Gene Structure and Expression of the High-affinity Nitrate Transport System in Rice Roots

Chao Cai^{1,2,3}, Jun-Yi Wang^{1,3}, Yong-Guan Zhu², Qi-Rong Shen⁴, Bin Li¹, Yi-Ping Tong^{1*} and Zhen-Sheng Li^{1*}

(¹State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, the Chinese Academy of Sciences, Beijing 100101, China;

²Research Center for Eco-environmental Sciences, the Chinese Academy of Sciences, Beijing 100085, China;

³Graduated University, the Chinese Academy of Sciences, Beijing 100049, China;

⁴College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing 210095, China)

Abstract

Rice has a preference for uptake of ammonium over nitrate and can use ammonium-N efficiently. Consequently, transporters mediating ammonium uptake have been extensively studied, but nitrate transporters have been largely ignored. Recently, some reports have shown that rice also has high capacity to acquire nitrate from growth medium, so understanding the nitrate transport system in rice roots is very important for improving N use efficiency in rice. The present study identified four putative *NRT2* and two putative *NAR2* genes that encode components of the high-affinity nitrate transport system (HATS) in the rice (*Oryza sativa* L. subsp. *japonica* cv. Nipponbare) genome. *OsNRT2.1* and *OsNRT2.2* share an identical coding region sequence, and their deduced proteins are closely related to those from mono-cotyledonous plants. The two *NAR2* proteins are closely related to those from mono-cotyledonous plants as well. However, *OsNRT2.3* and *OsNRT2.4* are more closely related to *Arabidopsis* *NRT2* proteins. Relative quantitative reverse transcription-polymerase chain reaction analysis showed that all of the six genes were rapidly upregulated and then downregulated in the roots of N-starved rice plants after they were re-supplied with 0.2 mM nitrate, but the response to nitrate differed among gene members. The results from phylogenetic tree, gene structure and expression analysis implied the divergent roles for the individual members of the rice *NRT2* and *NAR2* families. High-affinity nitrate influx rates associated with nitrate induction in rice roots were investigated and were found to be regulated by external pH. Compared with the nitrate influx rates at pH 6.5, alkaline pH (pH 8.0) inhibited nitrate influx, and acidic pH (pH 5.0) enhanced the nitrate influx in 1 h nitrate induced roots, but did not significantly affect that in 4 to 8 h nitrate induced roots.

Key words: high-affinity nitrate transport system; nitrate influx; *NRT2*; *NAR2*; *Oryza sativa*.

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Rice is the staple food for more than half of the world's population. Nitrogen (N) deficiency is the most common constraint to lowland rice production, and thus N fertilization is a key input in increasing rice production (Vlek and Byrnes 1986). However, N

use efficiency (NUE) in lowland rice is often low. Upland crops frequently use 40–60% of the applied N, while flooded rice plants typically use only 20–40% (Vlek and Byrnes 1986). The same is true for NUE in the lowland rice in China; loss of as much as 70% of the applied N fertilizers have been reported in high yielding rice fields in China (Zhu 2000). Therefore there is an urgent need to increase NUE for decreasing the costs of rice production, and the loss of N, which can cause eutrophication of surface water bodies and contribute to the emission of greenhouse gases (Tilman 1999). Understanding the mechanisms of how the crop takes up N at a molecular level will help to improve NUE in rice.

Ammonium is the main form of plant-available N in flooded rice soils where the bulk of the soil is anaerobic (Sasakawa and Yamamoto 1978; Yu 1985). Rice has been reported by many

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*Authors for correspondence.

Tel(Fax): +86 (0)10 6488 9381;

E-mail: <yptong@genetics.ac.cn> and <zsli@genetics.ac.cn>.

