ (Figure 1i, j). Although no significant difference in root K⁺ concentrations was observed among the lines with less than 0.1 mM K⁺ concentration (Figure 1j), the akt1 mutant displayed a significant decreased shoot K⁺ concentration compared with WS and the two transgenic lines (Figure 1i). The akt1 mutant also showed a Na⁺-sensitive phenotype under different NaCl concentrations, and this phenotype was rescued by overexpression of ZxAKT1 and AtAKT1 (Figure S3a–d). With the increase in external NaCl concentration, Na⁺ concentrations in shoots and roots significantly increased in all the lines, and the akt1 mutant possessed a significantly higher shoot Na⁺ concentration (under 75 mM NaCl) and root Na⁺ concentration (under 50 and 75 mM NaCl) than the WT or two transgenic lines (Figure S3e,f). In addition, K⁺ concentrations showed a decrease trend in all the lines, and the akt1 mutant showed significantly reduced K⁺ concentrations in both shoots and roots in comparison with WT and the two transgenic lines subjected to different concentrations of NaCl (Figure S3g,h).

Taken together, these results demonstrate that ZxAKT1 has a similar function in K⁺ uptake as AKT1 in Arabidopsis. The expression of ZxAKT1 was induced by KCl and NaCl treatments

With increased external KCl concentrations, the expression of ZxAKT1 in roots and stems increased significantly, peaking at 1 mM KCl, then decreased and remained stable under 5 or 10 mM KCl, respectively, while the expression level in leaves remained at a low level under different concentrations of KCl (Figure 2a). The highest expression level in roots and lowest expression level in leaves were always observed under different concentrations of KCl (Figure 2a).

In the absence of NaCl, the expression level of ZxAKT1 was higher in roots than that in leaves and stems (Figure 2b). The addition of 50 mM NaCl significantly increased the transcription of ZxAKT1 in roots by 4.5-fold, but did not affect the expression level in leaves and stems (Figure 2b). Along with the increase of external NaCl, the expression level of ZxAKT1 in roots significantly increased and reached peak levels at 50 or 100 mM NaCl, which was 4.8-fold higher than that under control condition (no additional NaCl). The expression level of ZxAKT1 then significantly decreased under 150 mM NaCl, but was still 2.5-fold higher than that under control conditions (Figure 2c). We further investigated the expression of ZxAKT1 in roots of plants exposed to 50 mM NaCl over a 120 h period. The expression levels of ZxAKT1 increased with the elongation of treatment time, and reached their peak values at 48 h, and then displayed a decreasing trend (Figure 2d).

Taken together, these results revealed that ZxAKT1 was preferentially expressed in roots, and the transcript was induced not only by KCl treatment but also by NaCl treatment.

ZxAKT1-silencing triggered a significant inhibition of the growth of Z. xanthoxylum

To further explore the function of ZxAKT1 in planta, ZxAKT1 was silenced by RNA interference (RNAi). Fourteen transgenic lines carrying the RNAi construct were identified by PCR amplification. Since the mRNA amount of ZxAKT1 was upregulated by salt and reached the highest transcript level under 50 mM NaCl at 48 h (Figure 2c), the expression level of ZxAKT1 in four randomly selected transgenic lines treated with 50 mM NaCl for 48 h was assessed. As expected, the expression level of ZxAKT1 in the transgenic lines was reduced by different degrees in comparison with WT (Figure S4), among which line 7 displayed the highest silencing efficiency and line 10 displayed the lowest silencing efficiency of ZxAKT1 (Figures S4 and 3a). These two lines were selected for further physiological assays.

Phenotypically, the silencing of ZxAKT1 induced a significant inhibition on the growth of Z. xanthoxylum (Figure 3b). In line 7, especially, the fresh weight, dry weight, shoot height and root length were significantly decreased by 65, 62, 52 and 79% under control condition, respectively, and by 66, 63, 50 and 78% under 50 mM NaCl, respectively, in comparison with those of the WT (Figure 3c–f).

