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Regulation of ion homeostasis in rice subjected to salt and alkali stresses

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Abstract

Alkali stress has shown a strong effect on K^+/Na^+ homeostasis than salt stress in many plants. In this study, rice seedlings were subjected to salt stress ($NaCl:Na_2SO_4$ = 9:1; pH 5.45) or alkali stress ($NaHCO_3:Na_2CO_3$ = 9:1; pH 9.05). The contents of Na^+, K^+, and inorganic anions and organic acids in the stressed seedlings were then measured. Then, the expression of some genes related to K^+/Na^+ metabolism such as *OsHKT*, *OsNHX* and *OsHAK* families, the *SOS* pathway and *OsAKT1* were assayed to investigate ion homeostasis and the regulative functions of these genes in rice alkali-tolerance. The results indicated that, alkali stress clearly reduced the contents of inorganic anions in rice, induced the massive influx of Na^+ and a deficiency of K^+ in roots, and disrupted Na^+/K^+ homeostasis and charge balance compared with salt stress ($NaCl:Na_2SO_4$ = 9:1; pH 5.45). Salt stress only has small effects on the *OsHAK*, *OsNHX* and *OsHKT* families and the *OsAKT1* and *SOS* pathways in rice, but alkali stress strongly stimulated their expression in roots and shoots. These findings suggested that the rice *OsHKT* family, *OsNHX* family and *SOS* pathway might play important roles in protecting shoots from high-Na^+ injury caused by alkali stress, especially in controlling Na^+ transport from roots to shoots. Under alkali stress, the overexpression of *OsHAKs* and *OsAKT1* might contribute to the release of K^+ from roots to shoots or the K^+ uptake by roots and maintain the potassium nutrition supply of shoots. The responses of these genes to alkali stress indicated that they might play important roles in rice alkali tolerance. Therefore, we propose that these genes have the potential for use in alkali tolerance and should be investigated further.

Keywords: Alkali stress; rice; ion balance; gene expression; salt stress.
Abbreviations: AKT–low affinity K^+ transporter, HAK–KUP/HAK/KT K^+ transporter**,** HKT–high affinity K^+ transporter, NHX–Na^+/H^+ exchanger, OA–organic acid**,** SOS–salt overly sensitive.

Introduction

Soil salinity is a widespread environmental problem and an important factor that limits agricultural productivity. There were 831 million hectares of soil in the world affected by salinization. Of this area, alkalinized soils underlie 434 million hectares, while saline soils underlie 397 million hectares (Wang et al., 2008). More than 70% of land area in northeast China is alkaline grassland (Kawanabe and Zhu, 1991), sometimes with a soil pH > 10 (Zheng and Li, 1999). Concomitant salt and alkali (high-pH) stress of these soils make difficult the plant growth. Only a few species of plants can survive on such soils. Alkaline salt stress ($NaHCO_3$ and/or Na_2CO_3) and neutral salt stress (NaCl and/or Na_2SO_4) are two distinct types of stress that affect plants. They should be referred to as alkali-stress and salt-stress, respectively (Shi and Yin, 1993). In fact, alkali stress has been shown to cause much stronger destructive effects on plants than salt stress (Yang et al., 2008a, b, c). However, to date, salt stress studies have generally emphasized NaCl (Munns and Tester, 2008), while little attention has been given to alkali stress (Yang et al., 2007; Yang et al., 2008 a, b, c; Shi and Wang, 2005; Shi and Sheng, 2005; Gao et al., 2008; Wang et al., 2008). Salt stress in soil generally involves osmotic stress and ion-induced injury (Ibraheem et al., 2011). Comparison of alkali stress with salt stress reveals an added high-pH effect due to alkali stress. The high-pH environment surrounding the roots can cause the loss of the normal physiological functions of the roots and destruction of the root cell structure (Yang et al. 2008a, b, c). Alkali stress can inhibit the absorption of ions such as Cl^-, NO_3^- and $H_2PO_4^-$, greatly affect the metabolism of K^+ and Na^+, and disrupt the homeostasis of K^+–Na^+ (Yang et al., 2007; Yang et al., 2008b). In many plants such as *Triticum aestivum* (Yang et al., 2008c), *Hordeum vulgare* (Yang et al., 2009), *Helianthus annuus* (Shi and Sheng, 2005), *Suaeda glauca* (Yang et al., 2008b), and *Aneurolepidium chinense* (Shi and Wang 2005), increases in Na^+ and decreases in K^+ under alkali stress are much greater than under salt stress. Alkali stress caused by these changes in Na^+ and K^+ may be the main reason of severe reductions of photosynthetic pigment and the net photosynthetic rate and a sharp increase in membrane permeability (Yang et al., 2009). Na^+ stress disrupts K^+ uptake by root cells. Additionally, when Na^+ enters cells and accumulates in high levels, it becomes toxic to enzymes (Zhu, 2003). Therefore, we believe that the maintenance of K^+ and Na^+ homeostasis is crucial for alkali tolerance. To prevent growth cessation or cell death, excessive Na^+ must be extruded or compartmentalized in the vacuole (Zhu, 2003). Many transporters of K^+ and Na^+ have been identified to date. In addition, the regulatory mechanisms that control the expression and activity of the transporters are beginning to be elucidated (Zhu, 2003; Munns and Tester, 2008). It is known that Na^+ enters plant roots through the high-affinity K^+ transporter (HKT) and non-selective cation channels (Zhu, 2003; Munns and Tester, 2008). In *Arabidopsis thaliana* (At), the salt overly sensitive (SOS) protein 1 functions in Na^+

