DOI: 10.1007/s11099-015-0079-4 PHOTOSYNTHETICA 53 (1): 55-65, 2015

Alkali tolerance in rice (*Oryza sativa* L.): growth, photosynthesis, nitrogen metabolism, and ion homeostasis

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Abstract

Alkali stress is an important agricultural problem that affects plant metabolism, specifically root physiology. In this study, using two rice cultivars differing in alkali resistance, we investigated the physiological and molecular responses of rice plants to alkali stress. Compared to the alkali-sensitive cultivar (SC), the alkali-tolerant cultivar (TC) maintained higher photosynthesis and root system activity under alkali stress. Correspondingly, the Na^+ content in its shoots was much lower, and the contents of mineral ions (*e.g.*, K^+, NO_3^-, and $H_2PO_4^-$) in its roots was higher than those of the SC. These data showed that the metabolic regulation of roots might play a central role in rice alkali tolerance. Gene expression differences between the cultivars were much greater in roots than in shoots. In roots, 46.5% (20 of 43) of selected genes indicated over fivefold expression differences between cultivars under alkali stress. The TC had higher root system activity that might protect shoots from Na^+ injury and maintain normal metabolic processes. During adaptation of TC to alkali stress, *OsSOS1* (salt overly sensitive protein 1) may mediate Na^+ exclusion from shoots or roots. Under alkali stress, SC could accumulate Na^+ up to toxic concentrations due to relatively low expression of *OsSOS1* in shoots. It possibly harmed chloroplasts and influenced photorespiration processes, thus reducing NH_4^+ production from photorespiration. Under alkali stress, TC was able to maintain normal nitrogen metabolism, which might be important for resisting alkali stress.

Additional key words: citrate; gas exchange; gene expression regulation; germination rate; malate; Na^+/K^+ ratio; survival rate.

Introduction

Soil alkalization frequently causes severe problems in some areas, *e.g.*, in northeast China where alkalinized grassland covers over 70 % of the land area and is expanding (Kawanabe and Zhu 1991). Previous studies have suggested that salt stress could be defined as the stress of neutral salts; alkali stress as the stress of alkaline salts (Shi and Sheng 2005, Shi and Wang 2005). Reports have clearly demonstrated that the effects of alkali stress on plants are more severe than those of salt stress (Shi and Yin 1993). Although some attention has been given to alkali stress (Shi and Sheng 2005, Shi and Wang 2005, Yang *et al*. 2007, Gao *et al*. 2008), the physiological and molecular mechanisms of alkali tolerance remain largely obscure.

Salt stress in soil generally involves osmotic stress and ion injury (Munns and Tester 2008). Alkali stress exerts the same stress factors but with the added influence of high-pH stress. Plant survival and growth in saline or alkaline environments is a result of adaptive processes, such as ion transport and compartmentation, and compatible solute synthesis and accumulation. Many of these compatible solutes are nitrogen (N)-containing compounds, such as amino acids and amides or betaines (Läuchli and Lüttge 2002). Interference between salinity and N nutrition

Received 30 December 2013, *accepted* 3 June 2014.
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Abbreviations: AKT – low affinity K^+ transporter; AS – asparagine synthetase; AST – alkali stress treatment; GDH – glutamate dehydrogenase; GOGAT – glutamate synthase; GS – glutamine synthetase; HAK – KUP/HAK/KT K^+ transporter; HKT – high affinity K^+ transporter; NHX – Na^+/H^+ exchanger; NiR – nitrite reductase; NR – nitrate reductase; OA – organic acid; P5CS – δ1-pyrroline-5-carboxylate synthetase; ProDH – proline dehydrogenase; SC – alkali-sensitive cultivar; SOS – salt overly sensitive; SST – salt stress treatment; TC – alkali-tolerant cultivar.
Acknowledgements: This study was supported by the National Natural Science Foundation of China Project (31300192), Project of the Jilin Provincial Government (No. 20106023), and Basic Research Project by Jilin Provincial Government (No. 20090567). We thank International Science Editing (ISE) for language editing.

is a very complex network affecting almost all processes in plant metabolism and development (Läuchli and Lüttge 2002). Thus, N metabolism is of central importance for adaptation of plants to salt stress. Our previous studies showed that alkali stress (high-pH) strongly influences N metabolism (Chen *et al*. 2009, Yang *et al.* 2009, Wang *et al.* 2011, 2012). The high-pH environment surrounding the roots may limit assimilation and/or uptake of NO_3^- (Yang *et al*. 2009, Wang *et al*. 2011, 2012), and may be the main reason that alkali stress is more harmful to plants than salt stress. It was recognized that NO_3^- uptake is mediated by an H^+/NO_3^- symport mechanism, which relies on the transmembrane H^+ gradient (Crawford and Glass 1998). The lack of external H^+ caused by alkali stress may weaken NO_3^- uptake (Fig. 1S, *supplementary material available online*). In addition, it is also 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 H^+ gradient achieved by H^+-ATPase (Zhu 2003). Under alkali stress, the lack of external H^+ may 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 concentrations (Fig. 1S). Thus, the decreased Na^+ exclusion and NO_3^- uptake may be the basis of alkali injury. However, to date, the physiological and molecular mechanisms underlying the ion homeostasis and N metabolism regulation in adaptation of plants to alkali stress remain largely unexplored.

Comparison among genotypes differing in alkali tolerance may be helpful for alkali tolerance research and identifying alkali-tolerant candidate genes. In this study, two rice (*Oryza sativa* L.) cultivars differing in alkali tolerance, Changbai-9 (tolerant, TC) and Jijing-88 (SC) were tested. The seedlings of the two cultivars were subjected to salt stress or alkali stress, and growth, photosynthesis, inorganic ions, and organic acids (OAs) were measured in the stressed seedlings. The expression of some critical genes involved in N metabolism and ion balance were also assayed to test their roles in alkali tolerance. To our knowledge, this study represents the first comparative investigation of genotypes differing in alkali tolerance. Finally, we found some candidate genes involved in rice alkali tolerance, which may be vital for molecular breeding and alkali tolerance research.