ZxAKT1 silencing altered K⁺, Na⁺ accumulation and uptake

Under control conditions, K⁺ concentrations in roots, stems and leaves of line 7 were significantly decreased by 40, 43 or 30%, respectively, compared with that of the WT, while no significant difference in Na⁺ concentration was observed between ZxAKT1-silenced lines and WT (Figure 4). The addition of 50 mM NaCl significantly increased the Na⁺ concentration but hardly affected K⁺ concentrations in any tissues of the WT or line 7 (Figure 4). Under 50 mM NaCl, line 7 accumulated less Na⁺ in roots, stems

and leaves by 30, 30 and 25% than that of WT, respectively (Figure 4a–c). Meanwhile, K+ concentrations in all tissues were also significantly reduced by 35, 41 and 20% in roots, stems and leaves, respectively, compared with that in the WT plant (Figure 4d–f).

Further analysis showed that the net K+ uptake rate in line 7 was sharply reduced by 42% compared with that of the WT, but no significant difference in net Na+ uptake rate was observed between line 7 and WT under control conditions (Figure 5a). The addition of 50 mM NaCl significantly decreased the net K+ uptake rate, while it increased the net Na+ uptake rate in both WT and ZxAKT1-silenced lines (Figure 5). Under 50 mM NaCl, line 7 exhibited decreased net K+ and Na+ uptake rate by 64 and 40% compared with the WT (Figure 5b), respectively.

ZxAKT1 silencing downregulated the expression level of ZxNHX in leaves, and ZxSKOR, ZxSOS1 and ZxHKT1;1 in roots of Z. xanthoxylum under 50 mM NaCl

To further explore the possible molecular mechanisms underlying the above-described observations, the expression levels of genes involved in K+ and Na+ uptake and transport were analyzed in WT and ZxAKT1-silenced line 7 under control conditions or with 50 mM NaCl.

As shown in Figure 6(a), the K+ outward-rectifying channel gene ZxSKOR was preferentially expressed in roots and to very low levels in leaves. Under control conditions, the expression level of ZxSKOR in roots of line 7 was the same as that of the WT (Figure 6a). The addition of 50 mM NaCl significantly increased the expression level of
ZxSKOR in roots by approximately three-fold in the WT but did not significantly affect the expression in roots of line 7 (Figure 6a), suggesting that ZxAKT1-silencing induced a significant downregulation of ZxSKOR in roots of Z. xanthoxylum under the salt treatments.

The significantly higher expression levels of the plasma membrane Na⁺/H⁺ antiporter gene ZxSOS1 and high-affinity K⁺ transporter gene ZxHKT1;1 were also observed in roots rather than leaves under both control condition and 50 mM NaCl treatment (Figure 6b,c). In the absence of NaCl, there was no significant difference in the expression levels of ZxSOS1 and ZxHKT1;1 in roots between line 7 and WT (Figure 6b,c). The addition of 50 mM NaCl significantly upregulated their expression in WT roots, while it did not induce any changes in their expression in roots of line 7 (Figure 6b,c). Under 50 mM NaCl, the transcripts of those two genes were lower by 60 and 58% in roots of line 7 compared with that of WT, respectively (Figure 6b,c). Although the expression levels of ZxSOS1 and ZxHKT1;1 were much lower in leaves than in roots, the expression levels of these two genes in leaves of both WT and line 7 were also induced by 50 mM NaCl, and the expression levels were significantly lower in line 7 than that of WT under control conditions or 50 mM NaCl treatment (Figure 6b,c). These results indicated that ZxAKT1 silencing affected the expression level of genes involved in Na⁺ transport in Z. xanthoxylum (especially in roots) when cultured in 50 mM NaCl.

The expression level of tonoplast Na⁺/H⁺ antiporter gene ZxNHX was significantly higher in leaves than that in roots under both control conditions and 50 mM NaCl (Figure 6d). Although the expression levels of ZxNHX in the leaves of WT and line 7 was significantly induced by the addition of 50 mM NaCl, the increasing trend in line 7 (67%) was significantly lower than that in WT (140%) (Figure 6d). These results indicated that ZxAKT1 silencing triggered a significant downregulation of the expression level of ZxNHX under 50 mM NaCl in leaves of Z. xanthoxylum.