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to have a preference for ammonium uptake (Kronzucker et al. 1998, references therein), and performs well when ammonium is provided as the sole N source (Wang et al. 1993; Colmer and Bloom 1998; Kronzucker et al. 1998). Consequently, studies on molecular mechanisms of N uptake have mainly focused on ammonium transporters in rice (Kumar et al. 2003; Sonoda et al. 2003a, 2003b; Loque and von Wiren 2004). Many reports show that rice can also take up nitrate (Sasakawa and Yamamoto 1978; Kronzucker et al. 1999; Kronzucker et al. 2000), and nitrate can enhance ammonium uptake and metabolism of rice (Kronzucker et al. 1999). Additionally, a mixed N supply can increase biomass production and grain yield of rice (Ta and Ohira 1981; Ta et al. 1981). However, the molecular mechanism of how rice takes up nitrate is not well understood. Presently, to our knowledge, only one nitrate transporter, *OsNRT1* encoding a low-affinity nitrate transporter, has been functionally characterized (Lin et al. 2000). The existence and expression characteristics of high-affinity nitrate transporter genes in rice are still not clear.

Physiological and molecular studies revealed that there are two kinetically distinct nitrate uptake systems in plant roots: the low-affinity transport system (LATS) encoded by the *NRT1* family, and the high-affinity transport system (HATS) encoded by the *NRT2* family (Crawford and Glass 1998; Daniel-Vedele et al. 1998; Forde 2000; Galvan and Fernandez 2001; Glass et al. 2001; Williams and Miller 2001). The LATS generally has a larger capacity than does the HATS, and mediates nitrate uptake under high-nitrate environments (Siddiqi et al. 1990; Glass and Siddiqi 1995; Crawford and Glass 1998), while HATS appears to play a major role in nitrate uptake when nitrate concentrations in the soil are very low (<250 μM) (for a review, see Crawford and Glass 1998). The first *NRT2* gene *nrtA* (formerly known as *crnA*) was isolated from *Aspergillus nidulans* (Johnstone et al. 1990; Unkles et al. 1991), and was identified to be a functional nitrate/nitrite transporter when expressed in *Xenopus* oocytes (Zhou et al. 2000a). After the *nrtA* was isolated, a number of homologous genes were cloned from other eukaryotes, including *Chlamydomonas reinhardtii* (Quesada et al. 1994), barley (Trueman et al. 1996; Vidmar et al. 2000a), wheat (Zhao et al. 2004), tobacco (Quesada et al. 1997), soybean (Amarasinghe et al. 1998), and *Arabidopsis* (Zhuo et al. 1999). In photosynthetic organisms (e.g. *Chlamydomonas* and barley), some *NRT2*-type transporters required a second protein, *NAR2*, to co-produce a functional HATS (Quesada et al. 1994; Zhou et al. 2000b; Tong et al. 2005). In *Arabidopsis*, disruption in a *NAR2*-like gene *AtNRT3.1* caused a decrease in high-affinity nitrate influx (Okamoto et al. 2006). These cloned *NRT2* and *NAR2* genes helped us to identify the related family members in the rice genome. In the present study, four *NRT2* and two *NAR2* genes were isolated from rice. The phylogeny of these genes and their expression patterns in response to nitrate supply were analyzed. The effects of external pH on high-affinity nitrate influx rates were also investigated

because pH may be an important factor in regulating the activity of the two components high affinity nitrate transport system (Tong et al. 2005).

Results

Sequence analysis of rice *NRT2* and *NAR2* genes

The protein sequences of barley *NRT2.1* (accession no. AAC49531) and *NAR2.3* (accession no. AAP31852) were used to identify related *NRT2* and *NAR2* genes in The Institute for Genomic Research (TIGR) rice genome database. Four putative *NRT2* genes and two putative *NAR2* genes were identified (Table 1). The six genes were distributed on three chromosomes, *OsNRT2.3* and *OsNRT2.4* on chromosome 1, *OsNRT2.1*, *OsNRT2.2* and *OsNAR2.1* on chromosome 2, and *OsNAR2.2* on chromosome 4. Alignment of the cDNA sequences of the four *NRT2* genes showed that *OsNRT2.1* and *OsNRT2.2* have the same nucleotide sequences in their coding-regions (Table 1). *OsNRT2.1* and *OsNRT2.2* share 66% and 56% of nucleotide sequence identity with *OsNRT2.3* and *OsNRT2.4*, respectively; the nucleotide sequence identity between *OsNRT2.3* and *OsNRT2.4* is 57% (Table 2). Further analysis of the genomic sequences of *OsNRT2.1* and *OsNRT2.2* revealed that the two genes are closely located in a "tail-to-tail" arrangement on chromosome 2 (Figure 1) and separated by another gene (LOC_Os02g02180). However, there are relative low values of the nucleotide sequence identity between the 5' upstream regions and between the 3' downstream regions of these two genes. The nucleotide sequence identity between the two *OsNAR2* genes is 72%. The gene structures differ among the four *OsNRT2* genes, and between the two *OsNAR2* genes. Of the four *OsNRT2* genes, only *OsNRT2.4* has intron (Figure 3). Although both of *OsNAR2* genes have one intron, *OsNAR2.1* has a much longer intron than *OsNAR2.2*.

