Enhanced OsNLP4-OsNiR cascade confers nitrogen use efficiency by promoting tiller number in rice

Jun Yu1,†, Wei Xuan2,†, Yunlu Tian1, Lei Fan1, Juan Sun1, Weijie Tang1, Gaoming Chen1, Baoxiang Wang3, Yan Liu3, Wei Wu1, Xiaolan Liu1, Xingzhou Jiang1, Cong Zhou1, Zhaoyang Dai1, Dayong Xu3, Chunming Wang1,4,*,15 and Jianmin Wan1,5,*,16

1State Key Laboratory of Crop Genetics and Germplasm Enhancement, Jiangsu Collaborative Innovation Center for Modern Crop Production, Nanjing Agricultural University, Nanjing, China
2MDA Key Laboratory of Plant Nutrition and Fertilization in Lower-Middle Reaches of the Yangtze River, Nanjing Agricultural University, Nanjing, China
3Lianyungang Academy of Agricultural Science, Lianyungang, Jiangsu Province, China
4Key Laboratory of Biology, Genetics and Breeding of Japonica Rice in the Mid-lower Yangtze River, Ministry of Agriculture, Jiangsu Plant Gene Engineering Research Centre, Nanjing, China
5National Key Facility for Crop Gene Resources and Genetic Improvement, Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing, China

Keywords: rice, tiller, yield, nitrogen-use efficiency.

Abstract

Increased use of nitrogen fertilizers has deleterious impact on the environment. Increase in yield potential at low nitrogen supply is regarded as a cereal breeding goal for future agricultural sustainability. Although natural variations of nitrogen transporters have been investigated, key genes associated with assimilation remain largely unexplored for nitrogen use efficiency (NUE) enhancement. Here, we identified a NIN-like protein NLP4 associated with NUE through a GWAS in rice. We found that OsNLP4 transactivated OsNiR encoding nitrite reductase that was critical in nitrogen assimilation in rice. We further constructed quadrupling NREs (Nitrate-responsive cis-elements) in the promoter of OsNiR (p4xNRE:OsNiR) and enhanced nitrogen assimilation significantly. We demonstrated that OsNLP4-OsNiR increased tiller number and yield through enhancing nitrogen assimilation and NUE. Our discovery highlights the genetic modulation of OsNLP4-OsNiR signalling cascade as a strategy for high NUE and yield breeding in rice.

Introduction

Excessive use of nitrogen (N) fertilizers has been put under intense spotlight recently due to the deleterious impact on the environment. Genetic modification of nitrogen-use efficiency (NUE) genes is promising approach to breed high NUE cultivars with reduced supply of N fertilizer. Plant NUE traits are polygenic because NUE is determined by multiple procedures involving nitrogen sensing, uptake, transport and assimilation (Fan et al., 2017).

Natural variations of nitrogen transporters have been intensively investigated. For instance, in OsNRT1.1B (OsNPF6.5), a single polymorphism in its coding region contributed to the divergence in NUE between indica and japonica subspecies of Asian cultivated rice (Oryza sativa L.); (Hu et al., 2015). We previously showed that an elite haplotype of OsNPF6.1, OsNPF6.1HAapl, differed in both the protein and promoter element with natural variations, which were differentially transactivated by a NUE-related transcription factor OsNAC42 to confer rice NUE under low N supply (Tang et al., 2019).

To accelerate the breeding of NUE, major genes and their interactions associated with both efficient transport and assimilation are indispensable for NUE enhancement (Xu et al., 2012b). Genetic variation from an assimilation gene OsNR2 encoding nitrate reductase was recently explored for NUE enhancement. The greater N-assimilative NR activity of indica OsNR2 (versus japonica OsNR2) confers enhanced tiller number, grain yield and NUE (Gao et al., 2019). However, the regulatory mechanism is incompletely understood on how the assimilation genes were transcriptionally regulated in NUE.

Transcription factor NIN-LIKE PROTEIN 7 (NLP7) was reported to be a primary regulator in nitrate response that binds to nitrate-responsive cis-element (NRE) in many nitrogen genes in model plant Arabidopsis (Liu et al., 2017). NLPs were shown to bind to NREs and activated the NRE-dependent gene expression (Konishi and Yanagisawa, 2014). Therefore, NLPs were speculated to regulate downstream transporters and nitrogen assimilation, though natural variation in NLPs has not been investigated that contributes to nitrogen-use divergence in rice core populations.