**DISCUSSION**

Transcriptional regulation of AKT1 gene might be a vital mechanism in response to various K⁺ concentrations and salt conditions in Z. xanthoxylum

Similar to previous reports on AKT1 from A. thaliana (AtAKT1, Lagarde et al., 1996), rice (OsAKT1, Fuchs et al.,
2005), *Puccinellia tenuiflora* (PutAKT1; Wang et al., 2015) and *Suaeda salsa* (*Suaeda maritima* subsp. *salsa*) (SsAKT1, Duan et al., 2015), the transcript level of ZxAKT1 was more abundant in roots than shoots (Figure 2a,b). In Arabidopsis, changes in external K⁺ availability do not significantly affect AtAKT1 transcript levels in roots (Pilot et al., 2003), and similar expression patterns were observed in the salt-secreting plant *Mesembryanthemum crystallinum* (Su et al., 2001) and the salt-excluding plant *P. tenuiflora* (Wang et al., 2015). This finding suggests that the regulation of external K⁺ on AKT1 in the above species is a post-transcriptional process (Wang and Wu, 2013; Wang et al., 2015). In Arabidopsis, the regulatory genes of the AKT1 channel (e.g. CIPK23, CBL10 and AtKC1) show significant transcriptional changes under K⁺-deficiency stress (Cheong et al., 2007; Wang et al., 2010; Ren et al., 2013), indicating that the transcriptional regulation of AKT1 regulatory genes, rather than AKT1 itself, may be a more important mechanism for Arabidopsis responding to external K⁺ supply (Wang and Wu, 2013). However, in *Z. xanthoxylum*, the expression of ZxAKT1 in roots increased significantly with increase in external K⁺ concentrations (Figure 2a). This finding was consistent with the expression pattern of SsAKT1 in the salt-accumulating plant *S. salsa* (Duan et al., 2015), demonstrating that in salt-accumulating species such as *Z. xanthoxylum* and *S. salsa*, the transcriptional regulation of AKT1 itself might be a crucial response mechanism to different K⁺ concentrations.

Some studies showed that the transcript level of AKT1 was significantly downregulated in roots under NaCl treatments, such as AKT1 from *M. crystallinum* (Su et al., 2001) and *A. thaliana* (Kaddour et al., 2009). However, the transcript level of ZxAKT1 was significantly upregulated by NaCl, especially in roots (Figure 2b–d). It is worth noting that under salt stress, the increase in Na⁺ content in leaves of *M. crystallinum* and *A. thaliana* is always accompanied by a very large decrease in K⁺ content (Adams et al., 1998; Zhu et al., 1998). In contrast, although Na⁺ concentration in

![Figure 4. Na⁺ and K⁺ concentrations in roots, stems and leaves.](image-url)

**Table 1.** Na⁺ (a–c) and K⁺ (d–f) concentrations in roots, stems and leaves of WT and ZxAKT1-silenced lines (7, 10) under control (no additional NaCl) and 50 mM NaCl for 7 days. Values are means ± standard deviation (SD) (n = 5) and bars indicate SD. Columns with different letters indicate significant differences at P < 0.05 (Duncan’s test). [Colour figure can be viewed at wileyonlinelibrary.com].

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leaves of *Z. xanthoxylum* significantly increased under salt conditions, K⁺ concentration only displayed a slight decreased trend (Wu et al., 2011). Therefore, the upregulated transcript of *ZxAKT1* is responsible for K⁺ homeostasis under salt conditions in *Z. xanthoxylum*.

