Overexpression of *NaKR3* enhances salt tolerance in *Arabidopsis*

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ABSTRACT. Salinity is a major abiotic stress in agriculture. Here, we report that *SODIUM POTASSIUM ROOT DEFECTIVE3* (*NaKR3*), which encodes a heavy metal-associated domain protein, is involved in salt tolerance in *Arabidopsis*. The results of quantitative reverse transcription-polymerase chain reaction analysis revealed that *NaKR3* was induced by high salinity and osmotic stresses, but not by Cu^{2+} stress. Transient expression of *NaKR3-GFP* in *Arabidopsis* protoplasts showed that the NaKR3 protein was localized in the cytosol. Transgenic *Arabidopsis* plants constitutively expressing *NaKR3* under the control of the cauliflower mosaic virus 35S promoter exhibited increased tolerance to salt treatment. Furthermore, overexpression of *NaKR3* increased the expression of *SOS1* and *SOS3*, but decreased the accumulation of salt-induced proline. Taken together, our results indicate that *NaKR3* is involved in the salt stress response in *Arabidopsis*.

Key words: Abiotic stresses; *NaKR3*; *Arabidopsis*; Salt tolerance

INTRODUCTION

Plants are sessile organisms and are therefore constantly challenged by various environmental stresses throughout their life cycle. Salinity is a major abiotic stress. Approximately 20% of the Earth land mass is affected by salinity, and >50% of all arable lands will be saline by the year 2050 (Wang et al., 2003). To cope with such unfavorable growth conditions, plants have developed unique defense mechanisms and processes.

When plants grow in high-salinity conditions, the ability to control the Na^+ concentration is an essential process for survival. The amount of Na^+ in plants is determined by Na^+ influx and efflux, and these processes include Na^+ uptake from the soil, long distance transport to the shoot via the xylem, and redistribution through the phloem (Tester and Davenport, 2003; Apse and Blumwald, 2007). Na^+ influx, transport to the shoots, and distribution from the shoots to other parts of the plant have been extensively investigated (Tester and Davenport, 2003; Apse and Blumwald, 2007). However, the extent and mechanism of Na^+ recirculation in the phloem remains unclear. Na^+ recirculation is known to alter the Na^+ concentration in the leaves, thereby promoting Na^+ tolerance (Munns and Tester, 2008). Berthomieu et al. (2003) proposed that *AtHKT1* is related to Na^+ recirculation in the phloem; however, the current consensus is that *AtHKT1* removes Na^+ from the xylem (Rus et al., 2006).

In *Arabidopsis*, a phloem-expressed gene, *NaKR1*, is thought to be related to Na^+ transport. Tian et al. (2010) reported that loss of *NaKR1* expression led to over accumulation of Na^+ and K^+ in the leaves. This finding implied that *NaKR1* functions in the recirculation of Na^+ to the roots via the phloem, thereby limiting Na^+ accumulation in the leaves. NaKR1 has a heavy metal-associated (HMA) domain of 59 amino acids at the C-terminal end (Tian et al., 2010). Based on the results of NCBI-BLAST analysis, NaKR1 has one homologous protein in *Arabidopsis*; this protein, which shares 72% amino acid identity with NaKR1 at the C-terminal domain, is designated NaKR3 (Tian et al., 2010). In the present study, we showed that *NaKR3* is related to salt resistance. Overexpression of *NaKR3* increased the tolerance of plants to salt treatment. Our results indicate that *NaKR3* is involved in salt stress-related signal transduction and provide a basis for further study the functions of *NaKR3*.

MATERIAL AND METHODS

Plant material and growth conditions

All *Arabidopsis* plants used in this study were the Columbia (Col-0) ecotype. Seeds were stratified at 4°C for 3 days and sterilized in 75% ethanol for 30 s, followed by $HgCl_2$ for 8 min. Sterilized seeds were thoroughly washed with sterile distilled water, and were then germinated either on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) or in soil. Germinated seedlings were cultivated in perlite/soil mixture in a growth chamber at 22°C under a 16-h light/8-h dark cycle and at 60% humidity.

