Spatial expression and regulation of rice high-affinity nitrate transporters by nitrogen and carbon status

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Abstract

The high affinity nitrate transport system (HATS) plays an important role in rice nitrogen acquisition because, even under flooded anaerobic cultivation when NH4+ dominates, significant nitrification occurs on the root surface. In the rice genome, four NRT2 and two NAR2 genes encoding HATS components have been identified. One gene OsNRT2.3 was mRNA spliced into OsNRT2.3a and OsNRT2.3b and OsNAR2.1 interacts with OsNRT2.1/2.2 and OsNRT2.3a to provide nitrate uptake. Using promoter–GUS reporter plants and semi-quantitative RT-PCR analyses, it was observed that OsNAR2.1 was expressed mainly in the root epidermal cells, differently from the five OsNRT2 genes. OsNAR2.1, OsNRT2.1, OsNRT2.2, and OsNRT2.3a were up-regulated by nitrate and suppressed by NH4+ and high root temperature (37 °C). Expression of all these genes was increased by light or external sugar supply. Root transcripts of OsNRT2.3b and OsNRT2.4 were much less abundant and not affected by temperature. Expression of OsNRT2.3b was insensitive to the form of N supply. Expression of OsNRT2.4 responded to changes in auxin supply unlike all the other NRT2 genes. A region from position –311 to –1, relative to the translation start site in the promoter region of OsNAR2.1, was found to contain the cis-element(s) necessary for the nitrate-, but not light- and sugar-dependent activation. However, it was difficult to define a conserved cis-element in the promoters of the nitrate-regulated OsNRT2/OsNAR2 genes. The results imply distinct physiological functions for each OsNRT2 transporter, and differential regulation pathways by N and C status.

Key words: GUS-reporter, NAR2.1, nitrate transporter, NRT2, Oryza sativa, promoter.

Introduction

Nitrogen (N) nutrition affects all levels of plant function, from metabolism to resource allocation, growth, and development (Crawford, 1995; Scheible et al., 1997; Stitt, 1999). The most abundant sources for N acquisition by plant roots is nitrate (NO3-) in natural aerobic soils, due to intensive nitrification of applied organic and fertilizer N. By contrast, ammonium (NH4+) in flooded paddy soils due to the anaerobic soil conditions is the main form of available N (Sasaki and Yamamoto, 1978).

Rice (Oryza sativa L.), one of the world most important staple food crops, has traditionally been cultivated under flooded conditions and takes up N mainly as NH4+.

However, it has been proved that rice can use either NH4+ or NO3- as a satisfactory source of N (Kronzucker et al., 2000; Rubinigg et al., 2002; Qian et al., 2004; Fan et al., 2007; Li et al., 2008). Rice roots have specialized aerenchyma cells and can transport oxygen from the leaf to the rhizosphere (Kirk, 2003), which is then used to oxidize NH4+ to NO3- in the rhizosphere, particularly at the surface of rice roots (Li et al., 2008). Therefore, barely measurable NO3- in the rhizosphere of flooded paddy soil is immediately taken up by rice root cells. It has been predicted that rice roots may take up 15–40% of total N in the form of NO3- in waterlogged paddy soil, and denitrification losses increase
 sharply as root NO\textsubscript{3} uptake decreases (Kirk and Kronzucker, 2005).

Plants have two different uptake systems to cope with low or high NO\textsubscript{3} concentrations in soil, the high affinity and low affinity NO\textsubscript{3}-uptake systems abbreviated to HATS and LATS, respectively (Crawford and Glass, 1998; Forde, 2002). It was suggested that two types of NO\textsubscript{3} transporters, known as the NRT1/PT1 and NRT2 families, contribute to LATS and HATS for both uptake and distribution within the plant (Forde, 2002; Orsel et al., 2002a, b; Miller et al., 2007). Within the Arabidopsis genome, 53 NRT1/PT1 and seven NRT2 family members have been identified. Some NRT2 transporters need a partner protein called NAR2 for their function (Quesada et al., 1994; Zhou et al., 2000; Tong et al., 2005; Okamota et al., 2006; Orsel et al., 2006).

It is reasonable to predict that, at the low rhizosphere concentrations found in flooded paddy soil, NO\textsubscript{3} will be taken up mainly by the HATS rather than the LATS of rice roots (Fan et al., 2005). In the rice (Oryza sativa L. subsp. japonica cv. Nipponbare) genome, four putative NRT2 and two putative NAR2 genes that encode HATS components have previously been identified (Araki and Hasegawa, 2006; Cai et al., 2008). OsNRT2.1 and OsNRT2.2 share an identical coding region sequence with different 5′- and 3′-untranscription regions (UTR). Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that the expression responses to NO\textsubscript{3} supply differed among the gene members (Araki and Hasegawa, 2006; Cai et al., 2008).

Several putative NO\textsubscript{3} response motifs have been reported in the genomes of higher plants (Rastogi et al., 1997; Hwang et al., 1997; Girin et al., 2007; Konishi and Yanagisawa, 2010). In spinach, a 30 bp region of GATAN24bpGATA from OsNRT2.2, and OsNRT2.3a, while OsNAR2.2 did not show this function.