The role of *ZxAKT1* in K⁺ uptake and transport in *Z. xanthoxylum*

In Arabidopsis and rice, AKT1 mediates root K⁺ uptake over a wide range of external K⁺ concentrations (Lagarde et al., 1996; Hirsch et al., 1998; Xu et al., 2006; Li et al., 2014). In the present study, *ZxAKT1* is abundantly expressed in roots (Figure 2), which is the precondition that *ZxAKT1* mediates K⁺ uptake from soils in *Z. xanthoxylum*. The complementation assays in both yeast and akt1 mutant indicated that, similar to the AKT1 channel from Arabidopsis, *ZxAKT1* is involved in K⁺ uptake (Figure 1). Furthermore, *ZxAKT1*-silencing triggered a significant decline in net uptake of K⁺, resulting in decreased concentrations of K⁺ in *Z. xanthoxylum* (Figures 4d–f and 5a). These results were consistent with the observations that Arabidopsis akt1 and rice osakt1 mutant plants display a decreased K⁺ uptake and reduced K⁺ content (Xu et al., 2006; Li et al., 2014), demonstrating that *ZxAKT1* mediates K⁺ uptake in *Z. xanthoxylum*.

In higher plants, K⁺ uptake in roots displays typical dual-affinity mechanisms: the high-affinity mechanism mediates K⁺ uptake at low external K⁺ concentrations (below 0.2 mM); while at relatively high external K⁺ concentrations (above approximately 0.5 mM), the low-affinity mechanism is involved in K⁺ uptake (Epstein et al., 1963; Maathuis and Sanders, 1996; Wang and Wu, 2013). It has been shown that AKT1 contributes to both high- and low-affinity K⁺ uptake, and accounts for a large portion of the low-affinity uptake (Hirsch et al., 1998; Spalding et al., 1999; Gierth et al., 2005; Rubio et al., 2008). In the present study, *ZxAKT1* expression levels were much higher under high K⁺ concentrations (0.5–10 mM) than that under low (0.1 mM) K⁺ concentration (Figure 2), suggesting that *ZxAKT1* plays a greater role in the low-affinity system of K⁺ uptake.

The K⁺ outward-rectifying channel SKOR functions in loading K⁺ from xylem parenchyma cells (XPCs) into xylem sap toward the shoots (Wegner and Raschke, 1994; Wegner and De Boer, 1997; Gaymard et al., 1998; Liu et al., 2006). Our recent investigation showed a positive correlation between *ZxSKOR* expression in roots and K⁺ accumulation in shoots, indicating that *ZxSKOR* in roots probably plays important roles in K⁺ accumulation and homeostasis in *Z. xanthoxylum* under salt conditions (Hu et al., 2016). In the present study, the transcript of *ZxSKOR* in roots of line 7 was significantly downregulated under 50 mM NaCl compared to that of WT (Figure 6a), thereby restricting the delivery of K⁺ from roots to shoots and consequently reducing K⁺ accumulation in stems and leaves of *ZxAKT1*-silenced lines than that of WT (Figure 4d–f). It has been found that K⁺ deprivation significantly repressed the expression of *ZxSKOR* in roots of *Z. xanthoxylum* (Hu et al., 2016), and *ZxAKT1*-silencing, to some extent, may trigger K⁺ deficiency in *Z. xanthoxylum* since *ZxAKT1*-silenced lines displayed decreased K⁺ uptake (Figure 5a). This might be the reason why *ZxAKT1*-silencing resulted in a significant reduction in the transcripts of *ZxSKOR* in roots under salt treatment. These results suggest that *ZxAKT1* not only mediates K⁺ uptake in roots, but is also essential for maintaining long-distance transport and homeostasis of K⁺ in *Z. xanthoxylum*. © 2016 The Authors. The Plant Journal © 2016 John Wiley & Sons Ltd, *The Plant Journal*, (2016), doi: 10.1111/tpj.13465 

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Figure 5. Net uptake rate of K⁺ and Na⁺.
Net uptake rate of K⁺ (a) and Na⁺ (b) in whole plants of WT and *ZxAKT1*-silenced lines (7, 10) under control condition (no additional NaCl) or 50 mM NaCl for 7 days. Values are mean ± standard deviation (SD) (*n* = 5) and bars indicate SD. Columns with different letters indicate significant differences at *P* < 0.05 (Duncan’s test).
ZxAKT1 is involved in modulating Na⁺ uptake, transport and homeostasis in *Z. xanthoxylum* under salt conditions

Although ZxAKT1 could not mediate Na⁺ uptake in yeast and the Arabidopsis akt1 mutant (Figure 1), the net uptake rate and concentrations of Na⁺ in ZxAKT1-silenced plants (line 7) were significantly lower than that of WT under 50 mM NaCl (Figures 4d–f and 5b), demonstrating that ZxAKT1 might take part in modulating Na⁺ uptake and transport under salt conditions.

