Effects of salt stress on ion balance and nitrogen metabolism in rice

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ABSTRACT

The aim of this study was to test the effects of salt stress on nitrogen metabolism and ion balance in rice plants. The contents of inorganic ions, total amino acids, and NO_3^- in the stressed seedlings were then measured. The expressions of some critical genes involved in nitrogen metabolism were also assayed to test their roles in the regulation of nitrogen metabolism during adaptation of rice to salt stress. The results showed that when seedlings were subjected to salt stress for 4 h, in roots, salt stress strongly stimulated the accumulations of Na^+ and Cl^-, and reduced K^+ content; however, in leaves, only at 5 days these changes were observed. This confirmed that the response of root to salt stress was more sensitive than that of leaf. When seedlings were subjected to salt stress for 4 h, salt stress strongly stimulated the expression of *OsGS1;1*, *OsNADH-GOGAT*, *OsAS*, *OsGS1;3*, *OsGDH1*, *OsGDH2*, *OsGDH3* in both leaves and roots of rice, after this time point their expression decreased. Namely, at 5 days most of genes involved in NH_4^+ assimilation were downregulated by salt stress, which might be the response to NO_3^- change. Salt stress did not reduce NO_3^- contents in both roots and leaves at 4 h, whereas at 5 days salt stress mightily decreased the NO_3^- contents. The deficiencies of NO_3^- in both roots and leaves can cause a large downregulation of *OsNR1* and the subsequent reduction of NH_4^+ production. This event might immediately induce the downregulations of the genes involved in NH_4^+ assimilation.

Keywords: ion accumulation; sodium; ammonia assimilation; gene expression; *Oryza sativa* L.

Excessive salinity is the most important environmental stress factor that greatly affects the plant growth and nutrition of plant in arid and semiarid regions. Salt tolerance of plants is a complex phenomenon and involves morphological and developmental changes as well as physiological and biochemical processes (Munns 2002, Ashraf and Harris 2004). Plant survival and growth in saline environments is a result of adaptive processes such as ion transport and compartmentation, osmotic solute synthesis and accumulation that lead to osmotic adjustment and protein turnover for cellular repair (Munns and Termaat 1986). Further, on exposure to osmotic stress as a result of high salinity, plants accumulate a range of metabolically benign solutes, collectively known as compatible solutes or osmolytes (Läuchli and Lüttge 2002). Many of these compatible solutes are N-containing compounds, such as amino acids and amides or betaines, hence the nitrogen metabolism is of central importance under stressful conditions (Läuchli and Lüttge 2002). It is significant to research the effects of salt stress on nitrogen metabolism in plants. It was well described that salt stress strongly affects nitrogen metabolism of plants (Parida and Das 2005, Munns and Tester 2008). In addition, high salt environments can break the ion homeostasis of plant cells, destroy the ionic balance, and affect the distributions of ion at cells, tissues, and whole plant levels (Niu et al. 1995). It is necessary to osmoregulate and re-establish the ion homeostasis in cells for plant

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living under salt-stress (Surabhi et al. 2008). Thus, the understanding of nitrogen metabolism response and ion balance regulation may be crucial for salt tolerance research. However, the underlying mechanism of nitrogen metabolism regulation in plants under salt stress remains obscure.

Rice (*Oryza sativa* L.) is one of the most important cereal crops in tropical and temperate regions of the world. In many agricultural areas, especially Asia, soil salinity is an important factor that limits rice productivity. It was reported that salt stress strongly affects the germination (Deng et al. 2011), the growth (Wang et al. 2011), solute accumulation (Hoai et al. 2003, Nemati et al. 2011), and gene expression (Pandit et al. 2011) of rice plants. The aim of this study was to test the effects of salt stress on nitrogen metabolism and ion balance in rice plants. In this study, the contents of inorganic ions, total amino acids, and NO_3^- in the stressed seedlings were measured. The expressions of some critical genes involved in nitrogen metabolism were also assayed to test their roles in the regulation of nitrogen metabolism during adaptation of rice to salt stress.

