Rice NIN-LIKE PROTEIN 4 plays a pivotal role in nitrogen use efficiency

Jie Wu1, Zi-Sheng Zhang1, Jin-Qiu Xia1, Alamin Alfatih1, Ying Song1, Yi-Jie Huang1, Guang-Yu Wan1, Liang-Qi Sun1, Hui Tang1, Yang Liu1, Shi-Mei Wang2, Qi-Sheng Zhu2, Peng Qin3, Yu-Ping Wang3, Shi-Gui Li3, Chuan-Zao Mao4, Gui-Quan Zhang5, Chengcai Chu5,*, Lin-Hui Yu1,*,# and Cheng-Bin Xiang1,*

1School of Life Sciences and Division of Molecular & Cell Biophysics, Hefei National Science Center for Physical Sciences at the Microscale, University of Science and Technology of China, The Innovation Academy of Seed Design, Chinese Academy of Sciences, Hefei, Anhui Province, China
2Rice Research Institute, Anhui Academy of Agricultural Sciences, Hefei, China
3Rice Research Institute, State Key Laboratory of Hybrid Rice, Sichuan Agricultural University, Chengdu, Sichuan, China
4State Key Laboratory of Plant Physiology and Biochemistry, College of Life Sciences, Zhejiang University, Hangzhou, China
5The State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, South China Agricultural University, Guangzhou, China
6State Key Laboratory of Plant Genomics and National Center for Plant Gene Research (Beijing), Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China

Received 27 February 2020; revised 8 August 2020; accepted 20 August 2020.
*Correspondence (Tel 86-551636-00429; fax 86-551636-01443; email xiangcb@ustc.edu.cn (C-BX) and Tel 1-631-344-2221; fax 631-344-2221; email yuhl@ustc.edu.cn (L-HY))
#Present address: Biology Department, Brookhaven National Laboratory, Upton, New York, NY, USA

Keywords: nitrogen use efficiency, NIN-like protein, OsNLP4, nitrate uptake and assimilation, nitrogen deficiency, grain yield.

Summary
Nitrogen (N) is one of the key essential macronutrients that affects rice growth and yield. Inorganic N fertilizers are excessively used to boost yield and generate serious collateral environmental pollution. Therefore, improving crop N use efficiency (NUE) is highly desirable and has been a major endeavour in crop improvement. However, only a few regulators have been identified that can be used to improve NUE in rice to date. Here we show that the rice NIN-like protein 4 (OsNLP4) significantly improves the rice NUE and yield. Field trials consistently showed that loss-of-OsNL4P dramatically reduced yield and NUE compared with wild type under different N regimes. In contrast, the OsNLP4 overexpression lines remarkably increased yield by 30% and NUE by 47% under moderate N level compared with wild type. Transcriptomic analyses revealed that OsNLP4 orchestrates the expression of a majority of known N uptake, assimilation and signalling genes by directly binding to the nitrate-responsive cis-element in their promoters to regulate their expression. Moreover, overexpression of OsNL4P can recover the phenotype of Arabidopsis nlp7 mutant and enhance its biomass. Our results demonstrate that OsNLP4 plays a pivotal role in rice NUE and sheds light on crop NUE improvement.

Introduction
Nitrogen (N) is an essential macronutrient for the growth, development and production of plants (Crawford and Forde, 2002). Utilizable N resources in soil are very limited and therefore N fertilizer has been widely used to maintain high yield of crops in agriculture (Li et al., 2018; Lian et al., 2005). However, only a small fraction of the applied N is absorbed by plants, and a large portion is lost to the environment, causing serious environmental pollution (Garnett et al., 2009). N use efficiency (NUE), an integration of NUpE (N uptake efficiency) and NUE (N utilization efficiency), is generally defined as the total amount of yield in the form of grain or biomass achieved per unit of available N. NUpE is the efficiency of roots to acquire N from the soil, and NUE is the efficiency of assimilation and mobilization of plant N to total biomass or grain yield (Han et al., 2015; Hawkesford, 2014; Xu et al., 2012). Improving NUE of crops is regarded as one of the most promising ways to solve the dilemma. However, the molecular mechanisms governing NUE is not well understood.