When Na⁺ is taken up into roots, the surface area of xylem vessels in contact with XPCs accommodates the large quantities of Na⁺ that pass from roots to shoots (Blumwald et al., 2000; Tester and Davenport, 2003; Apse and Blumwald, 2007). SOS1 located at the plasma membrane of XPCs in roots is mainly involved in the long-distance transport of Na⁺ from roots into the xylem (Shabala and Mackay, 2011; Adolf et al., 2013; Ma et al., 2014). The HKT1 transporter mediates Na⁺ unloading from xylem vessels to parenchyma cells under salt treatment (Sunarpi et al., 2005; Guo et al., 2012), and the opposite Na⁺ fluxes mediated by ZxSOS1 and ZxHKT1 in roots are finely coordinated to preserve the membrane integrity (Guo et al., 2012). This would synergistically modulate Na⁺ transport and homeostasis in *Z. xanthoxylum* (Yuan et al., 2015). In the succulent leaves of *Z. xanthoxylum*, Na⁺ is compartmentalized by ZxNHX into the vacuoles to cope with environmental stresses by using Na⁺ as an important osmoregulatory substance (Wang et al., 2004; Wu et al., 2011). A recent investigation indicated that ZxNHX also plays a vital role in controlling the uptake, long-distance transport, and homeostasis of Na⁺ at the whole plant level via feedback regulation of the expression of ZxSOS1 and ZxHKT1;1 in roots (Yuan et al., 2015). In this study, under 50 mM NaCl, the ZxSOS1, ZxHKT1;1 and ZxNHX transcript levels were significantly lower in line 7 than that of WT (Figure 6), indicating that ZxAKT1-silencing triggered a significant downregulation on the expression level of genes.

![Figure 6. Real-time quantitative PCR analysis.](image_url)
involved in Na⁺ transport and accumulation in *Z. xanthoxylum* under salt conditions and consequently restrained Na⁺ transport from roots to shoots. This would in turn inhibit Na⁺ uptake in roots (Yuan et al., 2015), and as a result, ZxAKT1-silenced plants (line 7) displayed a reduced net Na⁺ uptake rate (Figure 5b). Therefore, we propose that ZxAKT1 not only mediates K⁺ uptake, but also functions in modulating Na⁺ uptake and transport systems in *Z. xanthoxylum* under salt conditions.

Then, the question – How does ZxAKT1-silencing affect the expression of genes involved in Na⁺ transport and accumulation? – is worthy of further consideration. The possible explanations may be as follows. ZxAKT1-silencing reduced K⁺ uptake in roots and restricted the delivery of K⁺ from roots to shoots under salt treatments, and thus, led to a significant decrease in K⁺ accumulation in leaves (Figure 4d). It has been reported that NHX1 and NHX2 in Arabidopsis mediate K⁺ sequestration into the vacuoles of leaves for osmotic adjustment and turgor regulation (Bassil et al., 2011; Barragán et al., 2012; Andrés et al., 2014). ZxNHX may also mediate the vacuolar compartmentation of both Na⁺ and K⁺, since ZxNHX-silencing triggers a significant decrease in Na⁺ and K⁺ concentrations in *Z. xanthoxylum* (Yuan et al., 2015). Also, the overexpression of ZxNHX and ZxVP1 enhances Na⁺ and K⁺ accumulation in transgenic legumes under salinity and drought conditions (Bao et al., 2014, 2015). Interestingly, recent research indicated that K⁺-deficiency significantly reduces the transcripts of wheat *TaNHX2* in transgenic alfalfa under salt stress (Zhang et al., 2015). Therefore, the decrease in leaf K⁺ accumulation could result in a significant reduction in the transcripts of ZxNHX and ZxAKT1-silenced plants (Figure 6d), and consequently, the capacity for sequestering Na⁺ into vacuoles of leaves would be weakened in ZxAKT1-silenced plants, which in turn would restrict Na⁺ long-distance transport from roots to shoots through feedback regulation of ZxSOS1 and ZxHKT1;1 transcripts in roots (Yuan et al., 2015).

