Knockdown of a Rice Stelar Nitrate Transporter Alters Long-Distance Translocation But Not Root Influx[1][W][OA]

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Root nitrate uptake is well known to adjust to the plant's nitrogen demand for growth. Long-distance transport and/or root storage pools are thought to provide negative feedback signals regulating root uptake. We have characterized a vascular specific nitrate transporter belonging to the high-affinity Nitrate Transporter2 (NRT2) family, OsNRT2.3a, in rice (*Oryza sativa* ssp. *japonica* 'Nipponbare'). Localization analyses using protoplast expression, in planta promoter-β-glucuronidase assay, and in situ hybridization showed that *OsNRT2.3a* was located in the plasma membrane and mainly expressed in xylem parenchyma cells of the stele of nitrate-supplied roots. Knockdown expression of *OsNRT2.3a* by RNA interference (RNAi) had impaired xylem loading of nitrate and decreased plant growth at low (0.5 mM) nitrate supply. In comparison with the wild type, the RNAi lines contained both nitrate and total nitrogen significantly higher in the roots and lower in the shoots. The short-term $[^{15}N]NO_3^-$ influx (5 min) in entire roots and NO_3^- fluxes in root surfaces showed that the knockdown of OsNRT2.3a in comparison with the wild type did not affect nitrate uptake by roots. The RNAi plants showed no significant changes in the expression of some root nitrate transporters (*OsNRT2.3b*, *OsNRT2.4*, and *OsNAR2.1*), but transcripts for *nia1* (nitrate reductase) had increased and *OsNRT2.1* and *OsNRT2.2* had decreased when the plants were supplied with nitrate. Taken together, the data demonstrate that OsNRT2.3a plays a key role in long-distance nitrate transport from root to shoot at low nitrate supply level in rice.

In soil, inorganic nitrogen (N) is available for plants as nitrate in aerobic uplands and ammonium in flooded anaerobic paddy fields. In many plants, the nitrate acquired by roots is transported to the shoots before being assimilated (Smirnoff and Stewart, 1985). By contrast, ammonium derived from nitrate reduction or directly from ammonium uptake is preferentially assimilated in the root and then transported in an organic form to the shoot (Xu et al., 2012). To cope with varied concentrations of nitrate in soils, plant roots have developed at least three nitrate uptake systems, two high-affinity transport systems (HATS) and one low-affinity transport system (LATS), responsible for the acquisition of nitrate (Crawford and Glass, 1998). The constitutive HATS and nitrate-inducible HATS operate to take up nitrate at low nitrate concentration in external medium, with saturation in a range of 0.2 to 0.5 mM. In contrast, LATS functions in nitrate acquisition at higher external nitrate concentration. The uptake by LATS and HATS is mediated by nitrate transporters belonging to the families of Nitrate Transporter1 (NRT1) and NRT2, respectively (Forde, 2000; Miller et al., 2007). Uptake by roots is regulated by negative feedback, linking the expression and activity of nitrate uptake to the N status of the plant (Miller et al., 2007). Several different N metabolites have been proposed to be cellular sensors of N status, including Gln (Fan et al., 2006; Miller et al., 2008), and one model has root vacuolar nitrate as the feedback signal as these pools increase with plant N status.

Both electrophysiological and molecular studies have shown that nitrate uptake through both HATS and LATS is an active process mediated by proton/nitrate cotransporters (Zhou et al., 2000; Miller et al., 2007). In the Arabidopsis (*Arabidopsis thaliana*) genome, there are at least 53 and seven members belonging to the NRT1 and NRT2 families, respectively (Miller et al., 2007; Tsay et al., 2007). Several Arabidopsis NRT1 and NRT2 family members have been characterized for their functions in nitrate uptake and long-distance transport. AtNRT1.1 (CHL1) is described as a transceptor, playing multiple roles as a dual-affinity nitrate transporter and a

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sensor of external nitrate supply concentration (Liu and Tsay, 2003; Ho et al., 2009; Gojon et al., 2011) and auxin transport at low nitrate concentrations (Krouk et al., 2010). In contrast, AtNRT1.2 (NTL1) is a constitutively expressed low-affinity nitrate transporter (Huang et al., 1999). AtNRT1.4 is a leaf petiole-expressed nitrate transporter and plays a critical role in regulating leaf nitrate homeostasis and leaf development (Chiu et al., 2004). AtNRT1.5 is expressed in the root pericycle cells close to the xylem and is responsible for the loading of nitrate into the xylem for root-to-shoot nitrate transport (Lin et al., 2008). AtNRT1.6 is expressed only in reproductive tissues and is involved in delivering nitrate from maternal tissue to the early-developing embryo (Almagro et al., 2008). AtNRT1.7 functions in phloem loading of nitrate to allow transport out of older leaves and into younger leaves, indicating that source-to-sink remobilization of nitrate is mediated by the phloem (Fan et al., 2009). AtNRT1.8 is expressed predominantly in xylem parenchyma cells within the vasculature and plays the role in the retrieval of nitrate from the xylem sap (Li et al., 2010). AtNRT1.9 facilitates the loading of nitrate into the root phloem, enhancing downward transport in roots, and its knockout increases root-to-shoot xylem transport of nitrate (Wang and Tsay, 2011).

Among the seven NRT2 family members in Arabidopsis, both *AtNRT2.1* and *AtNRT2.2* have been characterized as contributors to nitrate-inducible HATS (Filleur et al., 2001). In addition, *NRT2.1* transport activity requires a second accessory protein, *NAR2.1* (or *NRT3.1*), in Arabidopsis (Okamoto et al., 2006; Orsel et al., 2006; Yong et al., 2010). Knockout of *AtNAR2.1* (*atnar2.1* mutant) had more severe effects on both nitrate uptake at low nitrate concentrations and growth than knockout of its partner, *AtNRT2.1* (*atnrt2.1* mutant), suggesting other functions for *AtNAR2.1* (Orsel et al., 2006). Interestingly, *AtNRT2.7* is expressed specifically in the vacuolar membrane of reproductive organs and controls nitrate content in seeds (Chopin et al., 2007). Recently, *AtNRT2.4* has been found to be a high-affinity plasma membrane nitrate transporter expressed in the epidermis of lateral roots and in or close to the shoot phloem (Kiba et al., 2012). *AtNRT2.4* is involved in the uptake of NO_3^- by the root at very low external concentration and in shoot NO_3^- loading into the phloem and is important under N starvation (Kiba et al., 2012).

The molecular mechanisms of nitrate uptake and translocation in rice (*Oryza sativa*) are not fully understood. Since the nitrate concentration in the rhizosphere of paddy fields is estimated to be less than 10 μM (Kirk and Kronzucker, 2005), NRT2 family members play a major role in nitrate uptake in rice (Araki and Hasegawa, 2006; Yan et al., 2011). In addition, rice roots have abundant aerenchyma for the transportation of oxygen into the rhizosphere, resulting in ammonium nitrification by bacteria on the root surface (Kirk, 2003; Li et al., 2008). Therefore, up to 40% of the total N taken up by rice roots grown under wetland conditions might be in the form of nitrate, and the rates of uptake could be comparable with those of ammonium (Kronzucker et al., 2000; Kirk and Kronzucker, 2005).