Plants have evolved effective mechanisms of N uptake and metabolism. Nitrate (NO3−) is the main N source absorbed by plants in well-aerated soil, which is mainly transported by nitrate transporter1 (NRT1), NRT2, chloride channel (CLC) and slow anion channel-associated homologues (SLAH) protein families (Krapp et al., 2014). After being uptaken into the cells, nitrate is reduced to ammonium (NH4+) by the action of nitrate reductase (NR, such as NIA1 and NIA2) and nitrite reductase (NIR), then assimilated into amino acids by glutamine synthetase (GS) and glutamate synthase (GOGAT) (Hirel et al., 2011, Masclaux-Daubresse et al., 2010, Wilkinson and Crawford, 1993). In addition to its role as a major nutrient for plants, nitrate also acts as an important signalling molecule for numerous developmental processes (Ho and Tsay, 2010; Krouk et al., 2010a, Zhang et al., 2020). Arabidopsis nitrate regulated1 (AtANR1) was the first identified nitrate regulator that functions in lateral root branching in response to nitrate (Zhang, 1998). AtNRT1.1 (CHL1), a dual-affinity transporter, acts as a NO3− sensor that regulates the primary NO3− response by changing the phosphorylation status of its threonine 101 residue (Park et al., 2009; Wang et al., 2009). The NO3− inducible protein kinase calcineurin B-like-interacting protein kinases23 (AtCIPK23) and AtCIPK8 could up-regulate or down-regulate the expression of CHL1 respectively, under different nitrate concentrations (Hu et al., 2009; Park et al., 2009). Furthermore, nitrate regulatory gene2 (AtNRG2) was found to mediate nitrate signalling in Arabidopsis through modulating AtNRT1.1 expression and interaction with NIN-like protein7 (NLP7) (Xu et al., 2016). A few transcription factors (TFs) have...
OsNLP4 controls nitrogen use efficiency

449

been identified in the regulation of nitrate metabolism. For example, transcription factors lateral organ boundary domain 37/38/39 (AtLBD37/38/39) are negative regulators of NO3 regulated genes (Rubin et al., 2009). Transcription factors, such as Arabidopsis squamosa promoter binding protein-like9 (AtSPFL9), TGACG sequence-specific binding protein1 (AtTGA1), AtTGA4, auxin signalling F-box3 (AtAFB3) and NAC domain containing protein4 (AINAC-4) have also been identified as nitrate regulators involved in early nitrate response signalling (Alvarez et al., 2014; Krouk et al., 2010b; Vidal et al., 2014; Vidal et al., 2010). Rice is one of the most important food crops in the world. N is a key factor affecting the growth and yield of rice. However, the molecular mechanisms underlying rice NUE is not well understood. The members of OsNRT1/2 family have received the most exploration and research to date. It has been reported that OsNRT1 encodes a constitutive component of the low-affinity nitrate uptake transporter (Lin et al., 2000). The variation in OsNRT2.1B largely explains nitrate-use divergence between indica and japonica and that NRT1.1B-indica can potentially improve the NUE of japonica. Moreover, NRT1.1B can interact with a phosphate signalling repressor SPX domain containing protein4 (SPX4) to coordinate utilization of N and phosphorus (Hu et al., 2019; Hu et al., 2015; Zhang et al., 2020). Overexpression of OsNRT1.1A in rice significantly improves NUE and grain yield, and maturation time is also significantly shortened (Wang et al., 2018). Another nitrate transporter OsNPF6.1HapB confers high NUE and increases yield under low nitrogen supply (Tang et al., 2019). Nitrate assimilation related2.1 (OsNAR2.1) regulates the balance of ammonium and nitrate by interacting with multiple NRT2 family members, including OsNRT2.1, OsNRT2.2 and OsNRT2.3a (Yan et al., 2011). In addition, other genes have been reported to affect N metabolism in plants, such as dense and erect panicle1 (DEP1), which interacts in vivo with both the Gx (RGAI) and Gjb (RGBI) subunits of the heterotrimeric G protein, and reduced RGAI or enhanced RGBI activity inhibits N responses (Huang et al., 2009; Sun et al., 2014). The balanced opposing activities and physical interactions of the rice growth-regulating factor 4 (OsGRF4) transcription factor and the growth inhibitor DELLa confer homoeostatic co-regulation of growth and the metabolism of carbon (C) and N (Li et al., 2018). The indica OsNR2 allele has superior grain yield and NUE than the japonica allele, in part via feed-forward interaction with OsNRT1.1B (Gao et al., 2019). A recent study found transcription factor nitrogen-mediated tiller growth response 5 (NGR5) facilitates nitrogen-dependent recruitment of polycomb repressive complex 2 to repress branching-inhibitory genes via H3K27me3 modification. Increased NGR5 activity consequently uncouples tillering from nitrogen regulation, boosting rice yield at low nitrogen fertilization levels (Wu et al., 2020).