exclusion from root epidermal cells into the rhizosphere. In *A. thaliana* and some plant species, the Na^+/H^+ exchanger (NHX) family has been shown to function in Na^+ compartmentation into vacuoles (Munns and Tester, 2008). In addition, some members of the high affinity K^+ transporter (HKT) family, such as OsHKT1;5 and AtHKT1;1, mediate Na^+ exclusion from leaves via Na^+ removal from the xylem sap (Horie et al., 2009). Rice is one of the most important cereal crops in tropical and temperate regions of the world. In many agricultural areas of Asia, especially north China, soil alkalinity (high pH) limits rice productivity. In this study, rice seedlings were subjected to salt stress ($NaCl:Na_2SO_4$ = 9:1; pH 5.45) or alkali stress ($NaHCO_3:Na_2CO_3$ = 9:1; pH 9.05). The contents of Na^+, K^+, and inorganic anions and organic acids in the stressed seedlings were then measured, and the expression of some genes related to K^+/Na^+ metabolism such as *low affinity K^+ transporter 1(AKT1)*, the *SOS* pathway, and some members of the *KUP/HAK/KT K^+ transporter (HAK)*, *NHX* and *HKT* families were assayed. Finally, we attempted to investigate K^+ /Na^+ accumulation and the regulative functions of the genes during the response of rice to alkali stress.

Results

Cation contents

Six hours after exposure to stresses, the Na^+, K^+ and Na^+/K^+ levels began to change. Both salt and alkali stresses induced increases in Na^+ and Na^+/K^+ in either the shoots or the roots (Fig. 1), whereas the increases under alkali stress were usually greater than under salt stress. Salt stress slightly affected K^+ accumulation; however, alkali stress reduced the K^+ content in the shoots and roots (Fig. 1C, D). In addition, under alkali stress, both K^+ content and the K^+/Na^+ ratio in the shoots were higher than in the roots (Fig. 1).

Inorganic anion contents

Under salt stress, the Cl^- contents in the shoots and roots increased with increasing duration ($P < 0.05$; Fig. 1G, H). Under alkali stress, the Cl^- contents decreased with increasing stress time ($P < 0.05$; Fig. 1G, H). Both salt and alkali stresses reduced NO_3^- content in shoots at 48 h. In roots, salt stress did not affect NO_3^- accumulation, but alkali stress reduced NO_3^- content (Fig. 1I-J). The effects of salt stress on the $H_2PO_4^-$ and SO_4^{2-} contents were small. However, the alkali stress decreased their contents at 48 h, especially in roots (Fig. 1).

Organic acid

Malate, citrate, oxalate were detected in rice. However, these compounds did not accumulate under salt stress. Under alkali stress, the levels of these OAs increased sharply with increasing stress time (Fig. 1O-V, $P < 0.05$).

Salt overly sensitive pathway

In shoots, salt stress only had a slight effect on the expression levels of *OsSOS1*, *OsCIPK24*, and increased the expression level of *OsCBL4*, but their expression were up-regulated by alkali stress at 6 h (Fig. 2). In roots, salt stress slightly enhanced their expression levels at 6 h or 24 h, whereas alkali stress strongly stimulated their expression from 6 h to 48 h (Fig. 2).

NHX family

In the rice genome, we searched for two members of the *NHX* family, *NHX1* and *NHX2*. The responses of *NHX1* and *NHX2* to both stresses were similar. Salt stress only had small effect on the expression of *NHX1* in shoots at 6h, while alkali stress increased its expression at 6 h (Fig. 2G). Both salt and alkali stresses increased the expression of *NHX2* in shoots at 6 h. However, the increase at 6 h under alkali stress was much greater than under salt stress (Fig. 2I). In roots, salt stress did not affect the expression of the two genes, but alkali stress strongly stimulated their expression (Fig. 2H and J).

HKT family

Salt stress did not increase the expression of *OsHKT1;1*, *OsHKT1;3*, *OsHKT1;5* or *OsHKT2;1* in shoots, whereas alkali stress stimulated their expression in shoots at 6 h or 24 h (Fig. 2). In roots, salt stress for $\geq$ 24 h enhanced the expression of *OsHKT1;5*, and strongly decreased the expression of *OsHKT2;1*. However, alkali stress strongly stimulated the expression of all members of the *HKT* family (Fig. 2). The expression levels of *OsHKT1;5* and *OsHKT2;1* in the roots were much higher than in the shoots (Fig. 2).

