Plant Cell Tiss Organ Cult (2015) 123:569–578
DOI 10.1007/s11240-015-0861-9

ORIGINAL ARTICLE

Two alternative splicing variants of maize *HKT1;1* confer salt tolerance in transgenic tobacco plants

Zhenjing Ren[1] · Yan Liu[2] · Dan Kang[1] · Kaijian Fan[1] · Cuiyun Wang[1] · Guoying Wang[1] · Yunjun Liu[1]

Received: 3 April 2015 / Accepted: 8 September 2015 / Published online: 12 September 2015

Abstract The high-affinity potassium transporter (*HKT*) genes have crucial roles in the regulation of sodium and potassium transportation in many species, however little is known about maize *HKT* genes. In this study, we obtained two alternative splicing transcripts of *ZmHKT1;1* gene. One was named as *ZmHKT1;1a* which has the intact coding sequence, and the other was named as *ZmHKT1;1b* which has a deficiency of the third exon and a retention of the second intron. The phylogenic tree analysis showed that both translation products of *ZmHKT1;1a* and *ZmHKT1;1b* belong to group I HKT proteins which prefer for Na^+ transport than other cations. *ZmHKT1;1a* and *ZmHKT1;1b* showed different response to stress treatment in maize. Overexpressing *ZmHKT1;1a* or *ZmHKT1;1b* in transgenic tobacco plants conferred high salt tolerance by increasing root length and fresh weight of plants. When treated with high concentration of salt, transgenic tobacco plants manifested a trend of reduced Na^+ content and increased K^+ content in both shoot and root, suggesting that *ZmHKT1;1* may involve in Na^+ unloading and indirectly affect other transporter activity. It was also found that overexpression of *ZmHKT1;1a* and *ZmHKT1;1b* caused different expression of stress-related genes. The results in this study indicate that two alternative splicing variants of *ZmHKT1;1* might be useful for the development of salt-tolerant transgenic crops.

Keywords Maize · HKT · Alternative splicing · Salt tolerance · Transgenic plant

Introduction

Soil salinity is one of the major factors affecting agriculture and crop productivity. Deleterious effects of salinity on plants tissues are mainly divided into three aspects: osmotic stress, ion toxicity and nutrient deficiencies (Munns and Tester 2008). To cope with the negative effects of high sodium levels, plants have evolved a series of strategies, including sodium exclusion and/or sodium intracellular compartmentalization into the large cell vacuoles. Na^+/H^+ antiporters (NHXs) could mediated the sodium exclusion and the NHXs from several plant species have been shown to enhance salt tolerance (Apse et al. 1999; He et al. 2005; Patel et al. 2015; Mishra et al. 2015). By these strategies, plants could maintain Na^+/K^+ balance by limiting the over-accumulation of sodium in the cytosol (Hasegawa et al. 2000).

The high-affinity potassium transporter (HKT) genes, belonging to the Trk/Ktr/HKT transporter family, have been shown to play a crucial role in the regulation of sodium and potassium transportation in many species (Corratgé-Faillie et al. 2010). Since the first discovery of *TaHKT2;1* in *Triticum aestivum* (Schachtman and Schroeder 1994), more and more HKT transporters from different species have been found. The HKT transporters have four pore domains (PDs), and HKTs are classified into two sub-families according to the amino acid difference in the first PD (Platten et al. 2006). Sub-family 1 HKTs exhibit a preference for sodium permeability; whereas

Electronic supplementary material The online version of this article (doi:10.1007/s11240-015-0861-9) contains supplementary material, which is available to authorized users.

✉ Yunjun Liu
liuyunjun@caas.cn

[1] Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing 100081, China

[2] College of Agriculture and Biotechnology, China Agricultural University, Yuanmingyuan West Road 2, Beijing 100094, China

members of sub-family 2 could select either sodium and/or potassium according to their external concentrations.

Both sub-family 1 and sub-family 2 HKTs have been shown to be able to enhance the salt tolerance of plants by overexpression in transgenic plants. Arabidopsis genome has only one *HKT* gene, *AtHKT1;1*, which can mediate the sodium exclusion from leaves under salt stress condition (Horie et al. 2009). It was shown that specifically expression of *AtHKT1;1* in the mature root stele of Arabidopsis decreased the Na^+ accumulation, whereas constitutively expression of *AtHKT1;1* driven by 35S promoter led to a high accumulation of Na^+ (Moller et al. 2009). When *AtHKT1;1* was specifically expressed in the root cortical and epidermal cells, transgenic plants also had significantly improved Na^+ exclusion (Plett et al. 2010). Transgenic barley plants overexpressing *HvHKT2;1* could tolerate high concentration of salt (Mian et al. 2011). In these studies, the salt tolerance of transgenic plants are correlated with the significantly lower accumulation Na^+ than WT plants.

Compared to only one *HKT* gene in dicot Arabidopsis, monocotyledon species have a much larger number of *HKT* genes. It was shown that wheat genome have five to eleven *HKT* genes (Huang et al. 2006) and there are at least nine *HKT* genes in rice (Garciadeblas et al. 2003). Recently, four sorghum *HKT* genes were identified (Wang et al. 2014). *OsHKT1;5* was identified by map-based cloning of the *SKC1* QTL locus in a segregating population, and the physiological analysis showed that OsHKT1;5 is involved in Na^+/K^+ homeostasis under salt stress (Ren et al. 2005). Another important crop *HKT* gene is *TmHKT1;5* which was cloned from a *Nax2* locus in the ancestral wheat germplasm, and field trials confirmed that the presence of *TmHKT1;5* reduced leaf Na^+ contents and increased wheat grain yield under salt stress condition (Munns et al. 2012). These two crop *HKT* genes are good candidates for improving salt tolerance of crops.

The characteristics and the function of maize HKT transporter is rarely known. In this study, we cloned two alternative splicing variants of maize *HKT1;1*. Both transcripts of *ZmHKT1;1* was constitutively overexpressed in transgenic tobacco plants, which exhibited better growth status, significantly decreased sodium contents in shoots and roots compared to the WT plants under salt stress condition, indicating that *ZmHKT1;1* could confer salt tolerance of transgenic tobacco plants.

Materials and methods

Maize growth, stress treatments and RNA isolation

Seeds from the maize inbred line B73 were germinated in pots containing sand, and the seedlings were grown in a growth chamber under a 16 h light and 8 h dark photoperiod at 22 °C for 3 weeks. For cold and heat treatment, the seedlings were transferred to a growth chamber at 4 and 42 °C, respectively. For ABA and salt treatment, seedlings were gently removed from the sand, transferred to water for 1 h and then transferred to 100 μM ABA, 250 mM NaCl, respectively. For dehydration treatments, seedlings were carefully removed from the sand, transferred to water for 1 h and then dehydrated on filter paper. Shoots were sampled at 0, 1, 3, 6, 12 and 24 h after treatment, respectively.

Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. The cDNA was prepared with 5 μg total RNA with a TransScript II first-strand cDNA synthesis kit (TransGen, China).