Of all the plants, only legume species have successfully evolved the capacity to deal with N limitation by developing a symbiotic interaction with rhizobium. The initiation of nodule development depends on a specific transcription factor, nodule inception protein (NIN) (Borisov et al., 2003; Schauer et al., 1999). Loss of NIN function hinders the infiltration of rhizobium and the nodule formation (Marsh et al., 2007). Interestingly, the NIN gene family is widely present in higher plants and algae, including species that do not fix gaseous N. CreNRT2, a NIN-like protein (NLP) gene in Chlamydomonas, acts as a central regulator required for nitrate signalling and assimilation. CreNRT2 binds the promoter of nitrate reductase gene in the presence of nitrate and to enhance its transcription (Camargo et al., 2007). Arabidopsis NLPs play a central role in N signalling (Konishi and Yanagisawa, 2013; Liu et al., 2017; Marchive et al., 2013; Mu and Luo, 2019). AtNLP7 functions as the master regulator in the dynamic and early N responses by initiating a rapid nitrogen response cascade (Alvarez et al., 2020). Disruption of AtNLP7 or suppression of AtNLP6 results in an N-starved phenotype and impaired nitrate signalling (Alvarez et al., 2020; Castaings et al., 2009; Konishi and Yanagisawa, 2013). AtNLP7 is regulated by nitrate via a nuclear retention mechanism, which is controlled by the phosphorylation of the conserved serine205 by Ca2+-sensor protein kinases10/30/32 (ATCKP10/30/32) in the N-terminal region of AtNLP7 (Liu et al., 2017). AtNLP8, which localizes in the nucleus constantly, can bind directly to the promoter of CYCP707A2 in a nitrate-dependent manner to reduce the ABA content in seeds and promote seed germination (Yan et al., 2016). Protein–protein interactions mediated by the PB1 domain were observed between a variety of combinations of different Arabidopsis NLPs (Konishi and Yanagisawa, 2019). Moreover, several NLPs, such as ZmNLP5, ZmNLP6 and ZmNLP8, were reported to modulate nitrate signalling and assimilation in maize (Cao et al., 2017; Ge et al., 2020).

We previously reported that AtNLP7 significantly improves plant growth by coordinately enhancing N and C assimilation (Yu et al., 2016). To continue the investigation on the roles of NLPs in NUE, we initiated research to analyse the function of rice NLPs in NUE. There are six NLP family members in rice (Chardin et al., 2014). However, their functions are still largely unknown. Here we report that OsNLP4, one homologous gene of Arabidopsis NLP7, plays a pivotal role in N uptake, assimilation and signalling. Genetic analyses and field trials showed that loss-of-OsNLP4 reduced biomass, yield as well as NUE, while constitutive overexpression of OsNLP4 significantly increased plant biomass, NUE and yield under different N conditions. OsNLP4 has a broad impact on the transcriptome responding to N and can coordinate a majority of the genes related to N utilization and signalling. Our results indicate that OsNLP4 is a pivotal regulator of NUE and a promising candidate for crop NUE improvement.

Results

OsNLP4 greatly influences vegetative growth under different N conditions

To investigate the function of OsNLP4 in rice, we generated two knockout mutants (japonica variety Zhonghua11 background, ZH11) with CRISPR-Cas9 technology and transgenic lines overexpressing OsNLP4 (OE) under the control of rice ACTIN1 promoter and obtained an additional T-DNA insertion mutant nlp4-3 (japonica variety Dongjin background, DJ; Fig. S1).

When grown in hydroponic culture with different concentrations of nitrate, the OE plants exhibited significant growth advantages, and the seedlings of the nlp4 mutants exhibited obvious growth retardation compared with wild type (WT), especially under low nitrate conditions (0.02 mM, 0.2 mM; Figure 1A, Fig. S2). The biomass of WT, nlp4 and OE lines were significantly affected by OsNLP4 under different N concentrations. The knockout mutants showed the lowest biomass (fresh weight per plant), while the OE lines showed the highest (Figure 1B). Both shoot and root biomass displayed similar differences to whole plant biomass among the genotypes (Figure 1C and D). Consequently, the shoot to root fresh weight ratio (S/R) was higher for OsNLP4-overexpressing plants and much lower for the mutants, except under 0.02 mM nitrate condition.
where the knockout mutants showed the highest S/R (Figure 1E). Shoot fresh weights of the mutants remained largely unaltered under 0.02 and 0.2 mM nitrate conditions, while root fresh weights of the mutants obviously decreased under 0.02 mM nitrate condition compared with 0.2 mM nitrate condition, indicating root growth defects in the mutants under 0.02 mM nitrate condition. The knockout mutants displayed much less and shorter lateral roots, especially under low N conditions, and their lateral roots were less sensitive to environmental N contents compared with WT and the OsNLP4-overexpressing lines (Figure 1A and F). However, primary roots of the mutants were much longer under 0.02 mM and 0.2 mM N conditions, and the length decreased in the mutants as N concentrations rise while it increased in WT (Figure 1G). Therefore, higher S/R in the mutants under 0.02 mM nitrate mainly due to lateral root growth defects in the mutants.