AKT1

Salt stress did not influence the expression of *OsAKT1* in shoots (Fig. 2S). However, its expression level in shoots was up-regulated by alkali stress particularly at 6 h, after which it decreased (Fig. 2S). However, during all durations, it was higher than in the control. Both salt and alkali stresses increased the expression of the *OsAKT1* in roots at 6 h; however, the expression under salt stress decreased with increasing stress time (Fig. 2T), while it increased under alkali stress ($P < 0.05$; Fig. 2T).

HAK family

Salt stress only had a small effect on the expression of the *HAK* gene family members in both shoots and roots, leading to slight increases or decreases in their levels (Fig. 3). Under alkali stress, the expression levels of *OsHAK7*, *OsHAK10* and *OsHAK16* in shoots increased at 6 or 48 h (Fig. 3). Alkali stress strongly stimulated the expression of *OsHAK7*, *OsHAK10* and *OsHAK16* in roots.

Discussion

Ion accumulation

Na^+ is the main toxic ion in salinized soil. Low Na^+ and high K^+ in the cytoplasm are essential for the maintenance of a number of enzymatic processes (Munns and Tester, 2008). Our results showed that alkali stress may greatly affect the transport of K^+ and Na^+ and disrupt the homeostasis of K^+–Na^+ in rice. Ion imbalance and pH instability of plants under salt stress or alkali stress is primarily caused by the influx of superfluous Na^+ (Blumwald, 2000; Yang et al., 2007). The pH stability of tissues is necessary for plants to maintain normal metabolism (Yang et al., 2007). The pH homeostasis of the internal environment is related to all free ions and all solutes with charge, and occurs via ion balance that includes organic and inorganic ions (Yang et al., 2007).

Table 1. Gene-specific primers used in real time PCR analysis.

Gene name	GeneBank/Accession No.	Forward primer (5'-3')	Reverse primer (5'-3')
OsNHX1	AB021878	GTTCAAGAGTTACAACAAAGCACG	CAGCGGGAATACAAAAGCAG
OsNHX2	AY360145	ACCAAGACGAAACACCCCTAC	AACCCAGCAACTACTCCAAGAA
OsHKT1;1	AJ491816	ATTAGCAGAGCACTGTGGAGGAA	CCGACGAACCCGTAGGAAG
OsHKT1;3	AJ491818	CAGTTCATCTACCAAAACAATCCA	AATACCTCACCACCAATCAGCA
OsHKT1;5	DQ148410	TGCCACCTTACACCACTTTCG	TGCCATACGCACTGATAACCTC
OsHKT2;1	AB061311	GCATATTCACCCATTCTGGATTCAGT	CGATGGTGATGAGGCTGGAAAGT
OsSOS1	AY785147	CTCCGTGCTCATAGAATCGC	ATACTCACTCAAGTGGGTCAATACC
OsCBL4	AK101368	GGCATCGTTCGGATTTCAC	GAGATTCGCCTTTCTGCTGTT
OsCIPK24	AK102270	AAGAAGCGGGTGGGGAGGT	GCGGTGGTTGAGGATGGTGT
OsAKT1	AY065970	TACGACCGCCGATACAGAA	CCAAATAAGCCACAAAGAAGG
OsHAK4	AF129485	CGTTCCCATCCGTCAGTAAA	CAGCCTCTGGTCTGGTTCGTC
OsHAK7	AJ427971	GAACTCCAACTTCCTCAAGACG	AGATCATGCCGACTTCGACGAG
OsHAK10	AJ427972	CGCTCTCGGCTGCTTTCCT	TAACCGCCAATCCTGACGC
OsHAK16	AJ427973	AGCGACTGTGTGCTAAACCC	CATAGATGCCAATCCCTGAGA
β-actin	AK101613	ATGCCATTCTCCGTCTT	GCTCCTGCTCGTAGTC

NHX, Na^+/H^+ exchanger; HKT, high affinity K^+ transporter; HAK, KUP/HAK/KT K^+ transporter; AKT, low affinity K^+ transporter; SOS, salt overly sensitive.

