The Plant Cell, Vol. 30: 638–651, March 2018, www.plantcell.org © 2018 ASPB.

Expression of the Nitrate Transporter Gene *OsNRT1.1A*/*OsNPF6.3* Confers High Yield and Early Maturation in Rice[OPEN]

Wei Wang,[a,1] Bin Hu,[a,1] Dingyang Yuan,[b] Yongqiang Liu,[a,c] Ronghui Che,[a] Yingchun Hu,[d] Shujun Ou,[e] Yongxin Liu, Zhihua Zhang,[a,c] Hongru Wang,[a] Hua Li,[a,c] Zhimin Jiang,[a] Zhengli Zhang,[a] Xiaokai Gao,[f] Yahong Qiu,[a,c] Xiangbing Meng,[a] Yongxin Liu,[a] Yang Bai,[a] Yan Liang,[a,c] Yiqin Wang,[a] Lianhe Zhang,[f] Legong Li,[g] Sodmergen,[d] Haichun Jing,[h] Jiayang Li,[a] and Chengcai Chu [a,2]

[a] State Key Laboratory of Plant Genomics and National Center for Plant Gene Research (Beijing), Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China
[b] China National Hybrid Rice Research and Development Center, Changsha 410125, China
[c] University of Chinese Academy of Sciences, Beijing 100049, China
[d] College of Life Sciences, Peking University, Beijing 100871, China
[e] Department of Horticulture, Michigan State University, East Lansing, Michigan 48824
[f] School of Agriculture, Henan University of Science and Technology, Luoyang 471003, China
[g] College of Life Science, Capital Normal University, Beijing 100037, China
[h] Key Laboratory of Plant Resources, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China

ORCID IDs: 0000-0002-7156-5928 (D.Y.); 0000-0001-5938-7180 (S.O.); 0000-0003-1832-9835 (Y.X.L.); 0000-0002-0487-6574 (J.L.); 0000-0001-8097-6115 (C.C.)

Nitrogen (N) is a major driving force for crop yield improvement, but application of high levels of N delays flowering, prolonging maturation and thus increasing the risk of yield losses. Therefore, traits that enable utilization of high levels of N without delaying maturation will be highly desirable for crop breeding. Here, we show that OsNRT1.1A (OsNPF6.3), a member of the rice (*Oryza sativa*) nitrate transporter 1/peptide transporter family, is involved in regulating N utilization and flowering, providing a target to produce high yield and early maturation simultaneously. *OsNRT.1A* has functionally diverged from previously reported *NRT1.1* genes in plants and functions in upregulating the expression of N utilization-related genes not only for nitrate but also for ammonium, as well as flowering-related genes. Relative to the wild type, *osnrt1.1a* mutants exhibited reduced N utilization and late flowering. By contrast, overexpression of *OsNRT1.1A* in rice greatly improved N utilization and grain yield, and maturation time was also significantly shortened. These effects were further confirmed in different rice backgrounds and also in *Arabidopsis thaliana*. Our study paves a path for the use of a single gene to dramatically increase yield and shorten maturation time for crops, outcomes that promise to substantially increase world food security.

INTRODUCTION

Nitrogen (N) application is one of the most effective means for crop yield improvement. However, N fertilizers can have deleterious effects on the environment, such as eutrophication of water bodies. N fertilization can also delay flowering and thus prolong the maturation times of crops, which significantly increases the risk of yield losses, especially in high-latitude regions where late-season low temperatures would severely restrict grain filling (Withrow, 1945; Scott et al., 1973; Davenport et al., 2001; Dielen et al., 2001; Castro Marín et al., 2011; Li et al., 2017). Early flowering, the major factor contributing to early maturation, is a prerequisite for the expansion of crop production to higher latitude regions with shorter growing seasons, and for double/triple cropping systems each year (Izawa, 2007; Li et al., 2009). Therefore, the ability to increase yields by improving N use efficiency (NUE) while simultaneously reducing maturation times is viewed as one of the most desirable goals in crop breeding (Li et al., 2017), although to date, breeders have had little success.

Functional and structural studies have demonstrated AtNRT1.1 (AtNPF6.3), a member of the nitrate transporter 1/peptide transporter family, is the central component in nitrate signaling in *Arabidopsis thaliana*, as in addition to mediating nitrate uptake and transport, AtNRT1.1 functions as a sensor to trigger the primary nitrate response (Ho et al., 2009). Monocots and eudicots differ in the number of their *NRT1.1* genes, with grass species typically having three to four *NRT1.1* members and most eudicot species having only one *NRT1.1* gene (Plett et al., 2010). Recently, Wen et al. (2017) reported that two putative homologs of AtNRT1.1 (AtNPF6.3) in maize (*Zea mays* ssp *mays*), ZmNPF6.4 and ZmNPF6.6, display distinct substrates affinity for nitrate and chloride, indicating a possible functional divergence among different members of the *NRT1.1* family in grasses. However, the biological functions and the possible divergence of the *NRT1.1*

[1] These authors contributed equally to this work.
[2] Address correspondence to ccchu@genetics.ac.cn.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Chengcai Chu (ccchu@genetics.ac.cn).
[OPEN]Articles can be viewed without a subscription.
www.plantcell.org/cgi/doi/10.1105/tpc.17.00809

IN A NUTSHELL

Background: To cope with an increasing global population and decreasing availability of arable land, improving crop yield is a major agricultural goal. Nitrogen (N) application is one of the most effective means for crop yield improvement. However, application of N at high rates brings detrimental effects not only to the environment, but also to crops, such as delayed flowering and thus prolonged maturation times, greatly increasing risks of yield loss, especially in high-latitude regions where late-season low temperature would severely restrict grain filling. Thus, the ability to increase yields by improving N use efficiency (NUE) while simultaneously reducing maturation times is viewed as one of the most desirable goals in crop breeding efforts, but very limited success has so far been achieved.

Question: We would like to solve the long-standing paradox of achieving high-yield and early-maturation traits simultaneously.

Findings: We revealed that OsNRT1.1A, a nitrate transporter in rice, is a potential target to manipulate both yield and maturation times via significantly improved NUE. We found that OsNRT1.1A displays functional divergence with OsNRT1.1B that we characterized previously. *OsNRT1.1A* is induced by ammonium, while *OsNRT1.1B* is induced by nitrate. This functional divergence is particularly important for rice production, as ammonium is the preferred N form for rice. Large-scale multi-location field tests showed that rice overexpressing *OsNRT1.1A* had an ~50% increase in NUE and grain yield, and maturation times shortened up to 18 days. Importantly, we found that overexpression of *OsNRT1.1A* in the eudicot Arabidopsis had an even more dramatic effect on yield improvement and early maturation (as high as ~90% increase in seed weight per plant), indicating a potentially enormous influence of this discovery for developing early-maturation high-yield varieties in a great many crop species.

Next steps: Given the vital role of *OsNRT1.1A* in determining N utilization and agronomic performance, it is of particular importance to identify upstream and downstream components that interact with it and discover how the pathway is controlled.

genes in grass species remain largely unknown. Previously, we showed that *OsNRT1.1B* (*OsNPF6.5*) is involved in nitrate utilization, and a single polymorphism in this gene contributes to the long-noted divergence in NUE between *indica* and *japonica* subspecies of Asian cultivated rice (*Oryza sativa*) (Hu et al., 2015). Similar expression and subcellular localization patterns, as well as conserved functions in nitrate uptake, transport, and signaling, strongly suggest that *OsNRT1.1B* is the functional homolog of *AtNRT1.1*. In this study, we revealed that *OsNRT1.1A* (*OsNPF6.3*), another member of rice *NRT1.1* family, displays different N-responsive expression and subcellular localization patterns with *OsNRT1.1B*, showing an example of functional divergence among *NRT1.1* paralogs in grass species. More importantly, overexpression of *OsNRT1.1A* dramatically increases grain yields by improving NUE, and strikingly, also shortens maturation times, providing a promising target for crop improvement.

RESULTS

OsNRT1.1A Displays Ammonium-Inducible Expression and OsNRT1.1A Predominantly Localizes to the Tonoplast

Rice has three putative homologs of *AtNRT1.1*: *OsNRT1.1A*, *OsNRT1.1B*, and *OsNRT1.1C* (*OsNPF6.4*) (Plett et al., 2010). Among these, OsNRT1.1A clusters closest to and shares the highest protein sequence identity with AtNRT1.1 (Figure 1A; Supplemental Figure 1 and Supplemental File 1). However, in contrast to *OsNRT1.1B*, which is strongly induced by nitrate, *OsNRT1.1A* displays an ammonium-induced expression pattern (Figure 1B). The ammonium-induced expression of *OsNRT1.1A* suggested that it is probably involved in ammonium utilization, which is particular important for rice, as ammonium is not only the major N form in the paddy field but also the preferred N source for rice (Arth et al., 1998; Liu et al., 2015). *OsNRT1.1A* shows a very similar tissue expression pattern to *OsNRT1.1B*, as seen by $OsNRT1.1A_{promoter}$:*GUS* transgenic plants with preferential expression in the epidermis and in the vascular tissues of roots, except that *OsNRT1.1A* is also highly expressed in the parenchyma cells of both culms and leaf sheaths (Figure 2).

OsNRT1.1A predominantly localizes to the tonoplast, as seen by colocalization assays with a tonoplast marker and immunogold analysis (Figures 3A and 3B). Furthermore, fluorescence observation in *Nicotiana benthamiana* leaves and vacuole release from rice protoplasts also consistently supported the tonoplast localization of OsNRT1.1A (Supplemental Figure 2), demonstrating a clear difference from plasma membrane-localized AtNRT1.1 and OsNRT1.1B (Ho et al., 2009; Hu et al., 2015). This indicated that the additional member of the NRT1.1 family in rice may have undergone functional divergence, although OsNRT1.1A also has nitrate transporter activity (Figure 4).