Five *NRT2* genes have been identified in the rice genome (Araki and Hasegawa, 2006; Cai et al., 2008; Feng et al., 2011). *OsNRT2.1* and *OsNRT2.2* share an identical coding region sequence with different 5′- and 3′-untranscribed regions and have high similarity to the *NRT2* genes of other monocotyledons, while *OsNRT2.3* and *OsNRT2.4* are more closely related to Arabidopsis *NRT2* genes. We found that *OsNRT2.3* mRNA is actually spliced into two gene products, *OsNRT2.3a* (AK109776) and *OsNRT2.3b* (AK072215), with 94.2% similarity in their putative amino acid sequences (Feng et al., 2011; Yan et al., 2011). *OsNRT2.3a* is expressed mainly in roots, and this pattern is enhanced by nitrate supply, while *OsNRT2.3b* is expressed weakly in roots and relative abundantly in shoots, with no effect of the N form and concentration on the amount of transcript (Feng et al., 2011). We have also detected that *OsNAR2.1* interacts with *OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3a* and affects the activities of both HATS and LATS (Yan et al., 2011). However, no member of the NRT2 nitrate transporter family has been functionally characterized yet in rice. In this study, we generated rice *osnrt2.3a* knockdown lines by RNA interference (RNAi) and demonstrated that *OsNRT2.3a* is specifically expressed in the root stelar tissue and functions in long-distance nitrate transport from roots to shoots, particularly at low external nitrate supply. These *osnrt2.3a* knockdown plants show that in rice, the root pools of nitrate do not feed back to regulate the expression of root uptake transporters. These data show, to our knowledge for the first time, that root nitrate pools are not the indicators of N status signals regulating uptake.

RESULTS

OsNRT2.3a Was Localized to the Plasma Membrane

To determine subcellular location, *OsNRT2.3a* was fused in frame with GFP and transiently expressed in rice protoplasts under the control of the cauliflower mosaic virus 35S promoter. Analyses of *OsNRT2.3a*-GFP-expressing rice protoplast cells by confocal microscopy showed that the green fluorescence was confined to the plasma membrane (Fig. 1). Thus, we confirmed that *OsNRT2.3a* is a plasma membrane-localized nitrate transporter.

OsNRT2.3a Was Mainly Expressed in Root Stelar Cells

We have shown previously that the transcripts of *OsNRT2.3a* are abundantly represented in nitrate-supplied roots and are suppressed by ammonium N, while the transcripts of *OsNRT2.3b* are mainly in shoots and very faint in the roots irrespective of the supplied N form and concentration (Feng et al., 2011). In order to clarify the tissue localization of *OsNRT2.3a*

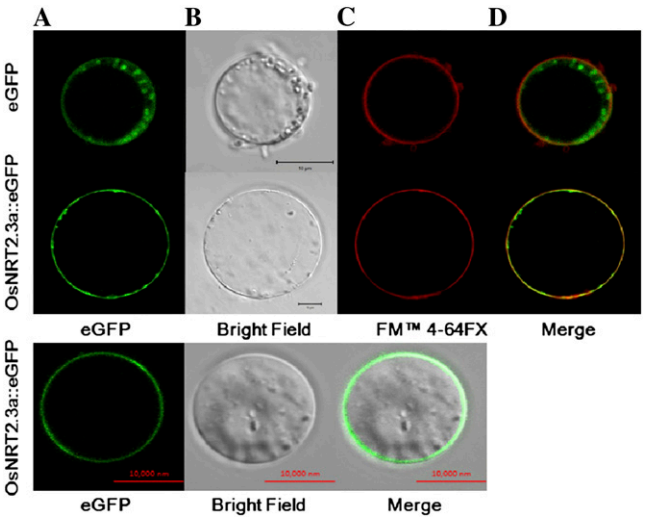

Figure 1. Localization of *OsNRT2.3a* in cell plasma membrane. A, Representative microscopy images of a rice protoplast expressing the NRT2.3a-GFP fusion protein. B, Bright-field images. C, FM4-64FX dye images. D, Overlap of the GFP (green) and FM4-64FX (red) fluorescence. Row 1 is the protoplast expressing GFP used as a control. Row 2 is the protoplast expressing OsNRT2.3a::GFP fusion protein with FM4-64 dye. Row 3 is the protoplast expressing OsNRT2.3a::GFP fusion protein without FM4-64 dye. FM4-64FX is a membrane-selective fluorescent vital dye. Bars = 10 μm.

and *OsNRT2.3b* in rice plants, the spatial distribution of their mRNAs in the root and leaf tissues was examined by in situ hybridization. *OsNRT2.3a* was abundantly expressed in the root stelar cells, particularly in the xylem parenchyma cells, while *OsNRT2.3b* was mainly detected in leaf phloem cells (Fig. 2, A–F). In addition, transgenic plants carrying the GUS gene under the control of the *OsNRT2.3* promoter (2.2 kb) showed that the GUS activity was detected specifically in cells of the stele close to the xylem vessels and pericycle of primary and lateral roots (Fig. 2, G and H; Feng et al., 2011). Combining the results with the in situ hybridization and the native promoter-GUS assay, we conclude that *OsNRT2.3a* is expressed predominantly in the xylem parenchyma cells of the root stele.

Generation of Rice *osnrt2.3a* Knockdown Lines by RNAi

We recently reported that a putative high-affinity nitrate transporter gene, *OsNRT2.3b* (AK072215), arises from alternative splicing of *OsNRT2.3a* (AK109776; Feng et al., 2011; Supplemental Fig. S1). Analysis of the *OsNRT2.3* genomic DNA sequence predicts an intron for *OsNRT2.3b* located between +190 and +280 bp from the ATG for translation initiation (Supplemental Fig. S1). To determine the specific role of *OsNRT2.3a* in planta, we generated an *OsNRT2.3a*-RNAi construct using a 482-bp fragment of *OsNRT2.3a* complementary DNA (cDNA), which contains the 90-bp intron for *OsNRT2.3b* that shares no similarity to any other sequence in the rice genome (Supplemental Fig. S1). More than 100 lines of hygromycin-resistant T0 transgenic rice seedlings were transferred into soil and grown in the greenhouse to obtain T2 progeny for genetic analysis.

We first screened the RNAi pool of T2 progeny grown in nitrate nutrient solution and obtained many different lines with *OsNRT2.3a* silencing in roots. We further used Southern-blot and thermal asymmetric interlaced (TAIL)-PCR analyses to obtain the stably inherited RNAi plants from the *OsNRT2.3a* knockdown lines. Two single-copy insertion lines in which the T-DNA insertion site was in the noncoding region of chromosome 3 or 12, respectively, were identified (Supplemental Fig. S2, A and B). Real-time quantitative reverse transcription (RT)-PCR was used to detect the efficiency of targeted knockdown of the *OsNRT2.3a* gene. The *OsNRT2.3a* transcript was found to be greatly suppressed (74.4% for line r1, 75.6% for line r2) in nitrate-supplied roots of RNAi plants (Supplemental Fig. S3A).

Using an *OsNRT2.3a*-specific monoclonal antibody for western-blot analysis, we observed that the expression of *OsNRT2.3a* was abundant in the wild-type roots, while it was very faint in the two *osnrt2.3a* RNAi knockdown lines grown in 0.5 mM nitrate solution (Supplemental Fig. S3B), confirming the true knockdown of *OsNRT2.3a* in the RNAi lines.

Effect of *osrt2.3a* Knockdown on Root-to-Shoot Nitrate Transport

To confirm that *osnrt2.3a* RNAi lines were defective in long-distance transport of nitrate, xylem exudates of

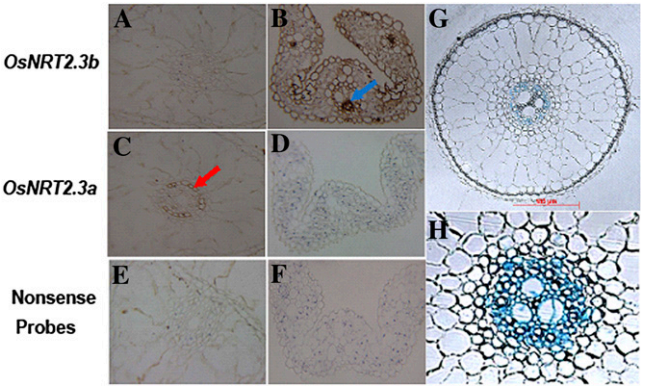

Figure 2. Detecting the tissue localization of *OsNRT2.3a* and *OsNRT2.3b* transcripts in nitrate-supplied root and leaf. The mRNA expression patterns of *OsNRT2.3* by in situ hybridization are shown. A and B, *OsNRT2.3b* probe hybridization. C and D, *OsNRT2.3a* probe hybridization. E and F, Nonsense probe hybridization as controls. A, C, and E show roots, and B, D, and F show leaves. The blue arrow indicates leaf phloem cells, and the red arrow indicates root stelar cells. G, Root branching zone (2 cm from the root tip) cross-section of rice transformed with the *OsNRT2.3a* promoter-GUS gene. H, Amplified cross-section of rice transformed with the *OsNRT2.3a* promoter-GUS gene. Plants were grown in IRRI solution containing 0.5 mM NO_3^- for 10 d.