The phylogenetic tree of *NRT2* proteins was created after alignment on the entire sequences of *NRT2* proteins including all of the four *NRT2* proteins in rice, all the seven *NRT2* proteins in *Arabidopsis*, and the *NRT2* proteins in other plants, algae, fungi, and *Escherichia coli*. The phylogenetic tree of *NRT2*s clearly shows that there are two distinct clusters, one for dicotyledonous and the other for monocotyledonous plants. The *NRT2* proteins from cereals are clustered together, within these the two rice sequences *OsNRT2.1* and *OsNRT2.2*. *OsNRT2.3* is more closely related to HvNRT2.5 and AtNRT2.5, and *OsNRT2.4* is more closely related to AtNRT2.7. These five plant *NRT2* proteins seem to be less related to other plant *NRT2* proteins and might be more closely related to the *NRT2* genes from lower eukaryotic organisms, or prokaryotic organisms (Figure 2A). The phylogenetic tree of *NAR2* proteins also contains two distinct clusters, with the two *OsNAR2* proteins in the monocotyledon cluster (Figure 2B).

Table 1. Polymerase chain reaction (PCR) primers used in this study

Gene	Locus ID ^a	Primer sequences for cDNA isolation	Product size (bp)
<i>OsNRT2.1</i>	LOC_Os01g50820	Forward 5'-CTTCGACGTCAACCTCCAGA-3' Reverse 5'-CGATCCACAGAAGTAAATCAAACC-3'	664
<i>OsNRT2.2</i>	LOC_Os02g02190	Forward 5'-ATCCTTCGGCATGGCCAACAT-3' Reverse 5'-CTCCATGACGACATACTCTAG-3'	731
<i>OsNRT2.3</i>	LOC_Os02g02170	Forward 5'-ATCCTTCGGCATGGCCAACATC-3' Reverse 5'-GCTGGTCTCGGTCGAGAATTGTT-3'	911
<i>OsNRT2.4</i>	LOC_Os01g36720	Forward 5'-GTCACCTACCTACTCCCGTG-3' Reverse 5'-ATCATCATCGTCATCAACATCGC-3'	798
<i>OsNAR2.1</i>	LOC_Os02g38230	Forward 5'-GCTGCTCTCTCGTTGGTGCT-3' Reverse 5'-ACCTTGAGGGAGGCGTGGAT-3'	518
<i>OsNAR2.2</i>	LOC_Os04g40410	Forward 5'-ACCTTAGCAGAGAACGATCATGGCT-3' Reverse 5'-CTGTCGTACACAGTCACACATTACAA-3'	711
<i>OsACT</i>	LOC_Os03g50890	Forward 5'-CCTCGTCTCGACCTTGCTGGG-3' Reverse 5'-GAGAACAAGCAGGAGGACGGC-3'	666
<i>Os18S rRNA</i>	LOC_Os09g00998	Forward 5'-CTGCGA AAGCATTTGCCA-3' Reverse 5'-AGAAAGAGCTCTCAGTCTGT-3'	320

^aThe Institute for Genomic Research (TIGR) Rice Genome Annotation Locus ID.