Cloning of *ZmHKT1;1a* and *ZmHKT1;1b* and plasmid construction

Based on the maize cDNA sequence (GRMZM2G047616), the primers *ZmHKT*-infusion-F(5′-GGGACTCTTACCATGGATCCTCTCCGTTTTGGGTTTCTTG-3′) and *ZmHKT*-infusion-R (5′GGGGAAATTCGAGCTGGACACCCAGGTTGATCGAGCGAGTTT-3′) were designed to amplify maize *HKT1;1* using cDNA from maize inbred line B73 as template. Two PCR fragments were obtained and cloned into plant expression vector pCAMBIA3301 by In-fusion technology.

Protein alignments and phylogenetic tree construction

Amino acids sequences were aligned using the Cluster X (http://www.clustal.org/) and phylogenetic tree was obtained by Cluster X and Mega (http://www.megasoftware.net/) software.

Quantitative real-time PCR assay

Quantitative real-time PCR (qRT-PCR) was performed using TransStart Green qPCR SuperMix kits (TransGen, China) according to the manufacturer's instructions under following conditions: initial denaturation at 95 °C for 2 min, 40 cycles at 95 °C for 10 s, 56 °C for 20 s and 72 °C for 30 s. At the end of the qRT-PCR cycles, the products were subjected to melt curve analysis to verify the specificity of PCR amplification. Three independent experiments were performed. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ formula with *β-tubulin* as a housekeeping gene (Livak and Schmittgen 2001). The used primers were shown in Supplemental Table 1.

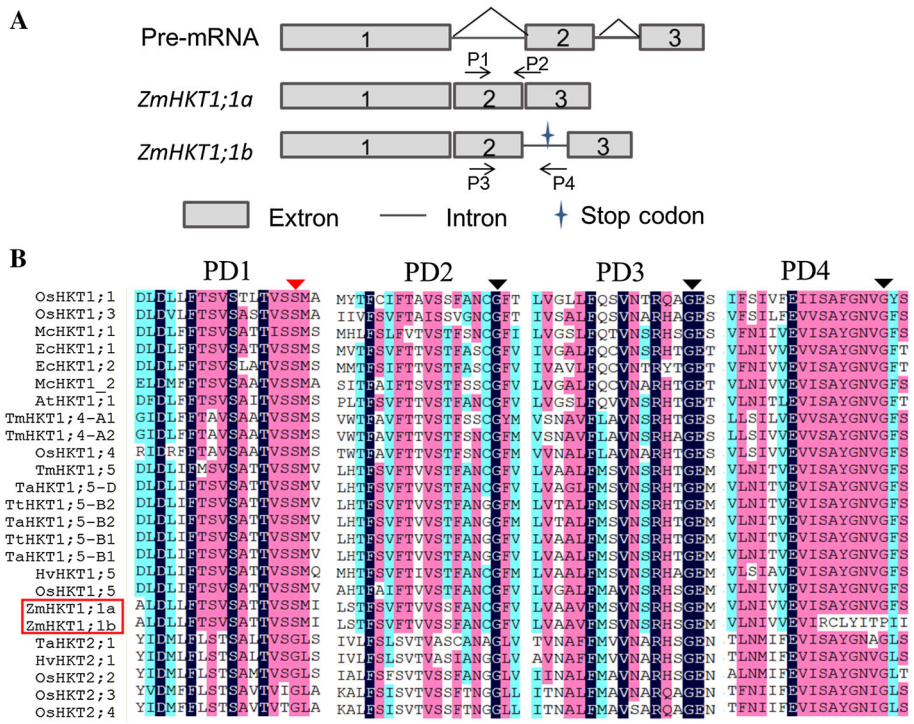

Fig. 1 Characteristics of *ZmHKT1;1a* and *ZmHKT1;1b*. **a** Structure of *ZmHKT1;1a* and *ZmHKT1;1b*; primers P1 and P2, P3 and P4 can specifically amplify *ZmHKT1;1a* and *ZmHKT1;1b*, respectively. **b** Alignment of the amino acid sequences of four pore domains (PDs) of maize HKT1;1 with other 18 HKT proteins

Tobacco transformation

Plant expression plasmids were transferred into competent cells of the *A. tumefaciens* strain EHA105 through freeze–thaw treatment, respectively. The transformed *A. tumefaciens* colonies were selected on YEB-agar plates containing 100 mg mL^{-1} of kanamycin and 125 mg mL^{-1} of streptomycin. The positive colonies were identified by PCR amplification of the inserted genes and used for the tobacco transformation as previously described (Horsch et al. 1985). The transgenic plants were confirmed by PCR amplification of the interested gene.

Salt tolerance evaluation of transgenic plants

Heterozygous tobacco seeds of the T_1 generation were germinated on MS medium containing 10 mg L^{-1} phosphinothricin and grown for 7 days at 100 µmol m^{-2} s^{-1} with a 16 h light/8 h dark period. The living seedlings with a similar size were transferred onto MS medium in plates containing different amounts of NaCl and grown vertically for another 1 week. The alive seedlings were also transferred to MS medium in plastic boxes containing different concentration of NaCl, and grown for another 1 month. The injury was observed and the fresh weight and main root length of transgenic plants were measured.

Na^+ and K^+ content analysis

The transgenic plants grown on the MS medium in plastic boxes containing different concentration of NaCl were used for measure the Na^+ and K^+ contents. The measurements were determined in shoot and root according to the described method with some modification (Shukla et al. 2012). Around 0.2 g tobacco plant tissues were digested in 5 ml of a mixed solution of nitric acids and perchloric acid (4:1). The solution was boiled on a hot plate at 175 °C until it turned colorless. The digested samples were then analyzed using a flame emission photometric method for Na^+ and K^+ concentration. Values from the flame photometer were converted into Na^+ and K^+ content values.

Results

Cloning of two alternative splicing variants of *ZmHKT1;1*

The BLAST analysis was performed in MaizeGDB using wheat *OsHKT1;5* sequence and a full-length cDNA (GRMZM2G047616) was found to have high identity with *OsHKT1;5*. This maize gene was named as *ZmHKT1;1* which contains two exons and one intron. When specific primers were designed to clone it, two *ZmHKT1;1* cDNA fragments with different size were amplified and sequenced. One was named as *ZmHKT1;1a* which contains 1482 bp in the open reading frame (ORF) and encodes a protein with 493 amino acids, and this cDNA is identical to the predicted sequence in MaizeGDB. The other was named as *ZmHKT1;1b* which contains 1308 bp in the ORF and encodes a truncated protein with 435 amino acids.

