A putative 6-transmembrane nitrate transporter OsNRT1.1b plays a key role in rice under low nitrogen

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Abstract OsNRT1.1a is a low-affinity nitrate (NO₃⁻) transporter gene. In this study, another mRNA splicing product, OsNRT1.1b, putatively encoding a protein with six transmembrane domains, was identified based on the rice genomic database and bioinformatics analysis. OsNRT1.1a/OsNRT1.1b expression in Xenopus oocytes showed OsNRT1.1a-expressing oocytes accumulated ¹⁵N levels to about half as compared to OsNRT1.1b-expressing oocytes. The electrophysiological recording of OsNRT1.1b-expressing oocytes treated with 0.25 mM NO₃⁻ confirmed ¹⁵N accumulation data. More functional assays were performed to examine the function of OsNRT1.1b in rice. The expression of both OsNRT1.1a and OsNRT1.1b was abundant in roots and downregulated by nitrogen (N) deficiency. The shoot biomass of transgenic rice plants with OsNRT1.1a or OsNRT1.1b overexpression increased under various N supplies under hydroponic conditions compared to wild-type (WT). The OsNRT1.1a overexpression lines showed increased plant N accumulation compared to the WT in 1.25 mM NH₄NO₃ and 2.5 mM NO₃⁻ or NH₄⁺ treatments, but not in 0.125 mM NH₄NO₃. However, OsNRT1.1b overexpression lines increased total N accumulation in all N treatments, including 0.125 mM NH₄NO₃, suggesting that under low N condition, OsNRT1.1b would accumulate more N in plants and improve rice growth, but also that OsNRT1.1a had no such function in rice plants.

Keywords: Nitrate; nitrogen accumulation; OsNRT1.1a/b; overexpression; rice; 6-transmembrane transport protein

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INTRODUCTION

Nitrate (NO₃⁻) is very important as a source of nitrogen (N) for plants. Plant root cells absorb NO₃⁻ via various transporters from the soil and then reduce it to ammonium (NH₄⁺), and assimilate NH₄⁺ into organic N via the GOGAT cycle (Forde 2000). Plant NO₃⁻ uptake requires efficient transport systems at both low and high NO₃⁻ concentrations, and numerous studies have demonstrated that NO₃⁻ influx involves high- and low-affinity NO₃⁻ transport processes (Miller et al. 2007; Xu et al. 2012). Many families of membrane proteins are involved in NO₃⁻ uptake, allocation, and storage in plants such as the NO₃⁻/peptide transporter family (NPF), NO₃⁻ transporter 2 family (NRT2), chloride channel family (CLC), and slow anion channel-associated homologs (SLAC/SLAH) (Krapp et al. 2014; Leran et al. 2014; Xia et al. 2014). The NPF transporters include the low-affinity NO₃⁻ transporter family (NRT1) and NO₃⁻/peptide transporter family (Leran et al. 2014; Xia et al. 2014).

NRT1 transporters in higher plants contain 12 putative transmembrane (TM) regions with a large hydrophilic loop between TM6 and TM7 whose position is unique in higher plant NRT1 and rat PHTS (Chiang et al. 2004). In most animal NRT1 transporters, the long loop is located between TM9 and TM10, while it is between TM7 and TM8 in fungi. However, the function of the long hydrophilic loop of NRT1 transporters remains unclear. AtNRT1.1 (or ChL1), as the first identified NRT1 gene in plants, was isolated from Arabidopsis in 1978 (Doddema et al. 1978) and further confirmed using a transferred DNA-tagged Arabidopsis mutant in 1993 (Tsay et al. 1993). Using the Xenopus oocyte expression system, Tsay et al. (1993) showed that AtNRT1.1 (CHL1) is a proton-coupled NO₃⁻ transporter (Tsai et al. 1993). In addition, AtNRT1.1 (CHL1) exhibits two phases of NO₃⁻ uptake, with a Km of 50 µM for the high-affinity phase and a Km of 4 mM for the low-affinity phase, indicating that CHL1 is a dual-affinity NO₃⁻ transporter (Liu et al. 1999); the reported NRT1 transporter had a Km above 4 mM NO₃⁻. Recently, structural studies showed that AtNRT1.1 functions in the membrane as a dimer (Sun et al. 2014). Parker and Newstead (2014) used a 6 TM model to study the structure of AtNRT1.1 and demonstrated that the NO₃⁻–proton symport model was a symmetry interaction of inward and outward open between the two 6 TM proteins in the AtNRT1.1 protein.