Sequence analysis

We identified 1150-bp putative cis elements in the promoter region of *NaKR3*, using a web-based signal scan search with the plant cis-acting regulatory DNA elements (PLACE) database (Higo et al., 1999).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of gene expression

Two-week-old seedlings cultivated on MS medium were treated with 50 μM $CuSO_4$, 250 mM NaCl, or 300 mM mannitol. Total RNA was isolated using RNAiso Plus (TaKaRa, Dalian, China) and was used for cDNA synthesis with the PrimeScript RT reagent Kit (TaKaRa, Shiga, Japan). DNA amplification was performed in the presence of FastStart SYBR Green Master (Roche, Mannheim, Germany) in an iCycler (Bio-Rad, Hercules, CA, USA), using the primer pairs ACTIN2-Q and NaKR3-Q shown in Table 1. The cycling conditions used were as follows: 95°C for 10 min; and 45 cycles each at 95°C for 15 s and 60°C for 30 s. All experiments were repeated three times independently. The raw data were quantitatively analyzed using the Bio-Rad iQ5 software. Data are reported as means ± SD.

Table 1. Primers used in this study.

Gene	Primer sequence 5'-3'
ACTIN2-Q (At1g49240)	F: GTTGACTACGAGCAGGAGATGG
	R: ACAAACGAGGGCTGGAACAAG
NaKR3-Q (At3g53530)	F: TTCTTAGAGTGTCTCTCCACTGTCA
	R: TGCAAAATCTATGTTGAAGGATGT
NaKR3-221 (At3g53530)	F: CGCTCTAGAATGAAAGCCGGTATGTTTTATTG
	R: CGCCCCGGGGGGGTTTCCGGATTTGCCC
NaKR3-121 (At3g53530)	F: CGCTCTAGAATGAAAGCCGGTATGTTTTATTG
	R: CGCGAGCTCTTAGGTTTCCGGATTTGCCC
SOS1-Q (At2g01980)	F: GCAAACACTTTGATATTTATCCTCAG
	R: CATGAATTCCCTTGGTAGGC
SOS3-Q (At5g24270)	F: CGAGCGAGAAGAATTGAAAGA
	R: CGTTTTGCGGTCTGCTT

Analysis of NaKR3 localization

The *NaKR3* coding region was cloned into the pBI221-GFP transient expression vector via the *Xba*I and *Sma*I sites. The recombinant plasmids were introduced into *Arabidopsis* mesophyll protoplasts using polyethylene glycol-mediated transformation (Yoo et al., 2007). Expression of fusion protein was monitored after transformation, and images were viewed using confocal microscopy (LSM 510 META, Carl Zeiss, Oberkochen, Germany). The primer (NaKR3-221) used in this assay is shown in Table 1.

Construction of transgenic plants

To produce 35S::NaKR3 plants, *NaKR3* cDNA was cloned into pBI121 (Clontech, Mountain View, CA, USA) via the *Xba*I and *Sac*I sites to replace the β-glucuronidase coding sequence. The obtained pBI121-NaRK3 construct was transformed into *Agrobacterium tumefaciens* strain EHA105 and was subsequently infiltrated into wild-type plants using the floral dip method (Clough and Bent, 1998). For phenotypic analysis, we used T3 homozygous lines. Data are reported as means ± SD. The primers (NaKR3-121, ACTIN2-Q, and NaKR3-Q) used in this assay are listed in Table 1.

Salt tolerance tests

For the salt tolerance test during seed germination, seeds of transgenic and wild-type plants were germinated on MS medium supplemented with 0, 50, 100, 150, or 200 mM NaCl. The germination characteristics were observed every 24 h. The experiments were repeated three times. Data are reported as means ± SD. For the salt tolerance test during the post-germination stage, four-week-old plants were watered with 400 mM NaCl for 10 days.