### Materials and methods

#### Generation of promoter–GUS transformation plants

The gene names and accession numbers or ID of the five NRT2 NO\textsubscript{3} transporters and the NAR2 member within the rice genome are OsNRT2.1 (AB008519), OsNRT2.2 (AK109733), OsNRT2.3a (AK109776), OsNRT2.3b (AK072215), OsNRT2.4 (LOC_Os01g3672), and OsNAR2.1 (LOC_Os02g38320). The regions of putative promoter of OsNAR2.1 and OsNRT2s immediately upstream of the translation start codon were PCR amplified from the genomic DNA of Oryza sativa L. subsp. japonica cv. Nipponbare, using the primers listed in Supplementary Table S1 at JXB online. The PCR products were first cloned into the pMD19-T vector (TaKaRa). After being confined by restriction enzyme digestion and DNA sequencing, each of the cloned fragments in the T-vector was cut and inserted upstream of the 5′ end of the GUS reporter gene in the binary vectors pStuAg-3 (kindly provided by Dr Delhaize, CSIRO Plant Industry, http://www.pi.csiro.au). The constructs pOsNAR2.1 (1698bp-GUS), pOsNAR2.2 (1311bp-GUS), pOsNRT2.1 (1652bp-GUS), pOsNRT2.2 (1000bp-GUS), pOsNRT2.3 (2228bp-GUS), and pOsNRT2.4 (1351bp-GUS) were obtained. All the constructs were transformed into callus initiated from mature wild-type plants (Oryza sativa L. subsp. japonica cv. Nipponbare) seeds by Agrobacterium tumefaciens strain (EHA105)-mediated transformation (Upadhyaya et al., 2000).

#### Plant materials and growth conditions

The seeds of all plants were sterilized using the method previously described by Li et al. (2006). Their seedlings were separately cultured in deionized water with or without 25 mg l\textsuperscript{-1} hygromycin (Roche, USA). IRRI nutrient solution (1.25 mM NH\textsubscript{4}NO\textsubscript{3}, 0.3 mM KH\textsubscript{2}PO\textsubscript{4}, 0.35 mM K\textsubscript{2}SO\textsubscript{4}, 1 mM CaCl\textsubscript{2}.2H\textsubscript{2}O, 1 mM MgSO\textsubscript{4}.7H\textsubscript{2}O, 0.5 mM Na\textsubscript{2}SiO\textsubscript{3}, 20 μM NaFeEDTA, 20 μM H\textsubscript{2}BO\textsubscript{3}, 9 μM MnCl\textsubscript{2}.4H\textsubscript{2}O, 0.32 μM CuSO\textsubscript{4}.5H\textsubscript{2}O, 0.77 μM ZnSO\textsubscript{4}.7H\textsubscript{2}O, and 0.39 μM Na\textsubscript{2}MoO\textsubscript{4}.2H\textsubscript{2}O, pH 5.5) was supplied as described previously (Fan et al., 2007), and replaced every 2 d. To inhibit nitrification, 7 μM dicyandiamide (DCD-C\textsubscript{2}H\textsubscript{4}N\textsubscript{4}) was mixed into all the solutions. All the plants were grown in a growth chamber (Thermoline Scientific Equipment Pty. Ltd, Smithfield, Australia) at 30/22°C day/night temperature with a 16/8 h light/dark regime (light starts at 06.00 h and dark starts at 22.00 h) with a light intensity of 400 μmol m\textsuperscript{-2} s\textsuperscript{-1}. The relative humidity was kept at 65–70%.

#### RNA extraction and semi-quantitative RT-PCR

Total RNA from tissues of the plants was isolated using the guanidine thiocyanate extraction method with Trizol reagent (Invitrogen, Shanghai, China). First-strand cDNA synthesis using M-MLV reverse transcriptase (Promega Madison, WI, USA) was performed with approximately 1 μg total RNA and oligo-dT primer (Invitrogen, Shanghai, China). The mRNA of OsACT (OsRac1, accession number AB047313) was used as the internal standard. Two independent plant cultivations were conducted and all samples were analysed twice by this technique.

#### GUS staining and quantitative measurement of GUS activity

Plant tissues were used in histochemical detection of GUS as described by Jefferson et al. (1987). The GUS reaction mix containing 0.05 mM sodium phosphate buffer, pH 7.0, 1 mM X-gluc, and 0.1% (v/v) Triton X-100. Samples were then incubated at 37°C overnight. To define the type of cells showing GUS signals, the stained root materials were embedded in Spurr resin and sectioned for optical microscopy. The detailed protocol was...
described by Ai et al. (2009). About 20 mg root samples were homogenized in 200 μl of extraction buffer (50 mM Na2HPO4, 10 mM dithiothreitol, 10 mM Na-EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100), followed by centrifugation. Enzyme reactions were carried out in 200 μl of extraction buffer containing 1 mM 4-methylumbelliferyl-6-D-glucuronide at 37 °C, and were stopped by the addition of 20 μl reaction buffer and 180 μl of 0.2 M Na2CO3. Protein concentrations were estimated with the colorimetric method of Bradford (1976). The 4-methylumbelliferyl-one fluorescence was measured using fluorometer.