In rice, OsNRT1.1 was identified as a low-affinity NO₃⁻ transporter gene with an affinity for NO₃⁻ of 9 mM (Km = 9
mM) (Lin et al. 2000). By searching the cDNA database, OsNRT1.1b, putatively encoding a protein with only six TM domains, was considered a putative mRNA splicing product of OsNRT1.1. To distinguish between OsNRT1.1b and OsNRT1.1 (Lin et al. 2000), we renamed OsNRT1.1 as OsNRT1.1a. Based on AtNRT1.1 structural data (Parker and Newstead 2014; Sun et al. 2014), we hypothesized that one 6 TM NO₃⁻ transporter could function in plants through symmetry interactions of inward and outward open between protein dimers. To support these hypotheses, we tested the NO₃⁻ uptake function of OsNRT1.1b in Xenopus oocytes and rice plants.

RESULTS

Sequence analysis and expression pattern of OsNRT1.1b

OsNRT1.1 was identified as a low-affinity NO₃⁻ transporter gene that is constitutively expressed in roots (Lin et al. 2000). Based on the rice genomic database and bioinformatics analysis, we searched OsNRT1.1 mRNA (AF140606) on the NCBI Web site and identified another mRNA splicing product, AK066920, which was further named OsNRT1.1b. To distinguish between OsNRT1.1b and OsNRT1.1 (Lin et al. 2000), we renamed OsNRT1.1 as OsNRT1.1a. OsNRT1.1a encoded a protein of 584 amino acids and 12 predicted TM domains, and a large hydrophilic loop existed between TM6 and TM7 (Figures 2A, S1). OsNRT1.1b consisted of 291 amino acids and six predicted TM domains, which shared the same amino acid sequences from 1 to 226 with OsNRT1.1a (Figures 2A, S1).

OsNRT1.1a is a root-specific gene with little or no expression in the shoot that remains relatively stable before and after NO₃⁻ induction (Lin et al. 2000). Furthermore, we detected OsNRT1.1b expression with different N supplies using reverse transcription–polymerase chain reaction (RT–PCR). Rice seedlings were hydroponically cultured in IRRI nutrient solution for 14 d, and then transferred to nutrient solutions containing different forms of nitrogen regimes, including standard N supply in IRRI solution 1.25 mM NH₄NO₃ solutions containing different forms of nitrogen regimes, nutrient solution for 14 d, and then transferred to nutrient was higher in 0.2 N and 1.25 AN than other forms of N regimes treatment; however, OsNRT1.1a did not show significant difference among 1.25 AN, 0.2 N, 5 N, 0.2 A, and 5 A conditions (Figure 2B, C).

OsNRT1.1b mRNA-injected oocytes showed NO₃⁻ uptake activity

To determine whether OsNRT1.1b encodes an NO₃⁻ transporter, OsNRT1.1b mRNA was injected into Xenopus oocytes. Two days after injection, the oocytes were used to record the membrane potential. Water-injected oocytes showed no response to NO₃⁻ (Figure 3A). However, when treated with 0.25 mM NO₃⁻, oocytes injected with mRNA encoding OsNRT1.1b displayed NO₃⁻-induced changes in membrane potential from −33 to −20 mV, representing a depolarization of 13 mV (Figure 3B). The membrane potential could be restored when NO₃⁻ was removed (Figure 3B).

To examine the NO₃⁻ uptake activity of OsNRT1.1b, OsNRT1.1b-injected oocytes were incubated in 0.25 mM ¹⁵NO₃⁻ (pH 7.4) 8 h for further NO₃⁻ uptake assays. Compared with water-injected oocytes, 100% of OsNRT1.1b-injected oocytes and 100% of OsNRT1.1a-injected oocytes exhibited ¹⁵N accumulation (Figure 3C). Data spread analysis showed that almost 100% of the OsNRT1.1b RNA injected oocytes showed higher ¹⁵N than OsNRT1.1a RNA injected oocytes (Figure 3C). As shown in Figure 3D, both OsNRT1.1a- and OsNRT1.1b-injected oocytes could absorb ¹⁵N–NO₃⁻, but OsNRT1.1a-expressing oocytes accumulated about half the ¹⁵N as compared with OsNRT1.1b-expressing oocytes.