Loss of Function of Os*NRT1.1A* Reduces the Utilization of Both Nitrate and Ammonium

To further investigate the function of *OsNRT1.1A* in rice, a homozygous loss-of-function mutant (*japonica* variety Dongjin background [DJ]) was characterized (Supplemental Figures 3A to 3C). The seedlings of the *osnrt1.1a* mutant exhibited significant growth retardation compared with the wild type when grown in hydroponic culture with sufficient N supply (Figure 5A). Notably, the growth retardation of the *osnrt1.1a* mutant was significant when N was supplied as ammonium or nitrate (Figure 5A). By contrast, the seedlings of the *osnrt1.1b* mutant did not display obvious

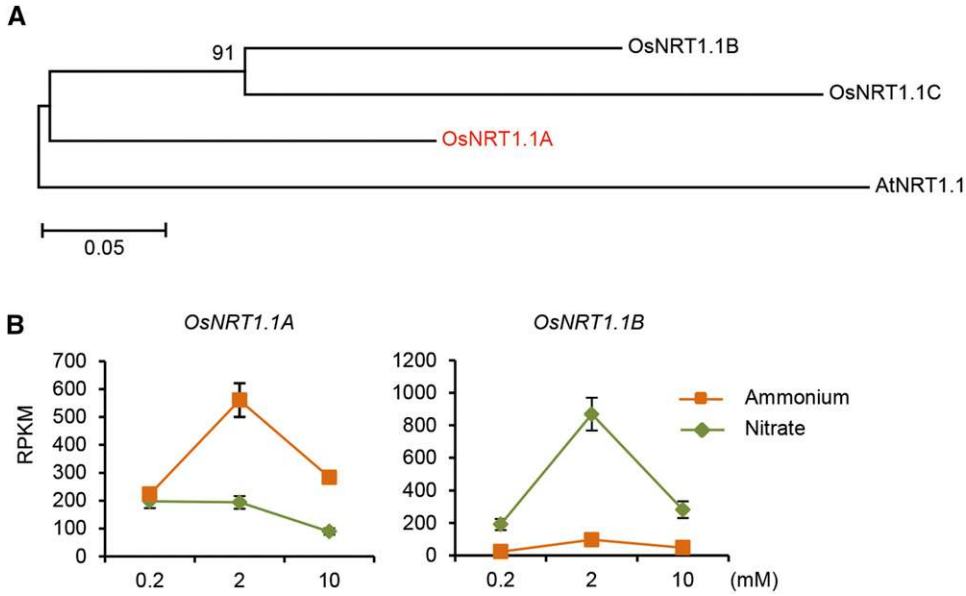

Figure 1. *OsNRT1.1A* Displays Ammonium-Inducible Expression.

(A) Phylogenetic analysis of amino acid sequences of AtNRT1.1 and OsNRT1.1A/B/C using maximum likelihood method by Mega 6.0. Bootstrap = 1000. **(B)** Expression analysis of *OsNRT1.1A* and *OsNRT1.1B* under different N sources (ammonium or nitrate) in roots of rice seedlings assessed by RNA-sequencing. RPKM, reads per kilobase per million mapped reads.

growth retardation compared with the wild type under ammonium or nitrate conditions (Figure 5B). Moreover, ^{15}N quantification following ^{15}N-nitrate or ^{15}N-ammonium feeding showed that acquisition of both nitrate and ammonium was repressed in the *osnrt1.1a* mutant (Supplemental Figure 3D).

Given that AtNRT1.1 and OsNRT1.1B act as transceptors triggering nitrate-responsive gene expression (Ho et al., 2009; Hu et al., 2015), we hypothesized that OsNRT1.1A may also function in regulating N utilization-related gene expression. Under normal hydroponic cultivation with nitrate and ammonium supply, the expression of genes for nitrate uptake and transport such as *OsNRT1.1B*, *OsNRT2.1*, and *OsNRT2.3a*, as well as genes for nitrate assimilation such as *OsNIA1* and *OsNIR1*, was significantly downregulated in the *osnrt1.1a* mutant (Figure 5C). Interestingly, the expression of genes for ammonium uptake and assimilation, such as *OsAMT1.1*, *OsGS1.2*, and *OsGOGAT1*, was also greatly repressed in the *osnrt1.1a* mutant (Figure 5C). By contrast, the expression of these N utilization-related genes was not repressed

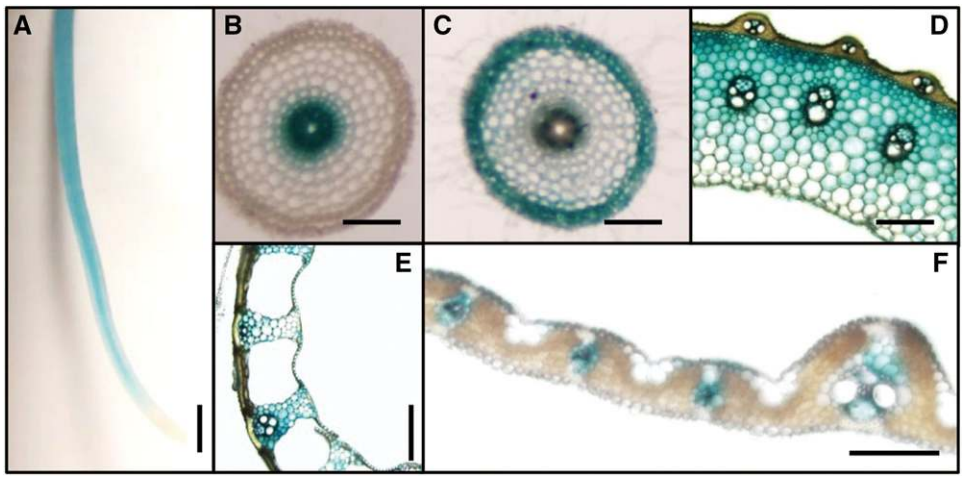

Figure 2. GUS Staining of *OsNRT1.1A*$_{promoter}$*:GUS* Transgenic Plants.

Tissues used for GUS staining include roots (**[A]** to **[C]**), culms **(D)**, leaf sheaths **(E)**, and leaf blades **(F)**, showing cross sections in **(B)** to **(F)**. **(B)** and **(C)** indicate the regions of root tip and root hair, respectively. Bars = 1 mm in **(A)**, 0.3 mm in **(B)** and **(C)**, and 1 mm in **(D)** to **(F)**.

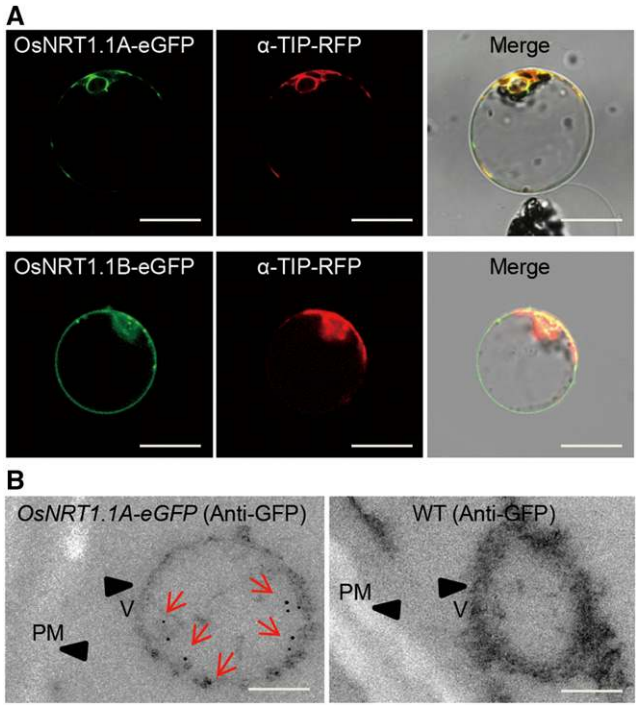

Figure 3. OsNRT1.1A Predominantly Localizes to the Tonoplast.

(A) The OsNRT1.1A-eGFP signal colocalizes with the tonoplast marker α-TIP (fused with RFP) in rice protoplasts. Bars = 20 μm.
(B) Immunogold analysis of OsNRT1.1A-eGFP in *35S:OsNRT1.1A-eGFP* transgenic rice and wild-type DJ (WT). Arrows indicate the immunodetection signal. PM, plasma membrane; V, vacuole. Bars = 100 nm.

in *osnrt1.1b* mutant (Supplemental Figure 3E). Under short-term nitrate treatment, the induction of typical nitrate-responsive genes such as *OsNRT2.1*, *OsNIA1*, and *OsNIA2* was greatly repressed in *osnrt1.1b* mutant (Figure 5D), whereas this primary nitrate-stimulated response was not repressed in *osnrt1.1a* mutant (Figure 5D). These results revealed that OsNRT1.1A plays a fundamental role in maintaining N utilization at high rates not only for nitrate but also for ammonium. OsNRT1.1B, closely related to AtNRT1.1, is responsive for sensing the nitrate stimulus and triggering downstream nitrate-induced gene expression in the short term.

Together, the altered patterns of N-responsive expression and subcellular localization of OsNRT1.1A indicated that a functional divergence has occurred between *OsNRT1.1A* and *OsNRT1.1B* and that *OsNRT1.1A* confers a substantial improvement in N utilization, especially for ammonium. To test whether *OsNRT1.1B* could complement the growth retardation of the *osnrt1.1a* mutant, we introduced *OsNRT1.1B* into *osnrt1.1a* mutant (*osnrt1.1a*/*OsNRT1.1B*) (Supplemental Figure 4A). Compared with the wild type, the seedlings of *osnrt1.1a*/*OsNRT1.1B* transgenic plants showed severe growth retardation (Supplemental Figure 4B), further supporting the functional divergence between *OsNRT1.1A* and *OsNRT1.1B*.

The *osnrt1.1a* Mutant Displays Severe Yield Loss and Late Flowering

In the field, *osnrt1.1a* mutants displayed substantial decreases in plant height, panicle size, seed-setting rate, and 1000-grain weight (Figure 6A; Supplemental Figure 4C), and grain yield thereby decreased by ~80% compared with the wild type (Figure 6B). The *osnrt1.1b* mutant also exhibited defects in these agronomic traits but much slighter than *osnrt1.1a*, with ~20% loss of grain yield (Supplemental Figure 4D). Since *osnrt1.1a* (DJ background) and *osnrt1.1b* (ZH11 background) were derived from different cultivars, the phenotype of the mutants might differ in different backgrounds. To exclude this possibility, we knocked down the expression of *OsNRT1.1A* in the ZH11 background using RNA interference (Supplemental Figure 4E); this also led to much more severe growth defects and yield loss compared with the *osnrt1.1b* mutant (Supplemental Figures 4D to 4G).

Notably, we also observed that *osnrt1.1a* mutant showed significant late-flowering and, thus, prolonged maturation times (Figures 6A and 6C; Supplemental Figure 4C), whereas the flowering time was not significantly altered in the *osnrt1.1b* mutant (Supplemental Figure 4D). All of the growth defects and the late-flowering phenotypes were completely rescued by the introduction of *OsNRT1.1A* into the *osnrt1.1a* mutant background

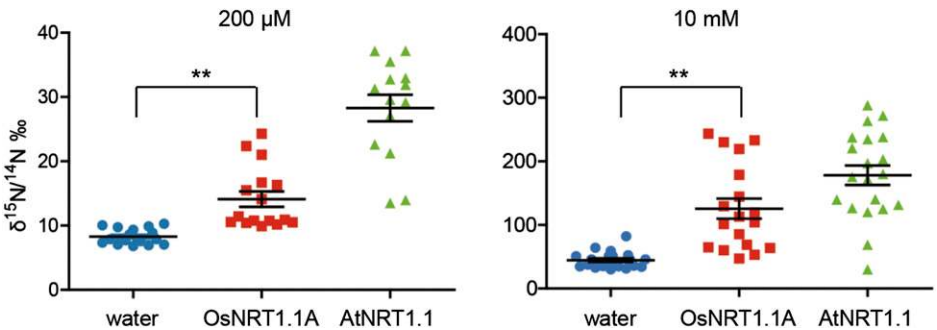

Figure 4. OsNRT1.1A Shows Nitrate Transport Activity in Vitro.