Analysis of the expression of salt stress-associated genes

We investigated the expression of two salt-associated genes salt overly sensitive 1 (*SOS1*) and *SOS3* in transgenic *Arabidopsis* plants using qRT-PCR analysis. Total RNA was isolated from two-week-old seedlings cultivated on MS medium. The primers (ACTIN2-Q, SOS1-Q, and SOS3-Q) used in this assay are listed in Table 1.

Determination of proline content

The proline content was determined as described previously (Bates et al., 1973). Four-week-old seedlings cultivated in soil were treated with 400 mM NaCl for 12 h. Proline was extracted from 300 mg leaves by boiling for 10 min in 3 mL 3% (w/v) 5-sulfosalicylic acid. The homogenate was centrifuged at 6000 rpm for 10 min to obtain the supernatant as a proline crude extract. The reaction mixture containing 2 mL proline crude extract, 2 mL acetic acid, and 2 mL 2.5% (w/v) acid-ninhydrin solution was boiled for 30 min. Next, 4 mL methanol was added into the samples and 3 mL of the upper methanol layer solution was obtained. The proline content was analyzed spectrophotometrically at 520 nm using a spectrophotometer (Model 754, Shanghai, China) with five replicates for each proline content assay; the proline content was quantified based on a standard curve of pure proline. Data are reported as means ± SD.

RESULTS

NaKR3 expression is induced by abiotic stresses

We analyzed the promoter region as far as 1150 bp upstream of the *NaKR3* gene using the PLACE database (Higo et al., 1999). We showed that a considerable number of potential cis-acting regulatory DNA elements exist. The predicted stress-responsive elements included a GT-1 box [involved in pathogen- and salt-induced gene expression (Park et al., 2004)], CCAAT box [involved in heat shock response (Chauhan et al., 2012)], MYC recognition site [binding site of ICE1, a major transcriptional activator of cold responsive genes (Lee et al., 2005)], and MYB recognition sites [important for dehydration-inducible expression (Abe et al., 2003)]. Our results suggest that *NaKR3* is a stress-related gene.

To examine the induction patterns of *NaKR3 in planta*, we conducted qRT-PCR analysis of the expression of *NaKR3* in *Arabidopsis* exposed to 50 μM $CuSO_4$, 250 mM NaCl, or 300 mM mannitol for different periods of time. We found that treatment with 50 μM $CuSO_4$ had no significant effect on *NaKR3* expression (Figure 1). In contrast, treatment with 250 mM NaCl or 300 mM mannitol led to increased transcript levels of *NaKR3*. After prolonged exposure to the 250 mM NaCl or 300 mM mannitol, the expression of *NaKR3* was markedly up-regulated in a time-dependent

manner. After 8 h of exposure, gene expression was significantly increased (Figure 1), possibly because of salt stress. Our results suggest that *NaKR3* is a salt- and osmotic stress-induced gene.

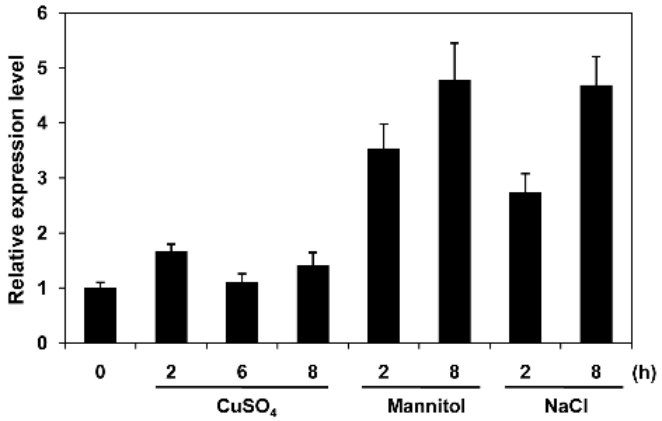

Figure 1. Quantitative reverse transcription-polymerase chain reaction analysis of the expression pattern of *NaKR3* in response to different abiotic stress conditions. Total RNA was isolated from two-week-old *Arabidopsis* seedlings that were treated with 50 μM $CuSO_4$ (2-8 h), 300 mM mannitol (2-8 h), or 250 mM NaCl (2-8 h). ACTIN2 was used as an internal control. Error bars indicate SD (N = 3).