In addition, the OsNLP4-overexpressing lines showed well-developed root system with increased root numbers and length compared with WT under 0.02 mM and 0.2 mM (Figure 1F and G). All these data suggest that OsNLP4 is involved in N regulated root development. The results of shoot length (Figure 1H) agreed with the data of shoot fresh weight (Figure 1C). Moreover, we also tested the growth response of different genotypes to ammonium (Fig. S3A). The results were similar to those of the nitrate treatment group with relatively slight differences (Fig. S3B). Taken together, these results demonstrate that OsNLP4 plays crucial roles in rice vegetative growth in response to nitrate.

OsNLP4 is a major determinant of rice NUE and crucial for yield

To investigate the role of OsNLP4 in rice NUE, we performed field trials for gain- and loss-of-OsNLP4 genotypes in different years at two different locations in China: Chengdu and Lingshui. In Chengdu, the field trials were performed under low (LN), normal (NN) and high N (HN) conditions as described in Methods. A representative plant (dug out from the field and potted) of each genotype grown under three N levels is shown in Figure 2A. Grain yield per plant and per plot were tremendously affected by OsNLP4. The knockout mutants exhibited a significant decrease of yield per plant by an average of 29.8% under LN, 30.5% under NN and 8.7% under HN compared with the wild type. In contrast, the OE lines showed a remarkable increase by an average of 38.0% under LN, 47.2% under NN and 26.8% under HN (Figure 2B). The same is true for actual yield per plot. The knockout mutants decreased yield per plot by an average of 29.5% under LN, 30.7% under NN and 9.0% under HN, while the OE lines increased by an average of 37.4% under LN, 46.1% under NN and 27.7% under HN compared with the wild type (Figure 2C).

Based on the above yield and the amount of N fertilizer applied, NUE was calculated and shown in Figure 2D. The OE lines remarkably enhanced NUE by an average of 37.3% under LN, 48.1% under NN and 24.5% under HN compared with the wild type. In contrast, the knockout mutants significantly
decreased NUE by an average of 28.9% under LN, 31.2% under NN and 10.1% under HN.

In another field trial with normal N level in Lingshui, Hainan Province, the impacts of OsNLP4 on yield and NUE were reproduced (Fig. S4). The OE lines also significantly increased grain yield by 25.1% (grain yield per plant), 23.9% (actual yield per plot) and NUE by 24.3% (Fig. S4C). The T-DNA insertion mutant osnlp4-3 showed dramatically decreased grain yield and NUE (Fig. S4D). Taken together, these results demonstrate that OsNLP4 is a major determinant of NUE and crucial for yield in rice.

OsNLP4 coordinates NUE-related gene expression

To investigate the molecular mechanism by which OsNLP4 controls NUE and yield, we explore the genome-wide transcriptional landscape controlled by OsNLP4 in the response to N availability by profiling the transcripts of wild type (WT), knockout mutant nlp4-1 (ko) and OE plants during a long-term hydroponic experiment with two N levels (LN with 0.02 mM nitrate and HN with 2 mM nitrate).

Transcriptomic differences between WT, ko and OE were determined by performing pairwise comparisons (WT vs. ko-LN, WT vs. ko-HN, WT vs. OE-LN and WT vs. OE-HN) of gene expression levels using the aligned reads so as to identify different expressed genes (DEGs) in each pair. Compared with the WT, DEGs with knockout or overexpression of OsNLP4 were significantly different, especially between WT and ko under HN condition, indicating that OsNLP4 has a profound and broad influence on the transcriptome in response to nitrate (Figure 3A and B, Fig. S5A).