**ZxAKT1 is essential for normal growth and development of *Z. xanthoxylum***

Our results showed that silencing of ZxAKT1 had strong negative effects on plant growth under salt treatment and even under normal conditions: the fresh weight, dry weight, shoot height, and root length of ZxAKT1-silenced lines were significantly lower than WT (Figure 3). These observations might be partly caused by the significantly reduced accumulation of K⁺ and Na⁺ (Figure 4), as K⁺ is one of the crucial nutrient elements in plant growth and development. Meanwhile, 50 mM NaCl could significantly promote the growth of *Z. xanthoxylum*, which is strongly related to Na⁺ accumulation (Ma et al., 2012; Yue et al., 2012). These results indicate that ZxAKT1 is important for maintaining growth and development of *Z. xanthoxylum*.

In conclusion, our results demonstrate that ZxAKT1 not only mediates K⁺ uptake and plays a crucial role in regulating long-distance transport of K⁺, but is also involved in modulating Na⁺ uptake and transport systems through feedback regulation in *Z. xanthoxylum*, thereby affecting plant growth.

**EXPERIMENTAL PROCEDURES**

**Isolation of ZxAKT1 from *Zygyophyllum xanthoxylum***

Seeds of *Z. xanthoxylum* were germinated and seedlings were cultivated with modified ½ strength Hoagland nutrient solution (containing 2 mM KNO₃, 0.5 mM NH₄H₂PO₄, 0.25 mM MgSO₄·7H₂O, 0.1 mM Ca(NO₃)₂·4H₂O, 50 μM Fe citrate, 92 μM H₂BO₃, 18 μM MnCl₂·4H₂O, 1.6 mM ZnSO₄·7H₂O, 0.6 mM CuSO₄·5H₂O, 0.7 μM (NH₄)₆Mo₇O₂₄·4H₂O) according to the described by Ma et al. (2012). Total RNA was extracted from roots of 4-week-old seedlings exposed to 150 mM NaCl for 48 h. A specific fragment of ZxAKT1 was amplified by RT-PCR using degenerate primers P1 and P2 (Table S1) designed according to the highly conserved regions of AKT1 genes from other plant species. The 5’- and 3’-ends of ZxAKT1 were obtained using the RNA Ligase Mediated Rapid Amplification of cDNA Ends (RLM-RACE) Kit (Invitrogen Co., Carlsbad, CA, USA) and primers P3 and P4 or P5 and P6, respectively (Table S1). These fragments were assembled to obtain the full-length of the ZxAKT1 cDNA, which was confirmed by PCR with primers P7 and P8 (Table S1).

Multiple sequence alignment was performed with DNAMAN6.0 software (Lynnon BioSoft, Vaudreuil, Canada). The phylogenetic tree was generated using MEGA 6.0 software.

**Real-time quantitative PCR analysis of ZxAKT1 responding to different treatments**

Four-week-old plants were used for the following treatments, respectively:

(i) Plants were treated with modified ½ strength Hoagland nutrient solutions deprived of KNO₃ for 3 days, where 2 mM KNO₃ was substituted with 1 mM NH₄NO₃. Different concentrations of K⁺ treatments were then applied by adding 0, 0.1, 0.5, 1, 5 or 10 mM KCl for 48 h.

(ii) Plants were treated with modified ½ strength Hoagland nutrient solutions supplemented with additional 0, 5, 25, 50, 100 or 150 mM NaCl for 48 h.

(iii) 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 or 120 h. The treatment solution was changed every day.