Materials and methods

Plant growth conditions: Two rice (*Oryza sativa* L.) cultivars, Changbai-9 (alkali tolerant, labeled as TC) and Jijing-88 (relatively sensitive compared to Changbai-9, labeled as SC), were chosen as test plants. Both cultivars are Japonica rice. Changbai-9 was provided by the Institute of Jilin Agricultural Science. Changbai-9 is widely grown in the moderately alkalinized fields of northeast China (Yang *et al.* 2010). The experiment was performed in 2011. All plants were grown in a hydroponic system. The seeds of both cultivars were germinated and grown in Petri dishes for 6 d in a greenhouse [28/22°C and 16/8 h of day/night, irradiance of 300 μmol(photon) m^{-2} s^{-1}]. Seedlings were then transferred to buckets containing 2,000 mL of sterile nutrient solution for solution culture. The nutrient solution was replaced daily. The nutrient solution used in this work contained 1.44 mM NH_4NO_3, 0.32 mM NaH_2PO_4, 0.6 mM K_2SO_4, 1.0 mM $CaCl_2$, 1.6 mM $MgSO_4$, 0.072 mM Fe-EDTA, 0.2 mM Na_2SiO_3, 9.1 μM $MnCl_2$, 0.154 μM $ZnSO_4$, 0.156 μM $CuSO_4$, 18.5 μM H_3BO_3, and 0.526 μM H_2MoO_4 at pH 5.3.

Stress treatments: Two neutral salts (NaCl and Na_2SO_4) and two alkaline salts ($NaHCO_3$ and Na_2CO_3) were selected based on the salt components and pH of 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 (SST). The two alkaline salts were also mixed in a 9:1 molar ratio ($NaHCO_3$:Na_2CO_3) as the alkali stress treatment (AST). SST and AST were set at the same total salt concentration (50 mM). This experiment was designed to apply the same Na^+ and total salt concentrations, but with different pH values for each stress. For the SST and AST, the pH values were 5.3 and 9.1, respectively. After 9 d of growth in hydroponic medium, rice plants were subjected to stresses by transferring them to another bucket containing 2,000 mL of the treatment solution amended with the above nutrients and 50 mM of the salts. A bucket including 20 seedlings represented one replicate, and there were three replicates per treatment. For each cultivar, nine buckets of seedlings were randomly divided into three sets, three buckets per set. Each bucket was considered as one replicate with three replicates per set, one set was used as control, one set was treated with SST and another set was treated with AST. Namely, the experiment included three biological replicates. Treatment solutions were replaced daily. The nutrient solution without stress salts was used as a control. The 20 seedlings in each bucket were harvested after treatment for 48 h.

Measurements of physiological indices: After treatment for 48 h, net photosynthetic rate (P_N), stomatal conductance (g_s), and transpiration rate (E) of 17-d-old seedlings were determined at 08:30–10:30 h on fully expanded blades, using a portable open flow gas exchange system *LI-6400* (*LICOR*, USA). The PAR was 1,200 μmol m^{-2} s^{-1} (*i.e.*, saturation). Leaf temperature and vapour pressure deficit were 29°C and 2.4–2.8 kPa, respectively. Membrane permeability was estimated by the electrolyte leakage rate (ELR), which was determined with the

ameliorated method of Lutts *et al*. (1996). One fresh leaf was taken from each bucket and washed three times with deionized water to remove surface adhered electrolytes. The leaves were placed in a closed cuvette containing 20 mL of deionized water. The cuvette was incubated at 25°C on a rotary shaker for 4 h, and electrical conductivity of the solution (EC1) was determined with a conductivity gauge. Then the cuvette was autoclaved at 120°C for 20 min and electrical conductivity of the solution (EC2) determined. ELR was defined as follows: ELR [%] = (EC1/EC2) × 100. The root activity was determined as described by Comas *et al*. (2000). The fresh roots were incubated for 60 min at 37°C in triphenyl tetrazolium chloride (TTC) solution (0.04% in phosphate buffer, pH 7.0). The red product in roots was extracted using ethyl acetate. The absorbances were determined by spectrophotometer (*1901*, *Puxitongyong Company*, China) at 485 nm. The activity of the root system was expressed relative to the control value.

The roots and shoots of ten seedlings in each bucket were separated, then immediately frozen in liquid nitrogen and then stored at −70°C for RNA isolation and the measurements of pigments. Another ten seedlings in each bucket were washed with distilled water, after which the roots and shoots were separated and freeze-dried. Dry samples of plant material were treated with 10 mL of deionized water at 100°C for 2 h, and the extract used to determine the contents of free inorganic ions and OAs (Wang 2001). The contents of NO_3^-, Cl^-, $H_2PO_4^-$, 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) with a mobile phase of $Na_2CO_3/NaHCO_3$ = 1.7/1.8 mM (*DIONEX*, Sunnyvale, USA). Other OAs 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)]. A flame photometer was used to determine K^+ and Na^+ contents (*6300X*, *Shangdong Instrument Company*, China). Ammoniacal N content was measured using the ninhydrin method of Zhang (2004).

Quantitative PCR analysis: We extracted the total RNA from the roots and shoots of seedlings grown under stress or control 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. The gene-specific primers are listed in Table 1S (*supplementary material available online*). PCR amplification was conducted with an initial step at 95°C for 1 min followed by 45 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. Amplification of the rice *UBQ5* (GenBank Accession No. AK061988) mRNA was used as an internal quantitative control (Jain *et al*. 2006, Quinet *et al*. 2010, Zang *et al*. 2010). This reference gene maintains relative stabile expression under stress conditions (Jain *et al*. 2006, Quinet *et al*. 2010, Zang *et al*. 2010). The relative expression of the target genes was calculated using the ΔCT method (Livak and Schmittgen 2001). In present study, we tested relative expression levels of 43 genes. The low relative expression level is considered to be unreliable. Thus, we only showed results of genes having high expression level in the text (expression value was about more than 0.1).

Survival rate under salt stress and alkali stress: The seeds of both cultivars were germinated and grown in Petri dishes for 6 d in a growth cabinet (28/22°C and 16/8 h of day/night, light at 300 μmol m^{-2} s^{-1}). The seedlings were cultured and stressed using the above methods. After 9 d of growth in hydroponic medium, rice plants were subjected to stresses by transferring them to another bucket containing 4,000 mL of the treatment solution amended with the above nutrients and 50 mM of the stress salts. A bucket including 50 seedlings represented one replicate, and there were three replicates per treatment. For each cultivar, nine buckets of seedlings were randomly divided into three sets, three buckets per set. Each bucket was considered as one replicate with three replicates per set, one set was used as control, one set was treated with SST and another set was treated with AST. The nutrient solution without stress salts was used as a control. The survival rates were calculated after treatment for 12 d.