In this study, we identified a rice NIN-like protein NLP4 associated with NUE through a genome-wide association study (GWAS) in rice core populations. Here, we show that the elite haplotype OsNLP4-HA0apl transactivates the NRE motif at the promoter of OsNiR encoding nitrite reductase that is a key enzyme determining nitrogen assimilation in rice. We demonstrate that our newly generated OsNLP4-OsNiR cascade confers nitrogen assimilation efficiency and promotes tiller number and yield in rice.

Results

Identification of a NIN-like protein in rice

Through three-year GWAS on NUE-related traits including plant height (PH), effective panicle number (EPN) and yield per plant
(YPP), we identified a major QTL in a linkage disequilibrium (LD) block on chromosome 9 ($P = 8.91 \times 10^{-5}$) in the region spanning bp 21,615,896 to 22,925,531 containing 180 SNPs missense mutations in 33 genes (Fig. 1a).

Meanwhile, we also performed an RNA sequencing (RNA-seq) to identify mRNA changes regarding nitrogen starvation. Seedlings were grown in hydroponics for 2 weeks, and urea was used as the sole source of nitrogen. Half of plants were then exposed to nitrogen-free medium for 7 days (Fig. 1b). The result showed that there were 2804 genes down-regulated and 2539 genes up-regulated after 7 days nitrogen starvation. These differentially expressed genes (DEGs) were identified with of log2 expression ratios being either $\geq 1$ or $\leq -1$ and $q \leq 0.05$.

By overlapping the N-starvation induced DEGs and 33 candidate genes identified from NUE-associated GWAS, we found five genes of Os09g0548400, Os09g0549450, Os09g0563200, Os09g0567366 and Os09g0567900 showed changes in mRNA level upon N-starvation and might be linked to NUE. Os09g0567900 is annotated as a uridine nucleosidase, which has rarely been reported in plant. It was found that the isoforms urh1 and urh2 were not necessary for plant growth (Riegler et al., 2011). The gene Os09g0549450 was annotated as an NLP family transcriptional factor gene OsNLP4 that was up-regulated by nitrogen starvation (Fig. 1c). We therefore focused on OsNLP4 in this study due to its critical role in NUE. RT-PCR was further performed to confirm the RNA-seq data, and the results showed that N-starvation could enhance OsNLP4 expression when supplied with ammonium or urea as a sole nitrogen source (Fig. 1d).

Furthermore, OsNLP4 was classified into two haplotypes based on eight SNPs in its cDNA containing five SNPs in untranslated region (UTR) and one missense SNP in the coding region (Fig. 1e). OsNLP4HapB was found to be an elite haplotype for its higher plant height ratio (LN/HN) and expression level than OsNLP4HapA (Fig. 1f, g). Further Dual-luciferase report system confirmed that transcriptional activation ability of OsNLP4HapB was stronger than OsNLP4HapA (Fig. 1h). These results suggested that OsNLP4 was the candidate gene underlying rice NUE.

Figure 1 Identification of a NIN-like protein in rice (a) Local Manhattan plot (top) of $\log_{10} P$-value for region on chromosome 9 and linkage disequilibrium heat map (bottom) showing an enrichment of significant association. (b) Treatment and sampling point of RNA-Seq. (c) Genes detected mRNA changes in 1.309 Mb LD block. (d) Validation of microarray results of OsNLP4 by RT-PCR. The seedlings were grown in IRRI nutrient solution supplied with sole nitrogen source either 1 mM NO$_3^-$, 1 mM NH$_4^+$ or 500 $\mu$M urea for 2 weeks, then moved to N-free solution for 7 days. (e) SNPs in OsNLP4 CDNA between HapA and HapB. (f-h) Comparison of HapA and HapB in (f) Plant height ratio (LN/HN). (g) Transcription level, every yellow and red bar presents a variety of NLP4 HapA or NLP4 HapB. (h) Comparison between NLP4 HapA and NLP4 HapB in transcription activation abilities for OsNiR. Data presented are mean values of three technical repeats with standard deviation ($n \geq 3$).
NLP4 directly regulates OsNiR by binding NRE

To validate the function of OsNLp4 in NUE, we investigated the agronomic traits of a osnlp4 tilling mutant, and observed a significantly decreased tiller number and plant height in osnlp4 mutant compared to wild type (Fig. 2a, b). Remarkably, the miRNA levels of genes involved in nitrogen uptake and assimilation such as OsNiR, OsNiR1, OsNiR2.1, OsNiR7.1B and OsAMT2.1 decreased in osnlp4 tilling mutant (Fig. 2c, Figure S2). These data suggested that OsNLp4 might affect nitrogen assimilation.