Nitrate uptake assay in *Xenopus laevis* oocytes injected with water, *OsNRT1.1A*, and *AtNRT1.1* using ^{15}N-nitrate. *AtNRT1.1* was used as the positive control. Twenty replicates were performed for each sample. Asterisks indicate the significant differences between water (negative control) and *OsNRT1.1A* as evaluated by Student's *t* tests: $^{**}P < 0.01$.

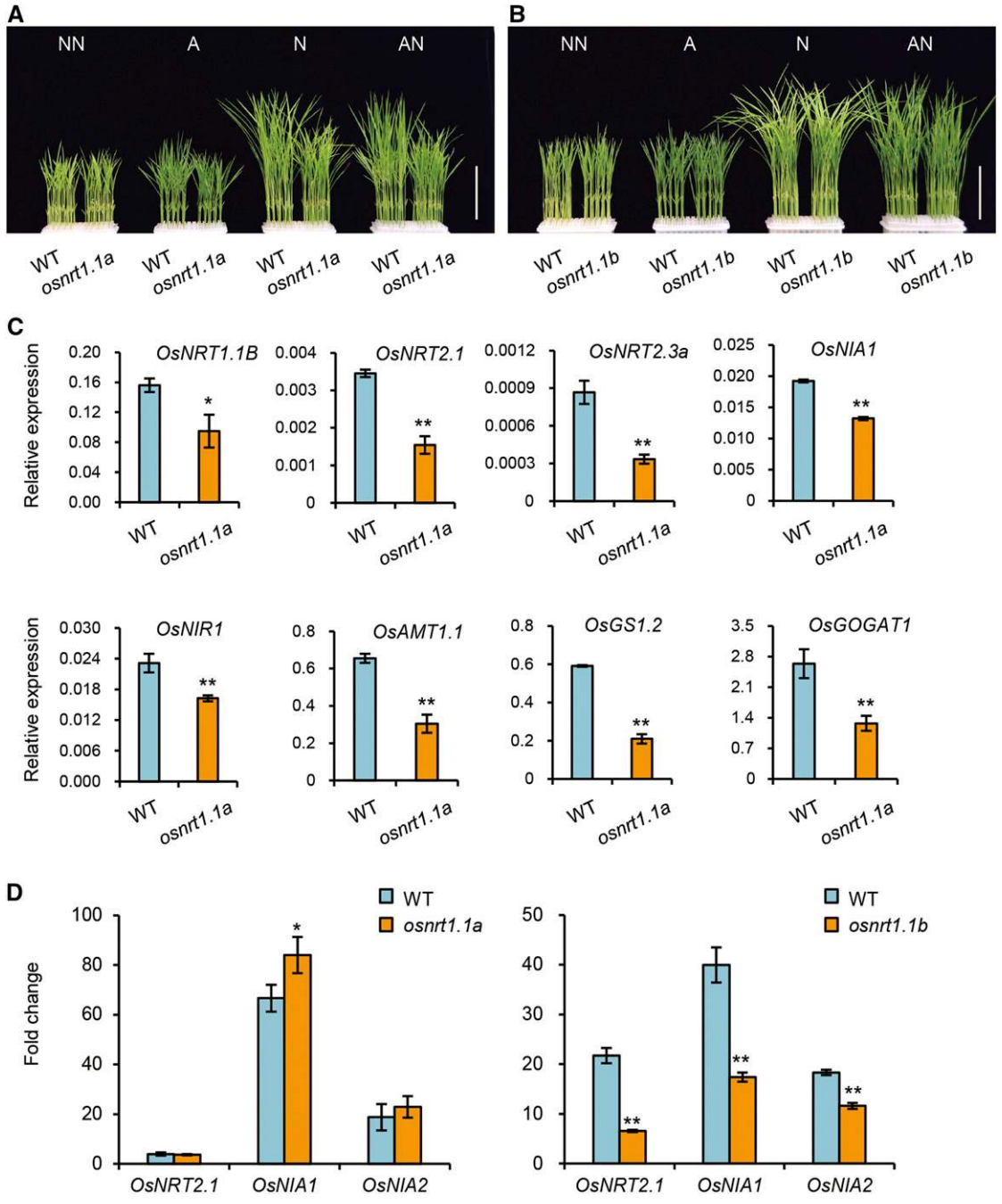

Figure 5. *OsNRT1.1A* Displays Functional Divergence with *OsNRT1.1B*.

(A) Growth of wild-type DJ (WT) and *osnrt1.1a* mutant seedlings under different N sources.

(B) Growth of wild-type ZH11 (WT) and *osnrt1.1b* mutant seedlings under different N sources. NN, no N; A, ammonium (2 mM); N, nitrate (2 mM); AN, ammonium and nitrate (1 mM for each). Bars = 8 cm.

(C) RT-qPCR-based expression analyses of genes involved in utilization of nitrate and ammonium in roots of wild-type DJ and *osnrt1.1a* mutant plants under normal hydroponic cultivation.

(D) RT-qPCR-based nitrate induction assay in roots of *osnrt1.1a* mutant and *osnrt1.1b* mutant. Values are the means ±SD ($n = 3$). Asterisks indicate significant differences between wild-type plants and according mutants as evaluated by Student's *t* tests: $^{*}P < 0.05$ and $^{**}P < 0.01$.

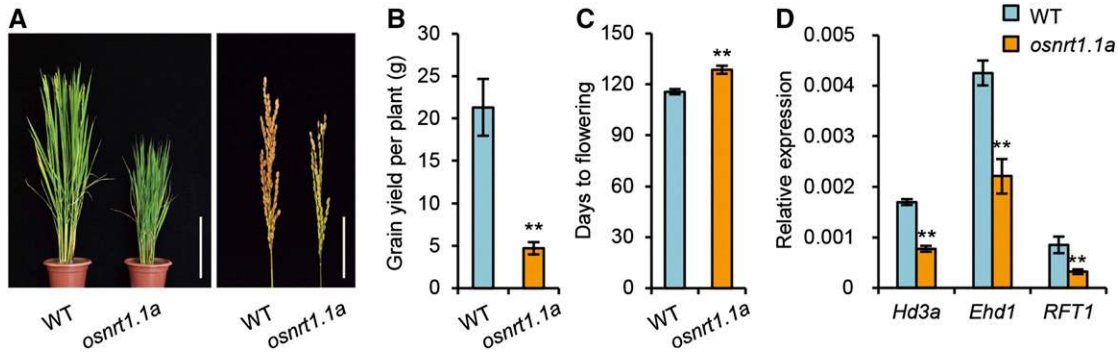

Figure 6. Loss of Function of *OsNRT1.1A* Results in Severe Grain Yield Loss and Late Flowering.

(A) Gross morphological phenotypes and panicle phenotypes of wild-type DJ (WT) and *osnrt1.1a* mutants grown in the field. Bars = 20 cm (gross morphological phenotypes) and 6 cm (panicle).

(B) Grain yield per plant of wild-type DJ and *osnrt1.1a* mutant.

(C) Days to flowering of wild-type DJ and *osnrt1.1a* mutant.

(D) RT-qPCR-based expression analysis of flowering-promoting genes in leaves of wild-type DJ and *osnrt1.1a* mutant. Values are the means ± SD (10 replicates for grain yield per plant, 10 replicates for days to flowering, and 3 replicates for RT-qPCR). Asterisks indicate significant differences between wild-type DJ and *osnrt1.1a* mutant as evaluated by Student's *t* tests: $^{**}P < 0.01$.

(Supplemental Figures 5A and 5B). We also observed that the expression of *Heading date 3a* (*Hd3a*), *Early heading date 1* (*Ehd1*), and *RICE FLOWERING LOCUS T1* (*RFT1*), critical genes known to promote flowering in rice (Doi et al., 2004; Komiya et al., 2009; Itoh et al., 2010), were significantly downregulated in *osnrt1.1a* mutants (Figure 6D). Furthermore, the expression of both *OsNRT1.1A* and *Hd3a* in the wild type was dramatically repressed by high N treatment, which is in accordance with the late-flowering phenotype caused by high N application (Supplemental Figures 5C to 5E). These data indicated that *OsNRT1.1A* is also involved in N-regulated flowering. Flowering time is one of the most important agronomic traits for determining the adaption to local environments and expansion of cultivation area, and high N application usually delays flowering (Withrow, 1945; Castro Marín et al., 2011; Li et al., 2017). Recalling the important effect of *OsNRT1.1A* in improving N utilization, our results suggested that *OsNRT1.1A* might provide a solution for rice to simultaneously promote N utilization and early maturation.

Overexpression of *OsNRT1.1A* Significantly Improves N Utilization

We next investigated whether *OsNRT1.1A* overexpression can facilitate N utilization. To this end, we generated transgenic rice overexpressing *OsNRT1.1A* under the control of either its native promoter or the constitutively active rice *ACTIN1* promoter. RT-qPCR analysis showed that the expression of *OsNRT1.1A* was greatly increased in the overexpression (OE) transgenic lines (OEnp-3 and OEnp-4, overexpression driven by the native promoter; OEa-6, overexpression driven by the *ACTIN1* promoter) (Supplemental Figure 6A). Compared with wild-type plants, *OsNRT1.1A*-OE plants exhibited significantly improved growth under long-term hydroponic culture, as indicated by increased plant height, chlorophyll content, and biomass (Figure 7A; Supplemental Figure 6B). The acquisition of nitrate and ammonium increased, as demonstrated by ^{15}N-nitrate and ^{15}N-ammonium feeding experiments, and the expression of genes for the utilization of nitrate and ammonium was also significantly upregulated in *OsNRT1.1A*-OE plants (Figures 7B and 7C), indicating that manipulation of *OsNRT1.1A* has great potential for improving N utilization of rice.

OsNRT1.1A Promotes Nuclear Localization of NLPs

The results from *osnrt1.1a* mutant and *OsNRT1.1A*-OE plants consistently demonstrated that OsNRT1.1A is involved in upregulating the expression of a great number of critical N utilization genes. In Arabidopsis, AtNLP7 is viewed as the central transcription factor in nitrate signaling and is involved in regulating the expression of N utilization genes. AtNLP7 is regulated by nitrate-dependent nuclear localization to trigger target gene expression (Marchive et al., 2013). We found that the two closest homologs of AtNLP7 in rice (Chardin et al., 2014), OsNLP3 and OsNLP4, also exhibited nitrate-promoted nuclear localization (Supplemental Figure 7). Notably, OsNRT1.1A can greatly promote the nuclear localization of OsNLP3 and OsNLP4 even under nitrate-absent condition (Figure 8). Moreover, the nuclear localization of OsNLP3 and OsNLP4 was also repressed in *osnrt1.1a* mutants compared with that in the wild type (Supplemental Figure 8), collectively indicating that OsNRT1.1A upregulates N utilization genes via facilitating cytoplasmic-nuclear shuttling of NLPs.

