Expression of Rice (*Oryza sativa* L.) Genes Involved in High-Affinity Nitrate Transport during the Period of Nitrate Induction

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When breeding crops that utilize nitrogen efficiently, it is important to reveal the regulation of nitrate uptake at the molecular level. We focused on the expression of two nitrate uptake-related genes, *NRT2* and *NAR2*, and nitrate uptake during the nitrate induction. Four rice *NRT2s* (*OsNRT2.1*~*2.4*) and two *NAR2s* (*OsNAR2.1*~*2.2*) were identified in the rice genome database. We analyzed the expression of the genes in the roots and shoots after supply of nitrate with or without ammonium pretreatment by the RT-PCR method. The apparent nitrate uptake was also measured. Differential expression of *OsNRT2.1* and *OsNRT2.2*, which have the same ORF with different 5'- and 3'-UTR, was observed in the seedlings pretreated with ammonium. The apparent nitrate uptake synchronously increased after supply of nitrate with the expression pattern of *OsNRT2.1* in roots either with or without ammonium pretreatment. The expression of *OsNAR2.1*, which is thought to be an activator gene for the function of *NRT2*, was also coordinated with that of *OsNRT2.1*. On the other hand, *OsNRT2.2* was already expressed after ammonium pretreatment, suggesting that *OsNRT2.2* would be transcribed to take up nitrate in flooded soils. The present results indicate that *OsNRT2.1*, *OsNRT2.2* and *OsNAR2.1* are candidate genes when breeding for efficient use of nitrate in rice.

Key Words: High-affinity nitrate transporter, *NRT2*, *NAR2*, *Oryza sativa*, nitrate induction, nitrate uptake.

Introduction

Breeding of crops that utilize nitrogen efficiently is one of the important goals of crop breeding, because nitrogen is the most important factor limiting growth or yield in crops. For most cultivated crops, nitrate is the major nitrogen source. In rice (*Oryza sativa* L.), the nitrate produced by nitrification has been shown to be taken up with the diffusion of oxygen through the roots in flooded soils (Kirk and Kronzucker 2005). In addition, rice grows better and utilizes nitrogen efficiently when nitrate and ammonium are supplied together (Ta and Ohira 1981, Kronzucker *et al.* 1999b). It is more important to study nitrate uptake than generally thought. On the other hand, the progress in the analysis of the rice genome (Sasaki *et al.* 2002, Sasaki *et al.* 2005, Yuan *et al.* 2005) has revealed that there is extensive synteny and gene homology between rice and cereal crops (Goff *et al.* 2002), indicating that rice can be a model plant for studying nitrate uptake mechanisms at the molecular level. Therefore, it is useful to reveal the regulatory mechanism of nitrate uptake in rice.

Nitrate uptake is mediated by the low affinity transport system (LATS) and high-affinity transport system (HATS) (Crawford and Glass 1998, Forde 2000), which are encoded

by *NRT1* and *NRT2*, respectively (Tsai *et al.* 1993, Krapp *et al.* 1998, Vidmar *et al.* 2000a, Cerezo *et al.* 2001, Filleur *et al.* 2001). HATS consists of two systems, the nitrate-inducible HATS (iHATS) and the constitutive HATS (cHATS) (Wang and Crawford 1996, Crawford and Glass 1998, Forde 2000). In *Arabidopsis thaliana*, seven genes were isolated as *NRT2* family gene members (Orsel *et al.* 2002b), suggesting that *NRT2* in higher plants is composed of several genes. The mechanism of iHATS for nitrate at the molecular level has been revealed by functional analysis using *A. thaliana* mutant, *atnrt2*, which is disrupted in *A. thaliana* *NRT2.1* (*AtNRT2.1*) and in a part of *AtNRT2.2* and showed a decrease of nitrate uptake ability only in iHATS (Cerezo *et al.* 2001, Filleur *et al.* 2001). However, the functional roles for the other members of *NRT2* remain uncertain. In rice, although the function of *NRT1* has been revealed (Tsai *et al.* 1993, Lin *et al.* 2000), investigations of *NRT2* have not been carried out. To develop efficient use of nitrogen in rice, it is important to reveal the roles of *NRT2* family genes.