plants grown in hydroponics were collected and analyzed. The nitrate concentrations of the xylem sap in the *osnrt2.3a* RNAi lines were significantly lower than that in the wild type when 0.5 mM NO_3^- was provided in the culture solution (Fig. 3A). For further investigation of the accumulation of nitrate in *osnrt2.3a* RNAi lines and wild-type plants, a ^{15}N-labeled nitrate-treated experiment for 24-h uptake and transport was carried out. The amount of ^{15}N translocated to the shoot from the roots was very significantly decreased in the RNAi lines supplied with low nitrate concentrations (Fig. 4A). The shoot-root [^{15}N]NO_3^- concentration ratio was approximately 0.87 in the wild type but only approximately 0.23 in the two RNAi knockdown lines (Fig. 4B).

Root Expression Profiles of Nitrate Reductase and *OsNRT2s* in the Wild Type and *osnrt2.3a* Knockdown Lines

With nitrate as the only N source, the root expression of nitrate reductase (*OsNia1*) was significantly increased in both knockdown lines relative to the wild type (Fig. 5A). We also analyzed whether the expression of *OsNAR2.1*, which interacts with *OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3a* for function (Feng et al., 2011; Yan et al., 2011), and other *OsNRT2s* in low-nitrate-supplied rice roots was altered in the two independent *osnrt2.3a* knockdown lines. The mRNA expression of *OsNRT2.3b* in roots was not affected by *OsNRT2.3a* RNAi in the two RNAi lines (Fig. 5B). The expression of both *OsNAR2.1* and *OsNRT2.4* transporters showed no difference between the two *osnrt2.3a* knockdown lines and wild-type plants (Fig. 5B). However, knockdown of *OsNRT2.3a* decreased the expression of *OsNRT2.1* by about 50% and *OsNRT2.2* by about 20% in the nitrate-supplied RNAi roots, relative to wild-type plants (Fig. 5A). Western-blot analysis confirmed the RNA expression pattern (Supplemental Fig. S4).

Rates of High-Affinity Nitrate Influx in the Wild Type and *osnrt2.3a* Knockdown Lines

To determine the effect of *OsNRT2.3a* gene silencing on high-affinity root nitrate influx into intact plants, short-term nitrate uptake was analyzed by exposing the plants to 0.25 mM $NH_4{}^{15}NO_3^-$ or $^{15}NH_4NO_3$ for 5 min. Both r1 and r2 lines showed no significant difference from wild-type plants in either nitrate or ammonium influx rate into roots (Fig. 6).

For further confirmation that the nitrate influx was not affected by knockdown of *OsNRT2.3a*, we investigated the temporal net NO_3^- fluxes in the primary root tips of three-leaf seedlings (3-d N-starved plants) in 11 min, using high-resolution scanning ion-selective electrode technique (SIET). In seedlings in which the whole root was supplied with 0.25 mM NO_3^-, the pattern of net NO_3^- fluxes in roots of RNAi lines did not show significant differences from wild-type seedlings from 1 to 8 min (Fig. 7A). The net NO_3^- influx rate of the RNAi lines was very significantly decreased from 9 to 11 min in comparison with the wild-type rate (Fig. 7A). There was no significant difference of net nitrate influx over the entire 11-min nitrate supply experiment between the wild type and the two RNAi lines (Fig. 7B). Thus, we conclude that *OsNRT2.3a* is not directly involved in nitrate uptake by rice roots.

Effects of *osrt2.3a* Knockdown on Rice Growth under Differing Forms of N Supply

The two *osnrt2.3a* knockdown lines were much smaller than the wild type when grown in 0.5 mM NO_3^- or 0.25 mM NH_4NO_3 as the sole N supply source (Fig. 8, A, B, D, and E). The RNAi lines had only about 57% root and 60% shoot dry weights of the wild type. When growing in an ammonium N supply, the RNAi lines and the wild type had very similar phenotypes (Fig. 8, C and F). When grown with only nitrate as the

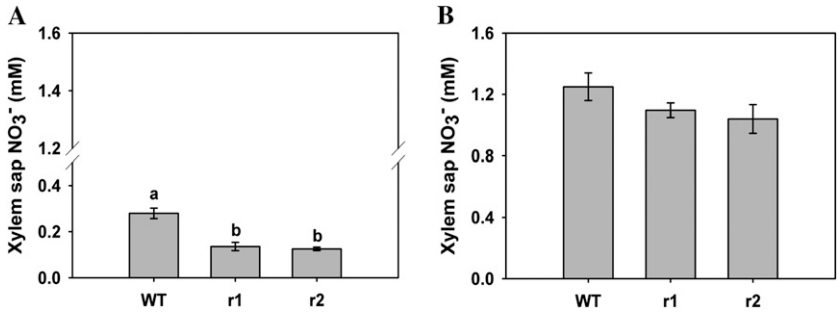

Figure 3. Nitrate concentrations in the xylem sap of the wild type (WT) and *osnrt2.3a* knockdown lines (r1 and r2). Rice seedlings of the wild type and RNAi lines were grown in IRRI solution containing 1 mM NH_4NO_3 for 8 weeks and then N starved for 3 d before treatment. The rice plants were cut 4 cm above the roots, and the roots were immediately transferred to either 0.5 mM NO_3^- or 5 mM NO_3^- and for 1-h xylem sap collection. A, N-starved seedlings were transferred to the nutrient solution containing 0.5 mM NO_3^- for 1-h xylem sap collection. B, N-starved seedlings were transferred to the nutrient solution containing 5 mM NO_3^- for 1-h xylem sap collection. Significant differences between the wild type and RNAi lines are indicated with different letters ($P < 0.05$, one-way ANOVA).

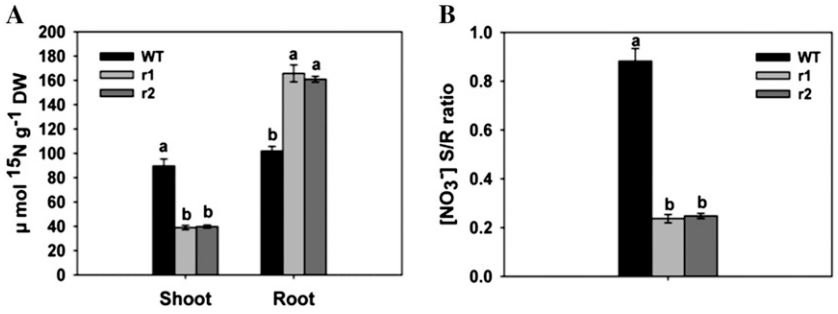

Figure 4. $[^{15}N]NO_3^-$ concentration and the distribution ratio of nitrate between the shoots and roots in the wild type (WT) and *osnrt2.3a* knockdown lines. A, $[^{15}N]NO_3^-$ concentration in the shoots and roots of the wild type and *osnrt2.3a* knockdown lines (r1 and r2). Rice seedlings were grown in IRRI solution containing 1 mM NH_4NO_3 for 4 weeks and then N starved for 1 week. N-starved seedlings were transferred to the nutrient solution containing 0.5 mM $[^{15}N]NO_3^-$ for 24 h before the sampling. B, The shoot-to-root ratio of the total nitrate content. Significant differences between the wild type and RNAi lines are indicated with different letters ($P < 0.05$, one-way ANOVA). DW, Dry weight.