Generation of the transgenic rice overexpressing OsNRT1.1a and OsNRT1.1b

OsNRT1.1a and OsNRT1.1b overexpression lines were constructed by transforming pUbi–OsNRT1.1a and pUbi–OsNRT1.1b constructs into rice. More than 15 lines of the T₂ generation for each gene were obtained. Three independent transgenic lines of the T₂ generation for OsNRT1.1a (OEa1, OEa2, and OEa3) and OsNRT1.1b (OEb1, OEb2, and OEb3) were selected based on Southern blotting and RT–PCR analysis (Figure 4). RT–PCR analysis showed that in OEa1, OEa2, and OEa3 lines, the expression of OsNRT1.1a increased, while OsNRT1.1b did not change compared with the wild type (WT) (Figures 4A, S2). In OEb1, OEb2, and OEb3 lines, OsNRT1.1b transcript levels increased, but the OsNRT1.1a mRNA level did not change compared with the WT (Figures 4A, S2). OEa1 and OEb1 transgenic lines had two copies, while OEa2, OEa3, OEb2, and OEb3 had a single copy based on Southern blotting analysis (Figure 4B).

Figure 1. Schematic diagram of the pUbi–OsNRT1.1a/b expression vector for rice transformation

OsNRT1.1a/b cDNA was inserted in place of the rice intron in pTCK303 with Kpnl and SpeI sites.
Overexpression of OsNRT1.1b significantly enhanced rice growth

Rice seeds of the WT, OsNRT1.1a, and OsNRT1.1b T2 transgenic lines were cultured with 1/2 IRRI nutrient solution for 14 d and then treated with 1.25 mM NH₄NO₃ (1.25 AN), no N (−N), 0.2 mM NO₃⁻ (0.2 N), 5 mM NO₃⁻ (5 N), 0.2 mM NH₄⁺ (0.2 A), and 5 mM NH₄⁺ (5 A) for another 14 d. Compared with the WT, OsNRT1.1a overexpression (OEa) lines showed increased shoot dry weight under all N supplies (Figure 5A) and increased root dry weight at 1.25 AN (Figures 5B, S3). Under 1.25 AN supply, the dry weight of OEa lines increased by 75 to 125% in shoots and 80 to 133% in roots compared with the WT (Figure 5A, B).

In OsNRT1.1b T2-overexpressed (OEb) lines, compared with the WT, shoot biomass was higher under all N supplies and root biomass was much higher in 1.25 AN and 2.5 A solutions (Figures 5C, D, S3). Shoot and root weight of OEb lines

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Figure 2. Amino acid sequence alignment and gene expression in OsNRT1.1a and OsNRT1.1b

(A) Alignment of OsNRT1.1a and OsNRT1.1b amino acid sequences. The picture was constructed using DNAMAN 5.2.2.

(B, C) Expression of OsNRT1.1a/b in response to different nitrogen (N) regimes by semiquantitative RT-PCR (B) and real-time PCR (C). Rice seedlings were cultured in IRRI nutrient solution for 14 d and then transferred to nutrient solutions containing 1.25 mM NH₄NO₃ (1.25 AN), no N (−N), 0.2 mM NO₃⁻ (0.2 N), 5 mM NO₃⁻ (5 N), 0.2 mM NH₄⁺ (0.2 A), and 5 mM NH₄⁺ (5 A) for another 14 d. Total RNA was extracted from roots and shoots. OsActin was used as an internal control.
increased respectively by 88 and 80% at 1.25 AN and 62 and 64% at 0.125 AN compared to the WT (Figure 5C, D). Both OEa and OEb lines showed no change in the root/shoot ratio under all N conditions (Figure 5E, F).