Table 2. Identity/similarity of the NRT2 family

Nucleotide identity	Amino acid similarity			
	NRT2.1	NRT2.2	NRT2.3	NRT2.4
<i>NRT2.1</i>	—	100	73	60
<i>NRT2.2</i>	100	—	73	60
<i>NRT2.3</i>	66	66	—	65
<i>NRT2.4</i>	56	56	57	—

Expression patterns of *NRT2* and *NAR2* genes in response to nitrate

The effects of two nitrate concentrations (0.2 and 2.0 mM) on the expression of *OsNRT2* and *OsNAR2* genes in rice roots were investigated using a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) approach. When the plants deprived of N for 5 d were exposed to 0.2 mM nitrate at pH 6.5 for 0 to 12 h (Figure 3A), the mRNA levels of *OsNRT2.1* and *OsNAR2.1* increased slowly but significantly to their highest level at 2–4 h of treatment, and thereafter decreased steadily and slowly. The transcripts of *OsNRT2.2* were rapidly upregulated and peaked at 1–2 h, and then decreased rapidly. The transcripts of *OsNRT2.4* and *OsNAR2.2* increased within 0.5–1.0 h after nitrate feeding, remained at elevated levels until 4 h after nitrate feeding, and then declined sharply. By contrast, *OsNRT2.3* had a transient inducible response to 0.2 mM nitrate; its expression peaked within 15 min and then slowly decreased during the experiment period. The overall expression patterns of the six genes in roots treated with 2.0 mM nitrate (Figure 3B) were similar to that treated with 0.2 mM nitrate, but the transcripts of these genes (except *OsNRT2.3*) reached their highest

Figure 1. Locations and structures of *NRT2* and *NAR2* genes in rice genome.

(A) Distribution of *NRT2* and *NAR2* genes on chromosomes of rice. The genes shown to the right of the chromosome are transcribed in the + orientation (from the top down); the genes shown to the left of the chromosomes are transcribed in the – orientation (from the bottom up). (B) Exons and introns of the genes are indicated by white and black boxes, respectively. The size of exons and introns is given in bp inside the boxes.

Figure 2. Phylogenetic trees of NRT2 (A) and NAR2 (B) families.

The entire protein sequences of NRT2 or NAR2 were aligned using ClustalW software, and then the alignments were used to create the unrooted, bootstrapped trees using MEGA version 3.0. Values indicate the number of times (in percent) that each branch topology was found during bootstrap analysis. The deduced protein sequences of the four *OsNRT2* and two *OsNAR2* genes from The Institute for Genomic Research (TIGR) Rice Genome Annotation Database were used in the present study (Table 1). Protein ID numbers of NRT2 are: barley (*Hordeum vulgare*) HvNRT2.1, AAC49531; HvNRT2.2, AAC49532; HvNRT2.3, AAD28363; HvNRT2.4, AAD28364; HvNRT2.5, ABG20828; HvNRT2.6, ABG20829; wheat (*Triticum aestivum*) TaNRT2.1, AAG01172; TaNRT2.2, AAK19519; TaNRT2.3, AAL11016; maize (*Zea mays*), ZmNRT2.1, AAN05088; ZmNRT2.2,

level earlier in roots treated with 2.0 mM nitrate than in the roots treated with 0.2 mM nitrate.

Effect of external pH on nitrate influx in nitrate induced roots

The plants deprived of N for 5 d were first induced with 0.2 mM nitrate at pH 6.5 for 0, 1, 4 and 8 h, then the nitrate influx rates of these plants were measured in a solution containing 0.2 mM ^{15}N -nitrate at pH 5.0, 6.5 and 8.0. The data in Figure 3C show that the nitrate influx rates increased with nitrate induction time from 1 h to 4 h and then did not increase any further at 8 h. The effects of pH on nitrate influx rates were measured and the lowest values at all of the three time points were obtained at pH 8.0. The nitrate influx rate measured at pH 5.0 was higher than that at pH 6.5 at 1 h, and was similar to that at pH 6.5 at 4 h and 8 h.