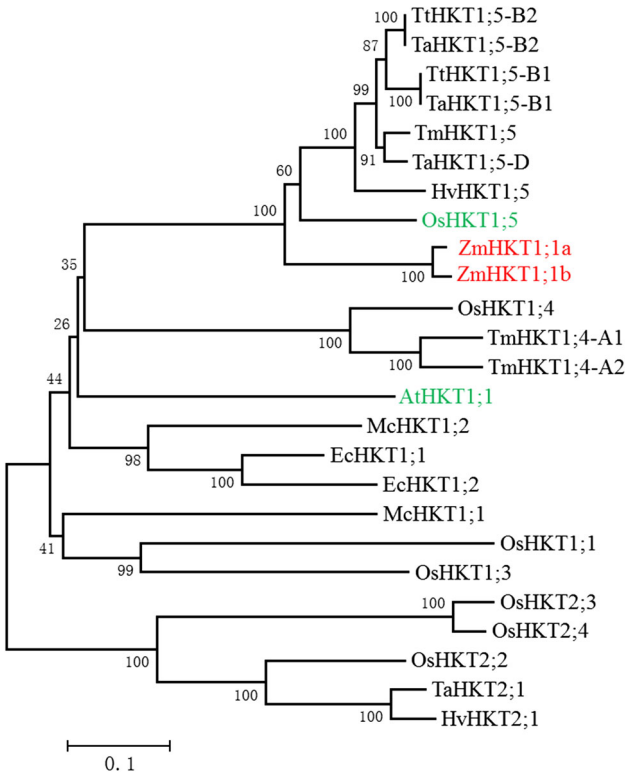

Fig. 2 The phylogenetic relationship of maize ZmHKT1;1 with HKTs from other plant species. The GenBank access numbers of the HKTs are: AAF68398 (AtHKT1;1), AAF97728 (EcHKT1;1), AAD53890 (EcHKT1;2), ABK58096 (HvHKT1;5), AAK52962 (McHKT1;1), AAO73474 (McHKT1;1), CAD37183 (OsHKT1;1), CAD37185 (OsHKT1;3), CAD37197 (OsHKT1;4), BAB93392 (OsHKT1;5), ABG 33947 (TaHKT1;5-B1), ABG33948 (TaHKT1;5-B2), ABG33945 (TaHKT1;5-D), ABK41858 (TmHKT1;4-A1), ABK41857 (TmHK T1;4-A2), ABG33946 (TmHKT1;5), ABG339460 (TtHKT1;5-B1), ABG33941 (TtHKT1;5-B2), CAJ01327 (HvHKT2;1), BAB61791 (OsHKT2;2), CAD37187 (OsHKT2;3), CAD37199 (OsHKT2;4), AAA 52749 (TaHKT2;1), DAA54361.1 (ZmHKT1;1a)

Compared with *ZmHKT1;1a*, the intron was not spliced in *ZmHKT1;1b*, and the termination codon in the intron leads to the translation of a truncated protein (Fig. 1a). The deduced amino acid sequences of *ZmHKT1;1a* and *ZmHKT1;1b* were aligned with the known HKT proteins. As other HKT proteins, ZmHKT1;1a has four membrane-pore-membrane (MPM) motifs, whereas the fourth MPM motif of ZmHKT1;1b was destroyed due to the pre-termination of translation (Fig. 1b).

To understand the phylogenetic relationship of ZmHKT1 and those from other species, a phylogenic tree of ZmHKT1;1 and other 18 known HKT proteins was constructed (Fig. 2). HKT transporters can be classified into two groups (Horie et al. 2009), and the phylogenetic tree clearly showed that ZmHKT1;1a and ZmHKT1;1b belong to group I (Fig. 2). So these two ZmHKT1;1 variants might be Na^+ transporters because group I HKT proteins prefer for Na^+ transport than other cations (Horie et al. 2009).

ZmHKT1;1a and *ZmHKT1;1b* showed different response to stress treatment in maize

The expression pattern of *ZmHKT1;1a* and *ZmHKT1;1b* under stress condition was investigated. One of the primers for *ZmHKT1;1b* was designed in the intron sequence which existed in *ZmHKT1;1b*, leading to the specific amplification of *ZmHKT1;1b* not *ZmHKT1;1a*. The primer specific for the amplification of *ZmHKT1;1a* was designed in the junction region of exon 2 and exon 3, leading to the specific amplification of *ZmHKT1;1a* (Fig. 1). When treated with 250 mM NaCl, the expression of *ZmHKT1;1b* in maize seedlings had 15 fold of increase 6 h after treatment, whereas the transcriptional level of *ZmHKT1;1a* was induced 12 h after treatment with threefold of increase (Fig. 3a). *ZmHKT1;1b* also showed faster response to heat stress than *ZmHKT1;1a*. 12 h after heat treatment, more than tenfold increase of *ZmHKT1;1b* was observed compared to the twice fold of increase of *ZmHKT1;1a* (Fig. 3c). The transcriptional level of both *ZmHKT1;1a* and *ZmHKT1;1b* were increased by ABA treatment, and 9- and 4-fold increase of transcriptional level were observed for *ZmHKT1;1a* and *ZmHKT1;1b*, respectively (Fig. 3b). 24 h after cold treatment, *ZmHKT1;1a* had 200 fold of transcript increase and *ZmHKT1;1b* had 62 fold of transcript increase (Fig. 3d). In dehydration treated maize seedlings, the transcriptional level of *ZmHKT1;1a* was not affected, whereas the transcriptional level of *ZmHKT1;1b* was decreased (Fig. 3e). These results clearly showed that *ZmHKT1;1a* and *ZmHKT1;1b* had different response to stress treatment in maize.

Overexpression of *ZmHKT1;1* increased the salt tolerance of transgenic tobacco plants

To further study the function of two alternative splicing *ZmHKT1;1* variants, transgenic tobacco plants overexpressing *ZmHKT1;1a* and *ZmHKT1;1b*, respectively, were obtained. Both genes were controlled by the CaMV 35S promoter and cloned into the plasmid pCAMBIA3301 to construct the vectors for plant transformation (Supplemental Fig. 1). The vectors were introduced into tobacco plants (*Nicotiana tabacum* var. *Samsum*) via *Agrobacterium*-mediated transformation. Transgenic tobacco plants were confirmed by PCR and RT-PCR analysis, and two transgenic lines (1–7 and 1–13) overexpressing *ZmHKT1;1a* and two transgenic tobacco lines (2–3 and 2–8) overexpressing *ZmHKT1;1b* were chosen for further analysis (Supplemental Fig. 1).