NaKR3 is localized to the cytosol

Subcellular localization prediction of *NaKR3* based on WoLF PSORT (http://wolfpsort.seq.cbrc.jp/) revealed that *NaKR3* may be localized to the chloroplast. To test this prediction, we transformed 35S:NaKR3-GFP and control 35S:GFP constructs into *Arabidopsis* mesophyll protoplasts using polyethylene glycol-mediated transformation. We found that NaKR3-GFP was localized to the cytosol (and not the chloroplast) (Figure 2), indicating that our subcellular localization prediction results require to be validated experimentally.

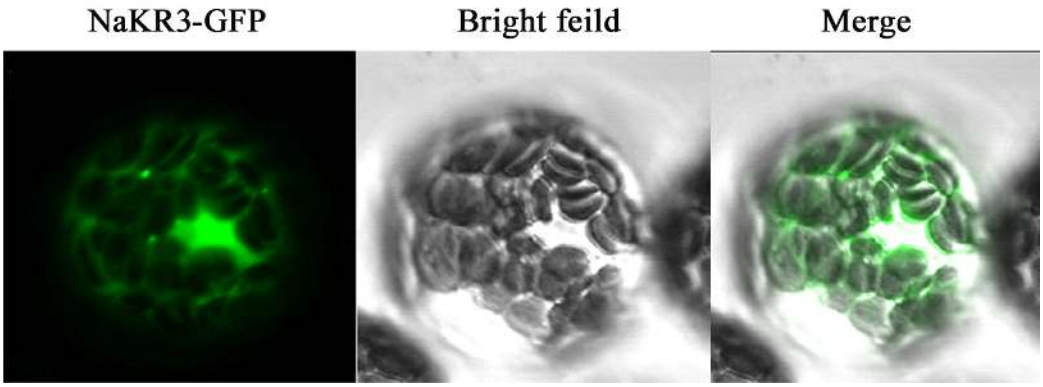

Figure 2. Subcellular localization of NaKR3. Confocal images of *Arabidopsis* protoplasts transiently expressing the *NaKR3-GFP* construct.

Overexpression of *NaKR3* enhances salt tolerance

To further investigate the function of *NaKR3 in vivo*, we generated *NaKR3* overexpressing (*NaKR3*-OE) lines. The coding region of *NaKR3* was introduced into the pBI121 vector under the control of the CaMV 35S promoter, and the construct was then transformed into wild-type *Arabidopsis* plants. We obtained five independent T3 homozygous lines and confirmed the mRNA levels of *NaKR3* expression in these lines using qRT-PCR analysis (Figure 3). We selected lines 4-1, 6-1, and 9-2 for further research.

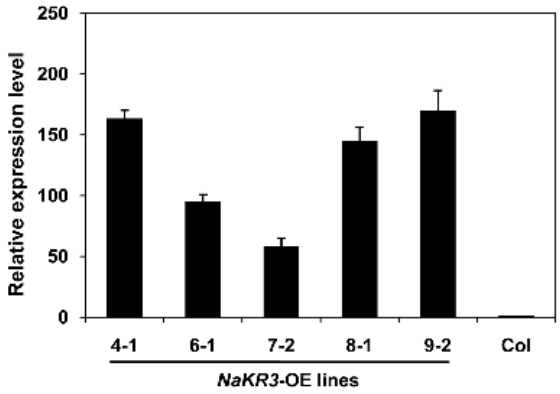

Figure 3. qRT-PCR analysis of *NaKR3* expression in wild-type and *NaKR3*-OE lines. Total RNA was extracted from two-week-old seedlings. ACTIN2 was used as an internal control. Error bars indicate SD (N = 3).