Discussion

The present study identified four *NRT2* and two *NAR2* genes in rice. It has been known that there are seven *NRT2* and two *NAR2* genes in *Arabidopsis* (Orsel et al. 2002), and six *NRT2* and three *NAR2* genes have been identified in barley (Figure 2). However, rice has more ammonium transporter (*AMT*) genes than *Arabidopsis*. According to the TIGR annotation database,

←

AAT66252; *Arabidopsis* AtNRT2.1, NP_172288; AtNRT2.2, NP_172289; AtNRT2.3, NP_200886; AtNRT2.4, NP_200885; AtNRT2.5, NP_172754; AtNRT2.6, NP_190092; AtNRT2.7, NP_196961; tomato (*Lycopersicon esculentum*) LeNRT2.1, AAF00053; LeNRT2.2, AAF00054; LeNRT2.3, AAK72402; *Nicotiana tabacum* NtNRT2.1, CAD89798; NtNRT2.2, CAD89799; *Brassica napus* BnNRT2, CAC05338; *Nicotiana plumbaginifolia* NpNRT2.1, CAA69387; *Lotus japonicus* LjNRT2, CAC35729; soybean (*Glycine max*) GmNRT2, AAC09320; *Chlamydomonas reinhardtii* CrNRT2.1, CAA80925; *Aspergillus nidulans* CRNA, AAA62125; *Hansenula polymorpha* YNT1, CAA93631; and *Escherichia coli* NARK, CAA34126. Protein ID numbers of NAR2 are: barley HvNAR2.1, AAP31850; HvNAR2.2, AAP31851; HvNAR2.3, AAP31852; wheat TaNAR2.1, AAV35210; TaNAR2.2, AAV35211; *Arabidopsis* AtNAR2.1, CAC36292; AtNAR2.2, CAC36942; bell pepper (*Capsicum annuum*), CaNAR2, AAM12786. The protein sequences of the *NAR2* genes from tomato, potato, *Medicago truncatula*, and *C. reinhardtii* were deduced from the expressed sequence tag (EST) sequences using the National Center for Biotechnology Information (NCBI) open reading frame (ORF) finding tool. The accession numbers of the ESTs are: tomato LeNAR2.1, BF113833; potato (*Solanum tuberosum*) StNAR2.1, BM109240; *Medicago truncatula* MtNAR2.1, BG584033; *C. reinhardtii* CrNAR2, BQ810103.

Figure 3. Expression patterns of the high-affinity nitrate transport system (HATS) genes and the effect of pH on nitrate influx in the roots pretreated with nitrate.

(A,B) Expression patterns of the HATS genes. Rice plants deprived of N for 5 d were resupplied with 0.2 mM **(A)** and 2.0 mM **(B)** nitrate at pH 6.5 for the time as shown. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was used to detect the mRNA levels in the roots. The constitutive gene *actin* and *18S rRNA* were used as a reference.

(C) The effect of pH on nitrate influx. Rice plants deprived of N for 5 d were resupplied with 0.2 mM nitrate at pH 6.5 for the time as indicated. The nitrate-induced plants were then used to measure nitrate influx in uptake solutions containing 0.2 mM nitrate at pH 5.0, 6.5 and 8.0 as described in "Materials and Methods". The nitrate influx rates are given as the mean of four replicates and SD.

there are 13 putative *AMT* genes of which eight are expressed in rice, while there are six *AMT* genes in *Arabidopsis*. The relatively small number of genes encoding HATS in rice relative to the number of putative ammonium transporters probably reflects the fact that rice grows in an ammonium richer environment. Rice has therefore been under evolutionary pressure to develop a more sophisticated ammonium rather than a nitrate uptake system and this is reflected in the number of genes available to transport each form of N.