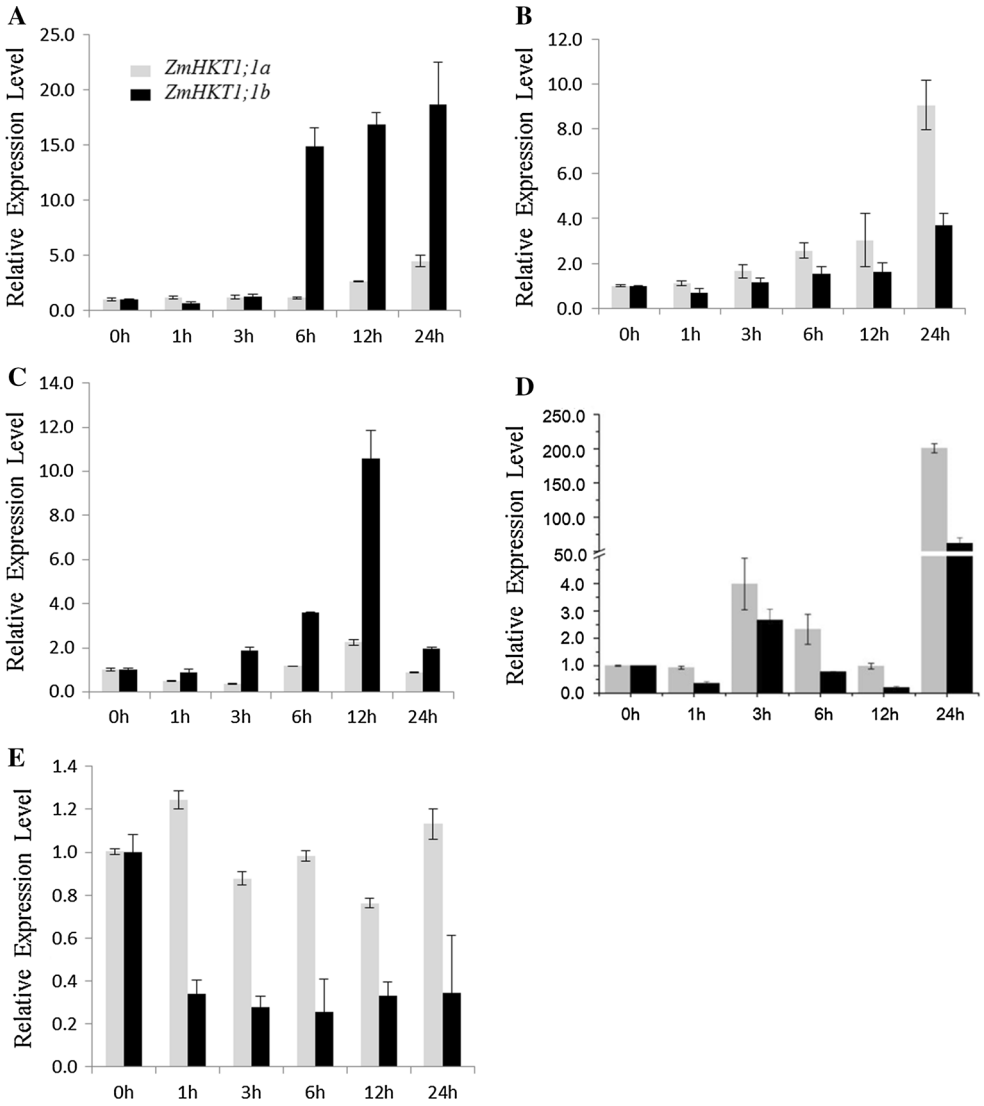

Fig. 3 Real-time PCR analysis of the expression of *ZmHKT1;1a* and *ZmHKT1;1b* in response to different stress treatment. **a** Treatment with 250 mM NaCl; **b** treatment with 100 μM ABA; **c** heat treatment at 42 °C; **d** cold treatment at 4 °C; **e** dehydration treatment. The relative transcriptional level was analyzed using $2^{-\Delta\Delta C_t}$ method, and the transcriptional level at 0 h was normalized as 1.00. The experiments were performed in triplicate, and values are the mean ± SE of three samples

T_2 progeny tobacco seeds were germinated on MS medium using 10 mg L^{-1} phosphinothricin as a selective reagent to eliminate non-transgenic plants. The germinated transgenic tobacco seedlings were moved on to the MS medium containing 0, 200 and 300 mM NaCl, respectively, and grown vertically for another 7 days. On the medium without NaCl, all transgenic plants grew similarly to the WT plants (Fig. 4a). Treatment with NaCl inhibited the growth of WT plants and the transgenic plants (Fig. 4a). The fresh weight (FW) and main root length (MRL) were measured to assess the inhibition of plant growth by NaCl. When treated with 200 and 300 mM NaCl, significantly higher fresh weight and root length were observed for transgenic plants overexpressing *ZmHKT1;1a* or *ZmHKT1;1b*, than those of WT plants (Fig. 4b, c). These results clearly showed that the over-expression of *ZmHKT1;1a* or *ZmHKT1;1b* in plants could offer high salt tolerance.

To further confirm the salt tolerance of transgenic tobacco plants over-expressing *ZmHKT1;1a* and *ZmHKT1;1b*, the germinated T_2 transgenic tobacco seedlings were moved on to the MS medium containing 0, 200 and 300 mM NaCl in plastic boxes, respectively, and grown for another 4 weeks. Transgenic plants showed better salt tolerance than non-transgenic plants (Fig. 5a), and had significantly higher fresh weight than non-transgenic plants (Fig. 5b). We also put tobacco leaf discs in the liquid 1/4 MS medium containing different concentration of NaCl, and transgenic plants showed more salt tolerance than WT plants (Supplemental Fig. 2).

Transgenic tobacco plants accumulated less Na^+ and more K^+ under salt treatment

Transgenic plants were treated with NaCl, and the accumulation of Na^+ and K^+ in shoot and root was measured.