Total RNA was extracted and first strand cDNA was synthesized from 2 μg of total RNA. Real-time quantitative PCR was performed on a thermal cycler (ABI PRISM 7500, Foster City, CA, USA) with the primer pairs P9 and P10 (Table S1). ZxAKT1 was used for RNA normalization, and the specific primers of ZxACTIN were A1 and A2 (Table S1). SYBR Green PCR master mix (Takara, Biotech Co., Ltd, Dalian, China) was used for PCR reactions. The relative expression levels of ZxAKT1 were normalized to ZxACTIN and calculated using the 2⁻ΔΔCt method (Duan et al., 2015). All reactions were performed with three replicates.

**Functional characterization of ZxAKT1 in yeast**

The full-length coding sequences of ZxAKT1, AtAKT1 (AKT1 from Arabidopsis) and AtHKT1;1 (HKT1 from Arabidopsis) were each
inserted into yeast expression vector p426 containing the GAL1 promoter.

The yeast (Saccharomyces cerevisiae) strains CY162 [MATa, trk1, trk2; pCK64, his3, leu2, ura3, trp1, ade2] defective in the K⁺ transporters TRK1 and TRK2 (Anderson et al., 1992) and G19 [MATa, his3, leu2, ura3, trp1, ade2, ena1::His3::ena4] disrupted in the ENA1-4 genes encoding Na⁺ export pumps (Quintero et al., 1996) were used for yeast complementation assays. Yeast transformations of the above constructed plasmids were performed using LiCl as described by Chen et al. (1992). Positive transformants were selected on Ura-selective medium [0.67% (w/v) yeast nitrogen base without amino acids, 0.077% (w/v) DO supplement, Ura, 2% (w/v) galactose, 100 mM KCl, and 1.5% (w/v) agar].

Yeast growth experiments were performed on arginine-phosphate (AP) medium containing 8 mM phosphoric acid, 10 mM L-arginine, 0.2 mM CaCl₂, 2 mM MgSO₄, 2% galactose, plus vitamins and trace elements, and 1.5% (w/v) agar (pH = 6.5) (Rodríguez-Navarro and Ramos, 1984). For growth tests of CY162 transformed with plasmids, AP medium supplemented with three concentrations of K⁺ (0, 0.2, 1 or 100 mM) were used. For growth assays of G19 transformed with plasmids, AP medium with added K⁺ (1 mM) and supplemented with various concentrations of NaCl (0, 10, 30, 50, 70, or 100 mM) were used. Yeast cells were plated on medium using 10-fold serial dilutions from OD₆₀₀ = 0.6 to OD₆₀₀ = 6 × 10⁻².

**Functional characterization of ZxAKT1 in Arabidopsis**

Full-length coding sequences of ZxAKT1 and AtAKT1 were each inserted into the vector pCAMBIA1301 containing the CaMV 35S promoter. The two constructs were transformed into an Arabidopsis akt1 mutant (Hirsch et al., 1998), using the floral-dip method with Agrobacterium tumefaciens (Clough and Bent, 1998). Transgenic lines of T₄ homozygous plants were used to examine the phenotype under low-K⁺ conditions or different NaCl treatments. The low-K⁺ medium was made by modification of MS medium, where KNO₃ and KH₂PO₄ were replaced by NH₄NO₃ and NH₄H₂PO₄, respectively (Xu et al., 2006). The final [K⁺] was 0.1 or 0.5 mM by adding the according concentrations of KNO₃. The medium for NaCl treatments was made by adding different concentrations of NaCl (0, 25, 50 or 75 mM) into MS medium. The K⁺ and Na⁺ concentrations were determined by atomic absorption spectrophotometry (Varian 220, Palo Alto, CA, USA).

**RNA interference silencing of ZxAKT1 in Z. xanthoxylum**

Stable gene silencing via Agrobacterium-mediated transformation was done using the pHANNIBAL vector (Wesley et al., 2001) designed for producing a hairpin RNA construct of ZxAKT1. A 665 bp PCR fragment as the target of RNA interference (RNAi) located in the region encoding the long hydrophilic tail in the C-terminal end of ZxAKT1 (nucleotides from 1728 to 2352 bp) was obtained by primer pairs P11 and P12 or P13 and P14 (Table S1, Xhol, KpnI, XbaI, ClaI restriction sites underlined). The whole Nof cassette bearing the RNAi construct was subcloned into the corresponding site of the binary vector pRT27, under the control of the CaMV 35S promoter. The construct was introduced into A. tumefaciens strain GV3101. The transformation was performed using the procedure as detailed described by Ma et al. (2014) and Yuan et al. (2015).