N source, the shoot nitrate concentration was about 33% less in the RNAi lines than in the wild type (Fig. 8G). However, nitrate concentrations in the roots were about 17% higher than in the wild type (Fig. 8G), and a similar pattern was observed when the plants were grown in a mixed ammonium and nitrate supply (Fig. 8H). Even when supplied with ammonium as an N source, a lower shoot nitrate concentration was detected in the RNAi lines relative to the wild type (Fig. 8I). This result suggests that even though a nitrification inhibitor was added to the ammonium supply, there was some nitrification occurring in the hydroponics. Total N analysis of the plants growing under the various N supplies confirmed the general pattern of less accumulation in shoots and more in roots of the two *osnrt2.3a* knockdown lines when compared with the wild type under nitrate or mixed nitrate and ammonium supply conditions (Fig. 8, J and K). The *osnrt2.3a* knockdown lines clearly show less long-distance transport of N from the root to the shoot, and this phenotype is increased when nitrate is supplied to the roots.

Under high nitrate (5 mM NO_3^-) supply, the visible phenotypic differences between the *osnrt2.3a* RNAi lines and wild-type plants were much less obvious (Supplemental Fig. S5). There was no significant difference in root-to-shoot biomass ratio between the RNAi lines and the wild type. Furthermore, knockdown of the expression of *OsNRT2.3a* did not have a statistically significant effect on root-to-shoot nitrate distribution at high nitrate supply (Supplemental Fig. S5).

DISCUSSION

Plant roots take up nitrate from the external medium and transport a large portion of nitrate from roots to shoots. A number of nitrate transporters belonging to the NRT1 family have been functionally characterized for their specific functions in the acquisition and

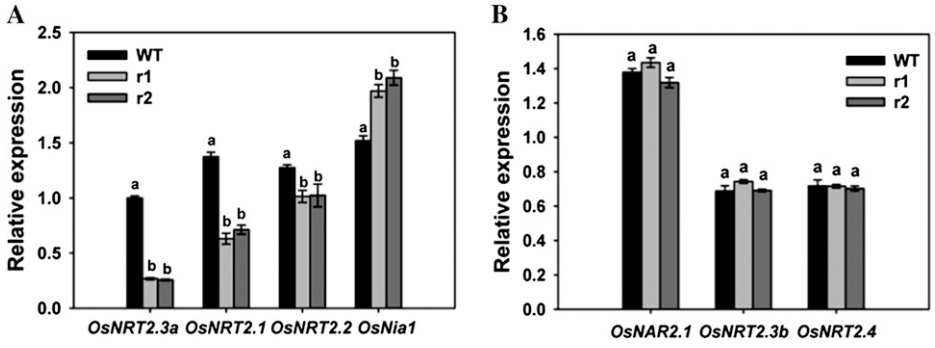

Figure 5. Comparison of the root mRNA expression pattern of other NRT2 nitrate transporters and nitrate reductase (*nia1*) in knockdown (r1 and r2) and wild-type (WT) plants. A, The mRNA expression patterns of *OsNRT2.3a*, *OsNRT2.1*, *OsNRT2.2*, and *OsNia1* in the roots of RNAi lines and wild-type plants under 0.5 mM nitrate supply. B, The mRNA expression patterns of *OsNAR2.1*, *OsNRT2.3b*, and *OsNRT2.4* in the roots of RNAi lines (r1 and r2) and wild-type plants under 0.5 mM nitrate supply. Total RNA was isolated from roots of RNAi lines (r1 and r2) and wild-type plants growing in IRRI nutrient solution containing 0.5 mM nitrate solution. Expression was analyzed using semiquantitative RT-PCR with rice actin (*OsActin*) used for comparison. The number of PCR cycles was 30 for the amplification of *OsNRT2.3a*, *OsNRT2.1*, *OsNRT2.2*, and *OsNia1* and 35 for *OsNAR2.1*, *OsNRT2.3b*, and *OsNRT2.4*.

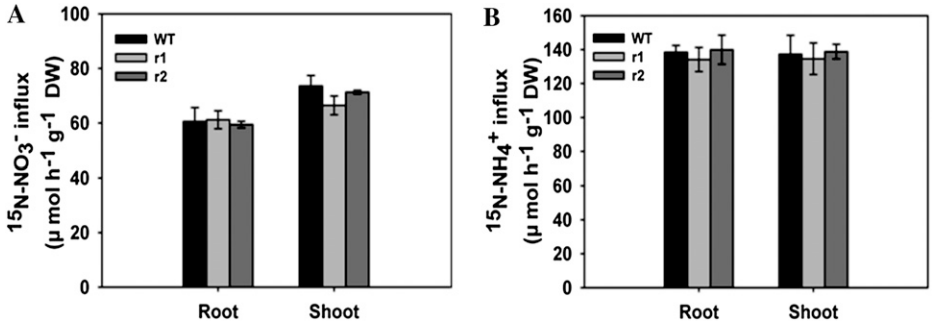

Figure 6. Nitrate and ammonium influx rates of *osnrt2.3a* knockdown lines (r1 and r2) and wild-type (WT) plants measured using ^{15}N-enriched sources. Rice seedlings of the wild type and RNAi lines were grown in IRRI nutrient solution containing 0.25 mM NH_4NO_3 for 4 weeks and then N starved for 1 week. Nitrate influx was then measured using 0.25 mM $NH_4{}^{15}NO_3^-$ (A) or 0.25 mM $^{15}NH_4NO_3$ (B) for 5 min. DW, Dry weight.

distribution of nitrate in Arabidopsis (Huang et al., 1999; Liu and Tsay, 2003; Chiu et al., 2004; Almagro et al., 2008; Lin et al., 2008; Fan et al., 2009; Li et al., 2010; Wang and Tsay, 2011). In the high-affinity NRT2 family, *AtNRT2.4* was found to be a root epidermis-expressing transporter and to play a role in uptake at low nitrate supply level (Kiba et al., 2012). However, it is not known if any NRT2 family members are involved in translocation inside the plant. In this study, we demonstrated that in rice *OsNRT2.3a*, the NAR2/NRT2 two-component nitrate transporter is expressed exclusively in the xylem parenchyma cells of nitrate-supplied roots and mediates the transport of root-acquired nitrate to the shoots in rice.

Evidence that *OsNRT2.3a* Functions in Root-to-Shoot Nitrate Transport

Like *OsNRT2.1/2.2*, *OsNRT2.3a* also requires *OsNAR2.1* for the transport of nitrate in rice, but its affinity for nitrate (K_m of 0.31 mM) is about 10-fold higher (Feng et al., 2011; Yan et al., 2011). In a previous study, *OsNRT2.3a* was mainly expressed in the stelar cells of both primary and lateral roots when the 10-d-old seedlings were cultured with only deionized water (Feng et al., 2011). In this experiment, we showed that *OsNRT2.3a* is a plasma membrane transporter and expressed abundantly in the xylem parenchyma cells throughout the primary roots when the 10-d-old seedlings were cultured with 0.5 mM nitrate (Figs. 1 and 2). No expression of *OsNRT2.3a* was detected in both epidermal and cortex cells (Fig. 2). Short-term [^{15}N]NO_3^- influx analyses of both whole plant roots and the root meristem zone showed that knockdown of *OsNRT2.3a* did not have a significant effect on root nitrate uptake at low external supply (Figs. 6 and 7). These data demonstrate that *OsNRT2.3a* is not directly involved in nitrate uptake from the external medium.