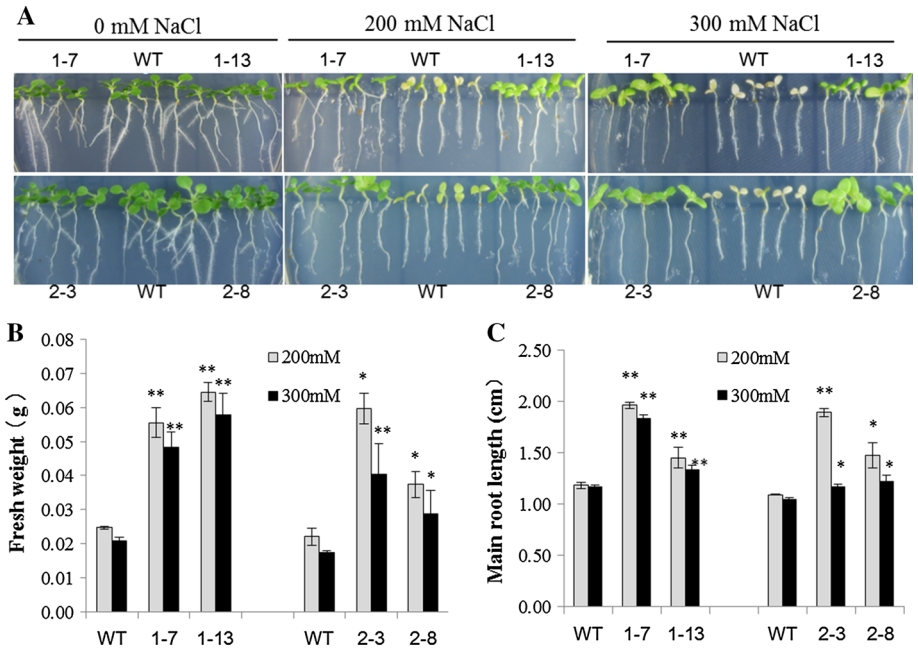

Fig. 4 The growth of transgenic tobacco seedlings on MS medium containing different concentration of NaCl. **a** Photograph of transgenic tobacco seedlings. Heterozygous tobacco seeds of the T_1 generation were germinated on MS medium containing 10 mg L^{-1} phosphinothricin and grown for 7 days at 100 μmol m^{-2} s^{-1} with a 16 h light/8 h dark period. The living seedlings with a similar size were transferred to MS medium in plates containing 0, 200, 300 mM of NaCl, respectively, and grown vertically for another 1 week. **b** The fresh weight of transgenic plants. **c** The main root length of transgenic plants. *1–7* and *1–13* are two independent transgenic lines expressing *ZmHKT1;1a*; *2–3* and *2–8* are two independent transgenic lines expressing *ZmHKT1;1b*. Data are shown as the average ± SE of 15 plants. Experimental data was analyzed by *t* test and the *asterisks* in columns mean significant difference from WT plants at $*P < 0.05$ or $**P < 0.01$ level

When plants grew under normal condition, there was no difference of Na^+ and K^+ concentration between WT plants and transgenic plants, indicating that overexpression of *ZmHKT1;1a* and *ZmHKT1;1b* did not affect the cation accumulation. Treatment with 200 mM NaCl led to significantly increase of Na^+ and K^+ contents in both WT plants and transgenic plants (Fig. 6). However, the transgenic plants had significantly less Na^+ contents than that in WT plants (Fig. 6a, c), indicating that overexpression of *ZmHKT1;1* reduced the accumulation of Na^+ under salt stress condition. The similar results were observed when plants grew in hydroponic culture with different concentration of NaCl (Supplemental Fig. 3). Compared to the decreased Na^+ contents, transgenic plants accumulated more K^+ in both shoots and roots than WT plants which were treated with NaCl (Fig. 6b, d), indicating that the increased K^+ could play roles in protecting plant cells from Na^+ stress.

Overexpression of *ZmHKT1;1* affected the expression levels of some stress-related genes

To further investigate the mechanism of salt tolerance of transgenic plants, qRT-PCR was performed to compare the expression of eleven stress-related genes (Fig. 7; Supplemental Table 2). It was observed that overexpression of *ZmHKT1;1b* decreased the transcription levels of *NtGPX*, *NtSPS*, *NtERD10B*, *NtRub-SS*, *NtAPX2*, *NtCAX3*, *NtNHX4*, *NtAPX1* and *AY554170* in transgenic tobacco leaves, whereas overexpression of *ZmHKT1;1a* only decreased the expression of *NtCAX3* and *NtERD10B*. When the plants were treated with 300 mM NaCl, higher expression levels of *NtGPX* in transgenic plants overexpressing *ZmHKT1;1a* or *ZmHKT1;1b* than that in WT plants was observed. The transgenic plants overexpressing *ZmHKT1;1b* also had higher *NtSPS* levels than WT plants when treated with 300 mM NaCl. These results indicate that *ZmHKT1;1b* might be more effective than *ZmHKT1;1a* to confer salt tolerance of transgenic plants.

Discussion

HKT genes, i.e. rice *OsHKT1;5* (Ren et al. 2005) and wheat *TmHKT1;5* (Munns et al. 2012), play important roles in the salt tolerance of crops. To clone the maize orthologue gene of *HKT1;5*, we performed BLAST analysis in MaizeGDB using rice *OsHKT1;5* sequence and a full-

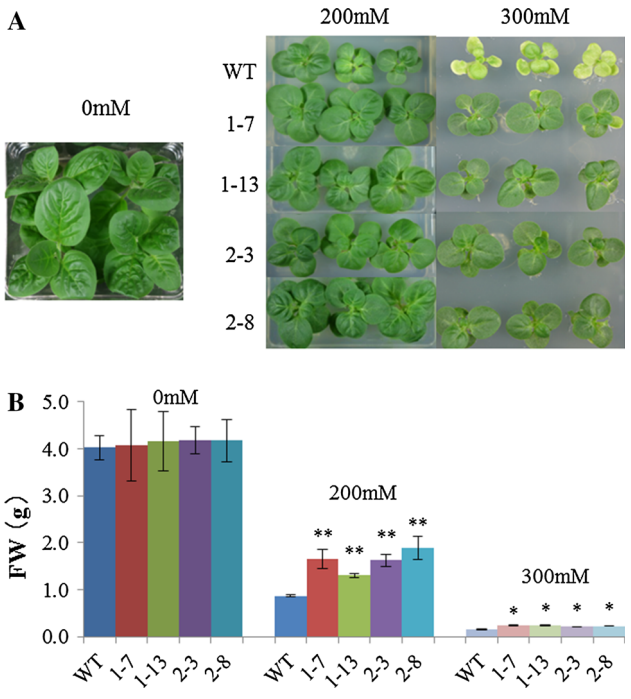

Fig. 5 The growth of transgenic tobacco plants under salt treatment for 4 weeks. **a** Photograph of transgenic tobacco plants. Heterozygous tobacco seeds of the T_1 generation were germinated on MS medium containing 10 mg L^{-1} phosphinothricin and grown for 7 days at 100 μmol m^{-2} s^{-1} with a 16 h light/8 h dark period. The alive seedlings were transferred onto MS medium in plastic boxes containing different concentration of NaCl, and grown for another 1 month. **b** The fresh weight (FW) of transgenic plants. *1–7* and *1–13* are two independent transgenic lines expressing *ZmHKT1;1a*; *2–3* and *2–8* are two independent transgenic lines expressing *ZmHKT1;1b*. Data are shown as the average ± SE of five samples each of which contains three plants. Experimental data was analyzed by *t* test and the *asterisks* in columns mean significant difference from WT plants at $*P < 0.05$ or $**P < 0.01$ level

length cDNA (GRMZM2G047616) was found to have high identity with *OsHKT1;5*. When specific primers were designed to clone it, two alternative splicing variants of *ZmHKT1* (named as *ZmHKT1;1a* and *ZmHKT1;1b*) were amplified. Compared with *ZmHKT1;1a*, the intron was not spliced in *ZmHKT1;1b*, and the termination codon in the intron leads to the translation of a truncated protein. It is known that *HKT* genes contain two introns, and sub-family 1 have significantly larger introns than sub-family 2 (Platten et al. 2006). It has been shown that in salt sensitive rice line IR29, the presence of K^+, Cs^+ or Rb^+ caused incomplete splicing of *OsHKT1* intron, leading to larger transcript which contains one intron and encodes a dysfunctional HKT protein (Golldack et al. 2002). This kind of incomplete splicing might be due to the stress treatment. There was a alternative splicing of the rice *OsHKT1;4* gene, and the spliced variants could be translated into a truncated protein due to the presence of translation stop codon in the second intron (Cotsaftis et al. 2012). It was suggested that the alternative splicing of *OsHKT1;4* might control the horizontal axis in the shoot system (Cotsaftis et al. 2012).