Overexpression of OsNRT1.1a enhanced N accumulation only at high N supplies, but OsNRT1.1b-overexpressing lines showed increased N accumulation at both high and low N supplies

To explore the cause of the enhanced biomass in OsNRT1.1a and OsNRT1.1b transgenic plants, we measured total N contents in WT and transgenic plants. Total N content in both shoots and roots showed no significant differences between OEa lines and the WT under different N treatments (Figure 6A, B). OEb lines showed a higher total N content in shoots and roots than the WT under 0.125 AN condition, except OEb1 root (Figure 6C, D). The pattern of nitrogen content in shoots and roots showed slightly different in OEb1 from OEb2 and OEb3 at this condition. Total plant N was calculated as the shoot N content multiplied by shoot biomass plus root N content multiplied by root biomass. Both OEa and OEb lines exhibited significantly greater accumulation in whole plants than the WT under all treatments, except OEa lines in 0.125 AN condition (Figure 6E, F). Under 0.125 AN condition, OEb lines accumulated more plant N compared with WT (Figure 6F); however, greater plant N accumulation of OEa was not observed in the same condition (Figure 6E). Under 0.125 AN supply, the N accumulation of OEb lines increased by 56 to 70% in plants compared with the WT and significantly more by 31 to 51% than OEa lines (Figure 6E, F).

The OSNRT1.1a overexpression lines showed no shift in the ratio of root N and shoot N from the WT at either 0.125 AN, 1.25 AN, 2.5 AN, or 2.5 N supplies (Figure 6G). However, OsNRT1.1b overexpression lines decreased root N transfer to the shoot at 0.125 AN (Figure 6H), indicating that under low N condition, OsNRT1.1b accumulated more N in roots but that OsNRT1.1a had no such function in rice plants. While, as root/shoot biomass ratio of OsNRT1.1b overexpression did not change under 0.125 AN (Figure 5F), which means that the shoot growth was also increased as much as root even though the root/shoot N ratio was upregulated in OsNRT1.1b overexpression lines. The possible reason for this may be the N assimilation efficiency in the shoot of OsNRT1.1b overexpression lines was higher than that in their roots. However, it needs to be investigated further in future.
OsNRT1.1a was identified as a NO\textsubscript{3}− transporter in *Xenopus* oocytes (Lin et al. 2000) and injected OSNRT1.1b mRNA could transport NO\textsubscript{3}− under low NO\textsubscript{3}− supply (Figure 3), its biological function in plants had not been investigated. In this study, T\textsubscript{2} overexpressed transgenic lines of OSNRT1.1a and OSNRT1.1b were obtained to test gene function in rice. Overexpression of OSNRT1.1a stimulated shoot growth under all N conditions and root growth only under 1.25 mM NH\textsubscript{4}NO\textsubscript{3} conditions (Figure 5A, B). However, OSNRT1.1b overexpression enhanced shoot growth in all N treatments and root growth in both 1.25 mM NH\textsubscript{4}NO\textsubscript{3} and 2.5 mM NH\textsubscript{4}NO\textsubscript{3} treatments, also increased the total N content in shoots and root under 0.125 mM NH\textsubscript{4}NO\textsubscript{3} conditions (Figures 5C, D, 6C, D). These results suggested that OEB absorbed more nitrogen from external solutions. However the constitutive overexpression of OSNRT2.1 in rice only enhanced vegetative growth, but did not affect NO\textsubscript{3}− uptake (Hisato et al. 2009).

It was very surprising that different nitrogen form treatments had significantly different effects on root growth for OEA and OEB lines, but not on shoots. In detail, we observed that OEA lines could show super root growth only under 1.25 AN, the mixture of nitrate and ammonium condition. None of the pure nitrate or ammonium or low nitrogen treatments could improve the OEA root growth, compared with WT. However, more interestingly we found the root growth of OEB lines could become greater not only under 1.25 AN but also under pure ammonium conditions. Whereas neither low nitrogen nor pure nitrate induced this super phenotype of OEB lines, compared with WT. Why pure nitrate failed to induce the root super growth of OEA and OEB

OsNRT1.1a is abundantly in roots and was not induced by NO\textsubscript{3}− (Lin et al. 2000). In our study, OSNRT1.1b showed similar expression patterns as OSNRT1.1a, which was constitutively expressed in roots (Figure 2B), even though the expression of OSNRT1.1a/b was quite low under N starvation condition, but was strongly induced under 0.2 mM nitrate or ammonium low nitrogen conditions (Figure 2B, C). The NRT1 family comprises both the constitutive and NO\textsubscript{3}−-inducible component of the low-affinity NO\textsubscript{3}− transport system in *Arabidopsis* (Tsai et al. 1993; Huang et al. 1996, 1999). AtNRT1.1 (CHL1), the NO\textsubscript{3}−-inducible component, is involved in both low- and high-affinity NO\textsubscript{3}− uptake (Wang et al. 1998; Liu et al. 1999). AtNRT1.2, the constitutive component, exhibits only low-affinity uptake activities (Huang et al. 1999; Liu et al. 1999), similar to OSNRT1.1a and OSNRT1.1b (Figure 2B).