The mRNA synthesis of OsNRT2s and NAR2s cDNAs, and analyses of 15N-NO3 uptake in oocytes

Full-length cDNAs were obtained from Dr Shoshi Kikuchi (National Institute of Agrobiological Sciences Rice Full-Length cDNA Project Team, Japan), the one exception was OsNAR2.1, which was cloned using the primers: OsNAR2.1F: 5'-CAATGCGC-GAGGCTAGCCGGCGTT-3'; OsNAR2.1R: 5'-CGATCTACTT-GTC CCTTCTGGCCTTCT-3' into the pGEM-T Easy vector (Promega). All of these cDNAs were then subcloned into the BigIII and SpeI sites of the oocyte expression vector pT7TS (Cleaver et al., 1996) using a directional cloning method. The PCR primers for subcloning are listed in Supplementary Table S3 at JXB online.

Oocyte preparation, mRNA injection, 15N-NO3 uptake were conducted as described previously (Tong et al., 2005; Orsel et al., 2006). Oocyte mRNA mixtures were injected as 50 ng OsNRT2.25 ng OsNAR2. Slight changes to the method for oocyte preparation were made. Collagenase (Sigma) was used at 0.03 to 0.05 g ml−1 to pre-treat the oocytes in Ca2+-free MBS for 8–15 min. Oocytes were incubated for 16 h in modified Barth’s saline (MBS) containing 0.5 mM 15N-NO3.

Expression of both OsNRT2.1 and OsNRT2.2 was obtained in most cell types of the roots (Fig. 2C–F). Abundant GUS expression driven by the OsNRT2.3 promoter was detected in the stelar cells of both primary and lateral roots, particularly at the site of lateral root emergence (Fig. 2G–I). The GUS staining showed that all these genes were expressed in the root–shoot junction (Fig. 1D) and only OsNRT2.4 was not expressed in the vascular tissues of adventitious root primordia (Fig. 1E) at this developmental stage. GUS staining for OsNRT2.1, OsNRT2.2, OsNRT2.3, and OsNRT2.4 expression was also detected in the leaves (Fig. 1F), consistent with the previous detection by RT-PCR (Araki and Hasegawa, 2006).

Differential expression profiles were observed for OsNAR2.1 and OsNRT2s in germinating seeds (Fig. 1G) and flowers (Fig. 1H, I). The expression was localized in the coleoptile for OsNAR2.1, at the base of the embryo for OsNRT2.1, both in the embryo and coleoptiles abundantly for OsNRT2.2, and in the germ tip for OsNRT2.3. No GUS staining was detected for OsNRT2.4, but note that, in comparison to the other images shown, the germination stage was earlier (Fig. 1G) and expression may be developmentally regulated. In the flowers, no GUS staining was detected for OsNAR2.1 and in both the hulls and anthers only weak staining for OsNRT2.1 was found. However, strong expression was observed for OsNRT2.2 in the hull and the anther. Expression of OsNRT2.3 was in the seed scutellum and OsNRT2.4 was found in both ends of the hull and in vascular tissue of the anther (Fig. 1H, I).

Expression of OsNAR2.1 and OsNRT2 genes was regulated by nitrate and ammonium

Seedlings were deprived of N for 2 weeks after germination and then supplied with 0.2 mM NO3 (LN), 5 mM NO3 (HN), 0.2 mM NH4 (LA) or 5 mM NH4 (HA) as N sources for 24 h before the expression analyses. The rice EST databases were searched in detail and another putative NRT2 family member was found: OsNRT2.3b (AK072215) encoding a 486 amino acid protein that might arise from the alternative splicing of OsNRT2.3 (AK109776) that encodes a 516 amino acid protein. OsNRT2.3 (AK109776) was named as OsNRT2.3a in this study. Both cDNA clones were deposited in the KOME database (http://cdna01.dna.affrc.go.jp/cDNA/). The transcriptional expression of OsNAR2.1, OsNRT2.1, OsNRT2.2, OsNRT2.3a, and OsNRT2.4 was much higher in roots supplied with NO3 than when compared with NH4+ solution, while no significant difference was found between LN and HN treatments (Fig. 3A–C). This result is different from the observation for low NO3-induced expression of NRT2/NAR2s in Arabidopsis (Nazoa et al., 2003; Krouk et al., 2006; Orsel et al., 2006). High NO3 supply greatly suppressed the expression of the NRT2 genes in Arabidopsis (Orsel et al., 2002b; Girin et al., 2007). OsNRT2.1 and OsNRT2.3a transcripts were not detected in HA treatments, while OsNRT2.1 transcript was low under LA supply (Fig. 3A, B). The expression of OsNRT2.3b was relatively stable in the
Fig. 1. Localization of pOsNAR2.1-GUS and pOsNRT2s-GUS expression in transgenic rice plants. (A–F) GUS staining pattern in tissues of 10-d-old seedlings cultured with de-ionized water with 25 mg l$^{-1}$ hygromycin. (A) Root tip; (B) root hair zones; (C) root lateral branching zones; (D) hand-cut cross-section of the root–shoot junction; (E) adventitious root primordia (ARP); and (F) leaves. (G) 3-d-old germinated seeds; (H) anthers; (I) caryopsis. (A, B, C, F, G, I) Bar=1 mm; (D, E, H) bar=0.5 mm.
roots and was independent of the N supply form and concentration (Fig. 3A–C). More PCR cycles were required for detecting the expression of OsNRT2.3b and OsNRT2.4 in the roots (Fig. 3A).