MATERIAL AND METHODS

Plant growth conditions. Rice (*O. sativa* L. cv. Nipponbare) seedlings were used in the experiments. Seeds were germinated and grown in petri dishes for 7 days in a growth cabinet (30°C during the day and 25°C during the night, 16/8 h photoperiod at 250 μmol/m^2/s). Seedlings were then transferred to buckets containing 2000 mL of aerated sterile nutrient solution for solution culture. The nutrient solution was replaced daily. The buckets were placed in a growth chamber that was maintained at 27.0 ± 1.5°C during the day and 21.0 ± 1.5°C during the night, under a 16/8 h photoperiod at 250 μmol/m^2/s. The nutrient solution used in this work corresponded to the components described by the International Rice Research Institute (Yoshida et al. 1976), and contained 1.44 mmol/L NH_4NO_3, 0.32 mmol/L NaH_2PO_4, 0.6 mmol/L K_2SO_4, 1.0 mmol/L $CaCl_2$, 1.6 mmol/L $MgSO_4$, 0.072 mmol/L Fe-EDTA, 0.2 mmol/L Na_2SiO_3, 9.1 μmol/L $MnCl_2$, 0.154 μmol/L $ZnSO_4$, 0.156 μmol/L $CuSO_4$, 18.5 μmol/L H_3BO_3 and 0.526 μmol/L H_2MoO_4 at pH 5.4.

Stress treatment and ion content determinations. After 12 days of growth in hydroponic medium, rice plants were subjected to salt stress (100 mmol/L NaCl) by being transferred to another bucket containing 3500 mL of the treatment solution amended with the above nutrients and 100 mmol/L NaCl (pH 5.45). A bucket including 20 seedlings represented one replicate, and there were three replicates per treatment. Treatment solutions were replaced daily. The nutrient solution without stress salts was used as a control. A bucket including 20 seedlings represented one replicate, and there were three replicates per treatment. 12 buckets of seedlings were randomly divided into 4 sets, three buckets per set. Each bucket was considered as one replicate with three replicates per set. Two sets were used as control (one set per time point); another two sets were treated with salt stress (one set per time point). Treatment solutions were replaced daily. The nutrient solution without stress salts was used as a control. The 20 seedlings were harvested after treatment for 4 h and 5 days, respectively. The roots and leaves of 10 seedlings in each bucket were separated and mixed, then 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 leaves were separated and freeze-dried. Dry samples of plant material were treated with 10 mL deionised water at 100°C for 1 h, and the extract was then used to determine the contents of free inorganic ions. The Na^+ and K^+ contents were assayed using an atomic absorption spectrophotometer (TAS-990, Purkinje General, Beijing, China). The contents of NO_3^- and Cl^- were determined by ion chromatography (DX-300, Sunnyvale, USA). Total free amino acids content was measured using the ninhydrin method (Zhang 2002).

Quantitative real time PCR. We extracted the total RNA from the leaves and roots of seedlings grown under stress or non-stress conditions using TRIzol reagent (Invitrogen, Carlsbad, USA). 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 1. PCR amplification was conducted with an initial 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. Amplification of the rice *UBQ5* (GenBank Accession AK061988) mRNA was used as an internal quantitative control (Quinet et al. 2010, Zhang et al. 2010). We optimized the PCR reaction system, after which