We further classified the DEGs based on Gene Ontology and KEGG pathway. Remarkably, genes involved in N uptake and metabolism were highly enriched in DEGs (Fig. S5B-C, Table S1), indicating that OsNLP4 may coordinately regulate the key genes in N utilization. Interestingly, as showed in Figure 3C, OsNLP4 appeared to have a preference to positively regulate the expression of high-affinity nitrate/ammonium transporters, such as OsNTR2.1, OsNTR2.2, OsNTR2.3, OsAMT1.2 (Hoque et al., 2006; Sonoda et al., 2003; Yan et al., 2011), dual-affinity nitrate transporters, such as OsNTR1.1B and OsNTR2.4 (Hu et al., 2015; Wei et al., 2018). Besides transporter genes, key genes involved in nitrate reduction, ammonium assimilation were dramatically down-regulated in nlp4-1 mutant while significantly up-regulated in the OsNLP4-OE line. Moreover, several N signalling genes, including OsNLP2, OsGRF4, NITRATE-INDUCIBLE GARP-TYPE TRANSCRIPTIONAL REPRESSOR1 (OsNIGT1), EARLY NODE 93 (OsENOD93), OsENOD93a were found differently expressed in OsNLP4 mutant and overexpression plants. In addition, our data show that OsNLP4 also affected the expression of some genes related to C metabolism (Fig. S5B-C, Table S2).

The expression pattern of the genes involved in N response and metabolism was verified by RT-qPCR, which was largely in agreement with the RNA-seq data (Fig. S6). In addition, we examined the transcription levels of these genes in response to different ammonium concentrations. Similar to the nitrate treated
group, the expression of many genes was still positively regulated by OsNLP4, especially the genes for ammonium absorption and assimilation in response to ammonium (Fig. S7). Taken together, these results suggest that OsNLP4 is a pivotal regulator that orchestrates the N metabolism and signalling in rice.

OsNLP4 directly modulates the expression of N metabolism genes by binding the NRE in their promoters

Consistent with the above results, the promoter regions of the key N metabolism genes harbour at least one NRE-Like cis-element for NLP binding (Table S3). To evaluate whether OsNLP4 directly binds to the NREs, we performed chromatin immunoprecipitation (ChIP) quantitative PCR assays and confirmed in vivo association of OsNLP4 with NRE-containing promoter fragments from the N assimilation genes, including OsNRT2.1, OsNRT2.2, OsNRT2.3, OsNRT2.4, OsNRT1.1B, OsNIA1, OsNIA3, OsAMT1.1 and OsGRF4 (Figure 4A-I). This result was further verified with yeast one-hybrid (Y1H) assay (Figure 4J). These data indicate that the OsNLP4 may directly regulate the transcription of N absorption and assimilation genes.
OsNLP4 directly modulates N uptake and assimilation

To confirm the above results of transcriptomic comparison, we investigated the role of OsNLP4 in regulating N absorption and assimilation. With a chlorate sensitivity assay, we found that the nlp4 mutants exhibited a much lower chlorate sensitivity while the OsNLP4-overexpressing lines showed significantly higher chlorate sensitivity than WT (Figure 5A and B), demonstrating that OsNLP4 positively modulates nitrate uptake. According to the transcriptomic and RT-qPCR analysis, OsNLP4 positively regulates the expression of nitrate transport genes as well as nitrate assimilation genes (Figures 3 and S6), thus increasing nitrate uptake and assimilation, consequently resulting to chlorate sensitivity in the OsNLP4-overexpressing rice. This point was further verified by directly measuring 15N-nitrate uptake. The 15N accumulation in OE plants was significantly higher than that in WT while much lower in the knockout mutants (Figure 5C). Moreover, total N and nitrate content were increased in OE lines but reduced in knockout mutants compared with those of the wild type (Figure 5D and E).

OsNLP4 expression and OsNLP4 localization are responsive to N availability

To have a comprehensive understanding of OsNLP4 expression in response to N availability, we made a detailed time-course analysis of OsNLP4 expression. As shown in Figure 6A, when seedlings grown in normal N conditions (NN) were shifted to N starvation conditions (0N), OsNLP4 transcript level was rapidly induced and plateaued with fivefold increase after 2-hour N starvation, which was maintained as long as N starvation was imposed. When N was resupplied in the form of KNO3, OsNLP4 transcript level was rapidly declined to its pre-induction level within 2 h. However, when resupplied with NH4Cl, OsNLP4 transcript level also decreased rapidly but maintained at a higher level compared with KNO3 treatment (Figure 6A), while the KCl control treatment did not alter its transcript level. Moreover, OsNLP4 promoter:GUS transgenic plants exhibited similar response with strong beta (β)-glucuronidase (GUS) induction by N starvation and reversion by resupplied N (Figure 6B). GUS activity was detected throughout the whole life of the reporter plant (Fig. S10A-K) with strong expression in coleoptile (a) (b) (c) (d) (e) (f) (g) (h) (i) (j) (k) (l) (m) (n) (o) (p) (q) (r) (s) (t) (u) (v) (w) (x) (y) (z).
of germinating seeds, leaves and roots (Fig. S10A-D). Further observation showed that GUS activity was mainly detected in the epidermis and vascular tissues of leaves and roots (Fig. S10C-D and G), and also in stem, node, spikelet and anther (Fig. S10H-K). However, OsNLP4 was not expressed in the root hairs (Fig. S10F).