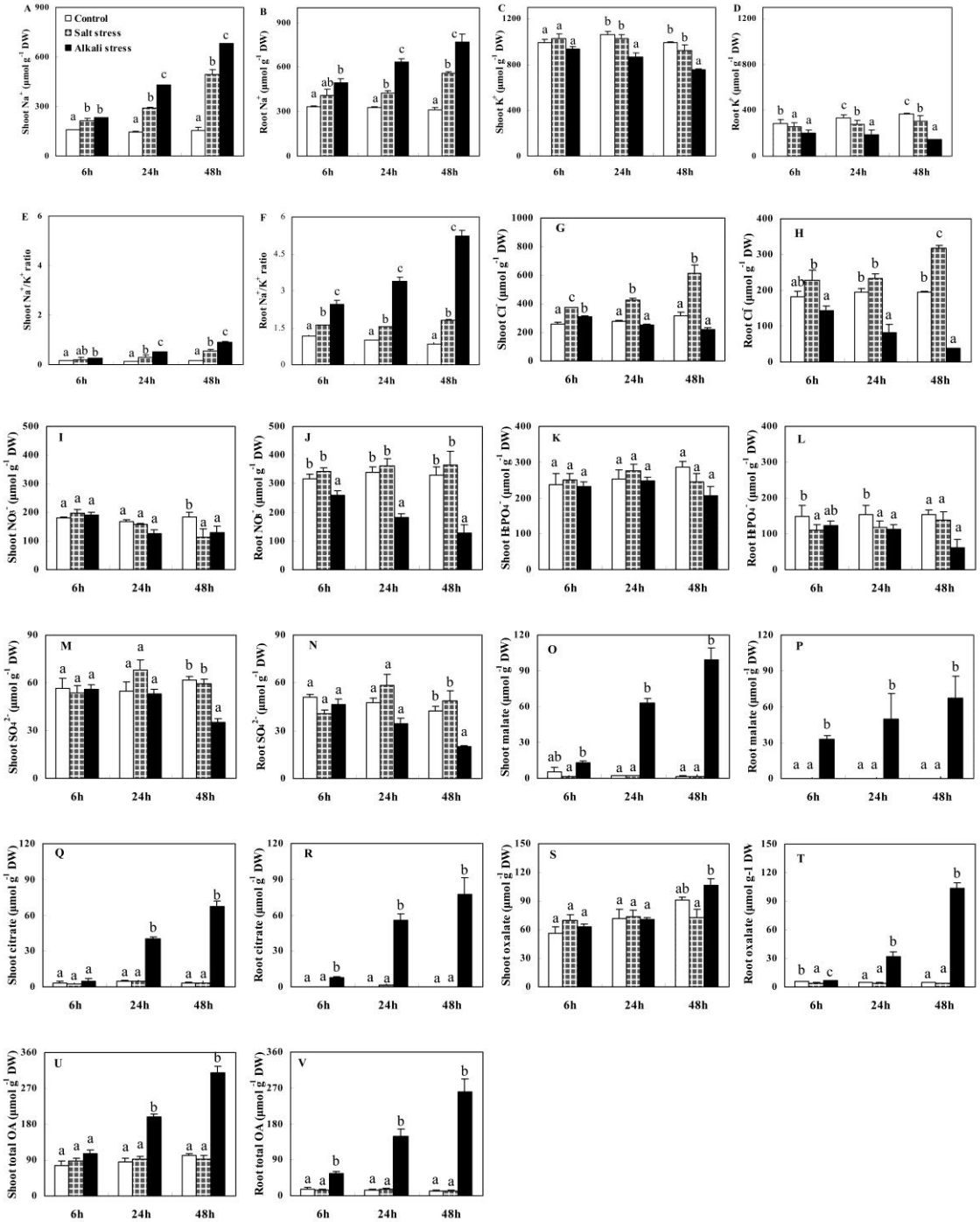

Fig 1. Effects of salt and alkali stresses on the contents of inorganic ions and organic acids (OA) in rice seedlings. 10-day-old seedlings were subjected to 45 mM salt ($NaCl:Na_2SO_4$ = 9:1; pH 5.45) or alkali ($NaHCO_3:Na_2CO_3$ = 9:1; pH 9.05) stresses for 6 h, 24 h and 48 h. Means followed by different letters among treatments at same time point are significantly different, according to least significant difference (LSD) test (P <0.05).

Under alkali stress, the accumulation of organic acids (OAs) in rice may be a response to increased Na+ and/or the reduction of inorganic anions (Fig. 1). The deficit of negative charge might be compensated by greatly accumulated OA (mainly oxalate, malate and citrate). Under alkali stress, the massive influx of Na+ and/or the reduction of inorganic anions may shock metabolism of OAs in rice, and may even affect the basal metabolic pathways involved in OAs such as the tricarboxylic acid cycle, glyoxylate cycle and photosynthesis.