The six rice genes locate on three chromosomes (Figure 1). *OsNRT2.1* and *OsNRT2.2* are closely located in a "tail-to-tail" arrangement on the short arm of chromosome 2 and are separated by another gene. These two genes share identical coding sequences, but diverge in the 5' upstream and 3' downstream sequences, suggesting that a duplication event occurred. Interestingly, a similar arrangement has been observed for two *Arabidopsis* *NRT2* genes, *AtNRT2.1* and *AtNRT2.2* (Orsel et al. 2002). Alignment of the entire protein sequences of rice *NRT2* and other known *NRT2* genes revealed that *OsNRT2.1* and *OsNRT2.2* belong to the monocotyledon cluster in the phylogenetic tree. Another two *NRT2* genes (*OsNRT2.3* and *OsNRT2.4*) locate on the long arm of chromosome 1. The protein sequences of these two *NRT2* genes together with *AtNRT2.5*, *AtNRT2.7* and *HvNRT2.5* are more closely related to the *NRT2* proteins from lower eukaryotic organisms or prokaryotic organisms than to other plant *NRT2* proteins, implying that these *NRT2* genes existed in higher plants before monocotyledon-dicotyledon split, or an ancient linkage before the divergence of monocotyledon from dicotyledon. Among the four rice *NRT2* genes, only *OsNRT2.4* has an intron. Therefore, most *OsNRT2* genes have different structure from *AtNRT2* genes that have one to three introns (Orsel et al. 2002). It is worth noting that *OsNRT2.4* not only encodes a putative protein closely related to *AtNRT2.7*, but also has a similar gene structure to *AtNRT2.7*. Both genes have an intron near the stop codon (Figure 1, Orsel et al. 2002). The two rice *NAR2* genes, *OsNAR2.1* and *OsNAR2.2*, are located on chromosomes 2 and 4, respectively. Although both *OsNAR2* genes have an intron near the start codon, *OsNAR2.1* has a much longer intron than *OsNAR2.2*. The deduced polypeptides of *OsNAR2.1* and *OsNAR2.2* are more closely related to *NAR2* proteins from monocotyledons than those from dicotyledons (Figure 2).

The investigation of the effects of nitrate treatment on individual members of the *OsNRT2* family and *OsNAR2* family revealed that these genes are differentially induced in root tissue treated with either low or high nitrate level (Figure 3A,B), indicating that these genes may have different roles in response to environmental nitrate. Although *OsNRT2.2* has an identical coding sequence with *OsNRT2.1* (Table 2), the mRNA levels of *OsNRT2.2* increased to their peak earlier and then decreased more rapidly to a low level than that of *OsNRT2.1* (Figure 3A). The different responses of *OsNRT2.1* and *OsNRT2.2* to nitrate could be attributed to their divergent 5' upstream and/or 3'

downstream sequences. Among the six investigated genes, only *OsNRT2.3* transiently responded to nitrate at the very beginning. The fact that high nitrate level more strongly induced *OsNRT2.3* than low nitrate level implies that a high nitrate level may be required to induce this gene (Figure 3A,B). It was described earlier that some NRT2 type transporters from photosynthetic organisms required a second protein, NAR2, to co-produce a functional HATS (Quesada et al. 1994; Zhou et al. 2000b; Tong et al. 2005; Orsel et al. 2006). As *OsNRT2.3* expression responded to nitrate very differently to the two *NAR2*s, it therefore can be assumed that *OsNRT2.3* does not require a second protein to function. Of the four rice *NRT2* genes, the expression pattern of *OsNRT2.1* best fitted with the change in pattern of the nitrate influx rates in response to nitrate induction (Figure 3), indicating that *OsNRT2.1* might be a major member of inducible HATS. The data presented in Figure 3(A,B) clearly demonstrate that external nitrate levels did not affect the overall expression patterns of these six genes in roots in our experiment conditions. Similar results have also been reported for wheat and barley *NRT2* genes (Vidmar et al. 2000a; Zhao et al. 2004).

High-affinity nitrate influx into rice root has been previously reported to be nitrate-inducible (Kronzucker et al. 2000) and this was also true in the present study (Figure 3C). Using 0.2 mM ^{15}N -nitrate to measure the nitrate influx in the roots pretreated with 0.2 mM nitrate at pH 6.5 for the time as indicated in Figure 3C, the nitrate influx increased 4.9-fold from the onset to 4 h after exposure to nitrate. The nitrate influx at 8 h was similar to that at 4 h, despite the fact that transcript abundances of all of the *NRT2* and *NAR2* genes decreased at 8 h (Figure 3A). This phenomenon (maintaining a high nitrate influx rate when *NRT2* transcript abundance is low) has also been reported in barley (Vidmar et al. 2000b), and was possibly because other transport systems might contribute to the measured influx, or *NRT2* or *NAR2* genes were subjected to post-transcriptional regulation. A 4.9-fold induction of the nitrate influx measured at pH 6.5 in present study was much lower than that previously reported. Using 0.1 mM nitrate to measure the nitrate influx in the rice (cv. IR72) roots pretreated with 0.1 mM nitrate at pH 6.5, Kronzucker et al. (2000) detected a 27-fold increase after 5 h of nitrate feeding. It has been reported in barley that the nitrate induction on high-affinity nitrate influx is genotype-dependent. The increase of influx associated with induction was less in *Steptoe* barley, which has higher constitutive high-affinity nitrate influx than in *Klondike* barley, which has lower constitutive high-affinity nitrate influx (King et al. 1993).