OsNRT1.1b can increase plant N accumulation under low N conditions

Although OSNRT1.1a was identified as a NO\textsubscript{3}− transporter in *Xenopus* oocytes (Lin et al. 2000) and injected OSNRT1.1b mRNA could transport NO\textsubscript{3}− under low NO\textsubscript{3}− supply (Figure 3), its biological function in plants had not been investigated. In this study, T\textsubscript{2} overexpressed transgenic lines of OSNRT1.1a and OSNRT1.1b were obtained to test gene function in rice. Overexpression of OSNRT1.1a stimulated shoot growth under all N conditions and root growth only under 1.25 mM NH\textsubscript{4}NO\textsubscript{3} conditions (Figure 5A, B). However, OSNRT1.1b overexpression enhanced shoot growth in all N treatments and root growth in both 1.25 mM NH\textsubscript{4}NO\textsubscript{3} and 2.5 mM NH\textsubscript{4}NO\textsubscript{3} treatments, also increased the total N content in shoots and root under 0.125 mM NH\textsubscript{4}NO\textsubscript{3} conditions (Figures 5C, D, 6C, D). These results suggested that OEB absorbed more nitrogen from external solutions. However the constitutive overexpression of OSNRT2.1 in rice only enhanced vegetative growth, but did not affect NO\textsubscript{3}− uptake (Hisato et al. 2009).

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lines is still unknown, even though it could be done at shoot growth. We guessed that the reason that low nitrogen treatment improved root growth of OEb lines rather than OEA lines was linked to the OsNRT1.1b function in oocytes, i.e. under low nitrate conditions, oocytes expressing OsNRT1.1b could respond to 0.25 mM nitrate quite well and absorbed more 15N-nitrate than OsNRT1.1a under 0.25 mM nitrate supply. However, it was still hard to explain that the root growth of OEb could be improved under pure ammonium condition but that did not happen on OEA lines.

Furthermore, we calculated plant N accumulation and found that when supplied with high N, both OsNRT1.1a and OsNRT1.1b overexpression lines showed higher plant N accumulation than the WT (Figure 6E, F). However, under 0.125 AN conditions, overexpression of OsNRT1.1b showed increased plant N accumulation compared to the WT (Figure 6F). One possible mechanism was OsNRT1.1b could accumulate more 15N than OsNRT1.1a under a low NO3⁻/C₃ supply in oocytes, and therefore OsNRT1.1b may contribute to plant N accumulation under a low N supply. However, for high N conditions, there was no such difference between OEA and OEB lines.

AtNRT1.1 is a NO3⁻ transporter present in the membrane as a dimer (Sun et al. 2014). The dimerization switch model was operated by the phosphorylation site at T101 of AtNRT1.1 (Sun et al. 2014). When AtNRT1.1 functioned as a high affinity...
transporter in plants, T101 site was phosphorylated and AtNRT1.1 dimer was decoupled. When T101 site of AtNRT1.1 was dephosphorylated the AtNRT1.1 protein was coupled into dimer and working as a low affinity transporter (Sun et al. 2014). We scanned the potential phosphorylation site in OsNRT1.1a and OsNRT1.1b by scansite software online (http://scansite.mit.edu/motifscan_seq.phtml). The prediction results showed that both OsNRT1.1a and OsNRT1.1b had two potential