Transgenic plants of pOsNAR2.1-GUS and pOsNRT2-GUS were used to investigate the effect of the N source on the promoter activity. Five independent transgenic lines were grown in nutrient solution containing 0.2 mM NO₃⁻ (LN) or
0.2 mM NH$_4^+$ (LA) as N sources for 1 week (Fig. 3D). The spatial pattern of *pOsNAR2.1-GUS*, *pOsNRT2.1-GUS*, and *pOsNRT2.2-GUS* expression remained similar, but with different intensities in the older parts of the roots between LN and LA treatments. When compared with low NO$_3^-$ treatment, low NH$_4^+$ supply decreased the GUS activity driven by the promoters of *OsNAR2.1* and *OsNRT2* genes (Fig. 3D). High (5 mM) NH$_4^+$ treatment further suppressed root expression of these genes. Furthermore, quantification of the change in GUS activity associated with altering the N supply form supported the results of semi-quantitative RT-PCR (comparing Fig. 3B, C, and D).

Expression of *OsNAR2.1* and *OsNRT2* genes was regulated by light change and sucrose

In response to the light/diurnal changes in photosynthesis, *OsNAR2.1* and *OsNRT2*s expression in roots varied between day and night (Fig. 4A–C). During the diurnal cycle, *OsNAR2.1* and *OsNRT2*s transcript level peaked after the first 4 h of the light period (10.00 h) then declined in the latter part of the day. The expression of *OsNRT2.4* in the roots was detected only after the first 4 h of the light period. For other genes, specifically *OsNRT2.2* and *OsNRT2.3a*, there was a second peak of transcript accumulation at the end of light period (22.00 h), but at a lower level than that measured after the first 4 h of light (Fig. 4A-C).

To test if these diurnal changes in root expression were directly dependent on the C status of the rice, the effect of supplying external sucrose on the transcriptional activation of *OsNAR2.1* and *OsNRT2*s (Fig. 4D) was also investigated. Ten-day-old seedlings were supplied with nutrient solution containing 0.2 mM NO$_3^-$ for 3 d before adding 3% sucrose in the dark period. The addition of external sucrose increased the accumulation of *OsNAR2.1*, *OsNRT2.1*, and *OsNRT2.3a* transcripts by about 3–5-fold, *OsNRT2.2* and *OsNRT2.3b* transcripts by about 2-fold, and *OsNRT2.4* transcripts from non-detectable to the similar level to that observed in the first 4 h of light period without external C supply (Fig. 4D, E, F).

Expression of *OsNAR2.1* and *OsNRT2* genes in response to changes of root temperature, solution pH, and exogenous hormones

As the expression of several rice NO$_3^-$ transporters was modified by changes in the light regime and form of N supply, it was investigated if other environmental factors and plant hormones can impact on the pattern of transcription. Changes in plant hormones such as auxin, may mediate the changes in transporter expression. Expression of *OsNAR2.1* and *OsNRT2* genes in response to high root temperature was examined by semi-quantitative RT-PCR. Seedlings were cultured with de-ionized water for 1 week at 23 °C, then were transferred into de-ionized water at 37 °C. Short exposure of the roots to the high temperature decreased the expression of *OsNAR2.1*, and almost completely suppressed expression of *OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3a* (Fig. 5A, B). By contrast, *OsNRT2.3b* and *OsNRT2.4* maintained their expression levels in the high temperature-treated roots (Fig. 5A, C).

It was also observed that transcriptional expression of the *OsNAR2.1*, *OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3a* genes was not sensitive to changes in the solution pH after 24 h (Fig. 5D–F). The transcript of *OsNRT2.3b* showed the lowest level when exposed to pH 5.5, lower than that at pH 3.0 and pH 8.0, while the opposite happened to the expression of *OsNRT2.4*. The expression of *OsNAR2.1* and *OsNRT2* except *OsNRT2.4* were not modified by treatment with 10 µM exogenous synthetic auxin (1-naphthalene acetic acid: NAA) or auxin transport inhibitor (N-1-naphthylphthalamic acid: NPA) (Fig. 5G–I). Expression of *OsNRT2.3b* and *OsNRT2.4* was up-regulated by NAA treatment, and *OsNRT2.4* was down-regulated by NPA treatment.

Functional assay of *OsNAR2*s and *OsNRT2*s in Xenopus oocytes

Gene expression profiles of *OsNAR2.1*, *OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3a* in the roots showed similar results in response to different N forms, C and environmental factors (Figs 3, 4, 5). To test whether *OsNAR2.1* is a partner in a two-component nitrate transport system with *OsNRT2.1*/OsNRT2.2 (both have the same ORF) and *OsNRT2.3*, both mRNAs were tested in the *Xenopus* oocyte expression system. The uptake of $^{15}$N-nitrate into oocytes injected with *OsNRT2.1* mRNA or combinations of *OsNAR2.1* and *OsNRT2.1* mRNA was increased compared with water or *OsNAR2.1* mRNA injected controls (Fig. 6A). The functional activity of *OsNRT2.1* was greatly enhanced by co-injection with *OsNAR2.1* mRNA (Fig. 6A). However, single *OsNAR2.1* mRNA injected oocytes did not show any nitrate transport activity. There was no effect of co-injecting *OsNRT2.2* mRNA on *OsNRT2.1* transport activity (Fig. 6A).