The transformed plants were screened by a PCR assay using pHANNIBAL-specific primers and DNA obtained from leaves in order to detect the presence of the RNAi construct. Positive plants were selected to study the expression level of ZxAKT1 by RT-PCR performed with primer pairs P9 and P10 (Table S1). Two ZxAKT1-silenced lines (7 and 10) with different reduced expression levels of ZxAKT1 were chosen for further analysis. Individual plants of WT and each ZxAKT1-silenced line were propagated from stem cuttings (ramets) as described by Ma et al. (2014) and Yuan et al. (2015).

For physiological analysis, 4-week-old plants were treated with modified ½ strength Hoagland nutrient solutions supplemented without (Control) or with additional 50 mM NaCl. After 7 days, roots was washed twice for 8 min in ice-cold 20 mM CaCl₂ to exchange cell wall-bound Na⁺, and leaves and stems were rinsed in deionized water to remove surface salts (Maathuis and Sanders, 2001). Fresh weight, shoot height and root length were measured. Finally, the tissues were immediately incubated in an oven at 80°C for 48 h to obtain dry weights.

Na⁺ and K⁺ were determined using a flame spectrophotometer (2655-00, Cole-Parmer Instrument Co., Vernon Hills, IL, USA) (Xu et al., 2015). 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/Δ time, where BT means before treatments.

To analyze the effect of ZxAKT1-silencing on the expression level of ZxNHX, ZxSKOR, ZxSOS1 and ZxHKT1;1 in Z. xanthoxylum, 4-week-old seedlings were grown with modified ½ strength Hoagland nutrient solution supplemented without (Control) or with additional 50 mM NaCl for 48 h. The expression level of each gene was analyzed by real-time quantitative PCR performed on a thermal cycler with primers described by Yuan et al. (2015). ZxACTIN was used for RNA normalization. The relative expression levels of ZxAKT1 normalized to ZxACTIN was calculated using the 2⁻ΔΔCt method (Duan et al., 2015). All reactions were performed with three replicates.

**Data analysis**

All data were presented as means with standard errors. Data analysis was conducted by one-way analysis of variance (ANOVA) using SPSS 13.0 statistical software (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.

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**AUTHOR CONTRIBUTIONS**

SMW conceived the project. SMW, SL and QM designed the research. QM, JH and XRZ performed the experiments. HJY and TK contributed to data analysis and discussion. QM, JH and SMW wrote the manuscript.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.


Primer sequences used in this study.

1. One-step transformation of yeast in stationary phase.

2. Two calcineurin B-like calcium sensors.

3. High salinity induces dehydrin accumulation in halophyte Puccinellia tenuiflora. 

4. Sodium chloride improves photosynthesis and water status in the succulent xerophyte. 

5. Sodium channel in Saccharomyces cerevisiae. 

6. Control of vacuolar homeostasis to regulate growth, flower development, and reproduction. 

7. Candidate involved in K+ uptake in the halophyte.

8. Ion exchangers NHX1 and NHX2 mediate vacuolar pH and K+ homeostasis in Arabidopsis. 


10. The Os-AKT1 channel is critical for K+ uptake in rice roots and is modulated by the rice CBL1-CIPK23 complex. 

11. Cotransporter AKT1-type potassium channel transcripts differently. 

12. Salt tolerance mechanisms in quinoa (Chenopodium quinoa Willd.). 

REFERENCES


AKT1 affects K⁺/Na⁺ transport in Z. xanthoxylum

under salinity by limiting unidirectional Na⁺ influx resulting in a high selectivity for K⁺ over Na⁺. Plant Cell Environ. 32, 488-496.