G**ermination rate under salt and alkali stresses**: The both SST and AST were designed with above salt proportion. Within each group, four total salt concentrations were applied: 100, 200, 300, and 400 mM. In the SST and AST groups, pH was 5.3–5.4 and 9.1–9.2, respectively. Three replicates with 50 seeds of both cultivars were used for each treatment. Seeds were sown on a filter paper in an 8.5-cm tight-fitting Petri dish and submerged in 6 mL of treatment solution for each dish. The dishes were placed in a growth cabinet (28/22°C and 16/8 h of day/night, light of 200 μmol m^{-2} s^{-1}). The percentage germination was recorded daily for 12 d, with emergence of the radicle considered as germination. The treatment solution was replaced daily. Ungerminated seeds were transferred to distilled water to test whether the seeds had been killed by stresses (recovery of germination). Seed death rate was estimated as follows: death rate [%] = 100 × number of ungerminated seeds after recovery/total seed number (50).

Statistical analysis: 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 (SE). Statistical significance was determined by *t*-test or least significant difference (LSD) test.

Results

Germination: At moderate salinity levels (100–200 mM), the effects of both stresses on seed germination were not significant (Fig. 1*A*). When salinity increased (300 and 400 mM), both stresses greatly reduced germination rates, with more reduction under AST than SST. Germination differences between cultivars were small under SST. However, the germination rate was greater in the TC compared to the SC under AST. After recovery of germination in distilled water, most ungerminated seeds from the 300 mM SST germinated. At 300 and 400 mM AST, most ungerminated seeds were killed; and the death rate was lower in the TC than the SC (Fig. 1*C*).

Growth and photosynthesis: Under control conditions, the SC showed lower biomass than TC (Fig. 2, Fig. 2S - *supplementary material available online*). SST (50 mM) affected similarly both cultivars, but effects of AST (50 mM) greatly differed. When seedlings were treated for 12 d, survival rates of both cultivars were 100% under SST (Fig. 2*H*). However, under AST, the survival rate only was 9.3% in the SC but almost all seedlings of the TC survived (92.7%) (Fig. 2*H*). AST had a stronger inhibitory effect on the growth and photosynthesis of the SC compared to the TC. AST decreased root activity and photosynthesis and increased the electrolyte leakage rate, but changes of the SC were greater than those of the TC (Fig. 2). Under AST, TC maintained much higher root activity than SC, indicating that TC has a strong respiratory vigour under AST.

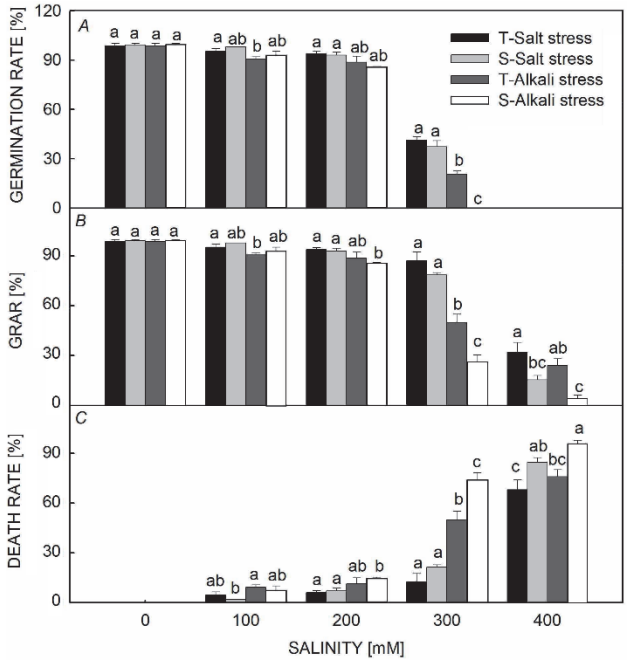

Fig. 1. Effects of salt and alkali stresses on germination of two rice cultivars differing in alkali tolerance (T – Changbai-9, alkali-tolerant cultivar; S – Jijing-88, alkali-sensitive cultivar). The values are means (± SE) of three replicates. Means followed by *different letters* among treatments at the same salinity are significantly different according to LSD test (P<0.05). GRAR – germination rate after recovery.

Ion accumulation: Under control conditions, the SC showed lower contents of NO_3^- and SO_4^{2-} and higher K^+ content in roots than that in TC (Fig. 3). AST exhibited a stronger effect on the accumulation of inorganic ions than SST. AST clearly increased Na^+ and Na^+/K^+, and decreased the contents of K^+, Cl^-, NO_3^-, $H_2PO_4^-$, and SO_4^{2-}; there were much higher contents in the roots of the TC compared to the SC under AST, whereas values were similar in shoots of both cultivars (Fig. 3). Malate, citrate, and oxalate were the dominant OA components, while only trace amounts of succinate, acetate, formate, and lactate were detected. Thus, we only listed the results of malate, citrate, oxalate, and the total OAs (Fig. 4). We found that AST strongly stimulated the accumulation of malate and citrate in both cultivars, with much higher concentrations of OAs in roots of the TC compared to the SC.

Gene expression differences between the cultivars: The relative gene expression levels (the ratio of TC to SC) between cultivars are shown in Fig. 5. We selected 43 key genes involved in rice alkali tolerance (Fig. 5, Table 1S) to examine gene expression differences between the cultivars. Roots showed much greater gene expression differences between cultivars than shoots. In roots, 46.5% (20 of 43) of selected genes exhibited over fivefold expression differences between cultivars under AST but only 16% under SST (Fig. 5*A*). It is interesting that many genes showed larger difference between both cultivars, *e.g.*, *GS2* gene in shoots and *NR1* and *HKT1;3* in roots (Fig. 5).

Ion balance: Diverse responses to AST were found in genes related to K^+/Na^+ metabolism. Under AST, the expression level of *OsSOS1* in the TC was much higher than that of SC (Fig. 6). However, the expression of *OsNHX2* in roots and the expression of *OsNHX1* were lower in the TC than that in the SC (Fig. 6).