As described above, *NaKR3* is up-regulated by NaCl. Hence, we examined whether overexpression of *NaRK3* would affect NaCl sensitivity. First, we investigated the germination of *NaKR3*-OE and wild-type seeds on MS medium supplemented with 0, 100, 150, or 200 mM NaCl. In the absence of NaCl, *NaKR3*-OE lines and wild-type plants showed similar germination rates (Figure 4A). In contrast, on medium supplemented with 150 or 200 mM NaCl, the germination rates of *NaKR3*-OE lines were significantly higher than those of wild-type plants. After 4 days of treatment with 150 mM NaCl, the germination rates of the 4-1, 6-1, and 9-2 transgenic lines were 86.4, 83.3, and 85.3%, respectively, whereas the germination rate of the control was only 60% (Figure 4B). We obtained similar results for plants cultivated on medium supplemented with 200 mM NaCl after 5 days. The germination rates of of the 4-1, 6-1, and 9-2 transgenic lines were 27.9, 20.8, and 56.3%, whereas the germination rate of the wild-type was only 12.5% (Figure 4C). After 7 days, the germination of *NaKR3*-OE lines are higher than those of wild-type on medium supplemented with 150 or 200 mM NaCl (Figure 4D). Next, we monitored cotyledon greening after germination. We found that on medium supplemented with 100 mM NaCl, most of *NaKR3*-OE lines displayed normal cotyledon greening, whereas less wild-type plants developed green cotyledons (Figure 5). To further investigate the effects of high salinity on post-germination growth, we treated soil-cultivated plants with 400 mM NaCl. We found that before treatment, *NaKR3*-OE lines and wild-type plants grew well; moreover, the growth of *NaKR3*-OE lines did not differ markedly from that of wild-type plants (Figure 6). In contrast, after NaCl treatment, *NaKR3*-OE lines grew better than wild-type plants. Taken together, our results indicate that overexpression of *NaKR3* enhances salt tolerance.

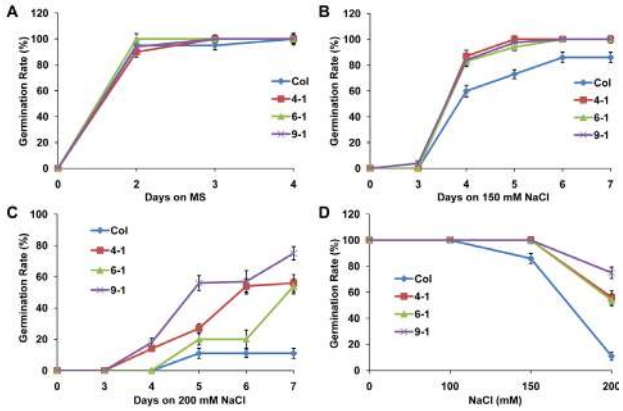

Figure 4. Seed germination in the absence and presence of NaCl. **A.** Seed germination on Murashige and Skoog (MS) medium. **B.** Seed germination on MS medium supplemented with 150 mM NaCl. **C.** Seed germination on MS medium supplemented with 200 mM NaCl. **D.** Seed germination after 7 days on MS medium supplemented with different concentrations of NaCl.

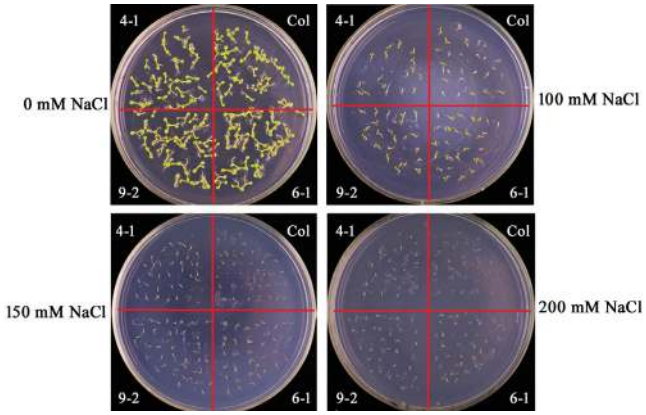

Figure 5. Seed germination on MS medium supplemented with 0, 100, 150, or 200 mM NaCl. All plates were photographed 7 days after imbibition.