OsNRT1.1A Overexpression Promotes Both High Yield and Early Maturation

As expected, the *OsNRT1.1A*-OE plants displayed improved growth in field trials with significantly earlier flowering and larger panicle (Figures 9A and 9B; Supplemental Figures 9A to 9C). In accordance with the early-flowering phenotype, the expression of *Hd3a*, *Ehd1*, and *RFT1* was also significantly upregulated in *OsNRT1.1A*-OE plants (Supplemental Figure 9D). The improved growth and early-flowering phenotypes and the upregulation of early-flowering genes in *OsNRT1.1A*-OE plants are consistent

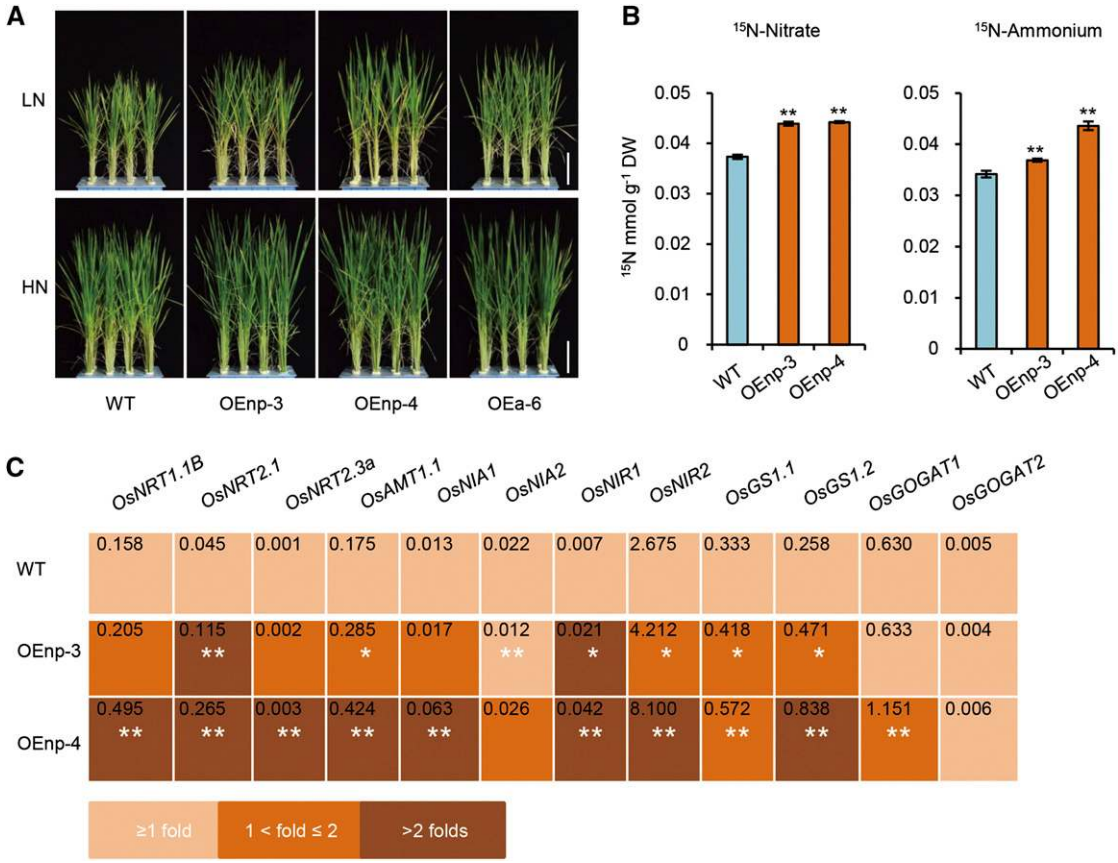

Figure 7. *OsNRT1.1A* Overexpression Activates N Utilization.

(A) Growth of wild-type DJ (WT) and *OsNRT1.1A*-OE plants in long-term hydroponic culture under LN (400 μM) or HN (2 mM) conditions. Bars = 20 cm.
(B) ^{15}N accumulation assays in shoots of wild-type DJ and *OsNRT1.1A*-OE plants labeled with ^{15}N-nitrate or ^{15}N-ammonium. Values are the means ± SD ($n = 4$).
(C) RT-qPCR-based expression analysis of N utilization genes in roots of wild-type DJ and *OsNRT1.1A*-OE plants. Numbers in table indicate the relative expression level, and the darker color represents the higher increased folds of gene expression in *OsNRT1.1A*-OE plants. Values are the means ± SD ($n = 3$). Asterisks indicate significant differences between wild-type DJ and *OsNRT1.1A*-OE plants as evaluated by one-way ANOVA with Tukey's test: $^{*}P < 0.05$ and $^{**}P < 0.01$.

with the results from the *osnrt1.1a* mutant, which collectively demonstrated that OsNRT1.1A plays important roles in promoting both N utilization and flowering.

To further test the potential of *OsNRT1.1A*, large-scale field trials of *OsNRT1.1A*-OE plants were performed in different years at three different locations across China: Beijing (E116°, N40°), Changsha (E112°, N28°), and Sanya (E108°, N18°). In Beijing, the field trials were performed in two years (2015 and 2016) under both low (LN) and high N (HN) conditions. In 2015, under the LN condition, *OsNRT1.1A*-OE plants showed significant increases in seed number per panicle, 1000-grain weight, and slight increase in tiller number, resulting in ~32 to 50% increases in grain yield per plant (Supplemental Table 1; Figure 9C). Under the HN conditions, *OsNRT1.1A*-OE plants displayed significant increases in the seed number per panicle and in the 1000-grain weight, resulting in ~25 to 45% increases in grain yield per plant (Supplemental Table 1; Figure 9D). Compared with wild-type plants, the actual yield per plot and the NUE (defined as the grain yield per unit of available N in the soil) (Moll et al., 1982, 1987) increased by ~38 to 54% and ~33 to 45% in *OsNRT1.1A*-OE plants under the LN and HN conditions, respectively (Figures 6C and 6D). More excitingly, the flowering time of *OsNRT1.1A*-OE plants was ~9 to 13 and ~10 to 18 d earlier than wild-type plants under LN and HN conditions, respectively (Figures 9C and 9D), which significantly shortened the maturation times (Supplemental Table 1). The results of the 2015 and 2016 field tests in Beijing were very similar (Supplemental Figure 10). In Changsha (2016), *OsNRT1.1A*-OE plants displayed significant improvements in grain yield and NUE (~27–37% in LN and ~16% in HN for both traits), these plants also exhibited earlier flowering than the wild type under both of the N conditions (~3–6 d in LN and ~4–6 d in HN) (Supplemental Figure 11A). Field tests in Sanya (tropical region) were performed from December 2015 to April 2016, and *OsNRT1.1A*-OE plants had greatly increased grain yields and NUE (~47–63% in LN and ~11–32% in HN for both traits) (Supplemental Figure 11B). However, there was no significant difference in flowering time between *OsNRT1.1A*-OE and wild-type plants grown in Sanya (Supplemental Figure 11B), which may be attributable to the short-day conditions in Sanya, as short

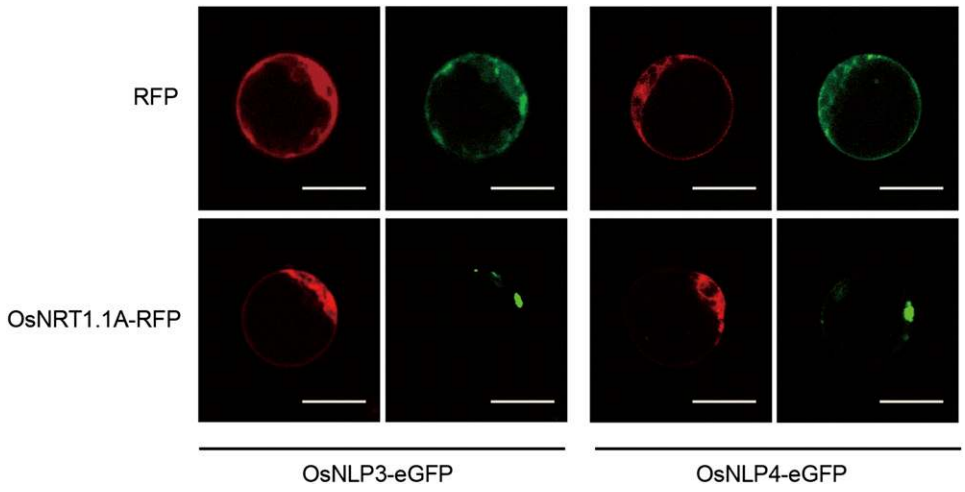

Figure 8. OsNRT1.1A Promotes Nuclear Localization of NLPs.

The effect of OsNRT1.1A on subcellular localization of OsNLP3/4-eGFP in rice protoplasts under nitrate-absent condition. *OsNLP3/4-eGFP* were co-transformed with *35S*:*RFP* (red fluorescent protein) or *35S*:*OsNRT1.1A-RFP*. Bars = 15 μm.

days strongly promote flowering in *japonica* rice (Brambilla and Fornara, 2013). We also overexpressed *OsNRT1.1A* in a different rice background, Hejiang 19 (HJ19), a *japonica* cultivar that developed for the northernmost rice-growing areas of China (E130°, N46°). When grown in Beijing (E116°, N40°) and Harbin (E127°, N48°), HJ19/*OsNRT1.1A*-OE transgenic plants consistently displayed markedly increased grain yields (Supplemental Figures 12A to 12C). These OE transgenic plants also exhibited an early-flowering phenotype, which is impressive as HJ19 already has a very short flowering time (Supplemental Figures 12A to 12C).

As eudicot species usually have one *NRT1.1* gene, it is possible that introduction of *OsNRT1.1A* would give rise to stronger effects. To test this, we overexpressed *OsNRT1.1A* in Arabidopsis. This resulted in dramatically increased plant size and earlier flowering (~6–15 d earlier than the wild type) (Figure 10A). Strikingly, the seed weight per plant and biomass increased up to 90% in the transgenic plants (Figure 10B). These results suggested that overexpression of *OsNRT1.1A* has enormous potential as a strategy to increase yields and shorten maturation times in both monocot and eudicot crops.

DISCUSSION

As a plasma membrane-localized transporter, besides mediating nitrate uptake and transport, AtNRT1.1 also functions as a sensor to perceive external nitrate and trigger the expression of nitrate-responsive genes in Arabidopsis (Ho et al., 2009). Given the central role of *AtNRT1.1* in nitrate utilization, it was very attractive to further explore the function and application of the *NRT1.1* homologs in NUE improvement, especially in crops. However, the function of *NRT1.1s* is still largely unknown in crops, even though it has been noted that the major grain crops like rice, wheat (*Triticum aestivum*), and maize possess additional members of the *NRT1.1* family (Plett et al., 2010). Our previous work demonstrated that *OsNRT1.1B* is the functional homolog of *AtNRT1.1* in rice (Hu et al., 2015). In this study, we found that *OsNTR1.1A*, another rice *NRT1.1*, displays an unexpected expression pattern with strong induction by ammonium. Additionally, functional characterization of the loss-of-function mutant and overexpression transgenic plants demonstrated that *OsNRT1.1A* can promote the utilization of both ammonium and nitrate by upregulating the expression of N utilization-related genes. In comparison with *AtNRT1.1* and *OsNRT1.1B* mediating the nitrate-stimulated primary response, *OsNRT1.1A* seems to play a more fundamental role in managing N utilization under a relative stable N supply with both ammonium and nitrate. Possibly, this functional divergence between OsNRT1.1A and OsNRT1.1B is associated with their subcellular localizations. The plasma membrane localization allows AtNRT1.1 and OsNRT11.B to sense the extracellular N status, whereas the tonoplast localization allows OsNRT1.1A to sense the intracellular N status. Rice is traditionally planted in waterlogged fields where ammonium is the major N source; however, up to 40% of total N taken up by rice is absorbed as nitrate because of nitrification in the rhizosphere (Arth et al., 1998; Li et al., 2008; Xu et al., 2012). Thereby, rice has evolved with the ability to utilize both ammonium and nitrate at high rates. Notably, the action of *OsNRT1.1A* seems to contribute to this specified N utilization manner of rice, suggesting a vital role of *OsNRT1.1A* in determination of rice NUE. The severe growth retardation and yield loss in *osnrt1.1a* mutants further supports this conclusion.