To understand the role of the *NRT2* family we need to investigate the relationship between the expression of *NRT2* and nitrate uptake during the period of nitrate induction (Amarasinghe *et al.* 1998, Krapp *et al.* 1998, Zhuo *et al.* 1999, Vidmar *et al.* 2000a). By the relative quantitative RT-PCR method, *AtNRT2* family genes have been grouped into three categories, nitrate-inducible, nitrate-repressible and nitrate-constitutive (Okamoto *et al.* 2003). Moreover, there

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is a clear positive correlation between nitrate uptake and the amounts of *AtNRT2.1* transcripts in nitrate induction (Zhao *et al.* 1999, Okamoto *et al.* 2003).

The differential repression of transcripts of NRT2 family genes except for nitrate constitutive genes, 3 to 12 h after supply of nitrate has been reported in barley (*Hordeum vulgare*) and *A. thaliana* (Vidmar *et al.* 2000a, Okamoto *et al.* 2003). However, most NRT2 family genes are induced coordinately by the nitrate treatment (Vidmar *et al.* 2000a, Okamoto *et al.* 2003). The negative feedback regulation for the expression of *NRT2* and nitrate uptake has been reported to be caused by the metabolites downstream, ammonium or glutamine (Youngdahl *et al.* 1982, Henriksen and Spanswick 1993, King *et al.* 1993, Quesada *et al.* 1997, Kronzucker *et al.* 1999a, Lejay *et al.* 1999, Zhao *et al.* 1999, Vidmar *et al.* 2000b, Glass *et al.* 2002, Orsel *et al.* 2002a). Therefore, the differential expression patterns of *NRT2s* may be attributed to a difference in the response to nitrogen status among the *NRT2* family members.

Furthermore, it is necessary to consider the activation and/or regulation factors for NRT2 function at the molecular level. In *Chlamydomonas reinhardtii*, NAR2 gives NRT2 the function of transporting nitrate through the membrane (Quesada *et al.* 1994, Zhou *et al.* 2000). Recently, Tong *et al.* (2005) found *NAR2*-like genes of *C. reinhardtii* in barley and reported that one of the barley *NAR2s* (HvNAR2.1~2.3), HvNAR2.3, could give functional nitrate transport activity to HvNRT2.1 in the *Xenopus laevis* oocyte. This result indicated that *NAR2s* are also important for the function of NRT2 with nitrate uptake in higher plants.

In this study, to reveal the regulation of HATS for nitrate at the molecular level in rice, we focused on the induction of nitrate uptake in relation to the time-course induction patterns of *OsNRT2* family genes and *OsNAR2* genes with or without ammonium pretreatment, based on the hypothesis that there is a difference in the response to nitrogen status among NRT2 family genes.

Materials and Methods

Plant materials and cultivation

Rice (*Oryza sativa* L. cv. Nipponbare) seeds were surface-sterilized for 30 min with 10% H₂O₂, and rinsed with de-ionized water twice. The seeds were germinated and cultured with de-ionized water in a biotron (LH-5-1205, Nippon Medical and Chemical Instruments, Osaka, Japan) controlled at 25 ± 1°C under continuous lighting (70.9 μmol m⁻² s⁻¹) with fluorescent lamps (FL20SS W/18, Toshiba, Tokyo, Japan) for 3 weeks.

Isolation and analysis of *OsNRT2s* and *OsNAR2s* from rice genome database

Four rice NRT2 family genes (*OsNRT2.1*~*2.4*) were extracted from the rice genome database (Sasaki *et al.* 2002, Sasaki *et al.* 2005) using DDBJ BLAST search (Altschul *et al.* 1997, Tateno *et al.* 2005) and BLAST search system in

the TIGR rice genome annotation (Yuan *et al.* 2005: <http://rice.tigr.org/>) with registered rice high-affinity nitrate transporter gene sequence (*OsNRT2*: GenBank accession No. AB008519). Extracted DNA sequences were analyzed with the TIGR rice genome annotation and Gramene (Jaiswal *et al.* 2002, Ware *et al.* 2002: <http://www.gramene.org/>) to determine the location of each *OsNRT2* on rice chromosomes. Two *NAR2s* in rice were also extracted from the TIGR rice genome annotation using the amino acid sequence of HvNAR2.3 (GenBank accession No. AAP31852). Plant *cis*-acting regulatory DNA elements in *OsNRT2s* and *OsNAR2s* were searched with PLACE (Higo *et al.* 1999). Transmembrane spanning regions of each *OsNRT2* and *OsNAR2* were determined using TMPred (Hofmann and Stoffel 1993), TopPred (von Heijne 1992, Claros and von Heijne 1994), MEMSAT (Jones *et al.* 1994, McGuffin *et al.* 2000) and HMMTOP (Tusnády and Simon 1998, Tusnády and Simon 2001). ClustalW was used for the phylogenetic analysis of *NRT2s* (Thompson *et al.* 1994) in DDBJ and Tree View (Page 1996).