Regulation of Na^+–K^+ metabolism

Our results revealed that alkali stress may strongly affect Na^+ transport or uptake of rice (Fig. 1). The increased Na^+ in rice shoots under alkali stress might also be related to decreased Na^+ exclusion (Fig. 1). It is well known that many plant species have a Na^+ exclusion mechanism that is dependent on a Na^+/H^+ antiport, such as SOS1, which exchanges cytoplasmic Na^+ with external H^+ (Zhu, 2003; Munns and Tester, 2008). This exchange activity relies on the transmembrane proton gradient achieved by H^+-ATPase (Zhu, 2003). Under alkali stress, the lack of external protons might weaken the exchange activity of the Na^+/H^+ antiport on the root plasma membrane, possibly reducing the exclusion of Na^+ into the rhizosphere and enhancing *in vivo* accumulation of Na^+, even to toxic levels. This may be the basis of alkali injury. Tolerance of plants to Na^+ stress relies on Na^+ compartmentation at the cellular and tissue levels, Na^+ exclusion and the control of long-distance transport in vasculatures. In roots, Na^+ influx, compartmentation and exclusion into the rhizosphere is mediated primarily by members of the HKT family, NHX family and SOS1, respectively. The land plants *HKT* gene family is divided into two classes based on their nucleic acid sequences and protein structures. HKT class 1 only transports Na^+, while HKT class 2 displays Na^+-K^+ symport (Horie et al., 2009). The Arabidopsis genome includes only one *HKT* gene, *AtHKT1;1*, while nine *OsHKT* genes are found in the rice genome (Horie et al., 2009). We tested the expression of *OsHKT1;1*, *OsHKT1;3*, *OsHKT1;5* and *OsHKT2;1* in rice in response to alkali stress. *OsHKT1;5* (*SKC1*) is a major rice quantitative trait locus (QTL) involved in regulation of Na^+/K^+ homeostasis under salt stress that is preferentially expressed in the parenchyma cells surrounding the xylem vessels in both shoots and roots (Ren et al., 2005) that also mediates Na^+ exclusion from shoots via Na^+ removal from the xylem sap (Horie et al., 2009). *In situ* hybridization experiments have shown that the tissue specificity of the expression of *OsHKT1;1*, *OsHKT1;3* and *OsHKT2;1* overlap in the vascular tissues of both roots and shoots (Jabnoune et al., 2009). When compared with salt stress, alkali stress strongly stimulated the expression of *OsHKT1;1*, *OsHKT1;3*, *OsHKT1;5* and *OsHKT2;1* in both roots and shoots (Fig. 2). Under alkali stress, the four members of rice *HKT* may primarily be expressed in stelar tissues in shoots and roots. One or perhaps several OsHKT transporters such as OsHKT1;5 may mediate Na^+ exclusion from shoots by unloading Na^+ from the ascending xylem sap, and may be important in protecting shoots from high-Na^+ injury caused by alkali stress (Fig. 1). Analogously, in Arabidopsis, AtSOS1 is preferentially expressed in parenchyma cells at the xylem/symplast boundary of roots, stems and leaves, and plays a role in retrieving Na^+ from the xylem stream under severe salt stress (Zhu, 2003; Shi et al., 2002). In Arabidopsis, the Ca^{2+}-responsive AtSOS3–AtSOS2 (AtCIPK24-AtCBL4) protein kinase pathway mediates regulation of the expression and activities of Na^+ transporters such as AtSOS1 and AtNHX, a Na^+/H^+ exchanger that mediates Na^+ compartmentation into vacuoles (Zhu, 2003). The rice SOS salt tolerance pathway has been identified and its functions have been shown to be similar to that of the SOS pathway in *Arabidopsis* (Martínez-Atienza et al., 2007). Our results revealed that salt stress only slightly enhanced the expression levels of *OsSOS1*, *OsCIPK24*, *OsCBL4*, *OsNHX1* and *OsNHX2* in rice roots, but that alkali stress strongly stimulated their expression in shoots and roots (Fig. 2). Under alkali stress, the overexpression of *OsSOS1* and *OsNHX* family members might be beneficial for the exclusion of Na^+ from roots into the rhizosphere and the Na^+ compartmentation into vacuoles, respectively. This may improve the reduction of Na^+ exclusion caused by loss of the transmembrane proton gradient under alkali stress. In addition, the roles of OsSOS1 in Na^+ homeostasis might be similar to At SOS1, and they may play important roles in controlling long-distance Na^+ transport under alkali stress. Under alkali stress, the rice HKT family, NHX family and SOS pathway might play important roles in the regulation on Na^+ transport, especially in controlling long-distance Na^+ transport from the roots to shoots. Many reports have shown that roots regulate ion transportation (Zhu, 2003; Ren et al., 2005; Mäser et al., 2002; Rus et al., 2001; James et al., 2006), whereas the main site of ion entry in roots is uncertain (Munns and Tester, 2008). As the broad aerenchyma in rice roots can weaken the regulation of ion entry, rice may have unique ion uptake mechanisms. Previous studies have indicated that apoplastic leakage, the transpirational by pass flow, makes a major contribution to sodium uptake in rice (Yeo et al., 1987; Yeo et al., 1999). If so, there may be a vital barrier or limitation for ion uptake in the root/shoot boundary or the parenchyma cells surrounding the xylem vessels of roots. Thus, most ion transporters or channels may be predominantly expressed in this area. These phenomena could explain why some ion transporters in rice such as OsHKT1;1, OsHKT1;3, OsHKT1;5 and OsHKT2;1 are expressed in vascular tissues, but not the epidermis (Ren et al., 2005; Jabnoune et al., 2009). Alkali stress greatly affects the accumulation of K^+ in rice. Transmembrane K^+ movements in plants are mediated by several types of channels, including the AKT family, and by transporters that belong to two families, KcsA-TRK (HKT) and KUP/HAK/KT (HAK) (Bañuelos et al., 2002; Amrutha et al., 2007).

Our results showed that salt stress only had a small effect on the expression of the *OsHAK* family and *OsAKT1* in rice, while alkali stress strongly stimulated the expression of rice *AKT1* and *HAK* family members (except for *HAK4*) in both roots and shoots (Figs. 7 and 8). OsHAK7 and OsHAK10 were also expressed in seedling roots and shoots. OsHAK10 is predominantly expressed in the tonoplast (Amrutha et al., 2007), while OsAKT1 is a dominant salt sensitive K^+ uptake channel in rice roots (Fuchs et al., 2005). Under alkali stress, in rice, OsAKT1 and one or perhaps several OsHAK transporters may mediate K^+ uptake or transport by roots, whereas some OsHAK transporters such as OsHAK10 are translocated to the tonoplast with the probable function of mediating the release of K^+ from the vacuoles (Bañuelos et al., 2002). Under alkali stress, the overexpression of the *OsHAK* family and *OsAKT1* might be an adaptive response to K^+ deficiency in rice roots (Figs. 1-2). We hypothesized that, under alkali stress, OsHAKs and OsAKT1 might also be expressed in the vascular tissues and mediate the release of K+ from the roots to shoots in order to supply shoots with potassium nutrition. If so, these data could explain why both the K+ content was much higher in shoots than in roots under alkali stress (Fig. 1).