Interestingly, the OsNiR promoter harbours NRE motifs (Konishi and Yanagisawa, 2014) that are supposed to be targeted by OsNiR. To determine whether OsNLP4 could bind to NRE motif in OsNiR promoter, we expressed and purified RWP-RK domain of OsNLp4 from bacterium. Electrophoretic mobility shift assay (EMSA) showed that RWP-RK domain of NLP4 was able to bind the NRE motif in OsNiR promoter, and in concert with the increased number of NRE motif, the binding activity of OsNLp4 to the promoter of OsNiR was strongly enhanced (Fig. 2d, e).

Subsequent chromatin immunoprecipitation (ChIP) assays followed by qPCR showed that the P3 region of the OsNiR promoter harbouring NRE was significantly enriched in immunoprecipitated DNA (Fig. 2f, g), confirming the binding of OsNLp4 to OsNiR promoter in vivo.

To further validate the transcription activation of OsNLp4 in vivo, we conducted a dual-luciferase reporter assay. The plasmid CaMV35S::OsNLp4, with NLp4 ORF from Nipponbare (Nip), was generated as an effector, while the reporter construct contained a firefly luciferase gene (LUC) driven by OsNiR promoter with different copy number of NRE (Fig. 2h). LUC activity slightly increased when driven by OsNiR promoter containing two NRE motifs (2× NRE) compared to one NRE motif (1× NRE). We next investigated the expression level of OsNiR in Koshihikari and Nipponbare with 1× NRE and 2× NRE, respectively, (Fig. 2i) and found that the expression level of OsNiR increased in Nipponbare under both KCl and KNO3 treatments compared to Koshihikari (Figure S5).

We next constructed luciferase reporter driven by Nip OsNiR promoter containing four NRE motifs. We found that LUC activity increased significantly when driven by four NRE motif (4× NRE). Therefore, NLp4 activated the transcription level of OsNiR by binding NRE, and the activation can be enhanced significantly by binding 4× NRE (Fig. 2j).

OsNiR with 4× NRE enhances NiR protein level, NiR activity and nitrogen assimilation

OsNiR encodes a nitrite reductase, which is responsible for reducing nitrite to ammonia (Xu et al., 2012a). Nitrite usually acts as a precursor to the formation of ammonium, meanwhile is poisonous to plants. To investigate the link between NRE copy number variation and OsNiR activity, we constructed transgenic lines carrying OsNiR with 4× NRE in promoter (p4xNRE:OsNiR), while receptor was Koshihikari with 1× NRE in OsNiR promoter (p1xNRE:OsNiR).

To examine whether NiR activity was elevated in transgenic lines, we treated Koshihikari and 4× NRE transgenic Koshihikari seedlings with 10 mM potassium nitrite (KNO3). We found that growth of Koshihikari was severely impaired by KNO3 compared with 4× NRE transgenic lines. The fresh weight of Koshihikari seedlings decreased significantly, while 4× NRE transgenic seedlings were not affected (Fig. 3a, b).

Because 4× NRE enhanced the activation of OsNiR by NLp4, we examined the transcription level of 4× NRE transgenic lines. The result showed that mRNA level of OsNiR in 4× NRE transgenic lines increased significantly (Fig. 3c). Meanwhile, we investigated the NiR protein abundance in 4× NRE transgenic lines by using ELISA and Western blot, and NiR protein level in 4× NRE lines was significantly higher than Koshihikari as expected (Fig. 3d, e). These results indicated that 4× NRE not merely elevated transcription level of OsNiR, but also increased the activity of NiR.

To further investigate the enhanced NiR function in nitrogen cycle, we detected the activity of GS (Glutamine synthetase), a downstream enzyme of NiR (Xu et al., 2012a). Compared to WT, activity of GS was significantly increased in 4× NRE transgenic lines (Fig. 3f).

15N uptake assay also showed that ammonium uptake was also increased in the 4× NRE transgenic lines (Fig. 3g). Furthermore, we investigated expression level of OsAMTs in wild type and 4× NRE transgenic lines and found that transcription level of the most OsAMTs increased significantly in 4× NRE transgenic lines, including OsAMT1.1, OsAMT1.3, OsAMT2.2 and OsAMT3.1 (Figure S4). While the nitrogen concentration was higher in flag leaves and secondary leaves of 4× NRE transgenic lines (Fig. 3h). These results showed that N uptake and assimilation were increased with higher NiR activity.

Overexpression of OsNiR increases rice tiller number

We next measured the effects of the overexpression of OsNiR on rice growth and found that tiller number was significantly increased in 4× NRE transgenic lines in both HN and LN fields (Fig. 4a, b).