Analysis of expression of *OsNRT2s* and *OsNAR2s*

For the molecular analysis, 3-week-old seedlings were treated with 200 μM KNO₃ containing 400 μM CaSO₄ solution after pretreatment with 400 μM CaSO₄ or 500 μM (NH₄)₂SO₄ containing 400 μM CaSO₄ for 20 h. Total RNA was extracted with TRI reagent® (Molecular Research Center, INC, Cincinnati, OH, USA) from the roots and shoots of 10 seedlings. Extracted total RNAs were treated with DNase (TaKaRa, Otsu, Japan) according to the manufacturer's manual. Total RNA concentrations were determined by UV spectrophotometry (FAS-III full system, TOYOBO, Osaka, Japan). First strand cDNA synthesis using RevaTra Ace® (TOYOBO, Osaka, Japan) was performed with 1 μg aliquots of total RNA and oligo dT(20) primer according to the manual.

Specific primers for each *OsNRT2* were as follows: 5'-GCTAGTAGTTGCCATGGACTCG-3' and 5'-ATCAGGATGTTCCCCGTTTG-3' for *OsNRT2.1*; 5'-TGGAGCGAACAGGAGAAGAG-3' and 5'-GCCTCTTAATTAGGCGTGC TC-3' for *OsNRT2.2*; 5'-GCCATCCACAAGATCGGTAG-3' and TGTGGAGCTTCCCGTAGTTG-3' for *OsNRT2.3*; 5'-ATCGTTCCCTTCGTCTGCAA-3' and 5'-TACCTGGA CCCGCTGAAGAA-3' for *OsNRT2.4*. *OsNAR2.1* and *OsNAR2.2* specific primers were as follows: 5'-AGGTGTT CCTCTCCAAGCCC-3' and 5'-CTTCACGCTCTTGAAC GCCG-3' for *OsNAR2.1*; 5'-CCTCGAGAACAAGAAGAA GAACAAGTGA-3' and 5'-TGCTGGGCGCTGTAGACAC-3' for *OsNAR2.2*. Specific primers for *Actin*, 5'-CTTCATGGAATGGAAGCTGCGGGTA-3' and 5'-CGACCACCTT GATCTTCATGCTGCTA-3' (McElroy *et al.* 1990), were used for the internal control as demonstrated by Sonoda *et al.* (2003).

The program of PCR was 95°C for 1 min for 1 cycle followed by 95°C for 5 sec and 60°C for 20 sec for 26~32 cycles as an amplification step. In addition, *OsNRT1.1*

(GenBank accession No. AF140606) were amplified using specific primers, 5'-ATCAGGATGTTCCCGTTTG-3' and 5'-GCCGATGTGTTTCTCCATCA-3', for comparison with the results of *OsNRT2s* expression patterns. The amplified DNA fragments were checked by electrophoresis on 3% (w/v) Certified™ Low Range Ultra Agarose (Bio-Rad, Hercules, CA, USA) and sequenced with an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) to confirm their identity. The gene expression in root and shoot was analyzed periodically 3 times.

Apparent nitrate uptake measurement

Seedlings cultivated for 3 weeks in de-ionized water were treated with 400 μ M CaSO₄ solution with or without 500 μ M (NH₄)₂SO₄ for 20 h (pretreatment). Then 10 seedlings were transferred into the 100 ml solution containing 200 μ M KNO₃ and 400 μ M CaSO₄ (experimental solution). The solution of 1.8 ml was taken up at the given times for 24 h after transferring to the experimental solution. Nitrate concentration was measured with an ion-chromatograph (DX-3000, Dionex, Sunnyvale, CA, USA) equipped with an anion-specific column (IonPacR AS12A, Dionex, Sunnyvale, CA, USA). Amounts of apparent nitrate taken up from the experimental solution were evaluated based on the nitrate uptake index as nitrate uptake taken up from the solution per fresh weight of 10 whole seedlings. Nitrate uptake was calculated from the depletion of experimental solution (Hasegawa and Ichii 1994, Araki *et al.* 2005). The experiment was repeated four to six times and five replicates were used in each treatment.