Only two maize HKT orthologous sequences (*ZmHKT1;1* and *ZmHKT2;1*) could be retrieved from maize genomes, and we could not amplify the sequence of ZmHKT2;1 from the cDNA of salt-treated plants (data not shown). So, the alternative splicing of *ZmHKT1;1* could provide maize plant with more *HKT* copy, which can increase the salt tolerance of maize. Generally, sorghum has high salt tolerance than maize, might due to that there are four *HKT* members in sorghum compared to the two genes in maize (Wang et al. 2014). We noticed that both alternative splicing variants of *ZmHKT1;1* exist under normal condition. However, *ZmHKT1;1b* showed faster response to salt and heat stress than *ZmHKT1;1a*. In dehydration treated maize seedlings, the transcriptional level of *ZmHKT1;1a* was not affected, whereas the transcriptional level of *ZmHKT1;1b* was decreased (Fig. 3e). These results clearly showed that *ZmHKT1;1a* and *ZmHKT1;1b* had different response to stress treatment in maize. The transcripts of these two variants also showed different levels in different maize tissues (Supplemental Fig. 4).

To further study the function of the alternative splicing variants of *ZmHKT1;1* genes, we performed transgenic tobacco plants. When treated with high concentration of NaCl, transgenic plants overexpressing *ZmHKT1;1a* or *ZmHKT1;1b* had significantly higher fresh weight and root length than WT plants (Figs. 4, 5), indicating the high salt tolerance of transgenic plants. It has been reported that transgenic plants overexpressing *AtHKT1;1* which was driven by constitutive promoter were relatively more salt sensitive than WT plants, whereas transgenic plants overexpressing the same gene specifically in root stele cells accumulated less salt in the shoot and became more salt tolerant (Moller et al. 2009). However, transgenic barley plants overexpressing *HvHKT2;1* which was driven by strong constitutive ubiquitin promoter had increased salt tolerance (Mian et al. 2011). In our experiments, both *ZmHKT1;1a* and *ZmHKT1;1b* were controlled by CaMV 35S promoter which is a strong constitutive promoter, and overexpression of both genes could confer plants with high salt tolerance, indicating that not all *HKT* genes need to be cell type specifically expressed in transgenic plants to improve the salinity tolerance. It has also been shown that the reduction of *TaHKT2;1* expression increased the salt tolerance of plants (Laurie et al. 2002). These results suggests that HKT proteins might play roles with multiple mechanism.

Exclusion of Na^+ from shoots and photosynthetic tissues is a mechanism of salt tolerance (Moller and Tester 2007). HKTs detoxify the salt stress mainly by decreasing

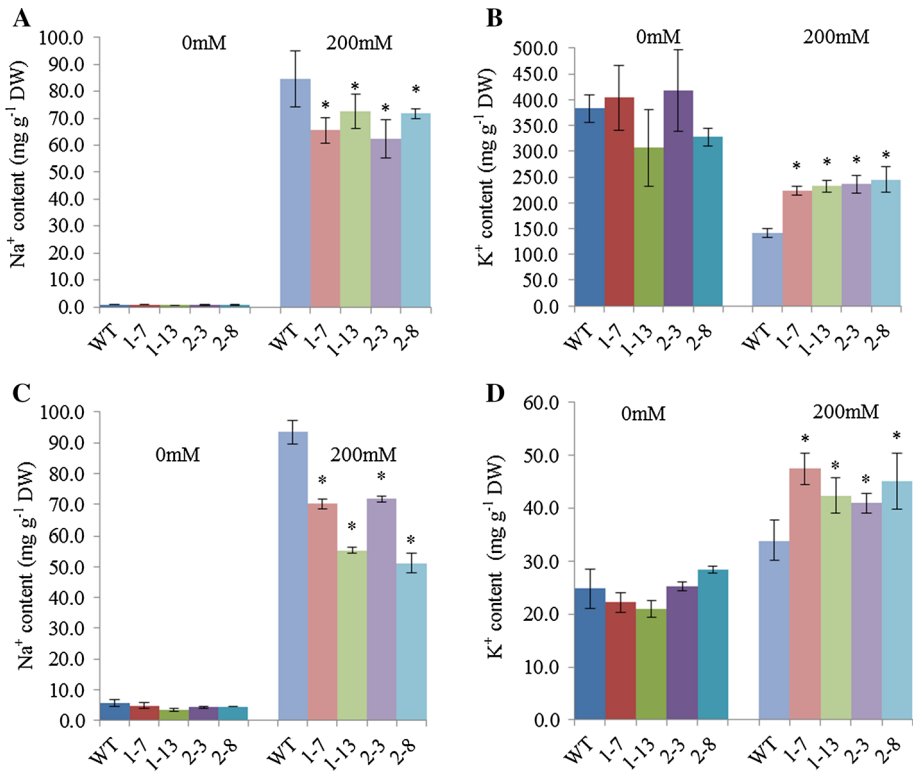

Fig. 6 Na^+ and K^+ contents in shoots (**a**, **b**), and roots (**b**, **d**) of transgenic plants. Heterozygous tobacco seeds of the T_1 generation were germinated on MS medium containing 10 mg L^{-1} phosphinothricin and grown for 7 days at 100 µmol m^{-2} s^{-1} with a 16 h light/8 h dark period. The alive seedlings were transferred onto MS medium in plastic boxes containing different concentration of NaCl, and grown for another 1 month. *1–7* and *1–13* are two independent transgenic lines expressing *ZmHKT1;1a*; *2–3* and *2–8* are two independent transgenic lines expressing *ZmHKT1;1b*. Data are shown as the average ± SE of three replicates. Experimental data was analyzed by *t* test and the *asterisks* in columns mean significant difference from WT plants at $P < 0.05$ level

the accumulation of Na^+ and it has been confirmed that the main role of AtHKT1;1 in transgenic plants is to avoid the accumulation of excessive Na^+ in the plant shoots (Almeida et al. 2013). When treated with salt, the transgenic plants overexpressing *ZmHKT1;1a* or *ZmHKT1;1b* had significantly less Na^+ contents in transgenic plants than that in WT plants (Fig. 6), indicating that overexpression of *ZmHKT1;1* reduced the accumulation of Na^+ under salt stress condition. The increased K^+ content could protect plant cells from Na^+ stress. Many studies have shown that increasing the K^+/Na^+ ratio is crucial for Na^+ tolerance in plants, and maintaining high K^+/Na^+ -ratio in shoots is highly correlated with salinity tolerance (Hauser and Horie 2010). So, it is not surprising that transgenic plants overexpressing *ZmHKT1;1* accumulated more K^+ in both shoots and roots than WT plants (Fig. 6).