When plants were exposed to relatively long-term nitrate supplies, the two independent *osnrt2.3a* RNAi lines accumulated much higher nitrate in their roots at low nitrate solution (0.5 mM) than the wild type (Figs. 4A and 8G). In contrast, their shoots had significantly lower nitrate than the wild type. This difference between the RNAi lines and the wild type was much less when the nitrate supply concentration was raised to 5 mM (Supplemental Fig. S5). Analyses of both [^{15}N]NO_3^- transport in xylem sap and the distribution of [^{15}N]NO_3^- in the roots and shoots (Figs. 3 and 4) clearly showed a strong decrease in nitrate translocation from the roots to shoots in the RNAi lines at low nitrate supply. Since OsNRT2.3a was an influx transporter (Feng et al., 2011) and was expressed abundantly in the xylem parenchyma throughout the primary and lateral roots, it could be concluded that OsNRT2.3a functions in loading nitrate into these cells, which are adjacent to the xylem. At high external nitrate supply, the RNAi lines and the wild type have similar phenotypes, suggesting that there are differing pathways operating for the root-to-shoot transfer of nitrate at these two different concentrations. Clearly, *OsNRT2.3a* mediates root-to-shoot transfer at low external concentrations, but at high external concentrations a

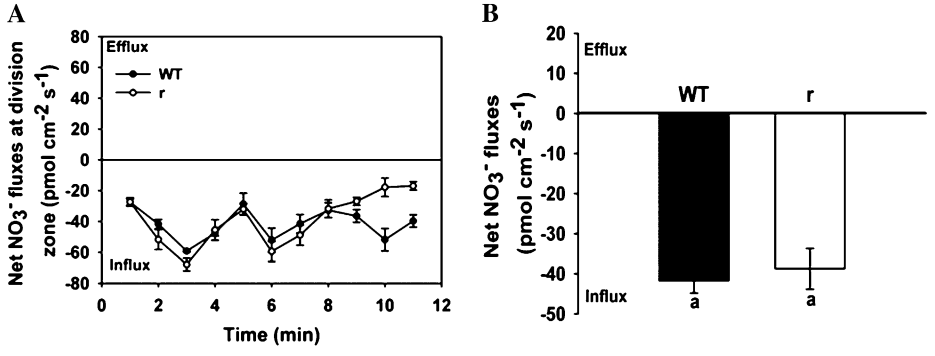

Figure 7. Influence of NO_3^- supply in the root on net plasma membrane NO_3^- fluxes at the root meristem. A, Net NO_3^- fluxes in primary root meristem of *osnrt2.3a* knockdown lines (r) and the wild type (WT) supplied with 0.25 mM NO_3^- for 11 min. B, Mean rate of NO_3^- fluxes during the entire 11 min. Each point represents the mean ± SE of more than five individual plants.

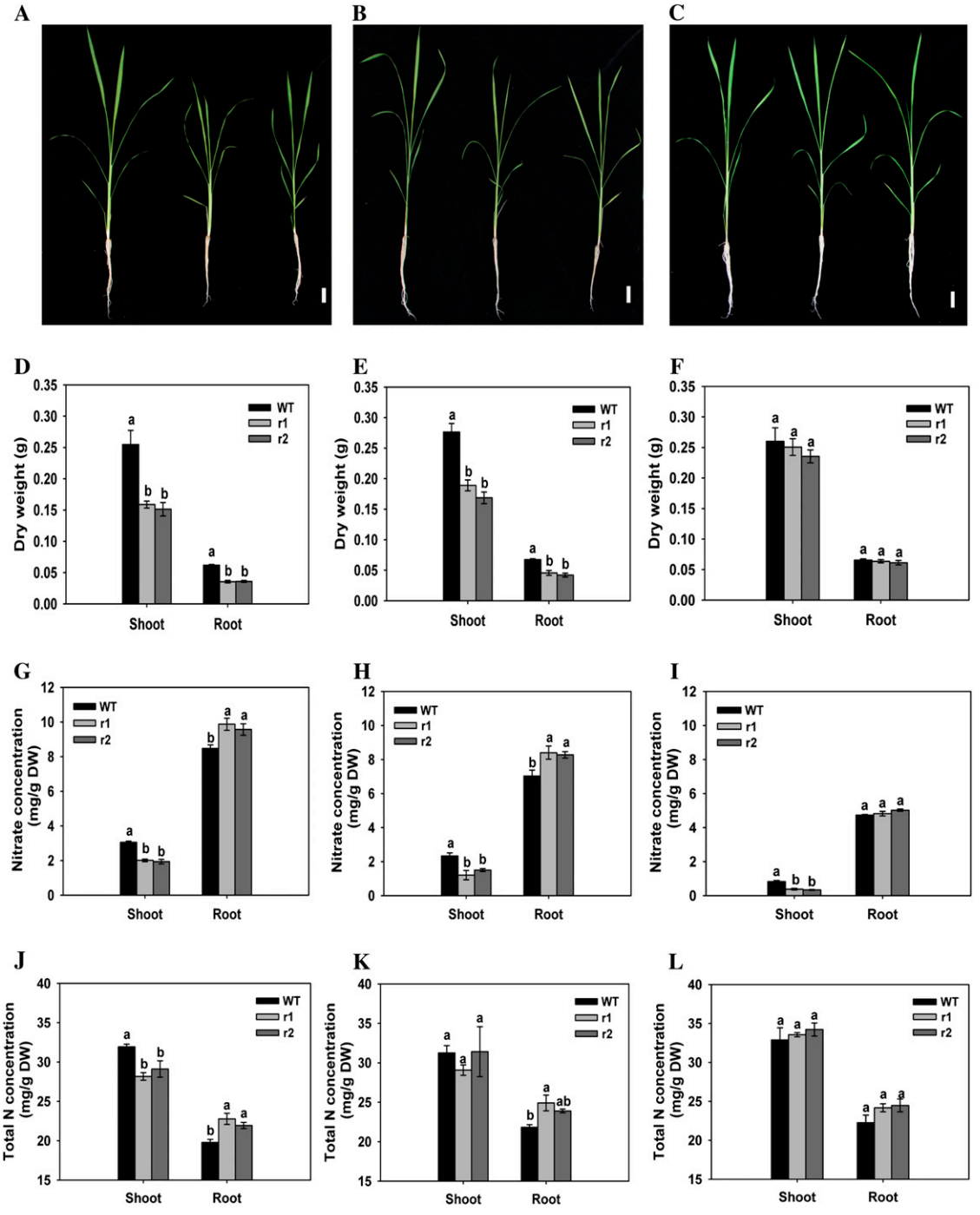

Figure 8. Comparison of growth, nitrate, and total N concentration of *osnrt2.3a* knockdown lines and wild-type plants. Rice seedlings of the wild type and RNAi lines were grown in IRRI solution containing 1 mM $(NH_4)_2SO_4$ for 4 weeks and then N starved for 3 d, then grown with different forms of N for another 1 week. A to C, Appearance of the seedlings treated with 0.5 mM NO_3^- (A), 0.25 mM NH_4NO_3 (B), and 0.5 mM NH_4^+ (C). D to F, The dry weight of shoots and roots of the knockdown lines and the wild type treated with 0.5 mM NO_3^- (D), 0.25 mM NH_4NO_3 (E), and 0.5 mM NH_4^+ (F). G to I, The nitrate concentration of shoots and roots of the knockdown lines and the wild type treated with 0.5 mM NO_3^- (G), 0.25 mM NH_4NO_3 (H), and 0.5 mM NH_4^+ (I). J to L, The total N concentration of shoots and roots of the knockdown lines and the wild type treated with 0.5 mM NO_3^- (J), 0.25 mM NH_4NO_3 (K), and 0.5 mM NH_4^+ (L). To inhibit nitrification, 7 μM dicyandiamide (DCD-$C_2H_4N_4$) was mixed into the solutions. Data represent means ± SE of three replicates. Significant differences between the wild type and RNAi lines are indicated with different letters ($P < 0.05$, one-way ANOVA). Bars = 3 cm.

bypass flow-type mechanism may predominate, like that reported for rice during salt stress (Faiyue et al., 2012). In wild-type plants, *OsNRT2.3a* contributed 74% of the nitrate transport from low-nitrate-supplied roots to the shoots (Fig. 4). Both the tissue localization of *OsNRT2.3a* expression and $[^{15}N]NO_3^-$ analyses clearly showed that *OsNRT2.3a* plays a key role in the xylem transport of nitrate from roots to shoots at low supply, resulting in nitrate accumulation in the roots of the RNAi lines.