Figure 6. Total nitrogen (N) content and N accumulation of wild-type (WT) and transgenic lines with different N supplies (A) Shoot N content and (B) root N content in OsNRT1.1a overexpression lines. (C) Shoot N content and (D) root N content in OsNRT1.1b overexpression lines. Plant N accumulation in OsNRT1.1a transgenic lines (E) and OsNRT1.1b transgenic lines (F). Root N accumulation/shoot N accumulation in OsNRT1.1a transgenic lines (G) and OsNRT1.1b transgenic lines (H). The treatments were labeled as 0.125 mM NH₄NO₃ (0.125 AN), 1.25 mM NH₄NO₃ (1.25 AN), 2.5 mM NH₄⁺ (2.5 A), and 2.5 mM NO₃⁻ (2.5 N). *Significant difference at the 0.05 probability level according to the LSD test (n = 4) estimated using one-tailed ANOVA between WT and T2 overexpression lines; data represent means ± SE.
phosphorylation sites at S199 and T204 (Figure S5). That meant for both OsNRT1.1a and OsNRT1.1b that they might be switched between dimer or mono protein in cells. Furthermore they might show a switch between low affinity and high affinity function. However, as Lin et al. (2000) presented that OsNRT1.1 had only one low affinity activity in oocytes no high affinity function, it defined the possibility of OsNRT1.1a could be switched between low and high affinity. But our oocytes data suggested this possibility may exist for OsNRT1.1b, since OsNRT1.1b could respond to low nitrate quite well (Figure 3B).

The NO3−–proton symport model of AtNRT1.1 was a symmetrical interaction of inward and outward open between the two 6 TM proteins in the AtNRT1.1 protein (Parker and Newstead 2014). Parker and Newstead (2014) used this 6 TM model to explain the mechanism for the effect of phosphorylation at T101 of this 6 TM on nitrate transport that was compared to the wild-type protein, the T101D of AtNRT1.1 increased nitrate uptake significantly. Therefore the phosphorylation site T101 controlled the functional activity of AtNRT1.1. Compared with the 12 TM topology of OsNRT1.1a, OsNRT1.1b contained only six TMs. However, our data suggested that OsNRT1.1b was expressed strongly under low nitrogen in rice (Figure 2B, C) and did function as an NO3− transporter during low nitrate supply (Figure 3). Overexpression of OsNRT1.1b could increase plant N accumulation in rice plants (Figures 5, 6). Based on structural studies of AtNRT1.1, we hypothesized that OsNRT1.1b may perform a NO3− transporter function as a dimer in the cell, through the phosphorylation on S199 or T204. Furthermore this phosphorylation should increase the nitrate uptake based on the finding of Parker and Newstead 2014. This hypothesis fitted well to our nitrate uptake data as OsNRT1.1b had a much better nitrate uptake than OsNRT1.1a (Figure 3D). More protein analysis is needed in the future in order to understand better the different behaviors of OsNRT1.1a and OsNRT1.1b in plants.

MATERIALS AND METHODS

Plant growth conditions

Seed sterilization and the basal IRRI nutrient solution composition for seedling growth were described previously (Li et al. 2006). Two-week-old seedlings with uniform size and vigor were transferred into pots. For different N treatments, 0.125 or 1.25 mM NH4NO3, 1.25 mM Ca(NO3)2, and 1.25 mM (NH4)2SO4 were added as N sources. Other nutrients were supplied as the irri nutrient solution with 0.3 mM KH2PO4, 0.35 mM K2SO4, 1 mM CaCl2·2H2O, 1 mM MgSO4·7H2O, 0.5 mM Na2SiO3, 20 mM NaFe-EDTA, 20 mM H3BO3, 9 μM MnCl2·4H2O, 0.32 μM CuSO4·5H2O, 0.77 μM ZnSO4·7H2O, and 0.39 μM Na2MoO4·2H2O, pH 5.5. To inhibit nitrification, 7 μM dicyandiamide (DCD–C4H4N4) was mixed into all solutions. All seedlings were grown in a greenhouse with a 16-h light (30°C)/8-h dark (22°C) photoperiod, and the relative humidity was approximately 70%. The nutrient solution was refreshed every 2 d.

Semiquantitative RT-PCR and real-time PCR assay

Total RNA extraction from different rice tissues and RT-PCR using specific primers for OsNRT1.1a and OsNRT1.1b and the internal standard gene OsActin were performed as described previously (Feng 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 PremixEx Tag TML; 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). All primers used for semiquantitative RT-PCR and real-time PCR are listed in Table S1.