Many attempts have been made to use the overexpression of N transporter genes to improve NUE and grain yields (Fang et al., 2013; Chen et al., 2016; Fan et al., 2016). All such efforts have required consideration of the influence of N in delaying maturation times. Our results demonstrate that overexpression of *OsNRT1.1A* can confer improvements in NUE and grain yield while simultaneously shortening maturation times. Therefore, *OsNRT1.1A* possibly provides a solution to the conflict between N nutrition and maturation time. Impressively, the effect of *OsNRT1.1A* was not only verified in different rice backgrounds but also in Arabidopsis,

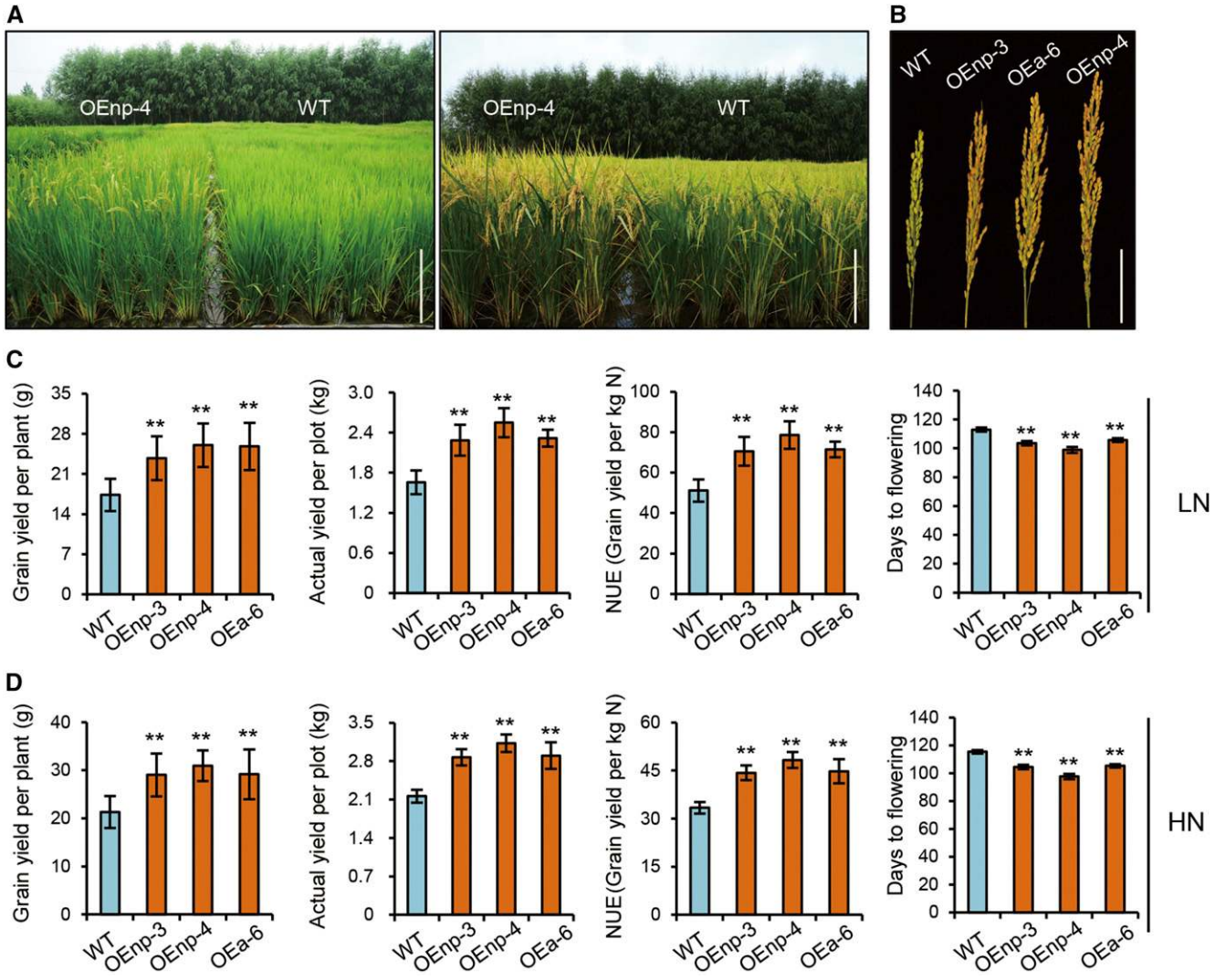

Figure 9. *OsNRT1.1A* Overexpression Promotes Early Flowering and Grain Yield Improvement in Rice.

(A) Growth of wild-type DJ (WT) and *OsNRT1.1A*-OE plants (OEnp-4) in the field at flowering (left) and grain-filling (right) stage. Bars = 50 cm.
(B) The panicles of wild-type DJ and *OsNRT1.1A*-OE plants. Bar = 8 cm.
(C) Grain yield per plant, actual yield per plot, NUE, and days to flowering of wild-type and *OsNRT1.1A*-OE plants under LN conditions in a field trial in Beijing (2015).
(D) As in **(C)**, for HN conditions in a field trial in Beijing (2015). Values in **(C)** and **(D)** are the means ± SD (18 replicates for grain yield per plant, 4 replicates for actual yield per plot and NUE, and 41 replicates for days to flowering). Asterisks indicate significant differences between wild-type and *OsNRT1.1A*-OE plants as evaluated by one-way ANOVA with Tukey's test: $^{**}P < 0.01$.

indicating the great potential for the use of this gene in crop improvement programs for multiple crop species. Further transgenic tests in eudicot crops such as soybean (*Glycine max*) and tomato (*Solanum lycopersicum*) will be greatly helpful to explore the application value of *OsNRT1.1A*.

OsNRT1.1A functions in promoting both high yield and early maturation by upregulating the expression of genes involved in N utilization and flowering. However, the regulating mechanism between OsNRT1.1A and these genes is still far from fully understood. We provide preliminary evidence to unveil the regulatory function of OsNRT1.1A, showing that OsNRT1.1A can facilitate the nuclear localization of NLP transcription factors, which play a central role in activating the expression of N utilization-related genes. Recently, Liu et al. (2017) revealed that the phosphorylation of AtNLP7 by Ca^{2+}-sensor protein kinases can promote its nuclear localization. Given the tonoplast localization, it is intriguing to speculate that OsNRT1.1A might serve as an intracellular scaffold to recruit NLPs and Ca^{2+}-sensor protein kinases together, which can facilitate the phosphorylation of NLPs and consequently promote their nuclear localization. To date, the mechanism underlying N-regulated flowering remains largely elusive. Recently, Yuan et al. (2016) reported that CRYPTOCHROME1 (CRY1), a blue

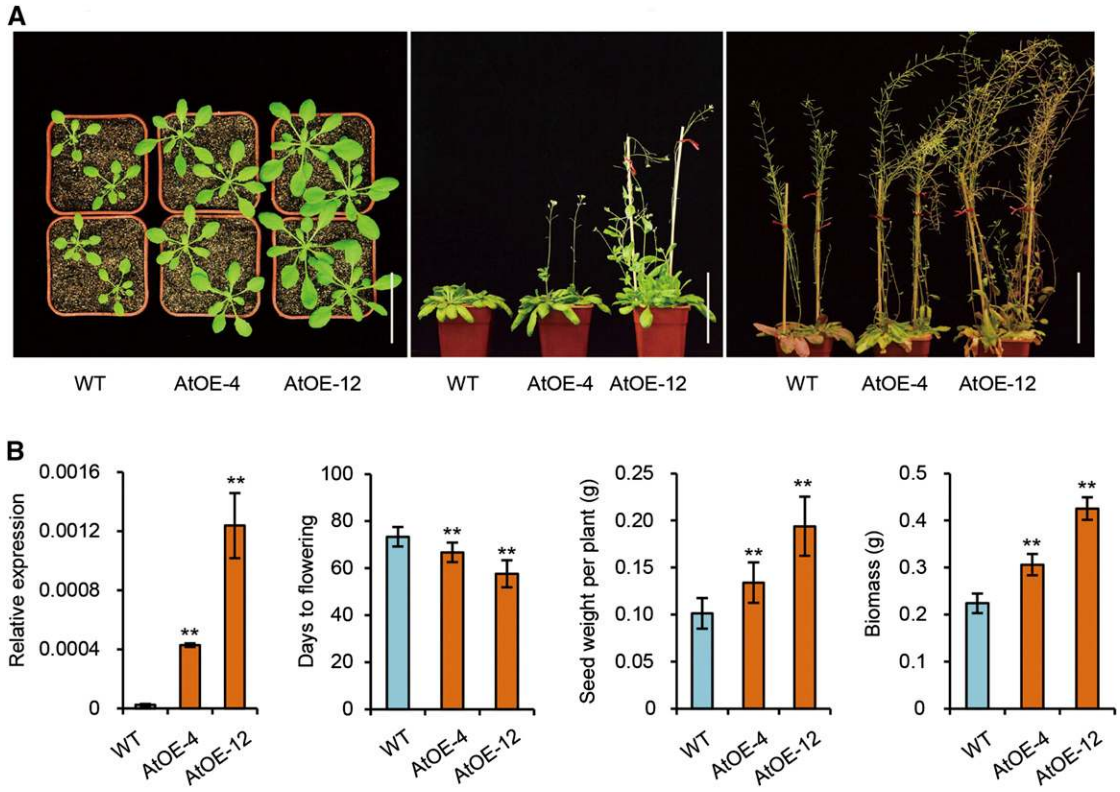

Figure 10. *OsNRT1.1A* Overexpression in Arabidopsis Also Improves Seed Weight and Affects Flowering Time.

(A) Growth of wild-type Arabidopsis (WT) and transgenic Arabidopsis overexpressing *OsNRT1.1A* (AtOE-4/12) at different growth stages. Bars = 5 cm (left) and 12 cm (middle and right).