High-affinity nitrate influx into 0.2 mM nitrate induced roots measured at 0.2 mM ^{15}N -nitrate and at various pHs revealed that the nitrate influx rates associated with nitrate induction were subjected to pH regulation (Figure 3C). As reported previously in maize and barley (Glass et al. 1990; McClure et al. 1990), inhibition of alkaline pH on nitrate influx into nitrate induced rice roots was observed (Figure 3C), reflecting the fact that

trans-membrane nitrate transport is mediated by a proton-nitrate symport (Ullrich and Novacky 1981; Glass et al. 1992, Miller and Smith 1996). However, the effect of acidic pH on inducible nitrate influx depended on the time of the rice roots induced by nitrate. Compared with the influx rates measured at pH 6.5, acidic pH (pH 5.0) enhanced the nitrate influx into 1 h nitrate induced roots, but slightly reduced (though not significantly) the nitrate influx into roots induced by nitrate for 4 h and 8 h (Figure 3C). It has been previously reported in barley that activities of different HATS systems are differentially regulated by external pH (Glass et al. 1990). Measurements of high-affinity ^{13}N -nitrate influx in 1 d nitrate-induced barley roots showed maximal influx at pH 6.0 that was 19% lower at pH 4.5 and 23% lower at pH 7.5, but the highest influx in uninduced roots was observed at pH 4.5 that was 16% lower at pH 6.0 and 34% lower at pH 7.5 (Glass et al. 1990). We also obtained similar results in barley with that observed by Glass et al. (1990) (Cai C. et al. unpubl. data, 2006). We measured high-affinity ^{15}N -nitrate influx in nitrate induced and uninduced barley roots at pH 5.0, 6.5 and 8.0 under approximately the same conditions used in the present study. The measured influx rates in uninduced roots at pH 5.0, 6.5 and 8.0 were 28.2, 22.6 and 14.4 $\mu\text{mol}\cdot\text{g}^{-1}\text{Dw}\cdot\text{h}^{-1}$, respectively; while the influx rates in 4–12 h nitrate induced roots were the highest at pH 6.5 that were 20–29% lower at pH 5.0 and 31–44% lower at pH 8.0. The above data from the present study and the published reports demonstrated that alkaline pH inhibits high-affinity nitrate influx in the roots of both rice and barley, and acidic pH inhibits high-affinity nitrate influx in nitrate-induced roots of barley, but not rice.

In summary, the present study identified four *NRT2* and two *NAR2* genes in rice roots. The protein sequence divergences within the *OsNRT2* family and within the *OsNAR2* family indicate their functional divergences. All six genes are nitrate-inducible, but they differentially respond to nitrate, indicating their different roles in adaptation to environmental conditions. Although the present study provided structure and expression data of the rice HATS components to understand the rice HATS, more detailed research is needed to analyze the kinetic characteristics of each *NRT2* member, and the temporal and spatial expression pattern of these six genes. Acidic pH, which has been shown to inhibit inducible high-affinity nitrate influx in barley, does not inhibit that in rice. Future research to understand this difference will help us to engineer crops with high activity of HATS under broad external pH values.

Materials and Methods

Preparation of plant materials

Rice (*Oryza sativa* L. subsp. *japonica* cv. Nipponbare) seeds were surface sterilized in 10% H_2O_2 for 10 min, then rinsed thoroughly with deionized water. The sterilized seeds were