Furthermore, $^{15}$N-enriched nitrate influx into oocytes injected with mRNA of *OsNAR2.1*, *OsNRT2.3a*, *OsNRT2.3b* or combinations of *OsNAR2.1* and *OsNRT2.3a* or *OsNRT2.3b* was measured. Interestingly, *OsNRT2.3b* alone could transport nitrate and co-injection of both *OsNRT2.3b* and *OsNAR2.1* mRNAs did not improve this function (Fig. 6B). However, there was no functional nitrate transport activity detected in *OsNRT2.3a* mRNA-injected oocytes. The co-injection of both *OsNRT2.3a* and *OsNAR2.1* mRNAs in the oocytes resulted in very large nitrate influxes (Fig. 6B). Co-injecting *OsNAR2.2* mRNA did not increase either *OsNRT2.3a* or *OsNRT2.3b* nitrate transport activity in oocytes (Fig. 6C).

Effect of 5′ deletions of the *OsNAR2.1* promoter on its activity in the responses to the regulation of N form and light/diurnal change

Since transcriptional expression of *OsNAR2.1* was regulated strongly by the form of N, external sucrose supply,
and diurnal changes of light, the regions of the putative OsNAR2.1 promoter which might contain key motifs, probably controlling root expression and responsiveness to the N form and sugar status, were investigated. The promoter fragments of 311 bp and 1698 bp upstream of the translational start codon were fused to the GUS reporter gene and introduced into transgenic rice. The transgenic lines for each construct were obtained and treated with different N forms and sucrose supply and sampled at various stages during the diurnal light cycle. At least five independent lines (biological replicates) were collected for the observation of their GUS-staining pattern. Overall, there were some changes of the intensity and tissue localization between pOsNAR2.1(1698bp)-GUS and pOsNAR2.1(311bp)-GUS plants. GUS staining was detected mainly in the root tip and root–shoot junction, but not in the root hair and lateral branching zone of the pOsNAR2.1(311bp)-GUS plants grown in NO_{3}^{-}/C_{0}^{+} solution (Table 1; Fig. 7). Nevertheless, expression of the GUS reporter driven by either pOsNAR2.1(1698bp) or pOsNAR2.1(311bp) in the transgenic lines was strongly induced by NO_{3}^{-} and almost completely repressed by NH_{4}^{+} (Fig. 7; Table 2). This result indicated that NO_{3}^{-} responsive cis-elements involved in the N source regulation are present in the first 311 bp of the OsNAR2.1 promoter sequence. Motif analysis showed three putative NO_{3}^{-}-responsive cis-elements [5′-GATA-3′ (Rastogi et al., 1997, Bi et al., 2005), 5′-A(C/G)TCA-3′...
(Hwang et al., 1997), and 5′-GAC(TTNT)AAG-3′ (Konishi and Yanagisawa, 2010)] are presented in this region of the OsNAR2.1 promoter (Fig. 8). However, the stimulation of the GUS reporter gene expression by sucrose and light was maintained in pOsNAR2.1 (1698bp)-GUS rice, but not in pOsNAR2.1(311)-GUS rice (Table 2). This result indicated that the cis-elements involved in the responses of OsNAR2.1 expression to light and sugar were present in the -1698 to -311 region of the promoter sequence. This analysis of the OsNAR2.1 promoter region has defined and separated N and C responsive elements.

Discussion

The expression profiles of the Arabidopsis NRT2 genes have been well described (Orsel et al., 2002a, b; Okamoto et al., 2003). The AtNRT2.1, AtNRT2.2, and AtNAR2.1 genes are all important contributors to HATS in Arabidopsis roots (Orsel et al., 2006). Since rice roots are commonly grown in flooded paddy soils and trace NO3 exists in its rhizosphere, the regulation and function of NAR2/NRT2 genes in rice are worthy of investigation. Although there are two NAR2-like genes in the rice genome (Araki and Hasegawa, 2006), only OsNAR2.1 interacts with OsNRT2.1/2.2 and
OsNRT2.3a to provide NO₃ uptake in the oocyte expression system (Fig. 6). The transporters OsNRT2.3b and OsNRT2.4 could function independently with no NAR2 requirement for NO₃ transport. No function for OsNAR2.2 in NO₃ transport could be identified in the

Table 1. Tissue specific localization of pOsNAR2.1(311)-GUS and pOsNAR2.1(1698)-GUS expression in rice detected by GUS staining

<table>
<thead>
<tr>
<th>Tissue</th>
<th>pOsNAR2.1 (311)-GUS</th>
<th>pOsNAR2.1 (1698)-GUS</th>
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<tbody>
<tr>
<td>Root tip</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Root hair zone</td>
<td>−</td>
<td>+++</td>
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<tr>
<td>Root lateral branching zone</td>
<td>−</td>
<td>+++</td>
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<tr>
<td>Root-shoot junction</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Leaf</td>
<td>−</td>
<td>−</td>
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<td>Flower</td>
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<td>Caryopsis</td>
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Table 2. A summary of regulation of pOsNAR2.1(311)-GUS and pOsNAR2.1(1698)-GUS by N supplies, light, and sucrose detected by GUS staining

For varying N supplies, rice seedlings were treated with 0.2 mM NH₄⁺, 0.2 mM NO₃⁻ for 7 d. For light experiments, rice seedlings were placed under light for 4 h. For sucrose treatment, rice seedlings were transferred into 0.2 mM NO₃⁻ in the presence of 3% sucrose for 8 h in the dark. Stained tissues were classified as − (not detectable) to ++++ (very abundant) according to the intensity of GUS staining.