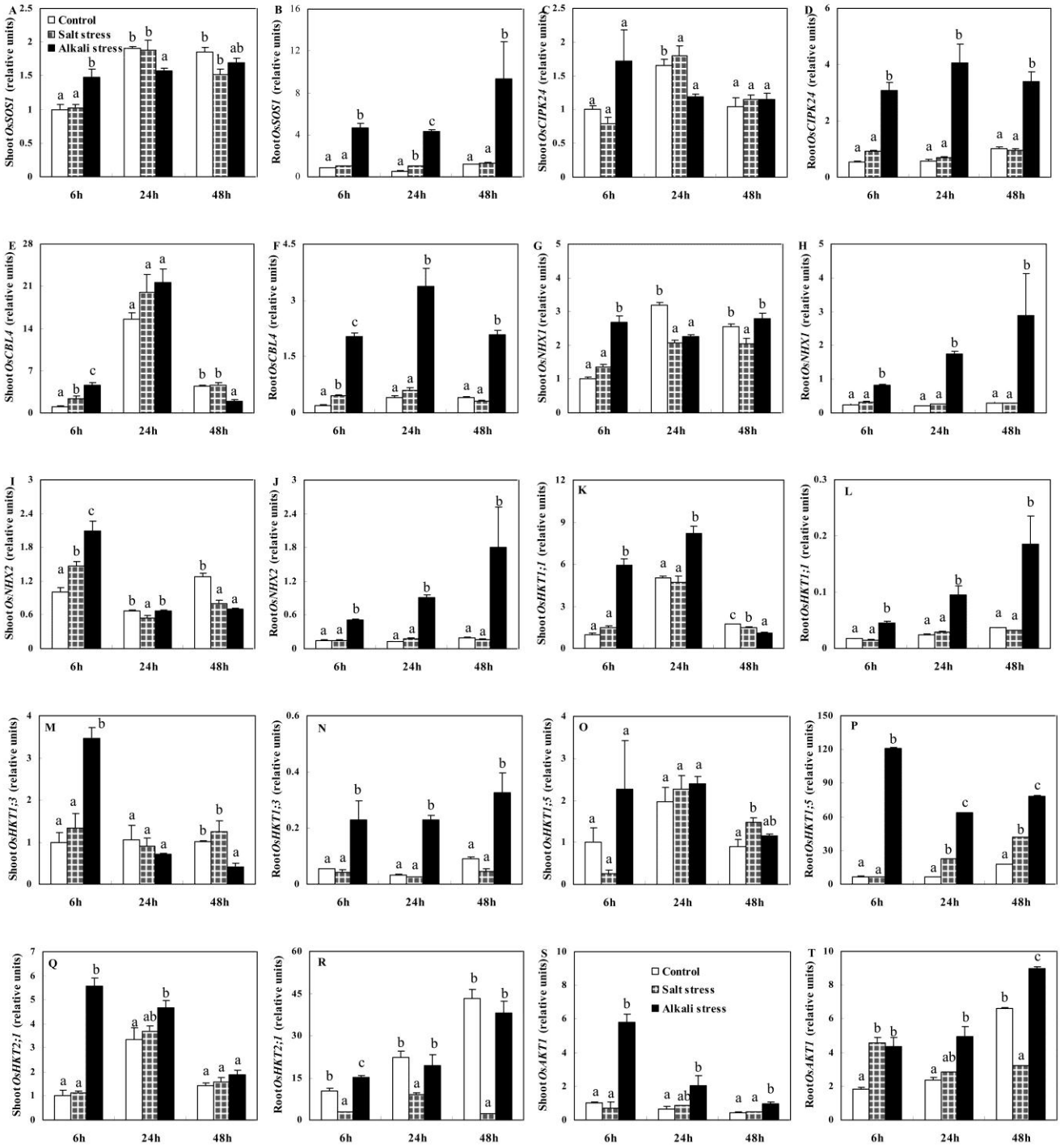

Fig 2. Effects of salt and alkali stresses on the expression of *OsHKT* and *OsNHX* gene families, *OsSOS* pathway, and *OsAKT1* in rice seedlings. 10-day-old seedlings were subjected to 45 mM salt ($NaCl:Na_2SO_4$ = 9:1; pH 5.45) or alkali ($NaHCO_3:Na_2CO_3$ = 9:1; pH 9.05) stresses for 6 h, 24 h and 48 h. Means followed by different letters among treatments at same time point are significantly different, according to least significant difference (LSD) test (P <0.05). NHX, Na^+/H^+ exchanger; HKT, high affinity K^+ transporter; AKT, low affinity K^+ transporter; SOS, salt overly sensitive.