To investigate the potential interaction between nitrogen and tiller outgrowth, expression level of genes associated with tiller outgrowth and cell proliferation was examined. Compared to WT, overexpression of OsNiR under the control of 4× NRE containing promoter resulted in the up-regulated expressions of LAX1 (Komatsu et al., 2003), MAD557 (Guo et al., 2013), RNC1 (Yasuno et al., 2007), PIN2 (Chen et al., 2012), CycD4;1, CycB2;1 (Renaudin et al., 1996) and CDKA1 and CAK1A (Joubès et al., 2000; Fig. 4g-n). As tiller outgrowth is regulated by plant hormones, especially strigolactones (SLs), we next measured the transcription level of genes involved in SL synthesis including D10 (Arite et al., 2007), D17 (Umehara et al., 2008), D27 (Lin et al., 2009) and MAX1 (Zhang et al., 2014), and SL signalling including D3 (Ishikawa et al., 2005), D14 (Arite et al., 2009) and D53 (Zhou et al., 2013). As a result, expressions of these genes decreased in 4× NRE transgenic lines significantly (Fig. 4e).

Enhanced OsNiR improves rice yield

We further evaluated breeding potential of enhanced OsNiR expression for yield potential by determining yield-related traits of the 4× NRE-driven OsNiR overexpression lines in both HN and LN fields. Strikingly, the 4× NRE-driven OsNiR overexpression lines showed enlarged panicle traits, increased the number of primary branches, secondary branches, yield per plant and yield per plot than WT, in both HN and LN fields (Fig. 4c, d, and Fig. 5c-i). Noticeably, yields per plot was increased by 51.94% and 62.57% in two 4× NRE-driven OsNiR overexpression lines under HN fields, respectively, which were largely enhanced as compared to the wild type (44.3%). It indicated that overexpression of OsNiR conferred a high NUE when its

Enhanced OsNLp4-OsNiR cascade confers to NUE

To further investigate the enhanced NRE function in nitrogen cycle, we detected the activity of GS (Glutamine synthetase), a downstream enzyme of NiR (Xu et al., 2012a). Compared to WT, activity of GS was significantly increased in 4× NRE transgenic lines (Fig. 3f).

15N uptake assay also showed that ammonium uptake was also increased in the 4× NRE transgenic lines (Fig. 3g). Furthermore, we investigated expression level of OsAMTs in wild type and 4× NRE transgenic lines and found that transcription level of the most OsAMTs increased significantly in 4× NRE transgenic lines, including OsAMT1.1, OsAMT1.3, OsAMT2.2 and OsAMT3.1 (Figure S4). While the nitrogen concentration was higher in flag leaves and secondary leaves of 4× NRE transgenic lines (Fig. 3h). These results showed that N uptake and assimilation were increased with higher NiR activity.

Overexpression of OsNiR increases rice tiller number

We next measured the effects of the overexpression of OsNiR on rice growth and found that tiller number was significantly increased in 4× NRE transgenic lines in both HN and LN fields (Fig. 4a, b).

To investigate the potential interaction between nitrogen and tiller outgrowth, expression level of genes associated with tiller outgrowth and cell proliferation was examined. Compared to WT, overexpression of OsNiR under the control of 4× NRE containing promoter resulted in the up-regulated expressions of LAX1 (Komatsu et al., 2003), MAD557 (Guo et al., 2013), RNC1 (Yasuno et al., 2007), PIN2 (Chen et al., 2012), CycD4;1, CycB2;1 (Renaudin et al., 1996) and CDKA1 and CAK1A (Joubès et al., 2000; Fig. 4g-n). As tiller outgrowth is regulated by plant hormones, especially strigolactones (SLs), we next measured the transcription level of genes involved in SL synthesis including D10 (Arite et al., 2007), D17 (Umehara et al., 2008), D27 (Lin et al., 2009) and MAX1 (Zhang et al., 2014), and SL signalling including D3 (Ishikawa et al., 2005), D14 (Arite et al., 2009) and D53 (Zhou et al., 2013). As a result, expressions of these genes decreased in 4× NRE transgenic lines significantly (Fig. 4e).