Nitrogen metabolism: The expression of *OsFd-GOGAT* in shoots was much more abundant than other *OsGOGAT* gene family members. The expression of *OsGS2* was also more abundant than other *OsGS* gene family members

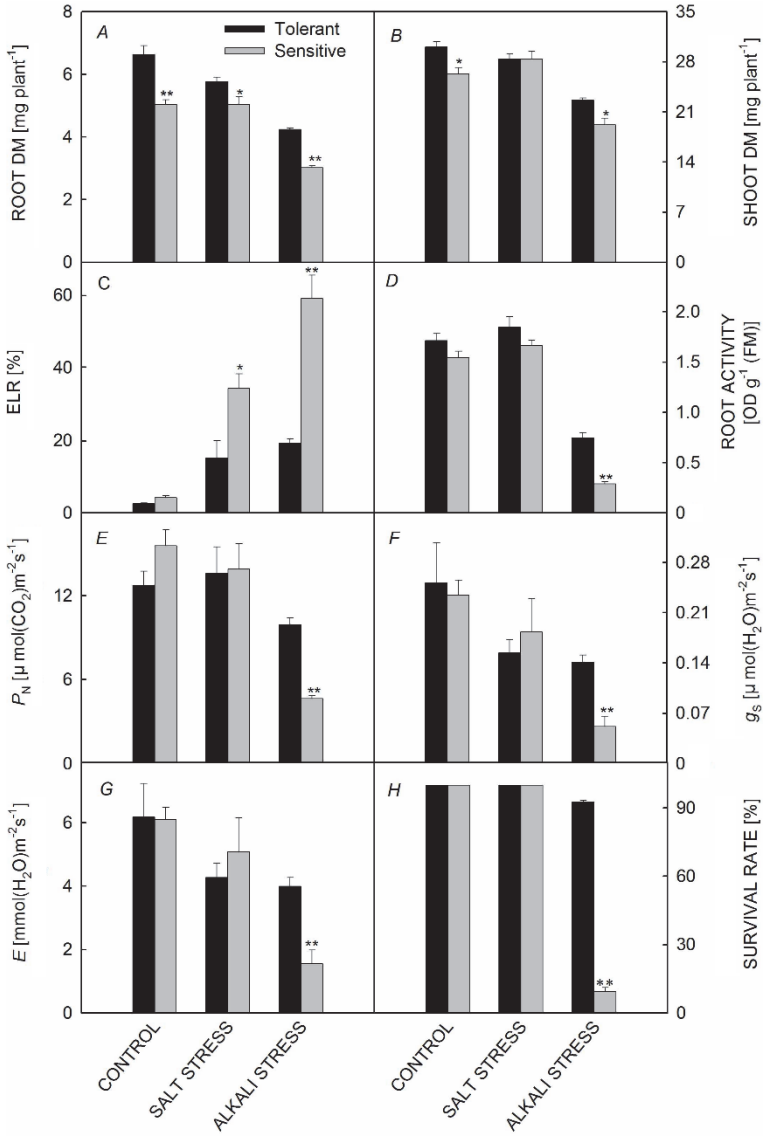

Fig. 2. Effects of salt and alkali stresses on the growth and photosynthesis in two rice cultivars differing in alkali tolerance. The values are means (± SE) of three replicates. When seedlings were subjected to 50 mM salt or alkali stresses for 48 h (*A*–*G*), the growth and photosynthesis were determined. When the seedlings were treated for 12 d, the survival rates were calculated (*H*). Statistical significance between cultivars under the same stress condition was determined by *t*-test, and marked as * (P<0.05) and ** (P<0.01). E − transpiration rate; ELR − electrolyte leakage rate; g_s − stomatal conductance; P_N − net photosynthetic rate.

(Figs. 7, 8). AST had only small effects on the expression of *OsGS2* and *OsFd-GOGAT* in shoots of the TC, but clearly reduced their expression in shoots of the SC (Figs. 7, 8). Similarly, AST upregulated the expression of *OsAS* in shoots of the TC, but slightly downregulated its expression in shoots of the SC. AST strongly stimulated the expression of *OsGS1;3* and *OsGDH2* in both roots and shoots of the SC, but had only a small effect on their expression in the TC (Fig. 8). AST decreased expression of *OsNR1* in both cultivars. The expression of *OsNR1* was similar in both cultivars under SST, but under AST the expression of *OsNR1* was much lower in the roots of the SC compared to the TC (Fig. 7).

Discussion

Ion balance: The inhibitory effects of AST on growth and photosynthesis have been reported to be stronger than those of SST (Yang *et al*. 2009). Our results revealed similar responses of both cultivars to SST, but the effects of AST greatly differed. Gene expression differences between the cultivars under SST were also lower than those under AST (Fig. 5). AST had a stronger injurious effect on germination, the membrane system, and photosynthesis of the SC compared to the TC (Figs. 1, 2, 2S). This revealed not only that salt and alkali stresses were distinct stresses but also that rice plants may have different adaptive strategies to these stresses. The metabolic regulation of roots might play a central role in rice alkali tolerance. Indeed, under AST, roots showed much greater gene expression differences between the cultivars than shoots (Fig. 5). Although AST clearly reduced the root system activities of both cultivars, the TC maintained a relatively high root activity (Fig. 2*D*). Correspondingly,