Consistent with the GUS staining result, RT-qPCR analyses of OsNLP4 transcript level revealed a similar pattern with high expression in leaf and root (Fig. S10L).

To investigate the subcellular localization of OsNLP4 protein, we generated transgenic rice lines expressing the fusion of full length CDS of OsNLP4 with GFP driven by the rice ACTIN1 promoter. We checked the subcellular localization of OsNLP4 protein under N starvation and N re-addition conditions. OsNLP4 mainly localized in cytosol with low protein levels under N starvation (Figure 6C). However, when nitrate was resupplied for 15 min, GFP signals were detected in the nucleus (Figure 6D), by 30 min GFP signals were dramatically increased in the nucleus (Figure 6E and F). This nuclear accumulation was reversed when nitrate was withdrawn for 60 min (Figure 6G). The dramatic change of GFP signal intensity in response to nitrate availability strongly suggests a tight regulation of OsNLP4 mRNA translation by N because the transcript level of OsNLP4-GFP remained constantly high regardless of N conditions (Figure 6H), while OsNLP4-GFP protein level showed a positive correlation with the nitrate levels (Figure 6I and J).

We also tested the response of the transgenic rice to NH4Cl. We found OsNLP4-GFP protein levels were slowly induced by ammonium. When NH4Cl was resupplied for 6 h after N starvation, the fluorescent signals of OsNLP4-GFP were low and mainly localized in the cytosol (Fig. S11A-C). Until 12 h later, slightly increased GFP signals were detected in the nucleus (Fig. S11D). Moreover, the seedlings grown in 2 mM ammonium hydroponic culture for 10 days produced more visible but still low GFP signals in both cytoplasm and nucleus (Fig. S11E).

OsNLP4 enhances N assimilation and growth in Arabidopsis

In order to investigate whether OsNLP4 can similarly modulate N assimilation in different plant species, we generated OsNLP4-overexpressing transgenic Arabidopsis in nlp7-1 background. We found overexpression of OsNLP4 not only fully rescued the N-deficient phenotypes of nlp7-1, but also significantly increased plant biomass under different nitrate conditions (Fig. S12A, C). Moreover, the overexpression plants had better developed roots (Fig. S12B, D) and grew better with larger rosette leaves and higher biomass when grown in soil (Fig. S12E and F). In addition, the expression of the genes involved in N assimilation and signalling was significantly up-regulated in overexpression lines compared with that of nlp7-1 and WT (Fig. S13). These results suggest that OsNLP4 is functionally conserved and, thus, has potential applications to improve N use and plant growth in both monocot and dicot crops.
In the present study, we show that the transcription factor OsNLP4 acts cooperatively instead of single step in a metabolic pathway. In the improvement plant growth and NUE by enhancing multiple steps coordinately regulate the expression of a set of genes so as to strategy for improve NUE, because they have the capacity to

Application of transcription factors might be a more efficient approach to boost crop yields in intensive agricultural systems. N fertilizer overuse leads to low NUE and serious environmental problems (Vitousek et al., 2009). Improving plant NUE is an important, cost-effective approach to N availability. Plants grown under NN condition were transferred to hydroponic medium without N for 8 h, and then transferred to hydroponic medium with 5 mM KNO₃, 5 mM NH₄Cl or 5 mM KCl. RT-qPCR data are mean ± SD (n = 3). (B) GUS staining of 7-day-old OsNLP4promoter::GUS transgenic plants cultured under different N conditions. NN: 2 mM KNO₃; ON: nitrogen free; N-resupply: nitrogen free for 6 days then resupply with 2 mM KNO₃ for 24 h. Bar = 1.0 cm. (C-G) Subcellular localization of OsNLP4. (C) Seedlings grown on nitrogen-free medium. (D, E) N-starved seedlings were transferred to a nitrate medium for 60 min, then to N-free medium again and observed after 60 min. Bar = 50 μm. (H) Transcript levels of OsNLP4 and OsNLP4-GFP in ACTIN1::OsNLP4-GFP transgenic rice plants under different N conditions revealed by RT-qPCR. NN: 2 mM KNO₃; ON: nitrogen free; -N: nitrogen free for 9 days then supplying 2 mM KNO₃ for 2 h; +N: nitrogen free for 9 days then supplying 2 mM KNO₃ for 2 h, and shifting back to nitrogen free for 2 h. (I) Band density of i calculated by ImageJ. Values are the mean ± SD (n = 3). The letters a and b indicate significant differences (P < 0.05).