Enhanced OsNiR improves rice yield

We further evaluated breeding potential of enhanced OsNiR expression for yield potential by determining yield-related traits of the 4× NRE-driven OsNiR overexpression lines in both HN and LN fields. Strikingly, the 4× NRE-driven OsNiR overexpression lines showed enlarged panicle traits, increased the number of primary branches, secondary branches, yield per plant and yield per plot than WT, in both HN and LN fields (Fig. 4c, d, and Fig. 5c-i). Noticeably, yields per plot was increased by 51.94% and 62.57% in two 4× NRE-driven OsNiR overexpression lines under HN fields, respectively, which were largely enhanced as compared to the wild type (44.3%). It indicated that overexpression of OsNiR conferred a high NUE when its
transcription was highly activated (Fig. 6a). We concluded that 4× NRE enhanced NLP4 transcriptional activation to OsNiR by increasing mRNA, protein levels and enzyme activity of OsNiR, in turn promoting ammonium uptake, decreased transcription level of genes related to strigolactones, and increased tiller number, as shown in the schematic model (Fig. 6b). Our newly generated OsNiR overexpression line under the control of quadrupling NRE motifs is highly beneficial in efforts to enhance NUE and yield in rice.

**Discussion**

Nitrogen transport and assimilation are two critical physiological pathways in plant NUE that is inherently complex trait. In this study, we identified a NIN-like protein OsNLP4 that promoted rice NUE through the transactivation of a key nitrogen assimilation gene OsNiR. Through quadrupling NRE motifs in the promoter of OsNiR and enhancing nitrogen assimilation in NUE, our enhanced OsNLP4-OsNiR cascade increased tiller number and yield.
More importantly, nitrogen-use efficiency is positively correlated with tiller outgrowth. Increase in tiller (lateral branch) numbers at low nitrogen supply is regarded as a key cereal breeding goal for future agricultural sustainability (Wu et al., 2020). Increased nitrogen enhanced transcription of the rice transcription factor encoded by an NGR5 (NITROGEN-MEDIATED TILLER GROWTH RESPONSE 5), and reduced the abundance of mRNAs specified by strigolactone signalling and other branching-inhibitory genes Dwarf14 (D14) and squamosa promoter binding protein-like 14 (OsSPL14). It was previously reported that lack of D14 or OsSPL14 function was epistatic to ngr5 in regulating rice tillering (Wu et al., 2020). We investigated the mRNA level of NGR5 in 4× NRE transgenic lines and found that mRNA level of NGR5 in 4× NRE transgenic lines slightly increased by ~1.3-fold, compared to wild type (Figure S3). As a negative regulator of tiller number, OsNiR with 4× NRE enhances NiR protein level, NiR activity and rice growth (a) Transgenic lines carrying OsNiR with 4× NRE in promoter grown in hydroponics for 14 days and then treated with 10 mM KCl or KNO₃ for 2 days. Bar ~ 5 cm. (b) Fresh weight not changed in the 4× NRE transgenic lines under KNO₃ treatment. (c) Gene expression of OsNiR of transgenic lines. (d) ELISA and (e) Western blot using anti-NiR antibody and Hsp90 acted as an internal control of protein level. (f) Activity of GS (Glutamine synthetase) (g) NH₄⁺ influx analysis in roots. The root NH₄⁺ influx was measured in 0.25 mM ¹⁵NH₄⁺. (h) Nitrogen concentration in different tissues. Data presented are mean values of three technical repeats with standard deviation (n ≥ 3).

Figure 3 OsNiR with 4× NRE enhances NiR protein level, NiR activity and rice growth (a) Transgenic lines carrying OsNiR with 4× NRE in promoter grown in hydroponics for 14 days and then treated with 10 mM KCl or KNO₃ for 2 days. Bar ~ 5 cm. (b) Fresh weight not changed in the 4× NRE transgenic lines under KNO₃ treatment. (c) Gene expression of OsNiR of transgenic lines. (d) ELISA and (e) Western blot using anti-NiR antibody and Hsp90 acted as an internal control of protein level. (f) Activity of GS (Glutamine synthetase) (g) NH₄⁺ influx analysis in roots. The root NH₄⁺ influx was measured in 0.25 mM ¹⁵NH₄⁺. (h) Nitrogen concentration in different tissues. Data presented are mean values of three technical repeats with standard deviation (n ≥ 3).
NGR5 does not seem to be involved in our OsNLP4-OsNiR module.

Interestingly, we measured the transcription level of genes involved in strigolactones synthesis (D10, D17, D27 and MAX1) and signalling (D3, D14 and D53) that decreased in 4× NRE transgenic lines. Previous study showed that SLs induced D53 degradation by the proteasome and abrogate its activity in promoting axillary bud outgrowth (Zhou et al., 2013). We found that the tiller number was increased in the 4× NRE transgenic lines because NH₄⁺ was much increased due to the enhanced enzyme activity of NiR. It will be intriguing to further investigate how the tiller number was regulated by the interaction between NH₄⁺ and D53 or the other genes involved in strigolactones synthesis.