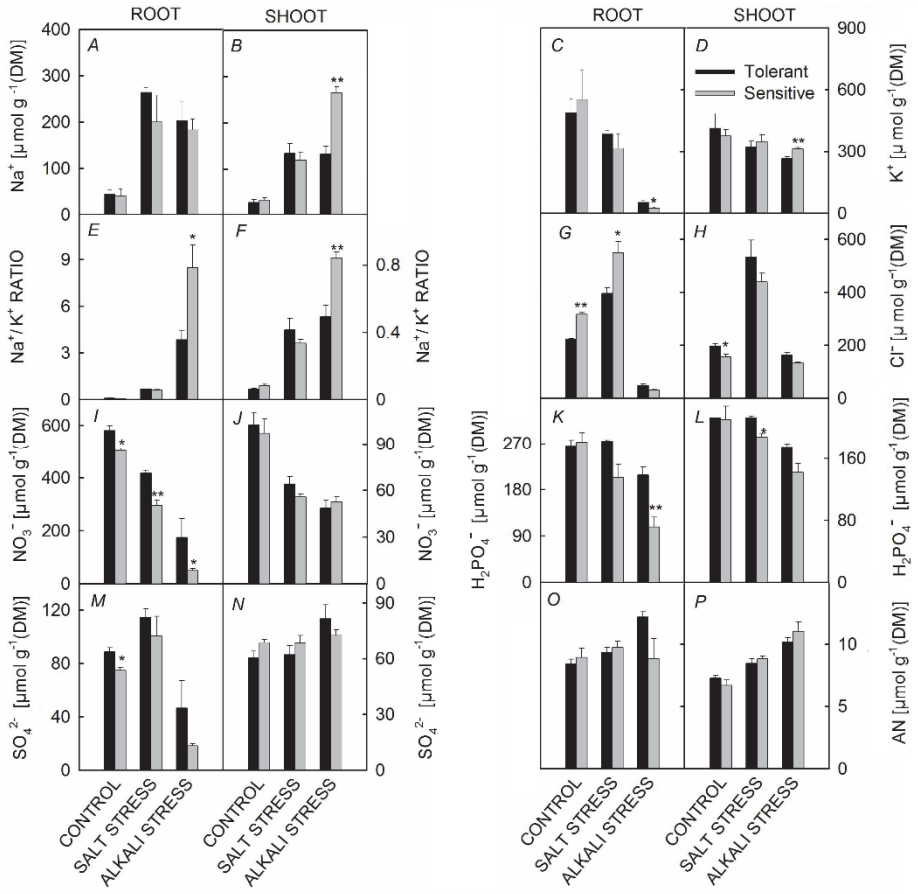

Fig. 3. Effects of salt and alkali stresses on the contents of inorganic ions and ammoniacal N in two rice cultivars differing in alkali tolerance. The values are means (± SE) of three replicates, each replicate consisted of a pool of ten plants. The seedlings were subjected to 50 mM salt or alkali stresses for 48 h. Statistical significance between cultivars under the same stress condition was determined by *t*-test, and marked as * (P<0.05) and ** (P<0.01). AN − ammoniacal nitrogen.

under AST, the Na^+ content and Na^+/K^+ ratio were much lower in shoots of the TC compared to the SC, and the K^+ content in roots higher in the TC than that of the SC (Fig. 3). 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). Under AST, TC rice maintained a lower Na^+ content and Na^+/K^+ ratio in shoots and a higher K^+ content in roots compared to the SC, which might be a central adaptive strategy by which TC resisted alkali stress.

Na^+ enters plant cells through the K^+ transporter pathways and through nonselective cation channels (Munns and Tester 2008). Under SST, the Na^+ metabolism of plants involves at least three processes: compartmentalization (at cellular and/or tissue levels), exclusion (from shoots into roots or from roots to rhizosphere), and transportation (in vasculature) of the ions. In *Arabidopsis*, the salt overly sensitive l (SOS1) protein functions in Na^+ exclusion from root epidermal cells into the rhizosphere. 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 (NHX) that mediates Na^+ compartmentalization into vacuoles (Zhu 2003). The rice SOS salt tolerance pathway has been identified and its functions was shown to be similar to those of the SOS pathway in *Arabidopsis* (Martinez-Atienza *et al*. 2007). It has been widely recognized that SOS1 plays an important role in the control of long-distance transport from roots to shoots and contribute to Na^+ exclusion from shoots to the roots (Munns and Tester 2008, Horie *et al*. 2009). Shi *et al*. (2002) clearly demonstrated that SOS1 is critical for controlling long-distance Na^+ transport from root to shoot, and SOS1 functions in retrieving Na^+ from the xylem stream under severe salt stress. The upregulation of *OsSOS1* can immediately decrease Na^+ content in shoots because their upregulation increased the frequency of Na^+ exclusion from shoots or roots.

In this study, we tested the expression of the *OsSOS1* and *OsNHX* gene family. Under AST, expression level of *OsSOS1* was higher in the TC compared to the SC. During the adaptation of rice to AST, *OsSOS1* might mediate Na^+ exclusion from shoots by unloading Na^+ from the ascending xylem sap, and *OsSOS1* mediated Na^+ exclusion from roots into the rhizosphere. This might be important in protecting shoots from high-Na^+ injury caused by AST (Fig. 3). Overexpression of *OsSOS1* could partly explain why the TC maintained the relatively low Na^+ content in the shoots, because their upregulation could increase the frequency of Na^+ exclusion from shoots or roots (Fig. 3*B*). Thus, *OsSOS1* may play important role in the regulation of Na^+/K^+ homeostasis of TC.