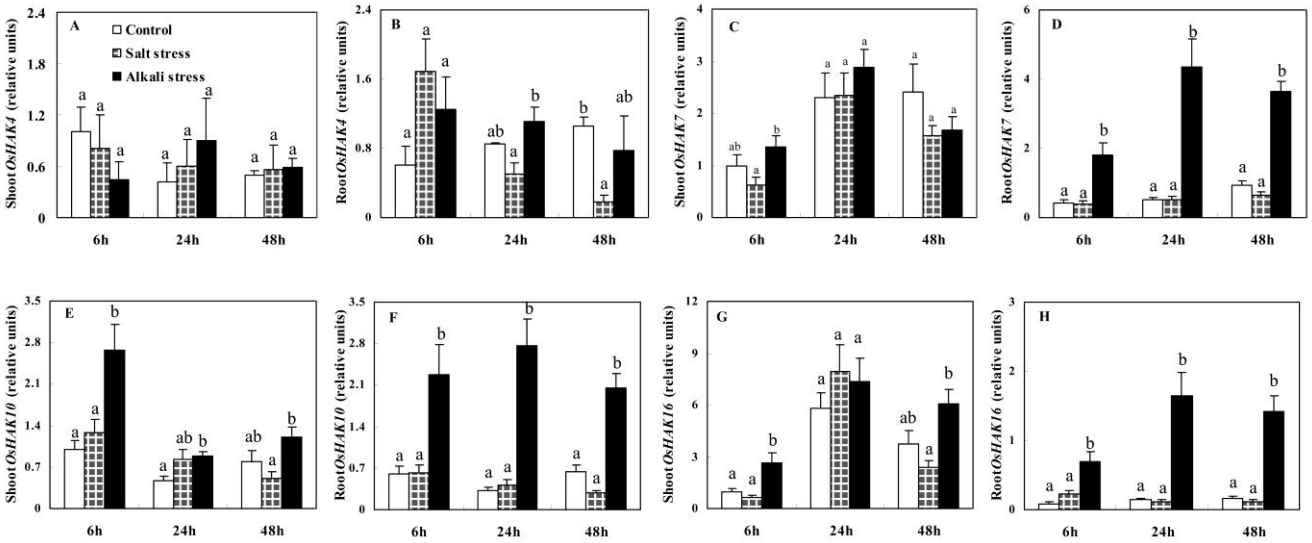

Fig 3. Effects of salt and alkali stresses on the expression of *OsHAK* gene family members in rice seedlings. 10-day-old seedlings were subjected to 45 mM salt ($NaCl:Na_2SO_4$ = 9:1; pH 5.45) or alkali ($NaHCO_3:Na_2CO_3$ = 9:1; pH 9.05) stresses for 6, 24 and 48 h. HAK, KT-HAK-KUP K^+ transporter. Means followed by different letters among treatments at same time point are significantly different, according to least significant difference (LSD) test (P <0.05).

Materials and methods

Plant growth conditions

Seeds of cultivar Longdun 97-1, a major rice cultivar in north China were germinated in Petri dishes for 5 d in a growth cabinet (30 °C during the day and 25°C during the night, 16/8 h photoperiod at 50 μmol m^{-2} s^{-1}). Longdun 97-1 as alkali tolerant rice cultivar is grown in the moderate-alkalinized field of northeast China. Seedlings were then transferred to buckets containing 1000 ml of aerated sterile nutrient solution for nutrient solution culture. The second-hand nutrient solution was replaced daily with new nutrient solution. The buckets were placed in a growth chamber that was maintained at 27.0 ± 1.5°C during the day and 20.0 ± 1.5°C during the night, under a 16/8 h photoperiod at 250 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR). The nutrient solution used in this work accorded to the components described by the International Rice Research Institute (Yoshida et al., 1976), and contained 0.715 mM NH_4NO_3, 0.16 mM NaH_2PO_4, 0.323 mM K_2SO_4, 0.5 mM $CaCl_2$, 0.83 mM $MgSO_4$, 0.036 mM Fe-EDTA, 0.1mM Na_2SiO_3, 4.55 μM $MnCl_2$, 0.077 μM $ZnSO_4$, 0.078 μM $CuSO_4$, 9.25 μM H_3BO_3, 0.263 μM H_2MoO_4, and with pH= 5.3-5.5.