In Arabidopsis, a member of the NRT1 family, the low-affinity transporter *AtNRT1.5*, is involved in nitrate loading into the root stele (Lin et al., 2008), and *AtNRT1.8* is involved in nitrate unloading from the root stele or from the shoot vasculature (Li et al., 2010). The expression of *AtNRT1.8* is induced mainly in roots by cadmium and is greater in shoots than in roots under nonstress conditions, which means that *AtNRT1.8* mainly functions in shoots to unload nitrate from the xylem vessels (Li et al., 2010). Compared with *AtNRT1.8*, *OsNRT2.3a* is almost constitutively expressed in the roots (Feng et al., 2011; Fig. 2). Although both *OsNRT2.3a* and *AtNRT1.8* are expressed in root xylem parenchyma cells, *OsNRT2.3a* knockdown decreased the nitrate concentration in xylem sap (Fig. 3), which is similar to the results of *AtNRT1.5* mutation (Lin et al., 2008), while *AtNRT1.8* disruption increased the nitrate concentration in xylem sap (Li et al., 2010). These results suggest that OsNRT2.3a and AtNRT1.8 may not be functionally orthologous. When comparing the physiology of rice and Arabidopsis, nitrate is generally less available as an external N source for rice, but both types of plants have root uptake systems to accumulate nitrate, so the vascular transport systems may be expected to operate with similar affinities for nitrate. We have clearly shown that rice has a high-affinity transport system mediating root-to-shoot transfer of nitrate, suggesting that under some environmental conditions, the internal root nitrate concentrations can be lower in rice compared with Arabidopsis. The nitrate-scavenging role of *AtNRT2.4* in Arabidopsis may be more functionally analogous to *OsNRT2.3a* in rice, although the former was expressed in the epidermis of lateral roots and in leaf vascular tissue (Kiba et al., 2012).

When plants encounter N deficiency, as a mobile element, N can be recycled within the plant from older to younger leaves to sustain the growth of developing tissues, and similar remobilization of potassium and phosphorus occurs. It has been reported that *AtNRT1.7* mediates phloem loading of nitrate in source leaves to remobilize nitrate from older leaves to N-demanding tissues in Arabidopsis (Fan et al., 2009). The *osnrt2.3a* RNAi lines contained very significantly lower nitrate in both old and young leaves in comparison with the wild type (Supplemental Fig. S6); however, the knockdown of *OsNRT2.3a* did not significantly affect the redistribution of nitrate among the leaves (Supplemental Fig. S6), suggesting that *OsNRT2.3a* is not involved in the nitrate remobilization from source to sink leaves in rice.

N Feedback Regulation in Rice, and the Activity and Expression of *NAR2/NRT2* Transporters

We have recently reported that a putative high-affinity nitrate transporter, *OsNRT2.3b* (AK072215), appears to arise from the alternative splicing of *OsNRT2.3a* mRNA (AK109776; Feng et al., 2011). No such splicing was reported in other known *NRT2* genes from other plants, including Arabidopsis (Orsel et al., 2002; Plett et al., 2010). However, in rice, the spliced forms have differing tissue localizations and responses to N supply form (Fig. 2; Feng et al., 2011; Yan et al., 2011). In contrast to the expression of *OsNRT2.3a*, which occurs exclusively in nitrate-supplied roots (Fig. 5; Feng et al., 2011), we confirmed that the expression of *OsNRT2.3b* is relatively weak in roots and abundant in leaves (Fig. 2, A and B). Suppression of *OsNRT2.3a* in the two independent RNAi lines did not affect *OsNRT2.3b* expression in the roots but down-regulated *OsNRT2.3b* expression at the lower nitrate supply in their shoots (Fig. 5; Supplemental Fig. S7). Since *OsNRT2.3b* expression is not sensitive to the N form and concentration in external solution (Feng et al., 2011), it is important to know the function of *OsNRT2.3b* and why the suppression of *OsNRT2.3a* could concurrently decrease *OsNRT2.3b* expression and only in the leaves.

In the rice NAR2/NRT2 two-component systems for nitrate transport, silencing of *OsNAR2.1* could result in the suppression of its interacting partner proteins, *OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3a* (Yan et al., 2011). In this study, we found that knockdown of *OsNRT2.3a* itself did not alter the expression of *OsNAR2.1* and *OsNRT2.4* (Fig. 5). Unlike the expression of *OsNRT2.3a* exclusively in the stele of roots, *OsNAR2.1* is expressed mainly in the epidermal cells of nitrate-supplied roots, and no common cis-element responding to nitrate supply was observed among the promoters of the rice NAR2/NRT2 family members (Feng et al., 2011). Therefore, we suggest that *OsNAR2.1* expression is independent of *OsNRT2.3a*, and possibly also *OsNRT2.1/2.2*, in rice.

OsNRT2.1 and *OsNRT2.2* have low sequence similarity to *OsNRT2.3a* in their coding and promoter regions. No similar region (482-bp fragment of *OsNRT2.3a* cDNA) for silencing of *OsNRT2.3a* by RNAi knockdown can be found in *OsNRT2.1/2.2*. Knockdown of *OsNRT2.3a* decreased the expression of *OsNRT2.2* slightly and *OsNRT2.1* strongly in the roots under low nitrate supply (Fig. 5). Since the suppression of *OsNRT2.3a* increased the nitrate concentration in the roots and decreased the nitrate concentration in the shoots of RNAi lines (Figs. 4 and 8), the data suggest that *OsNRT2.1* might be sensitive to the internal nitrate status in rice plants. These results indicate that the knockdown of *OsNRT2.3a* expression inhibited the transportation of nitrate from roots to shoots. This inhibition resulted in root nitrate accumulation, which indirectly down-regulated the expression of *OsNRT2.1* and, thus, could further decrease the acquisition of nitrate by the roots from the external medium in the long term (Figs. 5 and 8). In rice, it is

surprising then that plasma membrane $[^{15}N]NO_3^-$ uptake and root surface nitrate fluxes (Figs. 6 and 7) were not significantly different from the wild type. Possibly, the changes in expression were compensated by some posttranslational activation of the transporters, and this aspect is worthy of further investigation. This result clearly demonstrates a different effect in rice from barley (*Hordeum vulgare*) and Arabidopsis, both species that showed a well-established relationship between the root nitrate accumulation and the expression and activity of root high-affinity uptake transporters (Zhuo et al., 1999; Vidmar et al., 2000).

Furthermore, in rice, the increased accumulation of nitrate in the root of the RNAi lines (Fig. 8G) was also accompanied by increased nitrate reductase (*OsNia1*) expression (Fig. 5A). The expression pattern of nitrate reductase is well characterized and known to be low during nitrate deficiency, high in nitrate-replete plants, but low when downstream metabolites such as Gln accumulate (Klein et al., 2000). When compared with the wild type, the rice RNAi lines supplied with only nitrate as an N source accumulated more root nitrate and increased *OsNia1* expression, even though the shoot nitrate concentration was decreased (Fig. 8G). This result suggests that if cellular nitrate storage pools are indicators of N status, the root locally regulates gene expression in a way that is independent of signals coming from the shoot. Localized root responses to nitrate have been identified in rice and support this idea (Wang et al., 2002). In tobacco (*Nicotiana tabacum*), the accumulation of nitrate in the shoot acted as a signal to regulate shoot-root biomass (Scheible et al., 1997), but in rice, decreased shoot nitrate accumulation in the RNAi lines resulted in decreased root biomass (Fig. 8D). Taken together, these results suggest differences in the nitrate feedback signaling in rice, possibly resulting from the fact that ammonium is the predominant N supply form for this species. The RNAi approach has been successfully used to down-regulate the expression of *OsNRT2.3a*. The characterization of these knockdown plants shows how this high-affinity transporter has a specific role in transporting nitrate from the root to the shoot in rice.