Fig. 7. Effect of different N supplies on the GUS activity in the roots of pOsNAR2.1(311)-GUS and pOsNAR2.1(1698)-GUS rice. Seedlings were grown in nutrient solution containing 0.2 mM NH₄⁺, 0.2 mM NO₃⁻ Analysis was performed on five independent transgenic lines. Expression patterns of a representative transgenic line for each treatment is presented.

OsNRT2.3a to provide NO₃ uptake in the oocyte expression system (Fig. 6). The transporters OsNRT2.3b and OsNRT2.4 could function independently with no NAR2 requirement for NO₃ transport. No function for OsNAR2.2 in NO₃ transport could be identified in the
oocyte system (Fig. 6). The methods of promoter-GUS-reporter gene transformation and semi-quantitative RT-PCR identified the expression patterns of OsNRT2s and OsNAR2.1 in response to N source, light/diurnal change, sucrose, root temperature, solution pH, and exogenous hormones in rice. The results provided some insights into the role, regulation, and tissue localization of each individual high-affinity transport component during rice growth.

Differential tissue localization and regulation by N sources suggests different roles of OsNRT2s and OsNAR2.1 in the acquisition and translocation of N

In rice roots, expression of OsNAR2.1 was strongest in the epidermal cells, but included the same tissue types as obtained for OsNRT2.1, OsNRT2.2, and OsNRT2.3 (Fig. 2A, B). The RT-PCR results showed that the expression of OsNAR2.1, OsNRT2.1, OsNRT2.2, and OsNRT2.3a under NO3− treatment was significantly higher than under NH4+ treatment in roots (Fig. 3A–C). This result was consistent with the decreased GUS activity measured in the roots of pOsNAR2.1-GUS and pOsNRT2s-GUS transgenic plants treated with NH4+ (Fig. 3D).

The relative mRNA expression levels of OsNAR2.1, OsNRT2.1, OsNRT2.2, and OsNRT2.3a were similar (Fig. 3B), while the GUS activity driven by OsNRT2.1 or OsNRT2.2 promoters was stronger than that given by the OsNAR2.1 or OsNRT2.3 promoters in Fig. 3D. This result might be due to localized differences in their expression. As shown in Fig. 2, OsNAR2.1 promoter–GUS expression was very abundant in epidermal cells with much lower levels in the cortical and stelar cells (Fig. 2A, B), while the OsNRT2.3 promoter drove GUS expression only in the root stelar cells (Fig. 2G–I). By contrast, GUS expression driven by OsNRT2.1 or OsNRT2.2 was abundant in most cell types of the roots (Fig. 2C–F). Nevertheless, both RT-PCR and GUS expression analyses showed similar trends, for example, all these genes were down-regulated by ammonium supply in the external solution.

Previous studies had proved that some NRT2 transporters required a second protein, NAR2 to produce functional HATS (Quesada et al., 1994; Zhou et al., 2000; Tong et al., 2005; Orsel et al., 2006). The expression pattern of AtNAR2.1 almost exactly parallels that of AtNRT2.1 (Orsel et al., 2006; Wirth et al., 2007) and both genes are similarly repressed by NO3− (Krouk et al., 2006). Since OsNAR2.1 could co-operate with OsNRT2.1, OsNRT2.2, and OsNRT2.3a to provide NO3− uptake in oocytes (Fig. 6), and the consistent expression pattern of the gene partners in response to N source and environmental factors proved that the two-component NO3− uptake system occurs in rice. Furthermore, the very strong expression profiles in epidermal cells indicated that OsNAR2.1 and OsNRT2.1 (or OsNRT2.2) were playing a key role in NO3− uptake by roots. While OsNRT2.2 was strongly expressed not only in roots, but also in the caryopsis (Fig. 1G), suggesting it has another role in NO3− transport during seed development. The role of OsNRT2.2 in rice may be orthologous to that of AtNRT2.7 in Arabidopsis (Chopin et al., 2007), although this would require targeting of the transporter to the vacuolar membrane.

For the OsNRT2.3 genes, the root expression level of OsNRT2.3a of NO3−-treated seedlings was significantly higher than with NH4+ supply. Supply of 5 mM NH4+ more strongly inhibited the expression of OsNRT2.3a, when compared with a 0.2 mM NH4+ supply. However, OsNRT2.3b showed the same transcription level in NO3− and NH4+ treatments (Fig. 3A, C). Compared with OsNRT2.3b, OsNRT2.3a had additional nucleotide sequences of 90 bp in cDNA sequence. OsNRT2.3a and OsNRT2.3b shared the same upstream sequences before their translation start codon (ATG), but their 5′ UTR and 3′ UTR are different according to the cDNA sequences in the KOME website (http://cdna01.dna.affrc.go.jp/cDNA/). GUS staining in the transgenic plants showed that OsNRT2.3 was abundantly expressed in the stelar tissues of roots, root–shoot junctions, and shoots. This expression pattern suggested that OsNRT2.3 was responsible for NO3− transport inside the plant.