Results

Information of *OsNRT2s*

Four *OsNRT2* (*OsNRT2.1*–*OsNRT2.4*) sequences were extracted from the rice genome database using the BLAST search on DDBJ and TIGR rice genome annotation and Gramene (Table 1). The sequences of *OsNRT2.1*, *OsNRT2.2* and *OsNRT2.3* have been registered as putative *NRT2* cDNAs (GenBank accession numbers: AB008519, AK109733 and AK109776, respectively). Interestingly, *OsNRT2.1* and *OsNRT2.2* had the same ORF but with different sequences of 5'- and 3'-UTR. Neither *OsNRT2.1* nor *OsNRT2.2* had an intron. In addition, *OsNRT2.1* and *OsNRT2.2* were found on chromosome 2 in the region of 655,324–668,962 bp, holding

only a gene encoding hypothetical protein between *OsNRT2.1* and *OsNRT2.2*, being in the forward and the inverse direction, respectively. *OsNRT2.3* and *OsNRT2.4* were found on chromosome 1 (the distance between two genes is about 9,000,000 bp) (Table 1). Several putative TATA boxes were found in *OsNRT2.1*, *OsNRT2.2* and *OsNRT2.4*. TATABOX2 (Grace *et al.* 2004) was found at –152 and at –124 in *OsNRT2.1* and *OsNRT2.2*, respectively. TATABOXOSPAL (Zhu *et al.* 2002) was found at –156 in *OsNRT2.4*. In *OsNRT2.3*, a putative functional TATA box motif was not found.

The four *OsNRT2s* had the conserved amino acid motifs of [AG]-G-[WL]-G-N-M-G (Trueman *et al.* 1996), R-[PA]-x-G-G-x-x-[SA]-D and F-G-M-R-G-R-L-W (Okamoto *et al.* 2003) (Fig. 1). Membrane spanning regions of each *OsNRT2* were predicted 11 to 12 times using TopPred, MEMSAT and TMPred. MEMSTAT revealed 12 trans-membrane regions in all *OsNRT2s* (Fig. 1). These data indicated that the extracted sequences are genes encoding a high-affinity rice nitrate transporter.

The phylogenetic analysis for *NRT2s* based on the amino acid sequence demonstrated that *OsNRT2.1* and *OsNRT2.2* belonged to a monocotyledonous *NRT2* cluster, while *OsNRT2.3* and *OsNRT2.4* belonged to another *NRT2* cluster including a lower organism *NRT2* group (Fig. 2) as *AtNRT2.5* and *AtNRT2.7*. *OsNRT2.3* and *OsNRT2.4* have 58.3% and 43.3% similarity to *OsNRT2.1*, respectively. *OsNRT2.1* and *OsNRT2.2* showed higher similarity to the other higher plants *NRT2.1* than *OsNRT2.3* or *OsNRT2.4* (data not shown, refer to Fig. 2).

OsNAR2.1 and *OsNAR2.2*

Two *OsNAR2* genes were also extracted from the rice genome database (Table 1). *OsNAR2.1* and *OsNAR2.2* were located in chromosomes 2 and 4, respectively. A putative TATA box was found at –84 as TATABOX2 in *OsNAR2.1* and that at –33 as TATABOX4 (Grace *et al.* 2004) in *OsNAR2.2*. The deduced amino acid sequence of *OsNAR2.1* had 62.0% similarity to *OsNAR2.2*. *OsNAR2.1* and *OsNAR2.2* had 59.2% and 63.2% similarity to *HvNAR2.3*, respectively. The similarities of two *OsNAR2s* to several *NAR2s* of other higher plants were from 42.6% to 65.5%. *OsNAR2.1* and *OsNAR2.2* had 1 to 3 time(s) trans-membrane region(s) predicted by TMPred, TopPred and HMMTOP. MEMSAT predicted a 1 time trans-membrane

Table 1. Features of *NRT2s* and *NAR2s* in rice

Gene	Chromosome	Strand (+/–)	Accession No.	Gene ID
<i>OsNRT2.1</i>	2	+	AB008519	LOC_Os02g02170.1
<i>OsNRT2.2</i>	2	–	AK109733	LOC_Os02g02190.1
<i>OsNRT2.3</i>	1	+	AK109776	LOC_Os01g50820
<i>OsNRT2.4</i>	1	–	–	LOC_Os01g36720
<i>OsNAR2.1</i>	2	–	–	LOC_Os02g38230
<i>OsNAR2.2</i>	4	–	–	LOC_Os04g40410