**Discussion**

Plant growth and yield are usually limited by soil N availability in most agricultural cropping systems. N fertilizer overuse leads to low NUE and serious environmental problems (Vitousek et al., 2009). Improving plant NUE is an important, cost-effective approach to boost crop yields in intensive agricultural systems worldwide. Many genes, most of which are related to N uptake and primary assimilation, have been applied to increase N assimilation in various plants during the past decades. However, only very few of these studies have showed phenotypic effect on NUE or other growth parameters on the plant by individually ectopic expression of these genes, and some even showed negative pleiotropic effects, implying the notion of single-step rate-limiting regulation being oversimplified for improving NUE (Andrews et al., 2004; McAllister et al., 2012; Xu et al., 2012). Application of transcription factors might be a more efficient strategy for improve NUE, because they have the capacity to coordinately regulate the expression of a set of genes so as to improve plant growth and NUE by enhancing multiple steps cooperatively instead of single step in a metabolic pathway. In the present study, we show that the transcription factor OsNLP4 acts as a crucial regulator to simultaneously coordinate many processes in N utilization and signalling pathway to significantly improve rice yield and NUE under different N conditions.

Likely benefitting from the simultaneously coordinating the expression of N metabolism genes (Figure 3, Figs. S5–S7), OsNLP4-overexpressing rice significantly increased N uptake and assimilation (Figure 5, Figs. S8 and S9), thus enhanced plant growth (Figure 1, Figs. S2 and S3), grain yield and NUE (Figure 2 and Fig. S4) compared with wild type under all N conditions. On the contrary, plant growth, grain yield and NUE of the osnlp4 mutants are significantly impaired compared with wild type. However, under HN, there is no significant difference in NUE between wild type and the mutants, while decreased by 28.9% in osnlp4 mutants compared with wild type under LN and NN, respectively. Under HN, there is no significant difference in NUE between wild type and the mutants, while decreased by 28.9%–31.2% in osnlp4 mutants compared with wild type under LN and NN, respectively. However, under HN, there is no significant difference in NUE between wild type and the mutants, while OsNLP4-overexpressing lines shows a small but statistically significant increase compared with wild type (Figure 2). It is noteworthy that the field trial showed that grain yield per plot of the OsNLP4-overexpressing lines under LN and NN almost the same as that of WT under NN and HH, respectively (Figure 2A), which indicates, with the help of OsNLP4, farmers can harvest similar grain yield as
wild type with a 50% reduction of N fertilizer usage, thereby reducing the production cost and environmental pollution.

As one homolog of AtNLP7 in rice, OsNLP4 can fully recover the N starvation phenotype of the Arabidopsis nlp7-1 mutant (Fig. S12). OsNLP4 also orchestrates the N utilization and signalling, thus improve the NUE under both N-rich and deficiency conditions, as revealed by our transcriptomic and RT-qPCR data of plant under different N conditions (Figure 3, Figs. S5-S7). Many key genes involved in N transport, assimilation, as well as signalling were found significantly down-regulated in the loss-of-OsNLP4 mutant and up-regulated in the OsNLP4-overexpressing lines. Promoter sequence analysis showed that one or more NRE cis-element exist in the promoter of these genes, including OsNRT2.1, OsNRT2.2, OsNRT2.3, OsNRT2.4, OsNRT1.1B, OsAMT1.1, OsNIA1, OsNIA3 and OsGRF4. Through CHIP and Y1H assays, we showed that OsNLP4 directly bound to the promoter fragments of these genes to regulate their expression (Figure 4). Interestingly, OsNLP4 preferred to regulate the expression of high-affinity nitrate/ammonium transporters like OsNRT2 and OsAMT1 family members, which may well explain why the nlp4 mutant phenotype was more severe under low N conditions.