Most HKTs have eight transmembrane domains and four highly conserved P-loops. It was shown that the glycine residue in the first P-loop is crucial for K^+ selectivity and transport (Maser et al. 2002). Four TaHKT2;1 mutants in the second and third P-loop reduced the low affinity Na^+ uptake, indicating the important roles of certain residues in the second and third P-loops (Rubio et al. 1995, 1999). The E464Q mutation in the fourth P-loop lowered the affinity of HKT1 for Na^+, confirming the fourth P-loop plays important role in the binding and transport of Na^+ (Diatloff et al. 1998). The fourth P-loop of ZmHKT1;1b was destroyed due to the pre-termination of translation. We did not observe obvious difference of salt-tolerant level, Na^+ or K^+ contents between transgenic plants overexpressing *ZmHKT1;1a* and plants overexpressing *ZmHKT1;1b*, indicating that the un-integrity of the fourth P-loop did not affect its function of salt detoxification when it was overexpressed. However, we did not know whether the transgenic plants expressing both genes still have the similar salt tolerance when *ZmHKT1;1a* and *ZmHKT1;1b* were driven by their native promoter.

Although there was no difference of the salt tolerance of transgenic tobacco plants overexpressing *ZmHKT1;1a* or *ZmHKT1;1b*, we found that overexpression of these two variants caused different expression of stress-related genes (Fig. 7). It was observed that overexpression of *ZmHKT1;1b* decreased the transcriptional levels of more stress-related genes in transgenic tobacco leaves, compared to the plants overexpressing of *ZmHKT1;1a*. Alternative splicing of pre-mRNAs can increase the diversity in the proteomic world, and various environmental stresses affected the alternative splicing (Graveley 2001). Overexpression of *ZmHKT1;1b* which has intron retention might led to the stress simulation of transgenic plant cell, so more stress-related genes were induced. *ZmHKT1;1b* plays role in the detoxification of salt stress with somewhat difference with *ZmHKT1;1a* due to the lack of the fourth P-loop, which need further investigation.

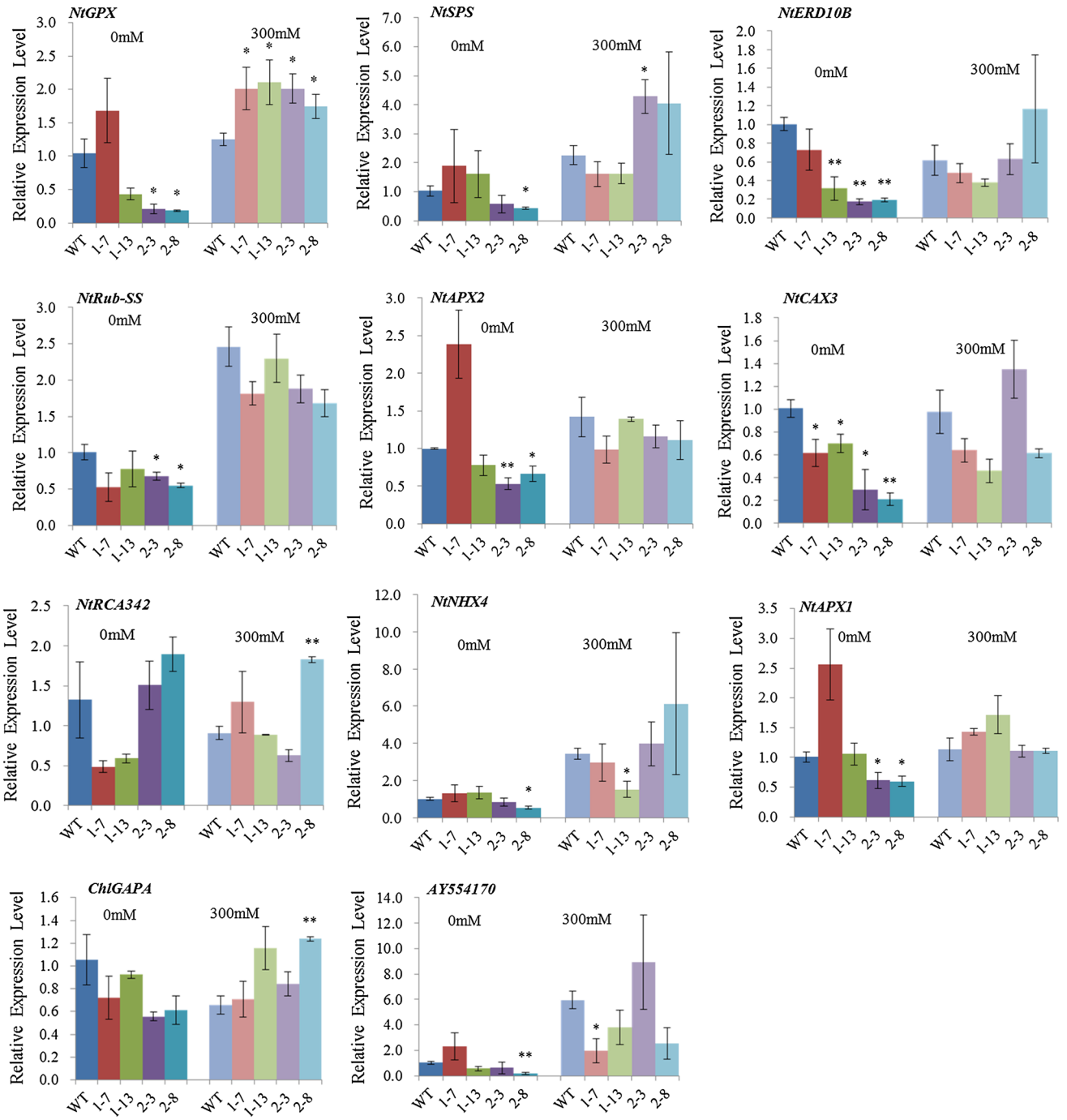

Fig. 7 The relative expression changes of stress-related genes in *ZmHKT1;1a* and *ZmHKT1;1b* transgenic tobaccos. 7-day old alive seedlings were transferred onto MS medium in plastic boxes containing different concentration of NaCl, and grown for another 1 month. *1–7* and *1–13* are two independent transgenic lines expressing *ZmHKT1;1a*; *2–3* and *2–8* are two independent transgenic lines expressing *ZmHKT1;1b*. The relative transcriptional level was analyzed using $2^{-\Delta\Delta C_t}$ method, and the transcriptional level of WT plants was normalized as 1.00. Data are shown as the average ± SE of three independent replicates. Experimental data was analyzed by *t* test and the *asterisks* in columns mean significant difference from WT plants at $*P < 0.05$ or $**P < 0.01$ level

Acknowledgments This work is financially supported by the National Basic Research Program of China (2014CB138202) and the Agricultural Science and Technology Innovation Program (ASTIP) of CAAS.