Fig. 1. Deduced amino acid sequences of OsNRT2 family (OsNRT2.1~2.4). Sequences were aligned with ClustalW. Shaded region shows the amino acid sequence homology to OsNRT2.1. Numbered lines above the sequences indicate predicted trans-membrane spanning regions, determined using MEMSTAT. Each OsNRT2 trans-membrane spanning region is indicated by italics. Conserved sequence regions (as described in results) are indicated by dotted lines under the sequences. Gaps are indicated by hyphens.

region for each OsNAR2.1 and OsNAR2.2.

OsNRT2s and OsNAR2s expression in roots

Pretreatment with 1 mM ammonium affected the expression of *OsNRT2s*. In the seedlings without ammonium pretreatment, transcripts of *OsNRT2.1*, *OsNRT2.2* and *OsNRT2.4* were detected from 0.5 h after the onset of 200 μ M nitrate treatment, while *OsNRT2.3* was already expressed before the nitrate induction (Fig. 3). When the seedlings were pretreated with ammonium, the induction of *OsNRT2.1* transcription was impaired from 0 to 2 h after the beginning of nitrate treatment (Fig. 3). The transcription of *OsNRT2.1* was increased from 2 h after the beginning of nitrate treatment but the transcript abundance of *OsNRT2.1* declined after 24 h. On the other hand, *OsNRT2.2* was expressed at the beginning of nitrate treatment following pretreatment with ammonium, and remained at elevated levels for the duration of nitrate treatment. *OsNRT2.3*

transcripts were already detected from beginning of nitrate induction. The expression pattern of *OsNRT2.4* was similar to that of *OsNRT2.1* (Fig. 3).

Nitrate induced the expression of *OsNAR2.1* within 0.5 h after transferring the seedlings to nitrate from an N-free solution, as it did *OsNRT2.1* and *OsNRT2.4* (Fig. 3). However, the expression level of *OsNAR2.1* at 0.5 h was lower than that of *OsNAR2.1* treated with nitrate for 1 h or more (Fig. 3). On the other hand, *OsNAR2.2* was expressed at the beginning of the treatment with nitrate from N-starvation. In the seedlings pretreated with ammonium, the expression of *OsNAR2.1* and *OsNAR2.2* was not clearly detected for 0.5 h after transfer to nitrate.

OsNRT1.1, which is involved only in LATS for nitrate (Lin *et al.* 2000), was constitutively expressed in roots under nitrate concentrations below 200 μ M.

Fig. 2. Phylogenetic analysis of NRT2 proteins. Deduced amino acid sequences of NRT2s were aligned by ClustalW, and Tree View was used for establishing the phylogenetic tree. Protein ID numbers are *Oryza sativa* OsNRT2.1, O82138; OsNRT2.2, Q6ZH34; OsNRT2.3, Q94JG1; OsNRT2.4, BAB90798; *Arabidopsis thaliana* AtNRT2.1, O82811; AtNRT2.2, O82412; AtNRT2.3, Q9FJH7; AtNRT2.4, Q9FJH8; AtNRT2.5, Q9LPV5; AtNRT2.6, Q9LXH0; AtNRT2.7, Q9LYK2; *Hordeum vulgare* HvNRT2.1, P93182; HvNRT2.2, P93183; HvNRT2.3, Q9XEG4; HvNRT2.4, Q9XEG5; *Triticum aestivum* TaNRT2.1, Q9ATG9; TaNRT2.3, Q940R8; *Phragmites australis* reed NRT2, Q84LK4; *Glycine max* GmNRT2, O64999; *Lotus japonicus* LjNRT2, Q9ARC5; *Nicotiana glauca* NpNRT2.1, O04431; NtNRT2.2, CAD89799; *Lycopersicon esculentum* LeNRT2.1, Q9SQG6; *Physcomitrella patens* PpNRT2.1, Q76C06; PpNRT2.2, Q76C05; PpNRT2.3, Q76C04; PpNRT2.4, Q76C03; PpNRT2.5, Q76C02; *Chlamydomonas reinhardtii* CrNRT2.1, Q39608; *Hansenula polymorpha* YNT1, Q92240; *Aspergillus nidulans* CRNA, Q5BEM2; *Escherichia coli* NARK, P10903.