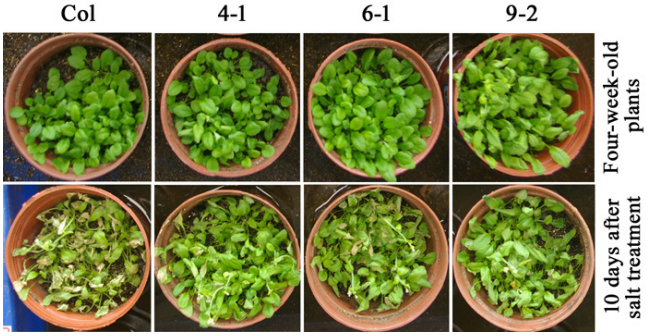

Figure 6. Salt tolerance test. Four-week-old plants were watered with 400 mM NaCl and were photographed after 10 days of treatment.

Overexpression of *NaKR3* increases the expression of salt stress-associated genes

To more fully elucidate the role of *NaKR3* in salt tolerance, we investigated the expression of *SOS1* and *SOS3* in transgenic *Arabidopsis* plants. We found that under normal growth conditions, the expression of *SOS1* and *SOS3* was induced in *NaKR3*-OE plants (Figure 7A). Moreover, in comparison with wild-type plants, even the lowest expression levels of *SOS1* and *SOS3* in transgenic plants were approximately 3- and 2-fold higher, respectively.

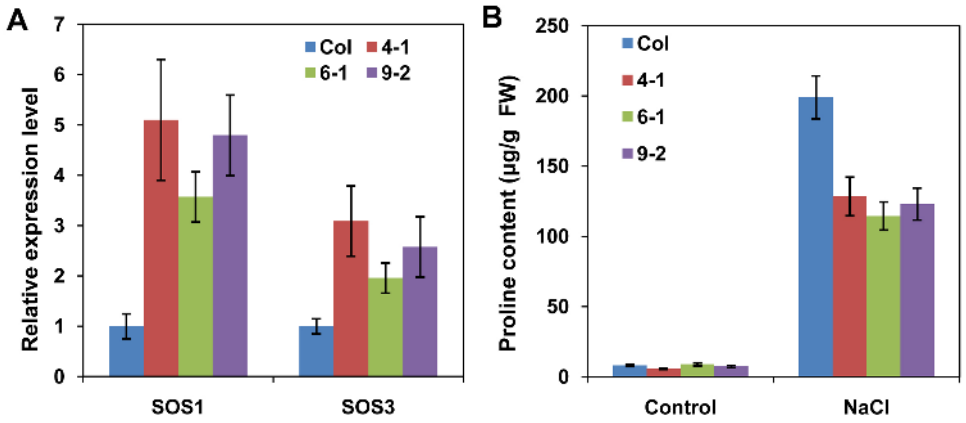

Figure 7. qRT-PCR analysis of *SOS1* and *SOS3* expression and measurement of proline content in wild-type and *NaKR3*-OE lines. **A**. qRT-PCR analysis of *SOS1* and *SOS3* expression in wild-type and *NaRK3*-OE lines. Total RNA was extracted from two-week-old seedlings. ACTIN2 was used as an internal control. Error bars indicate SD (N = 3). **B**. Proline content in the leaves of wild-type and *NaKR3-OE* lines before and after exposure to 400 NaCl for 12 h. Error bars indicate SD (N = 5).

Proline accumulation in the leaves is higher in wild-type plants than in transgenic plants

Proline accumulation is known to be critical for salt tolerance (Liu and Zhu, 1997); hence, we determined the proline contents in the leaves of wild-type and *NaKR3*-OE plants lines before and after exposure to 400 mM NaCl. We found that before NaCl treatment, the proline content in the leaves did not differ significantly between wild-type plants and transgenic lines (Figure 7B). However, after NaCl treatment, the proline content in the leaves of wild-type plants and *NaKR3*-OE lines increased markedly. Moreover, the proline content increased more rapidly in the leaves of wild-type plants than in the leaves of transgenic lines (Figure 7B), and the final proline content in the leaves of wild-type plants was approximately 1.5-fold higher than that in the leaves of transgenic lines.