Stress treatment and ion content determinations

Two neutral salts (NaCl and Na_2SO_4) and two alkaline salts ($NaHCO_3$ and Na_2CO_3) were chosen based on the salt components and pH in the majority of salt–alkaline soils in northeast China. The two neutral salts were mixed in a 9:1 molar ratio ($NaCl:Na_2SO_4$) as the salt stress treatment. Two alkaline salts were mixed in a 9:1 molar ratio ($NaHCO_3:Na_2CO_3$) as the alkali stress treatment. The total salt concentration of both treatments was set at 45 mM. This experiment was designed to apply the same Na^+ and total salt concentrations, but with different pH values for each stress. For the salt and alkali stresses, the pH values were 5.45 and 9.05, respectively. After 9 days of growth in hydroponic medium, rice plants were subjected to salt or alkali stresses by transferring them to another bucket containing 1000 mL of the treatment solution amended with the above nutrients and 45 mM stress salts. A bucket including 20 seedlings represented one replicate, and there were three replicates per treatment. 27 buckets of seedlings were randomly divided into 9 sets, three buckets per set. Each bucket was considered as one replicate with three replicates per set, Three sets were used as control (one set per time point); three sets were treated with salt stress (one set per time point); and the remaining three sets were treated with alkali stress (one per time point). Totally, the experiment had three biological replicates. Treatment solutions were replaced daily. The nutrient solution without stress salts was used as a control. The 20 seedlings were harvested after treatment for 6, 24, and 48 h, respectively. The roots and shoots of 10 seedlings in each bucket were separated and immediately frozen in liquid nitrogen and then stored at −70°C for RNA isolation. Another 10 seedlings in each bucket were washed with distilled water, after which the roots and shoots were separated and freeze-dried. Then the dry samples of plant material in each bucket were levigated and mixed for ion measurements. The dried samples of the roots and shoots were digested with HNO_3, and the Na^+ and K^+ contents were assayed using an atomic absorption spectrophotometer (TAS-990, Purkinje General, Beijing). Dry samples of plant material were treated with 10 mL deionized water at 100°C for 1 h, and the extract was then used to determine the contents of free inorganic anions and organic acids (OAs). The contents of NO_3^-, $H_2PO_4^-$, Cl^-, SO_4^{2-} and oxalic acid were determined by ion chromatography (DX-300 ion chromatographic system; AS4A-SC ion-exchange column,

CD M-II electrical conductivity detector, mobile phase: $Na_2CO_3/NaHCO_3$ = 1.7/1.8 mM; DIONEX, Sunnyvale, USA). Other OAs (malate, citrate, succinate, acetate, formate, tartrate and lactate) were also determined by ion chromatography (DX-300 ion chromatographic system; ICE-AS6 ion-exclusion column, CDM-II electrical conductivity detector, AMMS-ICE II suppressor, mobile phase: 0.4 mM heptafluorobutyric acid; DIONEX, Sunnyvale, USA).

Information of determined genes

In study, we evaluated the expression of some genes related to K^+/Na^+ metabolism such as *Na^+/H^+ exchanger (NHX)*, *high affinity K^+ transporter (HKT)*, and *KUP/HAK/KT K^+ transporter (HAK)* gene families, and *low affinity K^+ transporte (AKT)1*, and three genes of salt overly sensitive (SOS) pathway. SOS pathway contained three genes, *SOS1*, *CIPK24*, and *CBL4*. The name and GeneBank number of the genes are listed in Table 1.

Quantitative real time PCR analysis for genes

The total RNA was extracted from the shoots and roots of seedlings grown under stress or non-stress conditions using TRIzol reagent (Invitrogen). The RNA was treated with DNaseI (Invitrogen), reverse-transcribed using SuperScriptTM RNase H-Reverse Transcriptase (Invitrogen), and then subjected to real time PCR analysis using gene-specific primers (according to the method of manufacturer). The gene-specific primers are listed in Table 1. The primers are designed by software Primer 5.0. PCR amplification was conducted with an initial denaturation step at 95°C for 1 min followed by 40 cycles of 5 s at 95°C, 10 s at 60°C and 30 s at 72°C. Amplification of the target gene was monitored every cycle by SYBR Green mix according to the method of manufacturer (Sigma-Aldrich compnay). Amplification of the rice *β-actin* (GenBank Accession AK101613) mRNA was used as an internal quantitative control (Wang et al., 2010). We optimized PCR reaction system, after which the amplification efficiencies of each target gene and reference gene were approximately equal. The relative expression of the target genes was measured using the $\triangle\triangle$Ct method (Livak and Schmittgen, 2001). Real time PCR analyses were conducted at least three times for each sample.

Statistical Analysis

The experiment design was one factor-randomized complete design with three replications. When we compared control and the stress treatments, except for stress salt concentration in nutrient solution, all conditions of control and the stress treatments were similar. Statistical analysis of the data was performed using the statistical program SPSS 13.0 (SPSS, Chicago, USA). All data were represented by an average of the three replicates and the standard errors (S.E.). The treatment mean values at same time point were compared by least significant difference (LSD) test. The term significant indicates differences at P <0.05.

Conclusion

When compared with salt stress, alkali stress might shock the Na^+/K^+ and OA metabolisms. Under alkali stress, the rice HKT family, NHX family and SOS pathway play important roles in protecting shoots from high-Na^+ injury caused by alkali stress, especially when controlling Na^+ transport from roots to shoots. Also, under alkali stress, the *OsHAKs* and *OsAKT1* contribute to the release of K^+ from roots to shoots or the uptake of K^+ by roots, and maintain the potassium supply of shoots. In this study, we found many genes possibly involved in alkali tolerance. This is vital for the alkali stress physiology and molecular breeding of alkali-tolerant rice, and should be investigated in future studies.

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