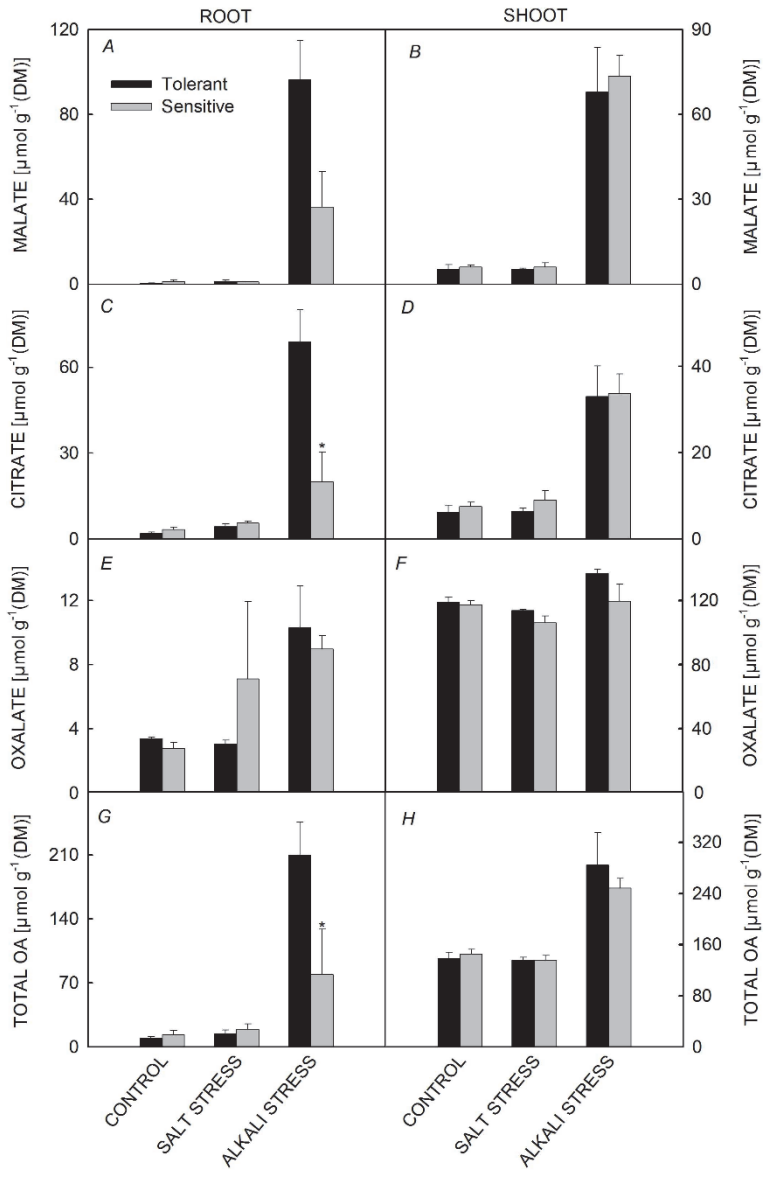

Fig. 4. Effects of salt and alkali stresses on the contents of organic acids (OA) in two rice cultivars differing in alkali tolerance. The values are means (± SE) of three replicates, each replicate consisted of a pool of ten plants. Statistical significance between cultivars under the same stress condition was determined by *t*-test, and marked as * (P<0.05) and ** (P<0.01). The seedlings were subjected to 50 mM salt or alkali stresses for 48 h.

Stable tissue pH is necessary for plants to maintain normal metabolism (Yang *et al.* 2007). As long as a living plant can adapt to the environment, its tissue pH should be stable, regardless of environmental pH (Touraine *et al*. 1988, Yang *et al*. 2007). The pH homeostasis of the internal environment is related to all free ions and also all solutes with charge, and is a result of ion balance that includes organic and inorganic ions (Yang *et al*. 2007). Ionic imbalance in plants is mainly caused by the influx of superfluous Na^+ (Yang *et al.* 2007). In the present study, we found that the effect of AST on the ion balance in roots was stronger than that in shoots. AST reduced the contents of $Cl^–$, $NO_3^–$, $SO_4^{2–}$, and $H_2PO_4^–$ in roots of both cultivars (Fig. 3), but the reductions in the TC were much smaller than in the SC. Under AST, the decreased inorganic anions in roots might cause a severe charge imbalance in rice roots (Fig. 4). We previously reported that OA accumulation resulted from a deficit of negative charge and OA metabolic regulation played an important role in maintaining ion balance and stable pH of rice (Wang *et al.* 2011). The data of the present study also supported this point – AST stimulated a massive accumulation of OAs in both rice cultivars (Fig. 4). However, in roots, a higher concentration of OA accumulated in the TC than in the SC. This revealed that TC might have a greater ability to maintain ion balance than the SC.

In summary, under AST, TC showed higher root system activity compared to the SC (Fig. 2*F*) and this may be the main reason for stronger alkali tolerance in TC. It is well known that mineral ion uptake and Na^+ exclusion in higher plants relies on the transmembrane H^+ gradient achieved by H^+-ATPase or other proteins (Zhu 2003). Under AST, the lack of external H^+ might break the transmembrane H^+ gradient of roots, possibly reducing the exclusion of Na^+ into the rhizosphere and limiting the uptake of mineral ions. This may be the basis of alkali injury. The TC showed

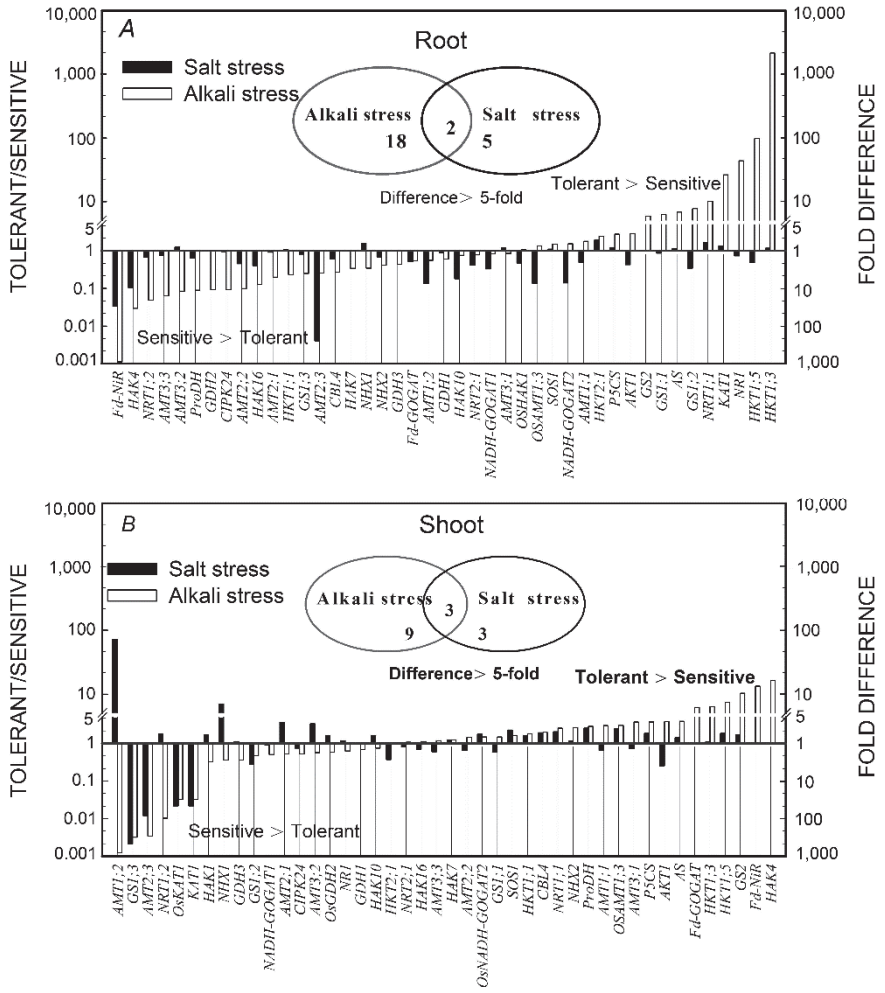

Fig. 5. Gene expression differences between cultivars (tolerant/sensitive). The relative expression levels between cultivars (ratio of alkali-tolerant cultivar to alkali-sensitive cultivar) were calculated according to means of each cultivar. The normalized ratio of tolerant cultivar to alkali cultivar is shown at the *left*; the corresponding fold expression changes are shown at the *right*. The numbers in large circles indicate the number of genes with fivefold difference between cultivars. The seedlings were subjected to 50 mM salt or alkali stresses for 48 h.

a relatively high root system activity, it was able to regulate the pH surrounding the roots, and maintained the exclusion of Na^+ and the accumulation of K^+, Cl^-, NO_3^-, SO_4^{2-}, and $H_2PO_4^-$ in its roots (Fig. 3).