MATERIALS AND METHODS

Transient Expression of *OsNRT2.3a* in Rice Protoplasts and Fluorescence Microscopy Imaging

The rice (*Oryza sativa* ssp. *japonica*) protoplast preparation and transfection followed previously described procedures (Bart et al., 2006; Miao and Jiang, 2007) with some modifications. Briefly, 0.2 mL of protoplast suspension (approximately 2×10^5 cells) was transfected with DNA for various constructs (10 μg each). After transfection, cells were cultured in protoplast medium (0.4 M mannitol, 4 mM MES [pH 5.7], 4 mM KC1, sterilized) overnight (approximately 12 h). For plasma membrane localization, a working staining solution of 5 $\mu g\ mL^{-1}$ membrane-selective fluorescent vital dye FM4-64 was used to stain the transfected protoplasts (Nelson et al., 2007). Protoplasts with or without FM4-64 were observed under a 60× objective. The fluorescence of FM4-64 (a membrane-selective dye) and GFP in the cells was analyzed with a 543-nm helium-neon laser and a 488-nm argon laser, respectively, using a confocal laser scanning microscope (LSM410; Carl Zeiss).

RNA in Situ Hybridization and Promoter-GUS Analysis

Four-week-old rice 'Nipponbare' plants were grown in 1.25 mM NH_4NO_3 for 2 weeks before in situ hybridization. RNA in situ hybridization was performed as described previously (Shanks et al., 1994). The sense *OsNRT2.3a* probe binding site was located in the 90-bp intron, which is not present in *OsNRT2.3b* (Feng et al., 2011; Supplemental Fig. S1). The sense probe sequence was 5′-CGTGGCCGTGAGCCCGAGGGTG-3′ and that of the nonsense probe was 5′-GCACCGGCACTCGGGCTCCCAC-3′. For the *OsNRT2.3b* probe, the binding site was in the *OsNRT2.3b*-specific 5′ untranscribed region (Supplemental Fig. S1); the sequence was 5′-CGATGGTTGGGTGCGGCGAGA-3′, and the nonsense sequence was 5′-GCTACCAACCCACGCCGCTCT-3′. All probes were labeled at the 5′ end with digoxigenin.

The regions of putative promoter of *OsNRT2.3a* immediately upstream of the translation start codon were PCR amplified from the genomic DNA of the rice cultivar (Nipponbare). The PCR products were first cloned into the pMD19-T vector (TaKaRa Biotechnology). After being confirmed by restriction enzyme digestion and DNA sequencing, the cloned fragments in the T-Vector were cut and inserted upstream of the 5′ end of the GUS reporter gene in the binary vector pS1aG-3 (kindly provided by Dr. Delhaize Schünmann, CSIRO Plant Industry; http://www.pi.csiro.au). The construct *pOsNRT2.3a* (2,228 bp)-GUS was obtained and transformed into callus initiated from the seeds of rice (cv Nipponbare) by *Agrobacterium tumefaciens* (strain EHA105)-mediated transformation (Upadhyaya et al., 2000).

RNAi Vector Construction and Rice Transformation

To generate the hairpin RNAi construct, a 482-bp highly specific fragment of *OsNRT2.3a* from 161 to 643 bp downstream of the translation start codon containing the intron of *OsNRT2.3b* was amplified by PCR using forward primer AS-F (5′-GCGTTCTAGACCTTCTTCTGCTGCTTCGTG-3′), with an *Xba*I site incorporated into the 5′ end, and reverse primer AS-R (5′-GGGAATCGATCGGGATGAAGAAGGCG-3′), which introduces a *Cla*I site at the 3′ end. After *Xba*I and *Cla*I digestion, the fragment was cloned into the *Xba*I/*Cla*I sites of vector pRNA69 (Chen and Slocum, 2008) in the antisense orientation. The same 482-bp fragment was amplified using forward primer S-F (5′-GTCTCTCGAGCTTCTGCTGCTTCGTGTCC-3′), with an *Xho*I site incorporated into the 5′ end, and reverse primer S-R (5′-AAATGGTACCCGGGATGAAGAAGGCG-3′), incorporating a *Kpn*I site into the 3′ end. The PCR product was *Xho*I/*Kpn*I digested and cloned in the sense orientation into the *Xho*I/*Kpn*I sites of the vector. A 3.5-kb *Pst*I/*Sac*I fragment containing the 35S: AS-Y5-S-3′-ocs expression cartridge was subcloned into the *Pst*I/*Sac*I sites of the binary vector pCambia1301 (CAMBIA). Then, the plasmid was transformed into *A. tumefaciens* strain EHA105 by electroporation. Rice embryonic calli were induced on N6 medium, and transformation was performed by *A. tumefaciens*-mediated cocultivation (Ai et al., 2009). Transgenic plants were selected on a medium containing 50 mg L^{-1} hygromycin (Roche). Hygromycin-resistant plants, defined as T0 generation transgenic plants, were transplanted into soil and grown for seed in the field.

Plant Growth Conditions

Rice 'Nipponbare' seeds and *OsNRT2.3a* knockdown lines (T2 generation) were surface sterilized with 10% (v/v) hydrogen peroxide for 30 min and then rinsed thoroughly with deionized water. The sterilized seeds were germinated on plastic supporting netting (mesh of 1 mm^2) mounted in plastic containers for 1 week. Uniform seedlings were selected and then transferred to a tank containing 8 L of International Rice Research Institute (IRRI) nutrient solution (1.25 mM NH_4NO_3, 0.3 mM KH_2PO_4, 0.35 mM K_2SO_4, 1 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM Na_2SiO_3, 20 μM NaFeEDTA, 20 μM H_3BO_3, 9 μM $MnCl_2 \cdot 4H_2O$, 0.32 μM $CuSO_4 \cdot 5H_2O$, 0.77 μM $ZnSO_4 \cdot 7H_2O$, and 0.39 μM $Na_2MoO_4 \cdot 2H_2O$, pH 5.5). All the plants were grown in a growth room with a 16-h-light (30°C)/8-h-dark (22°C) photoperiod, and the relative humidity was controlled at approximately 70%. The solution was refreshed every 2 d. For pot experiments, rice plants were grown in flooded soil in a greenhouse at 30° C with a 16-h photoperiod. The properties of the soil and N supply were as described previously (Li et al., 2006).

RT-PCR and Quantitative Real-Time RT-PCR

The protocols of total RNA isolation and concentration measurement, and the specific primers for semiquantitative RT-PCR of *OsNAR2.1*, each *OsNRT2*,

and *OsActin*, were described previously (Feng et al., 2011; Yan et al., 2011). Amplification of real-time quantitative PCR products was performed with a single Color Real-Time PCR Detection System (MyiQ Optical Module; Bio-Rad) in a reaction mixture of 20 μL of SYBR Green master mix (SYBR Premix Ex Tag TMII; TaKaRa Bio; http://www.takara-bio.com) according to the manufacturer's instructions (TaKaRa Biotechnology). The target genes and *OsActin* standards in 1:10, 1:100, and 1:1,000 dilutions were always present in the experiments (Tsuchiya et al., 2004; Jain et al., 2006). All of the primers used for RT-PCR and quantitative RT-PCR are listed in Supplemental Tables S1 and S2. All the PCR products were checked by electrophoresis and sequenced by Invitrogen to confirm their identities.

Southern-Blot and TAIL-PCR Analyses

The independent transgenic lines with gene knockdown of *OsNRT2.3a*, namely r1 and r2, were determined by Southern-blot analysis following the procedures described previously (Jia et al., 2011). A TAIL-PCR procedure was performed as described previously (Liu et al., 1995). The reaction volume for the first and second cycles was 20 μL, and this was increased to 50 μL for the third cycle. The reaction mixture contained 1× PCR buffer, 2.0 mM $MgCl_2$, 200 μM deoxyribonucleotide triphosphate, 2.0 μM degenerative primer (AD1, AD2, or AD3), 0.2 μM specific primer (SP1, SP2, or SP3), 1.0 μL of template DNA, and 1 unit of Taq DNA polymerase (TaKaRa Biotechnology). After the first or second PCR run, the PCR product was diluted at 1:100 with distilled, deionized water and was subsequently used as a template in the second or third PCR run. All TAIL-PCRs were carried out in an Applied Biosystems PCR System. Secondary and tertiary PCR products were separated on 1.5% agarose gels by electrophoresis with ethidium bromide staining. The PCR products were purified and then cloned into pMD-19 T vector (TaKaRa Biotechnology) for sequencing. Sequence identity was determined by BLASTing against the National Center for Biotechnology Information database. All of the primers used for TAIL-PCR and procedures are listed in Supplemental Table S3.