We analysed the differentiation of NLP4HapA and NLP4HapB varieties of cultivated rice in Rice3K Genome Project (Wang et al., 2018). NLP4HapA varieties accounted for a greater proportion, and most of them (91.14%) were indica species, while japonica accounted for the majority (76.91%) in NLP4HapB varieties (Fig. S1a). We also investigated the geographical distribution of NLP4HapA and NLP4HapB varieties in Asia rice populations and discovered that the varieties harbouring NLP4HapA have a larger proportion in East Asia, especially in Japan (92.45%) and South Korea (78.79%). Meanwhile varieties harbouring NLP4HapB mainly distributed in South China, India and South-East Asia (Fig. S1b, c). Therefore, NLP4HapB could be applied to improve NUE in japonica rice, especially in North China, Japan and South Korea. Although most of the indica varieties harbour elite haplotype NLP4HapB, we can further improve NUE of indica rice by applying 4× NRE elements in NiR for indica rice breeding improvement.

Our enhanced OsNLP4-OsNiR cascade increased tiller number and yield through enhancing N assimilation and NUE. The greater N-assimilative NR activity of indica OsNR2 (versus japonica OsNR2) also conferred enhanced tiller number, grain yield and NUE (Gao et al., 2019). We herein investigated the regulatory mechanism of the OsNLP4-OsNiR cascade and generated a novel OsNLP4-OsNiR cascade with quadrupling NREs in the promoter of OsNiR. We found the promotive effect of nitrogen on tiller bud outgrowth due to the assimilation capacity of OsNiR. We then showed genetic modulation of the novel OsNLP4-OsNiR cascade enabled increased grain yield and reduced nitrogen input. Furthermore, it
is intriguing to overexpress OsNLP4 to highly activate NiR for higher NUE. Together, these nitrogen assimilation genes will accelerate future efforts aimed at NUE and yield, and advance agricultural sustainability.

Materials and methods

Plant growth conditions

Rice plants were grown in the field at the experimental farm of Nanjing Agricultural University, Nanjing, China. For field experiments, the accessions were grown in a completely randomized block design with six replicates. The field experiments were carried out as a randomized block design with two N levels (+N, with 300 kg/ha N fertilizer, and 0 N fertilizer) in two blocks. P and K fertilizers were applied at 100 and 100 kg/ha, respectively. There were 20 cm and 17 cm between rows and individuals respectively.

Rice seedlings were cultured in IRRI nutrient solution (Yoshida et al., 1976). Daytime conditions in the greenhouse were 30 °C for 14 h; night-time conditions were 28 °C, and dark for 10 h.

Generation of 4× NRE transgenic lines

To construct 4× NRE transgenic lines, 2.3 kb regions upstream of NRE and 300 bp downstream of NRE were amplified using Nipponbare as template. The overlap extension PCR was then performed as described previously (Horton et al., 1989) using two PCR-amplified fragments as the templates, and the resultant 2.6 kb PCR product was inserted into the pCUBi1390 using HindIII and SacI producing the vector containing NiR promoter with 4× NRE. Then, fragments of OsNiR genomic DNA were amplified and inserted downstream of the 4× NRE. Constructed plasmids were transferred into japonica rice variety Koshihikari by the Agrobacterium mediated transformation method. The primer sequences used in the overlap PCR are listed in Table S1. Thirty-four transgenic lines were generated.

ChIP assay

Chromatin immunoprecipitation assays were carried out using the Agarose ChIP Kit from Thermo Scientific, according to the manufacturer’s guidelines. GFP-Trap magnetic beads were used for ChIP assay with the Millipore ChIP Assay Kit (17–295; Millipore). Briefly, 4 g rice seedlings were cross-linked by 1% formaldehyde for 10 min, and the reaction was stopped by the addition of glycine to a 125 mM final concentration. The samples were frozen in liquid nitrogen and ground into powder. Chromatin was sonicated to an average fragment size of 200–500 bp. GFP-Trap magnetic beads were used to immunoprecipitate the protein–DNA complex. Enrichment levels of genes were determined by real-time PCR using chromatin precipitated without antibody as an internal control.