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

Gene name	GeneBank Accession No.	Forward primer	Reverse primer
OsNR1	AK121810	CCTACTACTAAATTATACGCACCG	CAGGAAGGAATCAACCGCTA
OsGS1;1	AB037595	CCGTCTGTCGGCATTTCTG	GGGATGGGCTTGGGGTC
OsGS1;2	AB180688	GCCCACAGGGACCATACTACT	CGTTGATGCCACTGATGTTGAT
OsGS1;3	AB180689	CCGATTCCGACGAACAACC	GCTCCCGCCGCACAGT
OsGS2	X14246	AGTATGCGTGAAGATGGAGGAT	GCCCCACCCGAATAGAGC
OsNADH-GOGAT	AB008845	TTGCGGTTACAAGACACTCTACTG	GCTCCCGTCCCTCCATCA
OsFd-GOGAT	AJ132280	TGGTCTCCGCCCAGCAC	CAGTTTGTAGGTCAACCGTTATCAT
OsGDH1	AK071839	TTCTTCCTTCCCACTACCAAAC	TCCCAAGCAGCGAGCC
OsGDH2	AB189166	GGCCATTAACAACACTCATA	ACGCCGATCTATCTTGAAT
OsGDH3	AB035927	TATGCTACTGAGGCTTTATTGACTG	GCCACCTTTCTGATGGATGA
OsAS	D83378	GCCCTATTTACCTAAGCACATTC	AGGCTGCGTCCCATTCA
OsNRT1;1	AF140606	GGGCAGAGTTCAGCAATCG	GGAAGGACGCCGCAGGT
OsNRT1;2	AY305030	GCGGCGAGTCCCTGAG	CGACGGCGTAGATGAATGA
OsNRT2;1	AK072215	ACGGCACAAAGTACAAGACG	CCACTGCGGGAAGTAGATG
OsAMT1;1	AK073718	GCCTCCAACAGCAACAACC	CCAAACAGAAACTGGCAATCA
OsAMT1;2	NM_001053990	CACGGTGGCGATGAAAGG	TTGGAGATGGTGGTGAAGGAC
OsAMT1;3	AK107204	TCAAGCAGGTCCCACAGG	TGAGGAAGGCGGAGTAGATG
OsAMT2;1	AB051864	GATGAATCACGCCGAAACAC	GCACGGACGAATCGCTACTT
OsAMT2;2	AB083582	CGACCAAGGACAGGGAGA	CACGGCGAGCGAGGAG
OsAMT2;3	AK102106	GTTCACCCCGCTCTGGC	CCGCTCCCTGTCGCTCTT
OsAMT3;1	AK120352	CCAACTGCTGAAAAGTGAAAACG	TGCTTCGCATACGGCTGAC
OsAMT3;2	AK069311	CCCAGTTCGGCAAGCAG	TGGCGAGGCAGATGAGG
OsAMT3;3	AK108711	GAGATTCCCGCCCAACAA	TCCACCCAAGCCACAGC
OsUBQ5	AK061988	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT

NR – nitrate reductase; GOGAT – glutamate synthase; GS – glutamine synthetase; GDH – glutamate dehydrogenase; AS – asparagine synthetase; NRT – nitrate transporter; AMT – ammonium transporter

the amplification efficiencies of each target gene and reference gene were approximately equal. The relative expression of the target genes was measured using the ΔCT method (Livak and Schmittgen 2001). Real time PCR analyses were conducted at least three times for each sample.

Statistical data 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 (S.E.). The treatment mean values at the same time point were compared by *t*-test.

RESULTS AND DISCUSSION

Na^+ is the main poisonous ion in salinized soil. Low Na^+ and high K^+ in the cytoplasm are essential to maintain a number of enzymatic processes (Munns and Tester 2008). Na^+ enters plant cells principally through K^+ pathways (Munns and Tester 2008). Plants under salt stress usually absorb Na^+ and simultaneously inhibit K^+ absorption. We observed that salt stress increased Na^+/K^+ ratio and the contents of Na^+ and Cl^- in rice, reduced the contents of K^+ and NO_3^- (Table 2). Another interesting phenomenon was that the response of Na^+, Cl^- and K^+ in roots were more rapid than those in leaves during adaptation of rice to salt stress (Table 2). When seedlings were subjected to salt stress for 4 h, in roots, salt stress strongly stimulated the accumulations of Na^+ and Cl^-, and reduced K^+ content; however, in leaves, only when seedlings were subjected to salt stress for 5 days, these changes were observed. This confirmed that the response of root to salt stress was more sensitive than that of leaf. Plant root was the primary