(B) RT-qPCR-based *OsNRT1.1A* expression, days to flowering, seed weight per plant, and biomass of wild-type Arabidopsis and overexpression plants. Values are the means ± SD (3 replicates for RT-qPCR analysis, 17 replicates for days to flowering, and 10 replicates for seed weight per plant and biomass). Asterisks indicate the significant differences between wild-type Arabidopsis and AtOE plants as evaluated by one way ANOVA with Tukey's test: $^{**}P < 0.01$.

light receptor, is involved in N-regulated flowering in Arabidopsis. N supply can affect the nuclear CRY1 protein level, which modulates the amplitude of the circadian clock and in turn controls flowering. Given the direct involvement of OsNRT1.1A in N signaling and its function in promoting nuclear localization of NLPs, it is possible that OsNRT1.1A could also facilitate the nuclear localization of a CRY1 homolog in rice, thus mediating N-regulated flowering. Interestingly, *OsNRT1.1A* was also highly expressed in the developing panicles (Supplemental Figure 13), further suggesting OsNRT1.1A might be directly involved in regulating flowering or panicle development.

Alternative to direct effects on the clock, the function of OsNRT1.1A in regulating flowering could reflect its significant role in improving N utilization. In general, high N supply can greatly delay flowering, but it is not clear how high N application is responsible for delayed flowering. Possibly, high accumulation of the primary N source like nitrate and ammonium might convey the signal of a rich N status to plants, which favors the vegetative growth and delays flowering. As the assimilation of primary N sources, this repression of flowering will be released. Our results showed that OsNRT1.1A not only upregulates the expression of nitrate and ammonium transporters, but also activates the expression of a set of genes involved in nitrate and ammonium assimilation, including *NIA*, *NIR*, *GS*, and *GOGAT*, which greatly speeds up N assimilation and thereby eliminates high N accumulation, which is the cause of flowering repression. Despite such hypotheses, our understanding of the mechanism of OsNRT1.1A is far behind our recognition of its vital contribution in determining N utilization and agronomic performance in rice. Further efforts concentrating on identifying OsNRT1.1A-interacting proteins will be of particular importance to get new insight into its regulatory mechanism.

METHODS

Plant Materials and Growth Conditions

The rice (*Oryza sativa*) varieties Dongjin (DJ), Zhonghua 11 (ZH11), and Hejiang 19 (HJ19) were used in this study. The homozygous *osnrt1.1a* T-DNA insertion mutant (PFG_1E-00433.L, DJ background) was ordered from the Korea Rice Mutant Center (Pohang, Korea) (Jeong et al., 2002). The *osnrt1.1b* mutant was identified in a previous study (Hu et al., 2015). For short-term hydroponic culture, rice seedlings were grown in modified Kimura B solution in a growth chamber with a 12-h-light (30°C)/12-h-dark (28°C) photoperiod, ~200 μmol m^{-2} s^{-2} photon density, and ~70% humidity. The modified Kimura B solution contained the following macronutrients (mM): $(NH_4)_2SO_4$ (0.5), $MgSO_4 \cdot 7H_2O$ (0.54), KNO_3 (1), $CaCl_2$ (0.36), K_2SO_4 (0.09), KH_2PO_4 (0.18), and $Na_2SiO_3 \cdot 9H_2O$ (1.6); and

micronutrients (μM): $MnCl_2 \cdot 4H_2O$ (9.14), H_3BO_3 (46.2), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.08), $ZnSO_4 \cdot 7H_2O$ (0.76), $CuSO_4 \cdot 5H_2O$ (0.32), and Fe(II)-EDTA (40), with the pH adjusted to 5.8. The nutrient solution for culture was renewed every day. For long-term hydroponic culture, rice seedlings were grown in an artificial climate chamber (with 1 kW high-intensity discharge lamps) with a 12-h-light (28°C)/12-h-dark (25°C) photoperiod, ~300 μmol m^{-2} s^{-1} photon density, and ~40% humidity. The modified Kimura B solution [200 μM KNO_3 together with 100 μM $(NH_4)_2SO_4$ for LN and 1 mM KNO_3 together with 0.5 mM $(NH_4)_2SO_4$ for HN] with different N concentrations were used for long-term hydroponic cultivation. For *osnrt1.1a* mutant phenotyping under different N sources, the N was supplied with ammonium [1 mM $(NH_4)_2SO_4$], nitrate (2 mM KNO_3), or ammonium and nitrate [0.5 mM $(NH_4)_2SO_4$ and 1 mM KNO_3], respectively.

The *Arabidopsis thaliana* ecotype Columbia (Col-0) was used in this study. Arabidopsis seeds were surface-sterilized with 2.5% NaClO, rinsed five times with sterile distilled water, and placed on half-strength Murashige and Skoog medium containing 1.0% (w/v) sucrose and 0.7% (w/v) agar. After vernalization at 4°C for 2 d, seeds were germinated and grown in growth chambers at 22°C and 70% relative humidity. For measurement of flowering time, 7-d-old seedlings were transferred to pots containing a 2:1 vermiculite:soil mixture under the condition of an 8-h-light /16-h-dark photoperiod and 120 μmol m^{-2} s^{-2} photon density.

RNA-Sequencing Analysis

About 100 rice (*japonica* cultivar ZH11) seedlings of each treatment were grown hydroponically in a growth chamber with the condition described above. For long-term ammonium and nitrate treatments, the seedlings were cultured in modified Kimura B solution after germination with different ammonium or nitrate concentrations (0.2/2/10 mM), and the total concentrations of potassium in all treatments were adjusted for consistency. Roots of 10-d-old seedlings were sampled for RNA-sequencing. For each treatment, 15 seedlings were collected as a sample, and three independent biological replicates were conducted. RNA library construction and sequence analysis were conducted as described previously (Li et al., 2016).

^{15}N-Nitrate Uptake Assay in *Xenopus laevis* Oocytes

The coding region of *OsNRT1.1A* was amplified and cloned into the pCS2 + vector between the restriction sites *Bam*HI and *Eco*RI, and then was linearized by *Apa*I (Rupp et al., 1994). Complementary RNA of *OsNRT1.1A* was synthesized in vitro using the mMESSAGE mMACHINE kit (Ambion; AM1340) according to the manufacturer's protocol. *X. laevis* oocytes at stage V-VI were harvested and defolliculated in a Ca^{2+}-free solution (82 mM NaCl, 20 mM $MgCl_2$, 2 mM KCl, and 5 mM HEPES, pH 7.4) containing 1 mg/mL collagenase type II A (Sigma-Aldrich) for 30 min at room temperature (22°C). Oocytes were injected on the same day with 46 ng of *OsNRT1.1A* complementary RNA using a Nanoject II injector (Drummond Scientific). After injection, oocytes were cultured in ND-96 medium (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, and 50 μg/mL gentamicin sulfate, pH 7.4) at 18°C for 24 h and ready for ^{15}N-nitrate uptake assay. High- and low-affinity uptake assays in oocytes were performed using 200 μM and 10 mM ^{15}N-KNO_3, respectively, as described previously (Almagro et al., 2008). For each sample, one oocyte was analyzed as an independent sample, and 20 replicates were conducted. *AtNRT1.1* was used as the positive control. Primers used are listed in Supplemental Table 2.

Labeling with ^{15}N-Nitrate or ^{15}N-Ammonium for Determination of ^{15}N Accumulation

^{15}N-accumulation assay after ^{15}N-nitrate or ^{15}N-ammonium labeling was performed with ^{15}N-labeled KNO_3 (98 atom % ^{15}N; Sigma-Aldrich; No. 335134) or ^{15}N-labeled NH_4Cl (98 atom % ^{15}N; Sigma-Aldrich; No. 299251), respectively. For ^{15}N-nitrate accumulation assay, rice seedlings were cultured in the Kimura B solution for 10 d. Next, the seedlings were pretreated with the Kimura B solution for 2 h and then transferred to modified Kimura B solution containing 5 mM ^{15}N-KNO_3 for 3 h. At the end of labeling, the roots were washed for 1 min in 0.1 mM $CaSO_4$ and separated from the shoots. Roots and shoots were collected and dried at 70°C. Finally, the samples were ground and the ^{15}N content was evaluated by an isotope ratio mass spectrometer with an elemental analyzer (Thermo Finnigan Delta Plus XP; Flash EA 1112). For ^{15}N-ammonium accumulation assays, the treatment was conducted as above except that 5 mM ^{15}N-KNO_3 was replaced with 1 mM ^{15}N-NH_4Cl and 1 mM KNO_3. For each sample, 20 seedlings were collected as a sample, and four biological replicates were used.

Nitrate Induction Assay

Rice seedlings were first cultured in the modified Kimura B solution with 0.25 mM $(NH_4)_2SO_4$ as the sole N source for 3 weeks and then were induced with 5 mM KNO_3 for 0.5 h according to the method as previously described (Hu et al., 2015).

Subcellular Localization Assay

To investigate the subcellular localization of OsNRT1.1A, the *35S:OsNRT1.1A-eGFP* (enhanced green fluorescent protein) fusion constructs were produced by inserting the full open reading frame of *OsNRT1.1A* into the pCAMBIA2300-*35S:eGFP* vector. The gene-specific primers used for PCR amplification are listed in Supplemental Table 2. α-TIP was used as a tonoplast marker (Hunter et al., 2007). Plasmids were extracted and purified using the Plasmid Midi Kit (Qiagen; No. 12143) following the manufacturer's manual. The rice protoplasts were isolated and transformed according to the published methods described previously (Bart et al., 2006; Zhang et al., 2011). For vacuole releasing, intact rice protoplasts were treated by vacuole lysis buffer (10 mM EDTA, 10 mM EGTA, and 95 mM mannitol or sorbitol, pH 8.0 adjusted by 1 M Tris) for 3 to 5 min, and ready for microscopy observation. To confirm the subcellular localization, the construct was also transiently expressed in *Nicotiana benthamiana* leaves by *Agrobacterium tumefaciens*-mediated infiltration (strain GV3101) as described previously (An et al., 2017).

Moreover, for analysis the nuclear-cytoplasmic shuttling of OsNLP3/4 and the effect of OsNLP3/4 nuclear retention promoted by OsNRT1.1A, the full-length open reading frames of *OsNLP3/4* and *OsNRT1.1A* were cloned into pCAMBIA2300-*35S:eGFP* and pSAT6-*mRFP*-N1 (CD3-1108) vector, respectively. To investigate the nuclear-cytoplasmic shuttling of OsNLP3/4, the transformed rice protoplasts were treated for 30 min with 10 mM KCl or KNO_3, respectively. In addition, to test the OsNRT1.1A-mediated nuclear retention of OsNLP3/4, pCAMBIA2300-*35S:OsNLP3/4-eGFP* was cotransformed into rice protoplasts with pSAT6-*OsNRT1.1A-mRFP* or pSAT6-*mRFP*-N1, respectively. To further analyze the OsNLP3/4-eGFP subcellular localization patterns in the backgrounds of the wild type and *osnrt1.1a* mutant, rice seedlings were germinated and grown on the modified Kimura B solution with 0.25 mM $(NH_4)_2SO_4$ and 0.5 mM KNO_3 as the N sources for 10 d and used for rice protoplast isolation and transient expression analysis. The fluorescence images were captured via a confocal laser-scanning microscope (TCS SP5; Leica).