Protein purification and electrophoretic mobility shift assay

The sequence of RWP-RK domain in OsNLP4 was amplified using a BamHI-XbaI site-containing forward primer and an XhoI site-containing reverse primer, ligated into an EcoRI- and XhoI-digested pGEX-4T-2 vector, and transformed into competent Escherichia coli BL21(DE3) cells and then expressed at 16 °C for 20 h in the presence of 0.1 mM isopropyl-beta-D-thiogalactopyranoside. Recombinant protein was purified using glutathione magnetic beads. EMSA was performed using LightShift™ Chemiluminescent EMSA Kit (Thermo Fisher Scientific Inc.). For binding, 20 fmol of 5'-Biotin labelled oligonucleotide was incubated with 2 μg of purified proteins (GST-RWP-RK) in a total volume of 20 μL, and the samples were mixed with 5X Loading Buffer and loaded onto 6% polyacrylamide gel in 0.5X TBE. Electrophoresis was conducted at 100 V for 1.5 h in 0.5 × TBE buffer (44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA, pH 8.3) at 4 °C. Complexes were transferred to a positively charged Nylon Membranes (Roche) in 0.25 × TBE buffer at 200 mA for 45 min at 4 °C and UV cross-linked in a cross-linker. Gel shifts were visualized with a streptavidin–horseradish peroxidase followed by chemiluminescent detection.
Protoplast transfection and dual-luciferase reporter assay

Leaves from 14-day rice seedlings were excised and incubated in freshly prepared enzyme solution (1.5% cellulose R-10, 0.75% macerozyme R-10, 0.6 M mannitol, 10 mM MES, 1 mM CaCl$_2$, 0.1% bovine serum albumin, pH 5.7) and soaked for 3–4 h in the dark with gentle shaking (50 rpm). After adding an equal volume of W5 buffer (154 mM sodium chloride, 125 mM CaCl$_2$, 5 mM KCl, 2 mM MES, pH 5.7), the harvesting cells were resuspended in MMG buffer (0.6 M mannitol, 15 mM MgCl$_2$, 4 mM MES, pH 5.7). Ten µg of each vector was added to the protoplasts for 10 min using 40% PEG buffer (0.6 M mannitol, 15 mM MgCl$_2$, 4 mM MES, pH 5.7) and then were washed for two times using W5 buffer. At last, the protoplasts were resuspended in WI buffer (0.6 M mannitol, 4 mM MES, 4 mM KCl, pH 5.7).

In the protoplast transient expression experiments, vector pGreenII0800-LUC was used to analyse the activity of the different promoters, and the vector contains two luciferase genes, firefly luciferase (LUC) reporter gene driven by the target promoter and a Renilla luciferase (REN) reporter gene driven by 35S used as an internal control. The –2500 bp upstream of the start codon of OsNiR was cloned into vector pGreen II0800-LUC to generate reporters for the dual-luciferase assays. The full-length OsNL4 CDS was inserted into vector Pan580 to generate effector. The firefly luciferase (LUC) activity and Renilla luciferase (REN) activity were measured by the dual-luciferase reporter assay system (Promega, E1910) 20 h after transfection. The ratio between LUC and REN activities was measured three times.

ELISA and GS activity analysis

ELISA analysis was conducted using Plant NiR ELISA Kit (MeiMian Biotech Co., Ltd., Yancheng, China) according to manufacturer instructions. Samples were frozen in liquid nitrogen and grinded into powder and dissolved in lysis incubated at 4 °C for half an hour. Mixture was centrifuged for 10 min at 4 °C, 13 523 g. The supernatant was collected and used for ELISA analysis. OD values were measured at 450 nm wavelength.

GS activity was determined by GS test kit (Solarbio Biotechnology Co., Ltd, Beijing, China) follow the manufacturer’s instructions. Briefly, 0.1 g leaf material was taken and grinded into homologous slurry with 1 mL extraction solution under ice bath, then centrifuged for 10 min at 4 °C, 8000 g. The supernatant was collected and set on ice to be tested. Then, 70 µL of supernatant was taken to measure the absorbance at 540 nm.

Western blot assay

Proteins expression was examined using Western blot assay. For cell-derived samples, the total protein was extracted with lysis buffer. Samples were frozen in liquid nitrogen and ground into powder and dissolved in lysis incubated at 4 °C for half an hour. Mixture was centrifuged for 10 min at 4 °C, 13 523 g. A fraction of supernatant was collected and mixed with loading buffer before being boiled at 95 °C for 10 min. Equal amounts of protein were separated by SDS-PAGE and transferred to a PVDF membrane, which was activated by methanol alcohol for five minutes before. The membrane was blocked for 1–2 h at room temperature with blocking buffer (5% defatted milk in PBST); then, primary antibodies were diluted in blocking buffer, and incubated with the sample for 1 h at RT. Samples were washed three times with PBST, and incubated with secondary antibodies that are diluted in blocking buffer. One hour later, cells were washed three times with PBST to remove the unbound secondary antibodies. The signals were detected with a Pierce ECL Western blotting substrate (Thermo scientific), according the manufacturer instructions. 15NH$_4^+$ uptake

Rice seedlings were grown in IRRI nutrient solution for 3 weeks. Uniform seedlings were chosen for further treatments. The seedlings were grown in solution without N for 4 days; then, Plant roots were submerged in 0.1 mM CaSO$_4$ for 1 min and treated with 0.25 mM (15NH$_4$)$_2$SO$_4$ for 60 min, respectively. Finally, the seedlings were returned to 0.1 mM CaSO$_4$ for 1 min for nitrate influx rate determination using 15NH$_4^+$.