germinated and grown in moisture perlite (with nutrient solution, see below) for 25 d. Uniform seedlings were then transferred to a tank containing 45 L of nutrient solution. The nutrient solution was the modified Yoshida nutrient solution (Yoshida 1976) in which 2 mM KNO_3 was used as the sole N source. The solution pH was buffered at 6.5 ± 0.2 by adding 2 mM 2-(N-morpholino)ethanesulfonic acid (MES). The solution was refreshed every 2 d. After growing in the hydroponic tanks for 10 d, the plants were then removed to N-free solution (pH 6.5) for 5 d prior to analysis of gene expressions and nitrate influx rate measurements. The N-free nutrient solution contained 2 mM KCl instead of 2 mM KNO_3 , and was refreshed every day. In the present study, all plants were grown in a growth chamber with a 16:8 h light:dark cycle at $28.20 \pm 2^\circ\text{C}$. The relative humidity was kept at 65–70%, and the light intensity at the plant level was $400 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Expression patterns of *NRT2* and *NAR2* genes in response to nitrate

To analyze the expression patterns of *NRT2* and *NAR2* genes in response to nitrate, the plants deprived of N for 5 d were exposed to the nutrient solution containing 0.2 mM or 2.0 mM KNO_3 (pH 6.5) for the time as indicated in Figure 3(A,B). KCl, if needed, was used to keep potassium concentration at 2.0 mM as that used in the N-free nutrient solution. Three replicates for each time point were used. The treated root tissues were collected and stored at -80°C for future analysis.

Measurement of nitrate influx

Intact seedlings were used to measure nitrate influx rates according to the methods described previously (Siddiqi et al. 1989; Kronzucker et al. 1998). The plants deprived of N for 5 d were induced with the nutrient solution containing 0.2 mM KNO_3 at pH 6.5 for 0, 1, 4, and 8 h. Then eight nitrate-induced plants with similar size were transferred to a pot containing 0.5 L of 0.2 mM NO_3^- at pH 5.0, 6.5 or 8.0 for 5 min. After this pretreatment, the eight plants were transferred to a pot containing 0.5 L of 0.2 mM $^{15}\text{N}\text{-KNO}_3$ (98% atom $^{15}\text{N}\text{-KNO}_3$, Fluka-Aldrich Company, Miamisburg, OH, USA) at pH 5.0, 6.5 or 8.0 for 10 min. Thereafter, the plants were transferred to an unlabeled nutrient solution contain 0.2 mM KNO_3 for 3 min to remove tracer from the cell wall (Kronzucker et al. 1996). Four replicates for each treatment were used. The root and shoot samples were harvested separately, and dried at 70°C . The plant samples were ground carefully using a pestle and mortar after their dry weights were recorded. The $^{15}\text{N}/^{14}\text{N}$ ratio in the ground samples was measured using an isotope ratio mass spectrometer with elemental analyzer (Thermo Finnigan MAT DELTA^{plus} XP, Bremen, Germany). Nitrate influx rate was calculated according to micromoles of ^{15}N accumulated in the plant (root plus shoot) per gram root dry weight per h.

RNA isolation and relative quantitative RT-PCR

The protein sequences of barley *NRT2.1* and *NAR2.3* were used to identify related *NRT2* and *NAR2* genes in TIGR rice genome database, four putative *NRT2* genes and two putative *NAR2* genes were identified (Table 1). Relative quantitative RT-PCR was used to determine the expression of individual *NRT2* and *NAR2* genes in rice roots. Total RNAs were isolated from roots using TRIzol total RNA isolation reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First strand cDNA synthesis was carried out on 2 μg total RNA as a template using M-MLV RT (Invitrogen, Carlsbad, CA, USA) and 5 pmol of oligodT (Promega, Madison, WI, USA), following the manufacturer's recommended protocol. PCR reactions were carried out using one-twentieth of the first strand cDNA as templates. *Taq* DNA polymerase and dNTPs used in all reactions were provided by MBI Fermentas (Foster City, CA, USA). Gene specific primers were designed according to the predicated mRNA sequences or related EST sequences (Table 1). *Os18S rRNA* and *OsActin* (Table 1) were used as an internal control in the RT-PCR. PCR cycling conditions were as follows: an initial denaturation step at 95°C for 4 min, amplification 25–41 cycles (95°C for 30 s, 58°C for 30 s, and 72°C for 50 s), and a final elongation step at 72°C for 10 min. The PCR products were visualized on ethidium bromide stained 1.0% agarose gel, and confirmed by sequencing.

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