Electron Microscopy and Immunolabeling

Root tips of transgenic plants harboring 35S:*OsNRT1.1A-eGFP* were collected and fixed in 0.1 M phosphate buffer (pH 7.4), containing freshly prepared 4% (w/v) paraformaldehyde for 4 h at room temperature, and then overnight at 4°C. Root samples were dehydrated through a graded ethanol series and embedded in LR White resin (Sigma-Aldrich). Blocks were polymerized under UV light (360 nm) at −20°C for 24 h. Ultrathin sections

(80 nm) were cut with a diamond knife using an ultramicrotome (UC7; Leica Microsystems) and mounted on nickel grids with a single slot.

For immunolabeling, nickel grids carrying ultrathin root tip sections were incubated with anti-GFP antibody at room temperature for 1 h and then 4°C overnight. Goat anti-mouse IgG conjugated with 10-nm colloidal gold particles (Sigma-Aldrich; diluted 1:50) were used as secondary antibody. Grids were stained with 2% uranyl acetate and examined with FEI Tecnai G2 20 transmission electron microscope at 120 kV (Saito et al., 2009; Li et al., 2011).

Generation of Transgene Constructs and Plant Transformation

The *OsNRT1.1A* full-length coding region was amplified and cloned into pCAMBIA2301-*ACTIN1* to generate pCAMBIA2301-*ACTIN1*:*OsNRT1.1A* overexpression construct. A 2.0-kb promoter region and the full-length coding sequence of *OsNRT1.1A* were amplified and cloned into pCAMBIA2300 to generate pCAMBIA2300-$OsNRT1.1A_{promoter}$:*OsNRT1.1A* overexpression construct. To generate the *OsNRT1.1A*-RNAi vector, a specific sequence of the *OsNRT1.1A* coding region was amplified. The resulting PCR product was inserted into the pUCC-RNAi vector (Luo et al., 2006) in both sense and antisense orientation and then the fragment containing an artificial inverted-repeat sequence of *OsNRT1.1A* was transferred into pCAMBIA2301-*ACTIN1* for *OsNRT1.1A*-RNAi construct. All the primers used to generate the overexpression constructs above are listed in Supplemental Table 2, and all of the constructs were confirmed by sequencing. pCAMBIA2300-$NRT1.1B_{\text{Genomic fragment}}$ was used as described previously (Hu et al., 2015). The constructs were introduced into Agrobacterium strain AGL1. The wild type (DJ or HJ) was used as the recipient for Agrobacterium-mediated transformation as described previously to generate the transgenic rice (Hiei et al., 1994). The T-DNA copy numbers of relative transgenic rice plants were confirmed by RT-qPCR described previously (Bubner and Baldwin, 2004; Yang et al., 2005), and the results are presented in the Supplemental Table 3. Homozygous T3 or T4 plants were taken for the following field test.

For Arabidopsis transformation, the resulting construct pCAMBIA2300-*35S:OsNRT1.1A-eGFP* was transformed into Agrobacterium strain GV3101 and introduced into wild-type plants Col-0 by the floral dip method (Bent, 2006). The harvested seeds were selected on half-strength Murashige and Skoog plates containing 1% sucrose and 50 μg/mL kanamycin to acquire independent T1 transgenic lines, and then the T2 seeds based on the 3:1 segregation of the selection marker were obtained. The homozygous T3 lines were used for further phenotypic analyses.

RNA Extraction, cDNA Preparation, and RT-qPCR

Total RNA was extracted using TRIzol reagent (Invitrogen) from the indicated tissues of rice plants. Two micrograms of total RNA was used to synthesize cDNA using ReverTra Ace qPCR RT Master Mix (Toyobo). RT-qPCR assay was performed with SYBR Green Real-Time PCR Master Mix reagent (Toyobo) on a Chromo4 real-time PCR detection system according to manufacturer's instructions (Bio-Rad CFX96). Data were analyzed by Opticon monitor software (Bio-Rad). Three technical replicates from one of the three biological replicates were performed for each gene. Rice *Ubiquitin1* was used as the internal reference. The primers used for RT-qPCR are listed in Supplemental Table 2.

Promoter-GUS Assay

A 2.0-kb promoter region of *OsNRT1.1A* was amplified from DJ and cloned into pCAMBIA2391Z to generate $OsNRT1.1A_{promoter}$:*GUS*, and the resulting vector was transformed into DJ. For GUS staining, tissues from the root, leaf sheath, leaf blade, and culm of $OsNRT1.1A_{promoter}$:*GUS* transgenic rice were sampled and immersed in X-gluc (5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid/cyclohexyl ammonium salt) staining solution containing 100 mM sodium phosphate, pH 7.0, 0.1% Triton X-100, 10 mM EDTA (pH 8.0), 2% DMSO, 0.1% X-gluc, 1 mM $K_3[Fe(CN)_6]$, 1 mM $K_4[Fe(CN)_6]\cdot 3H_2O$, and 5% methanol (Jefferson, 1989). After staining for 1 h at 37°C, the samples were dehydrated in an ethanol series (70, 85, 95, and 100%) to remove the chlorophyll. The stained tissues were observed under a stereomicroscope (Olympus SZX16) and photographed using a digital camera (Nikon D700).

Field Tests of Rice

To investigate the application potential of *OsNRT1.1A,* large-scale field tests for rice using T4 generation *OsNRT1.1A*-OE plants in the DJ background were performed in the paddy field under natural growth condition during 2015-2016 at three experimental stations: the Institute of Genetics and Developmental Biology (IGDB, Beijing), the China National Hybrid Rice Research and Development Center (Changsha, Hunan Province), and the South China Experimental Station of IGDB (Sanya, Hainan Province). All the nutritional composition of the soils before rice was transplanted in each paddy field are summarized in Supplemental Table 4. For the field test in Beijing in 2015 (May to October), nitrate mixed with ammonium (60% nitrate mixed with 40% ammonium) was used as the N source with 1 kg N/100 m^2 for low N and 2 kg N/100 m^2 for high N. KNO_3 and $(NH_4)_2SO_4$ were used as sources for nitrate and ammonium, respectively. The plants were transplanted in 10 rows × 15 plants for each plot, and four replicates were used for each N condition. For field test in Sanya (December 2015 to April 2016), nitrate mixed with ammonium was used as the N source, which is the same as that used in Beijing in 2015, and the planting density was 8 rows × 20 plants with five replicates under each N condition. For field tests in Changsha and Beijing in 2016 (May to October), urea was used as the N source with 1 kg N/100 m^2 for low N and 2 kg N/100 m^2 for high N, respectively. The rice plants with four or five replicates were transplanted with a density of 8 rows × 15 plants or 10 rows × 20 plants in Changsha or Beijing, respectively. P_2O_5 was used as phosphorus fertilizer (0.5 kg P/100 m^2) in the paddy field before transferring, and the spacing between rice plants at all three locations was 20 cm. Plants were flooded throughout their growth period and grown in blocks with a completely random design for each plot. To reduce the variability in field test, the fertilizers are evenly applied to every plot for each N application level. For the final field test, the edge lines of each plot were removed to avoid margin effects.

For evaluating the grain yield per plant of *OsNRT1.1A*-OE in HJ19 background, field tests were conducted during 2016 and 2017 at two locations: the IGDB (Beijing) and Northeast Institute of Geography and Agroecology of Chinese Academy of Sciences (Harbin, Heilongjiang Province). The soil nutrient status in Harbin in 2017 is also presented in Supplemental Table 4. For field test in Beijing in 2016, T3 generation transgenic lines were transplanted as 8 rows × 15 plants along with wild-type plants. A field test was also conducted using T4 generation lines in Harbin in 2017, with a planting density of 10 rows × 20 plants. The spacing between rice plants at the two locations was 20 cm (row space) × 20 cm (plant space).

Analysis of Agronomic Traits

All the agronomic traits including plant height, seed number per panicle, seed-setting rate, tiller number per plant, and grain yield per plant were measured on a single-plant basis. Detailed methods for measurement of these agronomic traits were described previously (Hu et al., 2015).

Statistical Analysis

For the comparisons between two groups of data, the Student's *t* test was used. For the data sets of more than two groups, mainly for the transgenic plants phenotyping and large field tests, one-way ANOVA with Tukey's test

was used. The results of statistical analysis with ANOVA are listed in Supplemental Data Set 1.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis TAIR database (https://www.arabidopsis.org) or Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) under the following accession numbers: *AtNRT1.1*, *AT1G12110.1*; *OsNRT1.1A*, *LOC_Os08g05910*; *OsNRT1.1B*, *LOC_Os10g40600*; *OsNRT2.1*, *LOC_Os02g02170*; *OsNRT2.3a*, *LOC_Os01g50820*; *OsNIA1*, *LOC_Os08g36480*; *OsNIA2*, *LOC_Os08g36500*; *OsNIR1*, *LOC_Os01g25484*; *OsNIR2*, *LOC_Os02g52730*; *OsAMT1.1*, *LOC_Os04g43070*; *OsGOGAT1*, *LOC_Os01g48960*; *OsGOGAT2*, *LOC_Os05g48200*; *OsGS1.1*, *LOC_Os02g50240*; *OsGS1.2*, *LOC_Os03g12290*; *Ehd1*, *LOC_Os10g32600*; *Hd3a*, *LOC_Os06g06320*; and *RFT1*, *LOC_Os06g06300*.

Supplemental Data

Supplemental Figure 1. OsNRT1.1A is the closest ortholog to AtNRT1.1 in rice.

Supplemental Figure 2. OsNRT1.1A localizes to the tonoplast.

Supplemental Figure 3. Identification and characterization of *osnrt1.1a* mutant.

Supplemental Figure 4. *OsNRT1.1A* plays a more profound role in determining growth and grain yield than *OsNRT1.1B*.

Supplemental Figure 5. *OsNRT1.1A* is involved in N-regulated flowering.

Supplemental Figure 6. *OsNRT1.1A* overexpression promotes growth advantages under hydroponic cultivation.

Supplemental Figure 7. The effect of nitrate on subcellular localization of OsNLP3/4-eGFP in rice protoplasts.

Supplemental Figure 8. Subcellular localization of OsNLP3/4-eGFP in rice protoplasts prepared from wild-type DJ and *osnrt1.1a* mutant.

Supplemental Figure 9. *OsNRT1.1A* overexpression promotes early flowering and panicle elongation.

Supplemental Figure 10. Field trials of *OsNRT1.1A*-OE plants in Beijing.

Supplemental Table 1. Agronomic traits of wild-type DJ and *OsNRT1.1A*-OE plants in the field (2015 Beijing).

Supplemental Table 2. Primers used in this study.

Supplemental Table 3. T-DNA copy numbers in transgenic rice used in the field test deduced by real-time PCR.

Supplemental Table 4. Soil environment at different field trial locations.

Supplemental Data Set 1. ANOVA tables.

Supplemental File 1. Alignment used to produce the phylogenetic tree shown in Figure 1A.