DISCUSSION

NaKR1 is a phloem mobile metal binding protein that plays an important role in Na^+ transport (Tian et al., 2010). In the present study, we cloned *NaKR3*, which is a homolog of *NaKR1*, from *Arabidopsis*. We showed that *NaKR3* expression was induced by NaCl and mannitol treatment. Transient expression of *NaKR3-GFP* in *Arabidopsis* protoplasts indicated that NaKR3 is localized in the cytosol; this finding is consistent with the subcellular localization of NaKR1 (Tian et

al., 2010). Transgenic lines overexpressing *NaKR3* exhibited enhanced tolerance to salt stress, as well as decreased salt-induced proline accumulation. Our results suggest that *NaKR3* is involved in the salt stress response in *Arabidopsis*.

The results of protein domain analysis revealed that, similar to NaKR1, NaKR3 has a HMA domain at the C-terminus. The HMA domain exists in proteins that function in the transfer and/or binding of heavy metals; these proteins include heavy metal transporters, metallochaperones, and enzymes that use heavy metals as cofactors (Dykema et al., 1999; Tian et al., 2010). Tian et al. (2010) reported that Zn, Cu, Fe, Ni, and Co were associated with the NaKR1 protein. On the other hand, the HMA domain of NaKR3 shares low sequence similarity with the *Arabidopsis* HMA domain protein CCH; this protein is found in the phloem and is thought to transport Cu out of senescing tissues (Mira et al., 2001). This may explain why the expression of *NaKR3* was not affected by Cu^{2+} in our present study.

Previous studies have shown that Na^+ transport is related to salt tolerance. For example, HKT1 - a Na^+-K^+ co-transporter - decreases Na^+ accumulation by excluding Na^+ from the shoots, thereby leading to increased salt tolerance (Rubio et al., 1995; Rus et al., 2001, 2006). In the present study, we found that NaKR3, which is a homolog of NaKR1 - a Na^+ transport protein - was involved in salt tolerance. Overexpression of *NaKR3* enhanced the salt tolerance of transgenic *Arabidopsis* lines. Our findings provide further evidence for the relationship between Na^+ transport and salt tolerance.

The SOS pathway plays an important role in the plant response to salt stress (Zhu, 2002). Accordingly, *Arabidopsis* plants overexpressing *SOS1* and *SOS3* showed significantly increased salt tolerance (Liu and Zhu, 1998; Shi et al., 2000). In the present study, we demonstrated that overexpression of *NaRK3* increased the expression of *SOS1* and *SOS3*, indicating that *NaRK3* may be involved in salt tolerance through regulating downstream signal molecules such as *SOS1*and *SOS3* in the SOS pathway.

Proline acts as an organic solute in plants. Free proline levels increase when plants suffer from abiotic stresses such as cold, drought, and high salt (Hare et al., 1998). In the present study, we examined the free proline content in wild-type plants and *NaKR3*-OE lines. We showed that *NaKR3*-OE lines had lower free proline levels than wild-type plants. Our results are inconsistent with those of previous studies, in which overexpression salt tolerance genes was generally shown to confer higher proline levels (Liu et al., 2007; Gao et al., 2010; Xu et al., 2010). However, some salt tolerance genes play similar roles to *NaKR3*. For example, the *sosl* mutant of *Arabidopsis thaliana* is extremely sensitive to salt stress, but accumulates lower amounts of free proline than the wild-type (Liu and Zhu, 1997). In the present study, the observed increase in proline accumulation was likely a symptom of stress injury rather than an indicator of stress tolerance.

In conclusion, in the present study, we identified a new salt stress-related gene, *NaKR3*, in *Arabidopsis*. We showed that overexpression of this gene confers enhanced tolerance to salt stress. We propose that *NaKR3* represents a potential candidate for engineering of stress-tolerant crops.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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