GUS staining showed OsNRT2.4 was specially expressed in the base of lateral root primordia (Fig. 1A, B). Weak GUS activity was also found in the flowers of transgenic plants (Fig. 1H, I). Semi-quantitative RT-PCR results showed that OsNRT2.4 was expressed mainly in leaves and was down-regulated in roots by NH4+ supply (Fig. 3A, C). Furthermore, the synthetic auxin NAA enhanced OsNRT2.4 expression whereas the auxin transport inhibitor NPA decreased expression (Fig. 5G–I), suggesting that OsNRT2.4 function might be important for lateral root

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**Fig. 8.** Bioinformatic prediction of the nitrate-responsive cis-elements in the –311 bp to –1 bp region of the OsNAR2.1 promoter. Motif I [TATABOX (TATAAAT)] and motif II [CAATBOX (CAAT)] are conserved motifs involved in transcription regulation. Motif III [5′-GATA-3′ (Rastogi et al., 1997)], motif IV [5′- A/C/G)TCA-3′ (Hwang et al., 1997)] and motif V [5′- GACCTTN10AAG-3′ (Konishi et al., 2010)] were proposed to be putative nitrate-responsive cis-elements (NRE).
development and involve auxin signalling. Interestingly, the expression of none of the other rice NRT2/NAR2 genes was sensitive to these auxin treatments, implicating the specificity of OsNRT2.4 in NO$_3$/auxin responses.

The environmental regulation of OsNAR2.1 and OsNRT2 expression

Uptake of NO$_3$ is under diurnal regulation in many plant species (Delhon et al., 1995; Cárdenas-Navarro et al., 1998; Lejay et al., 1999). Furthermore, the circadian regulation of the expression of many genes for NO$_3$ transport (Cárdenas-Navarro et al., 1998) and assimilation (Pilgrim et al., 1993; Yang et al., 2005) has been demonstrated in plants. In Arabidopsis, both AtNRT2.1 and AtNRT1.1 transcript levels in roots were regulated by marked light/diurnal changes and were rapidly increased by sucrose supply at night (Lejay et al., 1999). Addition of sucrose stimulated the accumulation of AtNRT2.1 transcript and the activity of the pAtNRT2.1-GUS reporter gene (Girin et al., 2007). In tomato, LeNRT2 showed the highest peak in transcript levels 4 h after the onset of the light period and a second peak at the end of the light period (Ono et al., 2000). In our study, it was also observed that OsNAR2.1 and OsNRT2s in rice roots were induced by light and their transcripts rose to a maximum level after 4 h in the light (Fig. 4A–C), and increased greatly after sucrose treatment at night (Fig. 4D–F). The second peak of OsNAR2.1 and OsNRT2s mRNA abundance at the end of light (Fig. 4A) may reflect an endogenous circadian regulation. These results suggest that the accumulation of carbon at the end of the light period is stimulating rice nitrate transporter expression as observed for NRT2 transporters in Arabidopsis and other plant species. These data show that even though NH$_4$ is the main N supply form for rice in the field, a common regulatory link between carbon status and NO$_3$ uptake may exist in all plants. This finding confirms that, even in an anaerobic environment, some NO$_3$ N is available. Furthermore, since NH$_4$ supply suppresses the expression of many, but not all OsNRT2s (Fig. 3B, C), this result may argue for NO$_3$-specific regulation of some NRT2 family members.

Rice is grown in paddy soil in the hot summer season and experiences large environmental challenges. It was observed that a high root temperature almost completely suppressed the expression of OsNRT2.1, OsNRT2.2, and OsNRT2.3a genes in rice roots (Fig. 5A–C), indicating that, on a typical hot summer noon, the NO$_3$ uptake in paddy soil may be decreased by the high temperature of soil solution (Zhou et al., 2010). In addition, it was reported that high-affinity NO$_3$ influx rates in rice roots could be increased by acidic (pH 5) and decreased by alkaline (pH 8) nutrient solution pH over short periods of NO$_3$ supply (Cai et al., 2008). However, a large effect of the culture solution pH on the expression of the OsNAR2.1 and OsNRT2 genes was not found in the roots (Fig. 5D–F). Only OsNRT2.3b and OsNRT2.4 expression was slightly, but oppositely affected by both acidic and alkaline pH (Fig. 5D–F). Changes in transcriptional expression of the transporter components may not explain changes in influx as post-translational regulation and transporter turnover may be having a more direct influence.

The 311 bp sequence of OsNAR2.1 promoter is required for nitrate stimulation and ammonium repression, but not for sensing C status

Nitrate acts as a signal molecule (Tischner et al., 1993; Wang et al., 2000, 2009; Scheible et al., 2004), and its regulation has been described to occur at the transcriptional level (Vaucheret et al., 1992; Neininger et al., 1993; Vincentz et al., 1993) and the protein level (Wirth et al., 2007). However, at present, the trans-acting factors that directly regulate nitrate-responses in higher plants are not well characterized.