Table 2. Effects of salt stress on the contents of inorganic ions in rice seedlings. The seedlings were subjected to 100 mmol/L NaCl stress for 4 h and 5 days

	Shoot				Root			
	4 h		5 days		4 h		5 days	
	control	salt stress	control	salt stress	control	salt stress	control	salt stress
Na^+ (g/kg DW)	2.50 ± 0.06	3.08 ± 0.01	2.28 ± 0.007	15.9 ± 0.16**	2.62 ± 0.02	10.3 ± 0.27**	2.64 ± 0.01	8.90 ± 0.15**
K^+ (g/kg DW)	32.5 ± 0.55	32.4 ± 0.31	31.1 ± 0.10	24.1 ± 0.14**	19.3 ± 0.17	10.82 ± 0.26**	19.2 ± 0.39	9.33 ± 0.15**
Na^+/K^+ ratio	0.13 ± 0.002	0.16 ± 0.001	0.12 ± 0.0007	1.12 ± 0.01**	0.23 ± 0.001	1.61 ± 0.005**	0.23 ± 0.003	1.6 ± 0.003**
Cl^- (g/kg DW)	3.59 ± 0.76	3.03 ± 0.40	2.48 ± 0.50	11.02 ± 5.5*	1.78 ± 0.21	3.78 ± 0.39*	1.88 ± 0.13	4.18 ± 0.23**
NO_3^- (g/kg DW)	35.1 ± 12.1	28.0 ± 1.4	33.6 ± 1.9	13.3 ± 0.30**	40.9 ± 9.3	36.1 ± 7.1	39.1 ± 0.56	18.0 ± 2.2**
Total amino acids (mg/kg DW)	585 ± 42	627 ± 20	308 ± 19	421 ± 60**	502 ± 90	392 ± 27	491 ± 43	411 ± 49**

Statistically significant differences among salt stress and control treatments at same time point were determined by *t*-test, and marked as $^*P < 0.05$ and $^{**}P < 0.01$. The values are means (± SE) of three replicates

organ perceiving and responding to salt stress using signaling systems involved in stress tolerance.

It was well-known that NO_3^- is reduced to nitrite by nitrate reductase (NR) and then to NH_4^+ by nitrite reductase (NiR). NH_4^+ from both nitrate reduction and soil are incorporated into organic molecules by glutamine synthetase (GS) and glutamate synthase (Fd-GOGAT and NADH-GOGAT) or alternative glutamate dehydrogenase (GDH) pathway (reviewed by Shi et al. 2009). Glutamine synthetase (GS) primarily exists as two isozymes with different subcellular localisations: GS1 in the cytosol and GS2 in chloroplasts/plastids (Kusano et al. 2011). Another key protein, asparagine synthetase (AS), functions in transamination from glutamine to aspartic acid. Our results showed that *OsGS1;2*, *OsGS2*, *OsNR1*, and *OsFd-GOGAT*, all were slightly downregulated by salt stress at 4 h, but at 5 days their expression were strongly downregulated (Table 3). When seedlings were subjected to salt

Table 3. Effects of salt stress on the expression of the genes involved in NH_4^+ assimilation in rice seedlings. The seedlings were subjected to 100 mmol/L NaCl stress for 4 h and 5 days