References

Almeida P, Katschnig D, de Boer AH (2013) HKT transporters—state of the art. Int J Mol Sci 14(10):20359–20385

Apse MP, Aharon GS, Snedden WA, Blumwald E (1999) Salt tolerance conferred by over expression of a vacuolar Na^+/H^+ antiport in Arabidopsis. Science 85:1256–1258

Corratgé-Faillie C, Jabnoune M, Zimmermann S, Véry A-A, Fizames C, Sentenac H (2010) Potassium and sodium transport in non-animal cells: the Trk/Ktr/HKT transporter family. Cell Mol Life Sci 67(15):2511–2532

Cotsaftis O, Plett D, Shirley N, Tester M, Hrmova M (2012) A two-staged model of Na^+ exclusion in rice explained by 3D modeling of HKT transporters and alternative splicing. PLoS ONE 7(7):e39865

Diatloff E, Kumar R, Schachtman DP (1998) Site directed mutagenesis reduces the Na^+ affinity of HKT1, an Na^+ energized high affinity K^+ transporter. FEBS Lett 432:31–36

Garciadeblas B, Senn ME, Banuelos MA, Rodriguez-Navarro A (2003) Sodium transport and HKT transporters: the rice model. Plant J 34(6):788–801

Golldack D, Su H, Quigley F, Kamasani UR, Munoz-Garay C, Balderas E, Popova OV, Bennett J, Bohnert HJ, Pantoja O (2002) Characterization of a HKT-type transporter in rice as a general alkali cation transporter. Plant J 31(4):529–542

Graveley BR (2001) Alternative splicing: increasing diversity in the proteomic world. Trends Genet 17:100–107

Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol 51:463–499

Hauser F, Horie T (2010) A conserved primary salt tolerance mechanism mediated by HKT transporters: a mechanism for sodium exclusion and maintenance of high K^+/Na^+ ratio in leaves during salinity stress. Plant Cell Environ 33(4):552–565

He C, Yan J, Shen G, Fu L, Holaday AS, Auld D, Blumwald E, Zhang H (2005) Expression of an Arabidopsis vacuolar sodium/proton antiporter gene in cotton improves photosynthetic performance under salt conditions and increases fiber yield in the field. Plant Cell Physiol 46:1848–1854

Horie T, Hauser F, Schroeder JI (2009) HKT transporter-mediated salinity resistance mechanisms in Arabidopsis and monocot crop plants. Trends Plant Sci 14(12):660–668

Horsch R, Fry J, Hoffman N, Eichholz D, Rogers S, Fraley R (1985) A simple and general method for transferring genes into plants. Science 227(4691):1229–1231

Huang S, Spielmeyer W, Lagudah ES, James RA, Platten JD, Dennis ES, Munns R (2006) A sodium transporter (HKT7) is a candidate for *Nax1*, a gene for salt tolerance in durum wheat. Plant Physiol 142(4):1718–1727

Laurie S, Feeney KA, Maathuis FJ, Heard PJ, Brown SJ, Leigh RA (2002) A role for HKT1 in sodium uptake by wheat roots. Plant J 32(2):139–149

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25(4):402–408

Maser P, Hosoo Y, Goshima S, Horie T, Eckelman B, Yamada K, Yoshida K, Bakker EP, Shinmyo A, Oiki S, Schroeder JI, Uozumi N (2002) Glycine residues in potassium channel-like selectivity filters determine potassium selectivity in four-loop-per-subunit HKT transporters from plants. Proc Natl Acad Sci USA 99(9):6428–6433

Mian A, Oomen RJ, Isayenkov S, Sentenac H, Maathuis FJ, Very AA (2011) Over-expression of an Na^+-and K^+-permeable HKT transporter in barley improves salt tolerance. Plant J 68(3):468–479

Mishra S, Alavilli H, Lee B, Panda SK, Sahoo L (2015) Cloning and characterization of a novel vacuolar Na^+/H^+ antiporter gene (*VuNHX1*) from drought hardy legume, cowpea for salt tolerance. Plant Cell Tissue Organ Cult 120:19–33

Moller IS, Tester M (2007) Salinity tolerance of Arabidopsis: a good model for cereals? Trends Plant Sci 12(12):534–540

Moller IS, Gilliham M, Jha D, Mayo GM, Roy SJ, Coates JC, Haseloff J, Tester M (2009) Shoot Na^+ exclusion and increased salinity tolerance engineered by cell type-specific alteration of Na^+ transport in Arabidopsis. Plant Cell 21(7):2163–2178

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651–681

Munns R, James RA, Xu B, Athman A, Conn SJ, Jordans C, Byrt CS, Hare RA, Tyerman SD, Tester M, Plett D, Gilliham M (2012) Wheat grain yield on saline soils is improved by an ancestral Na^+ transporter gene. Nat Biotechnol 30(4):360–364

Patel MK, Joshi M, Mishra A, Jha B (2015) Ectopic expression of *SbNHX1* gene in transgenic castor (*Ricinus communis* L.) enhances salt stress by modulating physiological process. Plant Cell, Tissue Organ Cult 122:477–490

Platten JD, Cotsaftis O, Berthomieu P, Bohnert H, Davenport RJ, Fairbairn DJ, Horie T, Leigh RA, Lin H-X, Luan S (2006) Nomenclature for HKT transporters, key determinants of plant salinity tolerance. Trends Plant Sci 11(8):372–374

Plett D, Safwat G, Gilliham M, Skrumsager Moller I, Roy S, Shirley N, Jacobs A, Johnson A, Tester M (2010) Improved salinity tolerance of rice through cell type-specific expression of *AtHKT1;1*. PLoS ONE 5(9):e12571

Ren ZH, Gao JP, Li LG, Cai XL, Huang W, Chao DY, Zhu MZ, Wang ZY, Luan S, Lin HX (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. Nat Genet 37(10):1141–1146

Rubio F, Gassmann W, Schroeder JI (1995) Sodium-driven potassium uptake by the plant potassium transporter HKT1 and mutations conferring salt tolerance. Science 270:1660–1663

Rubio F, Schwarz M, Gassmann W, Schroeder JI (1999) Genetic selection of mutations in the high affinity K^+ transporter HKT1 that define functions of a loop site for reduced Na^+ permeability and increased Na^+ tolerance. J Biol Chem 274(11):6839–6847

Schachtman DP, Schroeder JI (1994) Structure and transport mechanism of a high-affinity potassium uptake transporter from higher plants. Nature 370:655–658

Shukla PS, Agarwal PK, Jha B (2012) Improved salinity tolerance of *Arachis hypogaea* (L.) by the interaction of halotolerant plant-growth-promoting rhizobacteria. J Plant Growth Regul 31(2):195–206

Wang TT, Ren ZJ, Liu ZQ, Feng X, Guo RQ, Li BG, Li LG, Jing HC (2014) *SbHKT1;4*, a member of the high-affinity potassium transporter gene family from Sorghum bicolor, functions to maintain optimal Na^+/K^+ balance under Na^+ stress. J Integr Plant Biol 56(3):315–332