Like the typical behaviour of other NO$_3$-induced genes, it was observed that OsNAR2.1 was up-regulated by NO$_3$ and down-regulated by NH$_4$ (Figs 3, 7), and that transcripts also responded to diurnal light/dark changes and increased sugar supply (Fig. 5). Furthermore, OsNAR2.1 is an essential partner for OsNRT2.1, OsNRT2.2, and OsNRT2.3a in NO$_3$ transport (Fig. 6). Therefore, the OsNAR2.1 promoter region was analysed to identify elements responding to N sources and C status. The GUS staining indicated that a short fragment of 311 bp upstream of the OsNAR2.1 translation start codon could respond to the N supply form. This result is like that for a longer (1689 bp) fragment (Fig. 7; Table 2), implying that the 311 bp promoter region contains the necessary cis-elements for combination with NO$_3$ signal factor(s). Four copies of the 5′-GATA-3′ motif and one copy of the 5′-A(C/G)TCA-3′ motif was found within this 311 bp region. The GATA-box and 5′-A(C/G)TCA-3′ motifs are also present within the 1000 bp promoter regions of all the OsNRT2 genes (see Supplementary Table S4 at JXB online). However, expression of OsNRT2.3b, unlike OsNRT2.3a, was not regulated by a change in N supply form (Fig. 3A, B). Since OsNRT2.3a and OsNRT2.3b shared the same upstream sequences before their translation start codon, it is hard to predict if GATA-box and 5′-A(C/G)TCA-3′ motifs are potential candidate fragments responding to NO$_3$ signals without separating the promoters of OsNRT2.3a and OsNRT2.3b genes.

Konishi and Yanagisawa (2010) used the upstream region of less than 300 bp from the start codon to compare alignments of the nitrite reductase gene (NIR1) promoters from different plants. They identified and proved that one conserved sequence of 5′-GCCCCTTN$_{10}$AAG-3′ was located in the region from −74 to −93 bp of the AtNIR1 promoter. One motif of 5′-GACCCCTTN$_{10}$AAG-3′, found at the region between −191 and −172 bp of the OsNAR2.1 promoter, showed a high similarity with 5′-GCCCCTTN$_{10}$AAG-3′; only one base was different, a change from A to C. However, there was no such conserved sequence in the −1000 bp promoter regions of OsNRT2 genes in rice, and AtNAR2.1/AtNAR2 genes in Arabidopsis (see Supplementary Table S4 at JXB online).
Since the -311 bp region with the 5'-GCCcCTTN{10}AAG-3' motif was necessary for NO\textsubscript{3} induction of OsNAR2.1 expression (Fig. 7; Table 2), OsNAR2.1 might be a key gene for the early sensing of the NO\textsubscript{3} supply and transferring this signal to the NO\textsubscript{3} uptake system by interacting with OsNRT2.1, OsNRT2.2, and OsNRT2.3a at the protein level (Fig. 6).

Plant NO\textsubscript{3} uptake is regulated by both N and C status (Krouk \textit{et al}., 2010). Previous reports and this study confirmed that the NO\textsubscript{3}-regulated \textit{NRT2/NAR2} genes were also affected by diurnal light/dark change, and that external sugar supply could overcome the dark-induced suppression of \textit{NRT2/NAR2} genes (Lejay \textit{et al}., 1999; Krouk \textit{et al}., 2010; Fig. 4). In \textit{Arabidopsis}, it has been shown that the activity of a \textit{cis}-acting 150 bp element was regulated by both nitrate and sucrose supply to the roots (Girin \textit{et al}., 2007). It was observed that the 311 promoter region of the \textit{OsNAR2.1} gene could drive low level expression of the GUS reporter in the roots, however, regulation by diurnal light/dark change and external sugar supply had disappeared (Table 2). These results indicate the possible location of different \textit{cis}-elements in the promoter region of the \textit{OsNAR2.1} gene that are required for responding to changes in N and C status. Even though paddy rice has NH\textsubscript{4}\textsuperscript{+} as a primary N source, the regulation of \textit{OsNAR2.1}/\textit{OsNRT2} gene expression by N sources and light/dark diurnal and sugar supply as in \textit{Arabidopsis} (Lejay \textit{et al}., 1999; Girin \textit{et al}., 2007) shows that there are common feedback regulatory pathways for C/N balance in both species.

**Supplementary data**

Supplementary data can be found at JXB online.

Supplementary Table S1. The primers for promoters of Os\textit{NAR2.1} and Os\textit{NRT2} genes.

Supplementary Table S2. The primers for semi-quantitative RT-PCR of Os\textit{NAR2.1} and Os\textit{NRT2} genes.

Supplementary Table S3. Primers for amplifying the full-length cDNA of Os\textit{NAR2s} and Os\textit{NRT2s} for mRNA synthesis.

Supplementary Table S4. Distribution of the CTTN\{0,15\}AAG motifs within the promoter sequences of the Os\textit{NAR2.1}, Os\textit{NRT2s}, At\textit{NAR2.1}, and At\textit{NRT2s}.

Supplementary Table S5. Putative motifs in Os\textit{NAR2.1} and Os\textit{NRT2s} promoters involved in N metabolism, light, and C metabolism.

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