OsNRT2s and OsNAR2s expression in shoots

OsNRT2s and OsNAR2s were also expressed in shoots (Fig. 4), but not as much as in roots. In particular, the expression level of OsNRT2.1 in shoots was very low either with or without ammonium pretreatment (Fig. 4). OsNRT2.2 and OsNRT2.4 were expressed after the nitrate treatment

Fig. 3. Expression patterns of OsNRT2s and OsNAR2s in roots from the beginning of nitrate treatment. Seedlings that had been pretreated with ammonium ($+NH_4^+$) or without ammonium (N-free) were treated with nitrate. The total RNAs were extracted from the seedlings sampled at the given times (hour), indicated by the numbers in the upper row, after nitrate treatment. *Actin* was the internal control gene. *OsNRT1.1* was used for comparison with the results of OsNRT2s expression patterns. This amplification step was repeated until signals were clear: 32 cycles for OsNRT2s, 30 cycles for OsNAR2s and 26 cycles for *Actin*.

Fig. 4. Expression patterns of OsNRT2s and OsNAR2s in shoots from the beginning of nitrate treatment. The procedures were the same as those in Fig. 3 except that the amplification step was repeated 30 cycles for *Actin*.

without ammonium pretreatment. OsNRT2.2 was expressed from 1 h after the beginning of nitrate treatment, and the transcript abundance of OsNRT2.2 peaked at 8 h then declined to a barely detectable level at 24 h (Fig. 4). However, in the seedlings pretreated with ammonium, no transcripts of OsNRT2.2 were detected at 0 to 1.5 h. OsNRT2.3 was expressed in shoots, as in roots (Fig. 3 and Fig. 4). The expression of OsNRT2.4 in shoots was similar to that of OsNRT2.1 as in roots.

In shoots, OsNAR2.1 was expressed slightly, while OsNAR2.2 was constitutively expressed (Fig. 4). OsNRT1.1 was also constitutively expressed in shoots (Fig. 4).

Apparent nitrate uptake

A difference in the apparent nitrate uptake index was observed between the seedlings pretreated with ammonium and those without pretreatment. When the seedlings were transferred to a nitrate solution from an N-free solution, the nitrate uptake index increased linearly following a 0.5 h

Fig. 5. Increase in apparent nitrate uptake index from 200 μM KNO_3 solution with time (A and B). The seedlings were immersed in the nitrate solution after pretreatment for 20 h with or without 500 μM $(\text{NH}_4)_2\text{SO}_4$. Open and closed squares indicate the amounts of nitrate uptake after pretreatment with NH_4^+ and that without NH_4^+ , respectively. A shows the data during the nitrate treatment for 24 h after the beginning of nitrate treatment. B shows the data from 0 h to 2 h. Points and bars are means and SE of four to six replications.

induction period (Fig. 5B) with increase in treatment duration. Apparent nitrate uptake was not clearly detected until 1.5 h after transfer to nitrate from an ammonium solution (Fig. 5B), suggesting an induction period of about 1 h for the uptake. After 1.5 h from the beginning of nitrate treatment, nitrate uptake index increased linearly with the prolongation of nitrate treatment (Fig. 5A).

Discussion

Of the four NRT2 family genes extracted from the rice genome database, three genes were nitrate inducible and one gene was expressed before the nitrate induction. In roots, nitrate induced the expression of both *OsNRT2.1* and *OsNRT2.2* and the apparent nitrate uptake within 0.5 h after N-starvation (Fig. 3). The amounts of nitrate uptake increased with prolongation of nitrate treatment (Fig. 5). This short induction period of nitrate uptake in rice is coincident with previous reports (Sasakawa and Yamamoto 1978, Hasegawa *et al.* 1989, Kronzucker *et al.* 2000). Nitrate induction occurred more rapidly in rice than in barley, which is known as a highly efficient nitrate user (reviewed in Kirk and Kronzucker 2005). On the other hand, the time-lag of apparent nitrate uptake induction in the seedlings pretreated with ammonium (Fig. 5) could be caused by transient negative feedback regulation.