	Shoot				Root			
	4 h		5 days		4 h		5 days	
	control	salt stress	control	salt stress	control	salt stress	control	salt stress
OsNR1	1.20 ± 0.19	0.78 ± 0.06	18.8 ± 0.64	5.23 ± 0.24**	0.02 ± 0.002	0.018 ± 0.0003	1.71 ± 0.05	0.086 ± 0.008**
OsGS1;1	0.31 ± 0.038	0.92 ± 0.03**	1.51 ± 0.053	1.10 ± 0.063**	0.15 ± 0.003	0.32 ± 0.019*	0.33 ± 0.007	0.22 ± 0.007**
OsGS1;2	0.039 ± 0.001	0.028 ± 0.001	0.24 ± 0.012	0.12 ± 0.016**	0.17 ± 0.001	0.066 ± 0.003*	1.67 ± 0.059	1.039 ± 0.009**
OsGS1;3(10^{-5})	9.9 ± 2.5	254 ± 19**	71.4 ± 20.3	922 ± 52**	78.5 ± 6.3	1165 ± 76**	53.8 ± 3.87	317 ± 4.2**
OsGS2	15.3 ± 0.33	13.0 ± 0.39*	59.8 ± 1.39	7.33 ± 0.16**	0.04 ± 0.0007	0.027 ± 0.001**	0.43 ± 0.03	0.14 ± 0.008**
OsNADH-GOGAT(10^{-2})	12.3 ± 1.5	254 ± 10**	44.5 ± 2.5	25 ± 6.8	10.6 ± 0.2	23.5 ± 0.4**	52.5 ± 2.1	35.6 ± 4.1*
OsFd-GOGAT	5.92 ± 0.21	4.18 ± 0.17**	31.6 ± 1.2	6.74 ± 0.09**	0.13 ± 0.005	0.16 ± 0.009	0.29 ± 0.03	0.16 ± 0.003*
OsGDH1	11.4 ± 0.5	15.8 ± 0.5**	27.3 ± 1.3	11.1 ± 0.7**	55.1 ± 0.5	65.6 ± 0.9**	141 ± 4.7	79.7 ± 2.9**
OsGDH2	12.6 ± 0.4	78.5 ± 2.3**	38.1 ± 5.4	59.0 ± 1.3	234 ± 3.7	833 ± 18**	66.1 ± 4.2	36.4 ± 2.5**
OsGDH3	3.2 ± 0.1	10.7 ± 0.9*	10.6 ± 0.9	9.0 ± 0.8	21.9 ± 0.2	88.9 ± 4.6**	4.9 ± 0.5	2.8 ± 0.4*
OsAS	74.8 ± 9.5	128 ± 3.5**	161 ± 1.0	70.8 ± 4.8**	27.1 ± 1.6	36.9 ± 2.3*	26.7 ± 0.5	19.0 ± 0.6**

Statistically significant differences among salt stress and control treatments at same time point were determined by *t*-test, and marked as $^*P < 0.05$ and $^{**}P < 0.01$. The values are means (± SE) of three replicates. The values of gene expression were the relative values to reference gene (*OsUBQ5*)

Table 4. Effects of salt stress on the expression of *OsAMT* and *OsNRT* gene families in rice seedlings. The seedlings were subjected to 100 mmol/L NaCl stress for 4 h and 5 days