Nitrogen nutrition: NH_4^+ from both NO_3^- reduction and soil are incorporated into organic molecules by glutamine synthetase (GS) and glutamate synthase (Fd-GOGAT and NADH-GOGAT) or the substituted glutamate dehydrogenase (GDH) pathway (Kant *et al.* 2010). Fd-GOGAT and GS2 are principally expressed in chloroplast and mediate assimilation of NH_4^+ from photorespiration or other metabolic processes (Kusano *et al.* 2011). GS1 is principally expressed in the cytosol, and GS2 in chloroplasts/ plastids (Kusano *et al.* 2011). In rice, *OsGS1;1*, *OsGS1;2*, and *OsGS1;3* encode GS1. *OsGS1;1* and *OsGS1;2* are especially abundant in the aerial parts and roots, respectively, whereas *OsGS1;3* is present only in the spikelets (Kusano *et al.* 2011). In the case of rice, the expression of *OsFd-GOGAT* in shoots was much greater than of other *OsGOGAT* gene family members, and the expression of *OsGS2* was also much greater than of other *OsGS* gene family members (Fig. 7). Under AST, in shoot, both *OsGS2* and *OsFd-GOGAT* expression levels were much lower in SC than in TC, but *OsGDH2* expression level was much higher in SC than in TC. Compared with control treatment, AST had only small effects on the expression of *OsGS2* and *OsFd-GOGAT* in the shoots of the TC, but exhibited clearly reduced expression in the shoots of the SC (Figs. 7, 8). Concurrently, AST strongly stimulated the expression of *OsGDH2* in shoots of the SC, but it did not affect their expression in shoots of the TC (Fig. 8). Under control and SST conditions, *OsGDH2* was only expressed in shoots of both cultivars, while under AST, the roots of SC showed extremely high expression level. This indicated that high organ-specific expression of rice *GDH2* gene was cultivar- and condition-dependent (Fig. 8). Similarly, the expression of *OsGS1;3* showed also cultivar- and condition-dependency (Fig. 7). The above data showed that AST may have changed the NH_4^+ assimilation pathway in shoots of the SC, weakened the frequency of NH_4^+ assimilation by the GS2/GOGAT

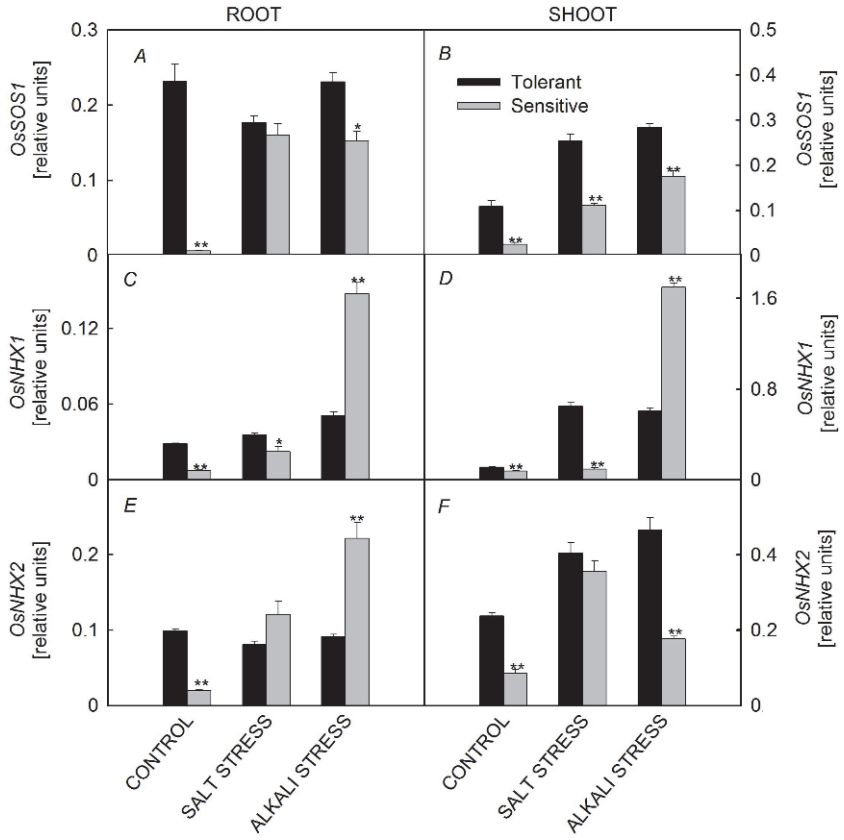

Fig. 6. Effects of salt and alkali stresses on the expression of *OsSOS* pathway genes in two rice cultivars differing in alkali tolerance. The values are means (± SE) of three replicates, and each replicate consisted of a pool of ten plants. Statistical significance between cultivars under the same stress condition was determined by *t*-test, and marked as * (P<0.05) and ** (P<0.01). The seedlings were subjected to 50 mM salt or alkali stresses for 48 h.