Western Blotting

The specific anti-*OsNRT2.3a* monoclonal antibody was generated using a peptide corresponding to the N-terminal 30 amino acids (64–93,which is the intron for *OsNRT2.3b*). The anti-*OsNAR2.1* antibody was generated using a peptide corresponding to the N-terminal 155 amino acids (Yan et al., 2011). The anti-*NRT2.1/2.2/2.3b/2.4* rabbit polyclonal antibody was generated using peptide corresponding to full-length amino acids. The cDNA fragment was amplified by PCR and then subcloned into the bacterial expression vector pGSX (Amersham). The amino acid product was purified, and its antibody was synthesized (Ye et al., 2008).

All tissue samples were homogenized and lysed in buffer containing 1% Nonidet P-40 and the protease inhibitors. Lysates were cleared by centrifugation, and protein concentration was measured spectrophotometrically at A_{595} using the Bradford reagent. Fifty micrograms of protein of each sample was boiled in gel loading buffer and then analyzed on 10% SDS-PAGE gels. Proteins were transferred to polyvinylidene difluoride membranes and incubated with *OsNRT2.1/2.2* (1:2,000), *OsNRT2.3a* (1:500), *OsNRT2.3b* (1:500), *OsNRT2.4* (1:1,000), *OsNAR2.1* (1:2,000), or *OsActin* (1:5,000) overnight at 4°C. The membrane was then incubated with the appropriate secondary antibody (1:20,000; Pierce), followed by chemiluminescence detection, and exposed to x-ray film.

Nitrate Content Analysis

Rice seedlings of the wild type and RNAi lines were grown in IRRI solution containing 1 mM $(NH_4)_2SO_4$ for 4 weeks and N starved for 3 d, then grown with different forms of N (0.5 mM NO_3^-, 0.25 mM NH_4NO_3, and 0.5 mM NH_4^+) for another 1 week. At each harvest, rice roots and shoots were separated and washed with 0.1 mM $CaSO_4$ for 1 min, then placed in an oven at 105°C for 30 min to inactivate the enzymes, and finally dried to a constant weight at 70°C. The dry weight was recorded. Nitrate concentration was determined in a boiled water extract of the sample on a continuous-flow autoanalyzer (Autoanalyzer 3; Bran & Luebbe) as described previously (Schortemeyer et al., 1993; Leleu and Vuylsteker, 2004). Total N concentration in plants was determined by the Kjeldahl method (Li et al., 2006).

For xylem sap nitrate measurement, rice seedlings of the wild type and RNAi lines were grown in IRRI solution containing 1 mM NH_4NO_3 for 8 weeks and then N starved for 1 week before the treatment. The rice plants were cut 4 cm above the ground level, and the roots were immediately transferred to either 0.5 mM NO_3^- or 5 mM NO_3^-; a preweighed absorbent cotton ball was attached to the cut surface and covered with plastic film for 1 h. Next, the collected xylem sap was squeezed from the cotton with a syringe, and the volume of the exudates was calculated from the increase in the weight of the cotton. Nitrate concentration was determined on a continuous-flow autoanalyzer (Autoanalyzer 3, Bran & Luebbe).

Determination of Nitrate Uptake and Accumulation Using ^{15}N

The influx rate of $^{15}NO_3^-$ was assayed as already described (Delhon et al., 1995) on plants grown hydroponically. Rice seedlings of the wild type and RNAi lines were grown in IRRI nutrient solution for 4 weeks and then deprived of N for 1 week. The plants were transferred first to 0.1 mM $CaSO_4$ for 1 min, then to a complete nutrient solution containing either 0.25 mM $^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$ (atom % ^{15}N: $^{15}NO_3^-$, 80.25%; $^{15}NH_4^+$, 40%) for 5 min, and finally to 0.1 mM $CaSO_4$ for 1 min. For analyzing the relatively longer term effect of the *OsNRT2.3a* knockdown on the uptake of different concentrations of nitrate, the N-starved seedlings were transferred to a nutrient solution containing 0.5 mM $[^{15}N]NO_3^-$ for 24 h before the harvest. After grinding in liquid N, an aliquot of the powder was dried to a constant weight at 70°C. About 10 mg of powder of each sample was analyzed using the Isotope Ratio Mass Spectrometer system (Thermo Fisher Scientific). Influx of $^{15}NO_3^-$ was calculated from the ^{15}N concentrations of the roots.

Measurement of Net NO_3^- Flux in Rice Plants with the SIET System

Net fluxes of NO_3^- in rice plants were measured noninvasively using SIET (BIO-003A; Younger USA Science and Technology). The working principle and measurement procedure of this method using the instrument were described in detail by Sun et al. (2009). Rice seedlings of the wild type and RNAi lines were grown in IRRI nutrient solution for 3 weeks and then deprived of N for 3 d, and the roots of seedlings were equilibrated in measuring solution for 20 to 30 min before measuring at room temperature (24°C–26°C). The equilibrated seedlings were then transferred to the measuring chamber, and a small plastic dish (6 cm diameter) was filled with 10 mL of fresh nutrient solution containing 0.25 mM NO_3^-. When the root became immobilized at the bottom of the dish, the microelectrode was vibrated in the measuring solution between two positions, 5 and 35 μm from the primary root surface, along an axis perpendicular to the root meristem zone. The background was recorded by vibrating the electrode in measuring solution not containing roots. The glass microelectrodes with 2- to 4-mm apertures were made by Xuyue Science and Technology. Prior to the flux measurements, the ion-selective electrodes were calibrated using NO_3^- concentrations of 0.05 and 0.5 mM. During the entire measurement process, the shoot was not in contact with the measuring solution. The net fluxes of NO_3^- at the meristem were measured individually. Each plant was measured once. The final flux values were the means of more than five individual plants. The measuring solution was composed of 0.2 mM $CaCl_2$, 0.1 mM NaCl, 0.1 mM $MgSO_4$, and 0.3 mM MES (pH 6.0, adjusted with 1 M NaOH). All measurements of net NO_3^- fluxes were carried out at Xuyue Science and Technology.

Statistical Analysis of the Data

All the data collected were tabulated and analyzed for significant differences using a statistical software (SPSS 13.0; SPSS, Inc.).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers *OsNRT2.3a* (AK109776) and *OsNRT2.3b* (AK072215).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Gene structure of *OsNRT2.3a/b*.

Supplemental Figure S2. Detection of T-DNA insertion site of *OsNRT2.3a*-RNAi lines.

Supplemental Figure S3. The mRNA and protein levels of OsNRT2.3a in *OsNRT2.3a*-RNAi lines.

Supplemental Figure S4. Protein level of OsNRT2s in roots of *OsNRT2.3a*-RNAi lines.

Supplemental Figure S5. Phenotype and nitrate contents of *OsNRT2.3a*-RNAi lines under 5 mM nitrate supply.

Supplemental Figure S6. Nitrate contents in different individual leaves of *OsNRT2.3a*-RNAi lines.

Supplemental Figure S7. Expression of *OsNRT2.3b* and *OsNRT2.4* in the shoots of *OsNRT2.3a*-RNAi lines.

Supplemental Table S1. Primers used for semiquantitative RT-PCR.

Supplemental Table S2. Primers used for real-time PCR.

Supplemental Table S3. Primers and procedures for TAIL-PCR.

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