Nitrogen concentration assay

Rice leaves were chopped and dried to a constant weight at 50 °C and then, the entirely dried and milled plant samples were digested with H$_2$SO$_4$–H$_2$O$_2$. Total plant N concentrations were determined using an automated continuous flow analyser (AA3, Bran and Luebbe, Norderstedt, Germany).

Acknowledgements

We wish to thank Professor Guohua Xu, MOA Key Laboratory of Plant Nutrition and Fertilization in Lower-Middle Reaches of the Yangtze River, for his help and guidance in our projects. The National Natural Science Foundation of China (31671269), National Key Research and Development Project (2016YFD0100700 and 2016YFD0101107), the Fundamental Research Funds for the Central Universities (JCY201901) and National Natural Science Foundation (No. 31672223) supported this study. The funding agencies had no role in the study design, data collection and analysis, decision to publish or paper preparation.

Competing interests

The authors declare no competing interests.

Author contributions

C.W. and J.W. directed the project. J.Y. performed the experiments. C.W., W.X. and J.W. conceived and designed the experiments. C.W. and J.W. directed the project. J.Y. performed the experiments, collected data and participated in paper writing. W.X. and J.W. conceived and designed the experiments. J.Y. performed the experiments. C.W., J.Y. and W.X. wrote the paper and finalized the paper. All the coauthors approved the paper.

References

Arite, T., Iwata, H., Ohshima, K., Maekawa, M., Nakajima, M., Kojima, M., Sakakibara, H., Kyoizuka, J. (2007) DWARF10, an RMS5/MAX4/DAD1 ortholog, controls lateral bud outgrowth in rice. Plant J. 51, 1019–1029.

Arite, T., Umehara, M., Ishikawa, S., Hanada, A., Maekawa, M., Yamaguchi, S., Kyoizuka, J. (2008) d14, a strigolactone-inensitive mutant of rice, shows accelerated outgrowth of tillers. Plant Cell Physiol. 50, 1416–1424.

Chen, Y., Fan, X., Song, W., Zhang, Y., Xu, G. (2012) Over-expression of OsPIN2 leads to increased tiller numbers, angle and shorter plant height through suppression of OsLAZY1. Plant Biotechnol. J. 10, 139–149.

Fan, X., Naz, M., Fan, X., Xuan, W., Miller, A.J. and Xu, G. (2017) Plant nitrate transporters: from gene function to application. J. Exp. Bot. 68, 2463–2475.
Gao, Z., Wang, Y., Chen, G., Zhang, A., Yang, S., Shang, L., Wang, D. et al. (2019) The indica nitrate reductase gene OsNRII allele enhances rice yield potential and nitrogen use efficiency. Nat. Commun. 10, 5207.

Guo, S., Xu, Y., Liu, H., Mao, Z., Zhang, C., Ma, Y., Zhang, Q. et al. (2013) The interaction between OsMADS57 and OsTB1 modulates rice tillering via DWARF14. Nat. Commun. 4, 1566.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K. and Pease, L.R. (1989) Nature Discovery of nitrate strigolactones, regulates rice tiller bud outgrowth. DWARF27, an iron-containing protein required for the biosynthesis of expression. and D-type cyclins based on sequence organization. et al Kouchi, H. 43

Renaudin, J.P. (2000) CDK-related protein kinases in plants. Plant Mol. Biol. (2005) Suppression of tiller bud activity in tillering dwarf mutants of rice. PNAS

Wang, W., Mauleon, R., Hu, Z., Chebotarov, D., Tai, S., Wu, Z., Li, M. et al. (2018) Genomic variation in 3,010 diverse accessions of Asian cultivated rice. Nature. 557, 43–49.

Wu, K., Wang, S., Song, W., Zhang, J., Wang, Y., Liu, Q., Yu, J. et al. (2020) Enhanced sustainable green revolution yield via nitrogen-responsive chromatin modulation in rice. Science, 367, eaaz2046.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.