Functional analysis of OsNRT1.1a and OsNRT1.1b in Xenopus laevis oocytes

OsNRT1.1a and OsNRT1.1b cDNAs were subcloned into the oocyte expression vector pT7T7s and linearized using XbaI. mRNA was then synthesized in vitro using the mMESSAGE mACHINE T7 Kit (Ambion, Austin, TX, USA). Oocytes were injected with 50 ng of mRNA, as described previously (Tong et al. 2000; Xia et al. 2014). Water-injected oocytes were used as controls. Oocytes were incubated in ND96 solution with antibiotics for 2 or 3 d (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, and 5 mM HEPES 50 μg mL−1 gentamycin and 100 μg mL−1 streptomycin, pH 7.4). Membrane potential recording was performed as described previously (Tong et al. 2000). Injected oocytes were incubated in 0.25 mM Na2NO3 ND96 solution for 8 h at pH 7.4, and then washed with cold 0.25 mM NaNO3 ND96 solution quickly four times. A single oocyte was transferred to an empty tin capsule and then dried at 60°C for 1 week to a constant weight. 15N was measured using a continuous-flow isotope ratio mass spectrometer coupled to a C–N elemental analyzer (ANCA-GSL MS; PDZ Europa, Northwich, Cheshire, UK).

Generation and identification of OsNRT1.1a and OsNRT1.1b overexpression rice lines

The open reading frames of OsNRT1.1a and OsNRT1.1b were amplified by PCR and ligated into the ubiquitin promoter of the pTCK303 vector (Figure 1). For OsNRT1.1a, the forward primer was 5′-taatggatccATCTCTGACATTAACCT and the right primer was 5′-taatagctcTTCCACCACATTATTTGC. For OsNRT1.1b, the forward primer was 5′-taatggatccTTGGAGCTCCTTTGAGCCTTCACCCGCC and the right primer was 5′-taatagctcCCTCCCCCTCGAAAGG.

The constructs were obtained and transformed into rice callus using Agrobacterium tumefaciens (strain EHA105), as described previously (Ai et al. 2009). More than 15 individual T0 transgenic lines were obtained to verify the levels of OsNRT1.1a or OsNRT1.1b overexpression. Three independent lines of the T0 generation were selected from both pUbI–OsNRT1.1a (OEa1, OEa2, and OEa3) and pUbI–OsNRT1.1b (OEb1, OEb2, and OEb3) transgenic lines, while one or two copies of the T-DNA insertion were used for further analyses. The copy numbers in the T1 generation were performed by Southern blotting (DIG High Prime DNA Labelling and Detection Starter Kit I; Roche, http://www.roche.com/index.htm). The fragment of the coding sequences of the hygromycin genes labeled with digoxigenin was used as a probe, which was prepared by PCR according to the manufacturer’s instructions (Roche).
Measurement of biomass, total N content, and accumulation

All plants (WT and T2 transgenic lines) were harvested after different treatments and then dried at 105°C for 30 min, after which shoots and roots were dried at 75°C for 3 d. The dry weight was recorded as biomass. The root to shoot ratio was calculated as the root dry weight divided by the shoot dry weight. Total N content was measured as described previously (Cai et al. 2012). The plant accumulation N = (shoot N content * shoot biomass) + (root N content * root biomass). Four replicates were used for the calculation.

Statistical analysis

Data were analyzed by ANOVA using the SPSS 10 program (SPSS, Chicago, IL, USA). The asterisks on the histograms between the transgenic plants and WT and/or different treatments indicate their statistical difference at P ≤ 0.05.

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AUTHOR CONTRIBUTIONS

X.F. and Q.M. performed most of the research. H.F. drafted the manuscript and part of oocyte expression experiment. Y.T. carried out gene expression experiments, Y.X. performed total N analyses. C.X. revised the manuscript. X.F. designed the experiment, supervised the study, and revised the manuscript.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of this article at the publisher’s web-site.

**Figure S1.** Transmembrane topology of OsNRT1.1a and OsNRT1.1b. The figure was generated using the ConPred II program (http://bioinfo.sii.hirosaki-u.ac.jp/~ConPred2/).

**Figure S2.** Expression of OsNRT1.1b in pUbi–OsNRT1.1a transgenic lines and OsNRT1.1a in pUbi–OsNRT1.1b transgenic lines compared to the wild type using RT-PCR in roots.

**Figure S3.** Plant growth phenotype of OsNRT1.1a overexpression lines under different nitrogen supply conditions.

**Figure S4.** Plant growth phenotype of OsNRT1.1b overexpression lines under different nitrogen supply conditions.

**Figure S5.** Phosphorylation site scanning of OsNRT1.1a and OsNRT1.1b.

**Table S1.** OsNRT1.1a and OsNRT1.1b primers for RT-PCR.