An interesting and important feature found is that *OsNRT2.1* and *OsNRT2.2* have the same ORF with different sequences of 5'-UTR and 3'-UTR and their expression in response to nitrogen status are different *in planta*. The expres-

sion patterns of *OsNRT2.1* and *OsNRT2.2* after transfer of the seedlings to nitrate solution from N-free solution were similar. Therefore, *OsNRT2.1* and *OsNRT2.2* are both nitrate-inducible genes as mentioned by Okamoto *et al.* (2003). However, the expression patterns in the seedlings pretreated with ammonium were different, suggesting a difference in the response to the negative feedback regulation by ammonium pretreatment between *OsNRT2.1* and *OsNRT2.2*. As *OsNRT2.1* and *OsNRT2.2* encode the same amino acid sequence, it is difficult to assess the contribution of their gene products for nitrate uptake. In several higher plants, nitrate uptake has been shown to be positively correlated with the amounts of NRT2 transcripts in nitrate induction (Krapp *et al.* 1998, Amarasinghe *et al.* 1998, Zhuo *et al.* 1999, Vidmar *et al.* 2000a, Okamoto *et al.* 2003). As the *OsNRT2.1* was expressed in parallel with the increase of apparent nitrate uptake index, it is concluded that *OsNRT2.1* is a key gene for the induction of nitrate uptake in rice. However, the result of the gene expression analysis implied that the products of *OsNRT2.2* also play an important role in nitrate uptake in the presence of nitrate and ammonium.

Nitrate has been proposed to be a signal molecule for transcription of genes related to nitrate assimilation (Crawford 1995, Wang *et al.* 2004). The difference in the expression of *OsNRT2.1* and *OsNRT2.2* might be attributed to the 5'-UTR up-stream region. Although, at present, the nitrate signal transduction pathway and its transcription factor in higher plants are still unclear, the high nitrogen status produced by ammonium pretreatment probably influences the transcription pathway of *OsNRT2.1* even where nitrate could be perceived as a signal by the seedlings.

The functional nitrate transport mediated by NRT2 has been shown to require the product of *NAR2* in *C. reinhardtii* and barley using the *Xenopus* oocyte expression system (Quesada *et al.* 1994, Zhou *et al.* 2000, Tong *et al.* 2005). The expression of *OsNAR2.1* was induced by nitrate treatment and repressed by ammonium pretreatment as demonstrated in *OsNRT2.1*. In addition, the expression of *OsNAR2.1* was clearly associated with an increase in the apparent nitrate uptake following pretreatment with or without ammonium (Fig. 5). However, the expression of *OsNAR2.1* and *OsNRT2.2* did not show a synchronous pattern when the seedlings were pretreated with ammonium (Fig. 3). These results suggest that *OsNAR2.1* is also one of the key genes in nitrate uptake in rice.

The difference in gene expression between *OsNRT2.1* and *OsNRT2.2* was also found in shoots. The transcripts of *OsNRT2.1* were barely detectable (Fig. 4), suggesting that *OsNRT2.1* is a root-specific gene. On the other hand, the expression of *OsNRT2.2* was induced by nitrate treatment, indicating that nitrate induction occurred in shoots (Fig. 4). Unlike the expression in roots, the repression of the *OsNRT2.2* expression by ammonium pretreatment was found in shoots. *OsNAR2.1* was barely detectable and expressed synchronously with *OsNRT2.1* in shoots, suggesting that *OsNAR2.1* is also a root-specific gene.

Differentiation of the NRT2 family was demonstrated in rice as shown in the AtNRT2 family (Fig. 2) (Orsel *et al.* 2002b). Phylogenetic analysis demonstrated that OsNRT2.1 and OsNRT2.2 were categorized in a monocotyledonous group. On the other hand, OsNRT2.3 and OsNRT2.4 were categorized in other NRT2 clusters including AtNRT2.5 and AtNRT2.7 (Orsel *et al.* 2002b), which are nitrate-repressible and constitutively expressed genes, respectively (Okamoto *et al.* 2003). *OsNRT2.3*, expressed constitutively, affected neither the nitrate uptake nor expression of *OsNRT2.4* in roots and shoots. Therefore, OsNRT2.3 and OsNRT2.4 are considered to be minor high-affinity nitrate transporters in rice.

In conclusion, it is important to consider *OsNAR2.1*, *OsNRT2.1* and *OsNRT2.2* when breeding for efficient use of nitrogen in rice. Further investigations on *OsNRT2.2* are necessary to understand the regulation of nitrate uptake mechanisms in rice.

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