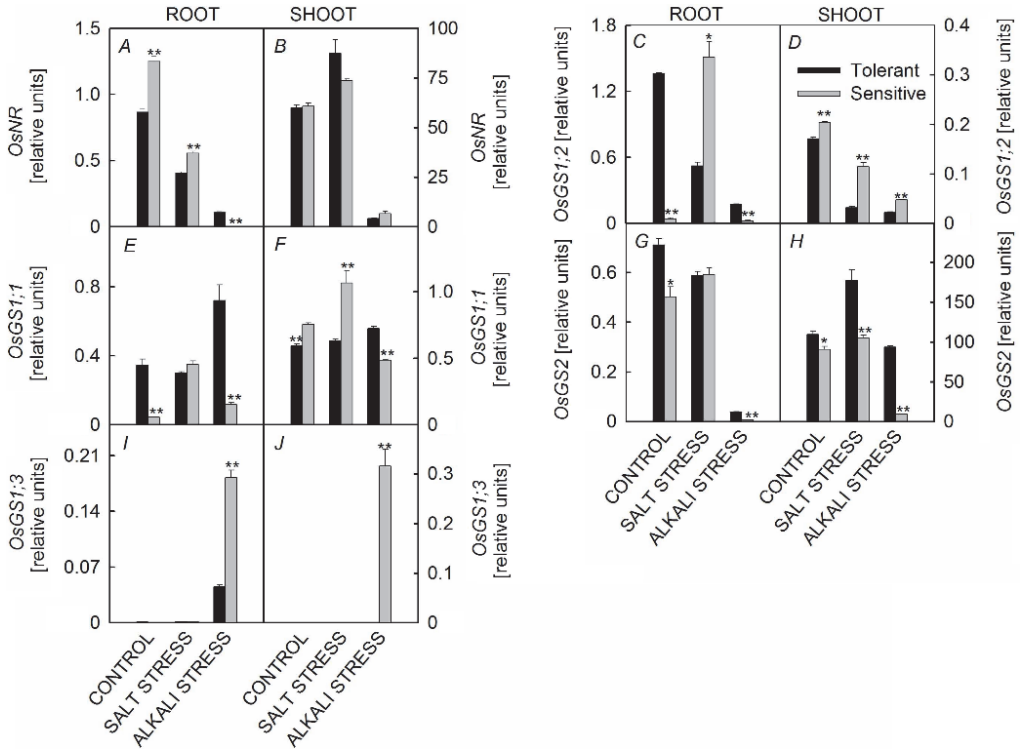

Fig. 7. Effects of salt and alkali stresses on the expression of *OsNR1* and *OsGS* family in two rice cultivars differing in alkali tolerance. The values are means (± SE) of three replicates, and each replicate consisted of a pool of ten plants. Statistical significance between cultivars under the same stress condition was determined by *t*-test, and marked as * (P<0.05) and ** (P<0.01). The seedlings were subjected to 50 mM salt or alkali stress for 48 h.

pathway, and elevated the frequency of NH_4^+ assimilation by the GDH pathway. Under AST, the downregulation of *OsGS2* and *OsFd-GOGAT* in shoots of the SC might occur due to the destruction of the photosynthetic system. Under AST, in shoots, SC might accumulate Na^+ to toxic concentrations (Fig. 3*B*), possibly harming chloroplasts,

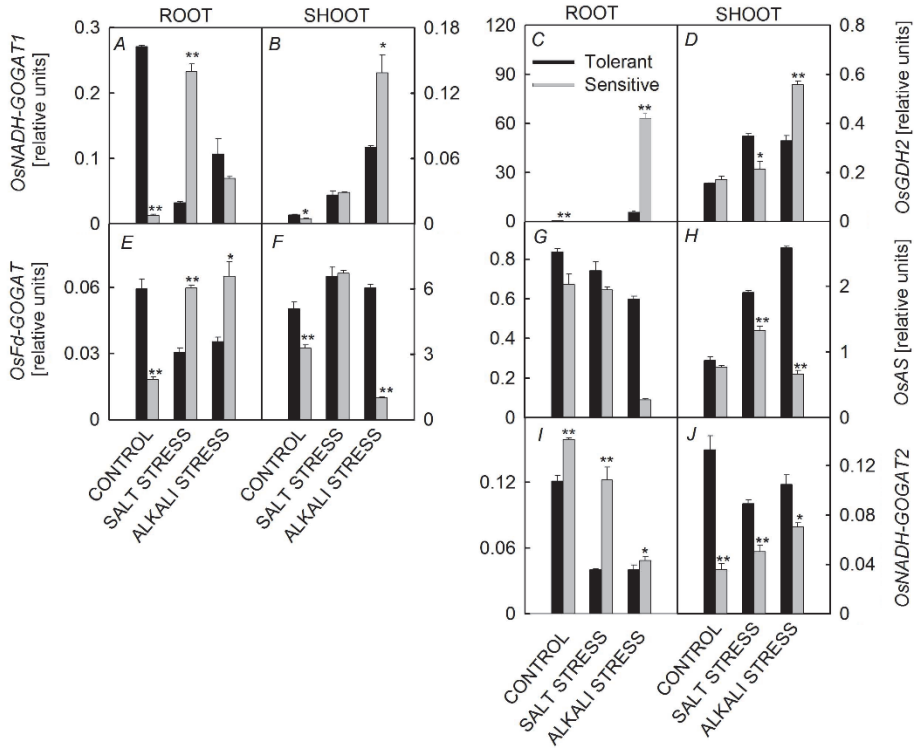

Fig. 8. Effects of salt and alkali stresses on the expression of *OsAS*, *OsGDH2* and *OsGOGAT* family in two rice cultivars differing in alkali tolerance. The values are means (± SE) of three replicates, and each replicate consisted of a pool of ten plants. Statistical significance between cultivars under the same stress condition was determined by *t*-test, and marked as * (P<0.05) and ** (P<0.01). The seedlings were subjected to 50 mM salt or alkali stress for 48 h.

disturbing metabolism, and immediately reducing P_N (Fig. 2*E*). The Na^+ excess in shoots might also influence photorespiration of the SC and reduce NH_4^+ production from photorespiration, which might immediately downregulate *OsGS2* and *OsFd-GOGAT* in shoots. Therefore, we propose that downregulation of *OsGS2* and *OsFd-GOGAT* might be a response of the SC to Na^+ excess in shoots caused by AST. Na^+ excess in shoots might even change the pathway of NH_4^+ assimilation in the SC, weaken the GOGAT/GS pathway and elevate the GDH pathway in roots and shoots (Figs. 7, 8). Under AST, TC was able to maintain normal N metabolism processes and relatively high expression levels of *OsGS2* and *OsFd-GOGAT* in the shoots. This might be important for synthesis of the N-containing compounds involved in alkali tolerance. Photosynthesis is closely related to nitrogen metabolism, and the negative effect of alkali stress on the nitrogen metabolism of SC may greatly influence its photosynthesis and growth. Our results suggested that the difference in alkali tolerance between both cultivars might result from expression regulation of key genes. However, more complex genetic and epigenetic mechanisms might be involved. For example, regulatory process mediated by microRNA and DNA methylation may play an important role in rice alkali tolerance, which should be investigated in future.

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