	Shoot				Root			
	4 h		5 days		4 h		5 days	
	control	salt stress	control	salt stress	control	salt stress	control	salt stress
OsNRT1;1(10^{-3})	35.2 ± 2.3	107 ± 5**	90.0 ± 10.1	97.9 ± 5	46.1 ± 0.03	47.9 ± 3.8	125.3 ± 3	67.9 ± 3.7**
OsNRT1;2(10^{-4})	18.1 ± 4.3	3.2 ± 0.4	1.6 ± 0.8	21.5 ± 1.1**	1176 ± 244	885 ± 45	410 ± 31	220 ± 14**
OsNRT2;1(10^{-4})	23.3 ± 9.2	2.5 ± 0.5	48.3 ± 7.6	56.2 ± 1.3	25.4 ± 1.0	17.6 ± 2.2*	20.6 ± 1.0	25.2 ± 2.2
OsAMT1;1	4.36 ± 0.6	0.87 ± 0.05**	4.81 ± 0.08	2.96 ± 0.05**	0.11 ± 0.001	0.31 ± 0.01**	0.30 ± 0.05	0.38 ± 0.06
OsAMT1;2(10^{-5})	10.9 ± 1.4	8.46 ± 3.2	19.7 ± 6.3	13.4 ± 3.3	12.2 ± 8.8	16.7 ± 1.7	241 ± 11	154 ± 2.2**
OsAMT1;3(10^{-3})	6.8 ± 2.2	3.9 ± 0.3	10.9 ± 0.8	2.2 ± 0.2**	21.9 ± 1.0	7.7 ± 0.4**	157 ± 10.2	77.2 ± 2*
OsAMT2;1(10^{-4})	83.4 ± 5.5	96.4 ± 15.4	120.7 ± 8.3	136.0 ± 4.1	3.1 ± 0.4	22.8 ± 1.3**	10.8 ± 1.8	9.5 ± 0.4
OsAMT2;2(10^{-4})	82.7 ± 33.1	31.9 ± 7.0	74.9 ± 17.9	48.3 ± 4.3	7.81 ± 2.5	18.5 ± 8.6	9.60 ± 1.48	24.1 ± 14
OsAMT2;3(10^{-5})	2.27 ± 0.8	6.22 ± 3.0	16.5 ± 8.4	58.7 ± 27.1	2.67 ± 0.3	0.60 ± 0.3**	1.12 ± 0.3	5.05 ± 2.4
OsAMT3;1(10^{-3})	18.0 ± 1.4	24.9 ± 1.0*	44.0 ± 4.4	21.6 ± 0.8*	9.12 ± 0.6	15.1 ± 0.1**	6.80 ± 0.3	7.0 ± 0.5
OsAMT3;2(10^{-3})	2.72 ± 0.3	14.6 ± 1.7**	8.48 ± 1.2	28.9 ± 0.4**	8.0 ± 0.3	13.0 ± 0.4**	5.5 ± 0.2	6.5 ± 0.9
OsAMT3;3(10^{-5})	117.6 ± 8.5	372.9 ± 40.2*	417.6 ± 59	316.8 ± 31.1	6.6 ± 6.1	5.42 ± 1.7	20.6 ± 1.1	6.11 ± 0.3**

Statistically significant differences among salt stress and control treatments at same time point were determined by *t*-test, and marked as $^{*}P < 0.05$ and $^{**}P < 0.01$. The values are means (± SE) of three replicates. The values of gene expression were the relative values to reference gene (*OsUBQ5*)

stress for 4 h, salt stress strongly stimulated the expression of *OsGS1;1*, *OsNADH-GOGAT*, *OsAS*, *OsGS1;3*, *OsGDH1*, *OsGDH2*, *OsGDH3* in both leaves and roots of rice, after this time point their expression decreased (Table 3). In summary, when seedlings were subjected to salt stress for 5 days, most of genes involved in NH_4^+ assimilation were downregulated by salt stress. The downregulations of these genes might be the response to NO_3^- change. Salt stress did not reduce NO_3^- contents in both roots and leaves at 4 h, whereas at 5 days salt stress mightily decreased its contents (Table 2). The deficiencies of NO_3^- in both roots and leaves can cause two baneful consequences, the large downregulation of *OsNR1* expression and the subsequent reduction of NH_4^+ production in both leaves and roots. This event might immediately induce the downregulations of the genes involved in NH_4^+ assimilation (Table 3). When rice seedlings were subjected to salt stress for 4 h, fleetly upregulated expression of *OsGS1;1*, *OsNADH-GOGAT*, *OsAS*, *OsGS1;3*, *OsGDH1*, *OsGDH2*, and *OsGDH3* in both leaves and roots of rice might show a quick adaptative response to salt stress, and these genes might be helpful for the synthesis of the N-containing compounds related to salt response. The responses of *OsAMT* and *OsNRT* gene families to salt stress were diversiform. There might be complex regulatory mechanisms, which should be investigated in future studies (Table 4). Our results indicated that the interference between salinity and nitrogen nutrition might be a very complex network, even may affect most metabolism processes in plant. The understanding of the nitrogen metabolism response to salt stress may be crucial for salt tolerance research, and will be an important research direction for salt stress physiology in future.

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