ACKNOWLEDGMENTS

We thank Yi-Fang Tsay (Institute of Molecular Biology, Academia Sinica) and Wenfeng Qian (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences) for their critical comments and suggestions on this manuscript. This work was supported by grants from the Ministry of Science and Technology of the People's Republic of China (2016YFD0100700 and 2015CB755702), by the Ministry of Agriculture (2014ZX08001-004), and by the Chinese Academy of Sciences (XDA08010400).

AUTHOR CONTRIBUTIONS

W.W. and B.H. performed the experiments and analyzed the data. B.H., W.W., and C.C. designed the research. B.H., C.C., and W.W. wrote the manuscript. D.Y., Y.Q.L., R.C., Y.H., H.L., Z.H.Z., Z.J., Z.L.Z., S.O., H.W., X.G., Y.Q., X.M., Y.X.L., Y.W., L.Z., L.L., S., and H.J. conducted the experiments. B.H., W.W., Y.X.L., and Y.B. analyzed the data. C.C. supervised the project.

Received October 19, 2017; revised January 16, 2018; accepted February 22, 2018; published February 23, 2018.

REFERENCES

Almagro, A., Lin, S.H., and Tsay, Y.F. (2008). Characterization of the *Arabidopsis* nitrate transporter NRT1.6 reveals a role of nitrate in early embryo development. Plant Cell **20:** 3289–3299.

An, C., Li, L., Zhai, Q., You, Y., Deng, L., Wu, F., Chen, R., Jiang, H., Wang, H., Chen, Q., and Li, C. (2017). Mediator subunit MED25 links the jasmonate receptor to transcriptionally active chromatin. Proc. Natl. Acad. Sci. USA **114:** E8930–E8939.

Arth, I., Frenzel, P., and Conrad, R. (1998). Denitrification coupled to nitrification in the rhizosphere of rice. Soil Biol. Biochem. **30:** 509–515.

Bart, R., Chern, M., Park, C.-J., Bartley, L., and Ronald, P.C. (2006). A novel system for gene silencing using siRNAs in rice leaf and stem-derived protoplasts. Plant Methods **2:** 13.

Bent, A. (2006). *Arabidopsis thaliana* floral dip transformation method. Methods Mol. Biol. **343:** 87–103.

Brambilla, V., and Fornara, F. (2013). Molecular control of flowering in response to day length in rice. J. Integr. Plant Biol. **55:** 410–418.

Bubner, B., and Baldwin, I.T. (2004). Use of real-time PCR for determining copy number and zygosity in transgenic plants. Plant Cell Rep. **23:** 263–271.

Castro Marín, I., Loef, I., Bartetzko, L., Searle, I., Coupland, G., Stitt, M., and Osuna, D. (2011). Nitrate regulates floral induction in *Arabidopsis*, acting independently of light, gibberellin and autonomous pathways. Planta **233:** 539–552.

Chardin, C., Girin, T., Roudier, F., Meyer, C., and Krapp, A. (2014). The plant RWP-RK transcription factors: key regulators of nitrogen responses and of gametophyte development. J. Exp. Bot. **65:** 5577–5587.

Chen, J., Zhang, Y., Tan, Y., Zhang, M., Zhu, L., Xu, G., and Fan, X. (2016). Agronomic nitrogen-use efficiency of rice can be increased by driving *OsNRT2.1* expression with the *OsNAR2.1* promoter. Plant Biotechnol. J. **14:** 1705–1715.

Davenport, T., Rao, R., Zheng, Q., and Li, Y. (2001). Nitrogen, flowering and production of lychee in Florida. Acta Hortic. **558:** 221–224.

Dielen, V., Lecouvet, V., Dupont, S., and Kinet, J.M. (2001). *In vitro* control of floral transition in tomato (*Lycopersicon esculentum* Mill.), the model for autonomously flowering plants, using the late flowering uniflora mutant. J. Exp. Bot. **52:** 715–723.

Doi, K., Izawa, T., Fuse, T., Yamanouchi, U., Kubo, T., Shimatani, Z., Yano, M., and Yoshimura, A. (2004). *Ehd1*, a B-type response regulator in rice, confers short-day promotion of flowering and controls *FT-like* gene expression independently of *Hd1.* Genes Dev. **18:** 926–936.

Fan, X., Tang, Z., Tan, Y., Zhang, Y., Luo, B., Yang, M., Lian, X., Shen, Q., Miller, A.J., and Xu, G. (2016). Overexpression of a -pH-sensitive nitrate transporter in rice increases crop yields. Proc. Natl. Acad. Sci. USA **113:** 7118–7123.

Fang, Z., Xia, K., Yang, X., Grotemeyer, M.S., Meier, S., Rentsch, D., Xu, X., and Zhang, M. (2013). Altered expression of the *PTR/NRT1* homologue *OsPTR9* affects nitrogen utilization efficiency, growth and grain yield in rice. Plant Biotechnol. J. **11:** 446–458.

Hiei, Y., Ohta, S., Komari, T., and Kumashiro, T. (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. **6:** 271–282.

Ho, C.H., Lin, S.H., Hu, H.C., and Tsay, Y.F. (2009). CHL1 functions as a nitrate sensor in plants. Cell **138:** 1184–1194.

Hu, B., et al. (2015). Variation in *NRT1.1B* contributes to nitrate-use divergence between rice subspecies. Nat. Genet. **47:** 834–838.

Hunter, P.R., Craddock, C.P., Di Benedetto, S., Roberts, L.M., and Frigerio, L. (2007). Fluorescent reporter proteins for the tonoplast and the vacuolar lumen identify a single vacuolar compartment in Arabidopsis cells. Plant Physiol. **145:** 1371–1382.

Itoh, H., Nonoue, Y., Yano, M., and Izawa, T. (2010). A pair of floral regulators sets critical day length for *Hd3a* florigen expression in rice. Nat. Genet. **42:** 635–638.

Izawa, T. (2007). Adaptation of flowering-time by natural and artificial selection in *Arabidopsis* and rice. J. Exp. Bot. **58:** 3091–3097.

Jefferson, R.A. (1989). The GUS reporter gene system. Nature **342:** 837–838.

Jeong, D.H., An, S., Kang, H.G., Moon, S., Han, J.J., Park, S., Lee, H.S., An, K., and An, G. (2002). T-DNA insertional mutagenesis for activation tagging in rice. Plant Physiol. **130:** 1636–1644.

Komiya, R., Yokoi, S., and Shimamoto, K. (2009). A gene network for long-day flowering activates *RFT1* encoding a mobile flowering signal in rice. Development **136:** 3443–3450.

Li, C., Wang, Y., Liu, L., Hu, Y., Zhang, F., Mergen, S., Wang, G., Schläppi, M.R., and Chu, C. (2011). A rice plastidial nucleotide sugar epimerase is involved in galactolipid biosynthesis and improves photosynthetic efficiency. PLoS Genet. **7:** e1002196.

Li, H., Hu, B., and Chu, C. (2017). Nitrogen use efficiency in crops: lessons from Arabidopsis and rice. J. Exp. Bot. **68:** 2477–2488.

Li, H., Hu, B., Wang, W., Zhang, Z., Liang, Y., Gao, X., Li, P., Liu, Y., Zhang, L., and Chu, C. (2016). Identification of microRNAs in rice root in response to nitrate and ammonium. J. Genet. Genomics **43:** 651–661.

Li, J., Xin, Y., and Yuan, L. (2009). Hybrid Rice Technology Development. Ensuring China's Food Security. (Washington, DC: International Food Policy Research Institute).

Li, Y.L., Fan, X.R., and Shen, Q.R. (2008). The relationship between rhizosphere nitrification and nitrogen-use efficiency in rice plants. Plant Cell Environ. **31:** 73–85.

Liu, K.H., et al. (2017). Discovery of nitrate-CPK-NLP signalling in central nutrient-growth networks. Nature **545:** 311–316.

Liu, Q., Chen, X., Wu, K., and Fu, X. (2015). Nitrogen signaling and use efficiency in plants: what's new? Curr. Opin. Plant Biol. **27:** 192–198.

Luo, A., et al. (2006). *EUI1*, encoding a putative cytochrome P450 monooxygenase, regulates internode elongation by modulating gibberellin responses in rice. Plant Cell Physiol. **47:** 181–191.

Marchive, C., Roudier, F., Castaings, L., Bréhaut, V., Blondet, E., Colot, V., Meyer, C., and Krapp, A. (2013). Nuclear retention of the transcription factor NLP7 orchestrates the early response to nitrate in plants. Nat. Commun. **4:** 1713.

Moll, R., Kamprath, E., and Jackson, W. (1982). Analysis and interpretation of factors which contribute to efficiency of nitrogen utilization. Agron. J. **74:** 562–564.

Moll, R., Kamprath, E., and Jackson, W. (1987). Development of nitrogen-efficient prolific hybrids of maize. Crop Sci. **27:** 181–186.

Plett, D., Toubia, J., Garnett, T., Tester, M., Kaiser, B.N., and Baumann, U. (2010). Dichotomy in the *NRT* gene families of dicots and grass species. PLoS One **5:** e15289.

Rupp, R.A., Snider, L., and Weintraub, H. (1994). *Xenopus* embryos regulate the nuclear localization of XMyoD. Genes Dev. **8:** 1311–1323.

Saito, Y., Kishida, K., Takata, K., Takahashi, H., Shimada, T., Tanaka, K., Morita, S., Satoh, S., and Masumura, T. (2009). A green fluorescent protein fused to rice prolamin forms protein body-like structures in transgenic rice. J. Exp. Bot. **60:** 615–627.

Scott, R., Ogunremi, E., Ivins, J., and Mendham, N. (1973). The effect of fertilizers and harvest date on growth and yield of oilseed rape sown in autumn and spring. J. Agric. Sci. **81:** 287–293.

Wen, Z., Tyerman, S.D., Dechorgnat, J., Ovchinnikova, E., Dhugga, K.S., and Kaiser, B.N. (2017). Maize NPF6 proteins are homologs of Arabidopsis CHL1 that are selective for both nitrate and chloride. Plant Cell **29:** 2581–2596.

Withrow, A.P. (1945). The interrelationship of nitrogen supply and photoperiod on the flowering, growth and stem anatomy of certain long and short day plants. Butler Univ. Bot. Stud. **7:** 40–64.

Xu, G., Fan, X., and Miller, A.J. (2012). Plant nitrogen assimilation and use efficiency. Annu. Rev. Plant Biol. **63:** 153–182.

Yang, L., Ding, J., Zhang, C., Jia, J., Weng, H., Liu, W., and Zhang, D. (2005). Estimating the copy number of transgenes in transformed rice by real-time quantitative PCR. Plant Cell Rep. **23:** 759–763.

Yuan, S., et al. (2016). *Arabidopsis* cryptochrome 1 functions in nitrogen regulation of flowering. Proc. Natl. Acad. Sci. USA **113:** 7661–7666.

Zhang, Y., Su, J., Duan, S., Ao, Y., Dai, J., Liu, J., Wang, P., Li, Y., Liu, B., Feng, D., Wang, J., and Wang, H. (2011). A highly efficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast-related processes. Plant Methods **7:** 30.