Besides direct regulation, it is worth noting that OsNLP4 can indirectly modulate N utilization through regulating other N signalling regulators. Several regulators, such as OsNRT1.1B, OsGRF4, OsNIGT1 and OsENOD93, were highly expressed in OsNLP4-overexpressing rice, while down-regulated in the mutant under HN or LN condition (Figure 4C). These key regulators had been also proved to significantly improve rice yield and NUE. For example, compared with NRT1.1B-japonica, NRT1.1B-indica with a single amino acid substitution, results in increased nitrate uptake, root-to-shoot transport and expression of nitrate-responsive genes, and a consequent increase in grain yield and NUE (Hu et al., 2015). Antagonistically working with DELLA, OsGRF4 boost rice yield and NUE by promoting and integrating N assimilation, carbon fixation and growth (Li et al., 2018). In addition, transgenic rice plants overexpressing the OsENOD93-1 gene improve NUE with increased shoot dry biomass and seed yield (Bi et al., 2009).

N and C metabolic processes are closely interrelated and tightly co-regulated (Stitt et al., 2002). NUE is not only dependent on N metabolism and manipulating C metabolism is useful in some cases to improve NUE (Chardon et al., 2012). In addition to modulating N metabolism, OsNLP4 may also participate in many C metabolism processes, such as C fixation and reductive pentose phosphate cycle as implicated by transcriptomic analyses (Fig. S5, Table S2), indicating that OsNLP4 coordinates N and C assimilation simultaneously.

According to the publicly available transcriptome data (Hruz et al., 2008; Jagadhesan et al., 2020), rice NLPs are expressed in almost all organs, with OsNLP1 and OsNLP3 preferentially expressed in source organs, and OsNLP6 expressed at very low level. Different from the other 5 OsNLPs, expression of OsNLP4 is repressed by many abiotic stresses and induced by low phosphate availability (Chardon et al., 2014). In our study, we found OsNLP4 is widely expressed in all organs, with the highest in leaf and followed by root (Fig. S10). Its expression in root and leaf is rapidly and dramatically induced by nitrogen starvation, and repressed by nitrate replenishment. Ammonium can also depress the expression of OsNLP4, but with a weaker suppression than nitrate, suggesting that OsNLP4 plays a more extensive role in nitrogen metabolism (Figure 6A). According to the report of Cao et al. (2017), low nitrate induces the expression of ZmNLP3 and ZmNLP4 in maize. The transcription level of ZmNLPS is significantly up-regulated shortly after the supply of nitrate on the nitrate-deprived plants (Ge et al., 2020). However, all the NLPs in Arabidopsis are not significantly induced or repressed in the N-starved seedlings resupplied with nitrate for one hour. Arabidopsis NLP6- and NLP7-regulated N assimilation and signalling is mostly at post-translational level (Alvarez et al., 2020; Konishi and Yanagisawa, 2013; Marchive et al., 2013). The different responses to environmental N between OsNLP4, ZmNLP3/4/5 and Arabidopsis NLPs imply that there may also exist transcriptional regulation in rice and maize NLPs. As an important modulator of N utilization, it is reasonable for NLPs to respond at both transcriptional and post-transcriptional levels to maximize N utilization.

Arabidopsis NLP7 has been proved as key transcription factor of nitrate signalling and primary responses through nuclear retention mechanisms (Marchive et al., 2013). Similar as in Arabidopsis, we found that OsNLP4 also showed nuclear retention in response to nitrate. Under nitrate starvation, weak OsNLP4-GFP signals were found in cytosol. However, after nitrate was resupplied, OsNLP4-GFP proteins were quickly and dramatically accumulated in the nucleus in 30 min and moved to cytosol 1 h after nitrate deprivation (Figure 6C-G). As OsNLP4 is strongly induced by N-free condition, while less OsNLP4 protein exists without the presence of N (Figure 6H-J). This dis-correlation between transcription and translation implies that OsNLP4 prepares for N deficiency by responding rapidly at the transcriptional level, priming the OsNLP4 mRNA capacity and readiness for protein synthesis. Once N is available in the growth environment, OsNLP4 mRNA can be rapidly translated to promote N absorption and utilization. OsNLP1, which is also induced by N deficiency, has very similar functions as OsNLP4 in N utilization. However, unlike OsNLP4, OsNLP1 protein retained in the nucleus under different N conditions (Alfath et al., 2020). Our quantitative RT-PCR data also showed that OsNLP4 does not affect OsNLP1 expression under different N conditions, indicating that the strong phenotypes of OsNLP4 mutants and overexpression lines are not contributed via OsNLP1 (Fig. S14). It is likely that OsNLP1 plays as a housekeeper to maintain the basal N utilization while OsNLP4 responds to different N levels through nucleocytosolic shuttling to maximize N utilization. This may be an economic and effective response mechanism for plants to adapt to the changes of nitrogen sources in the environment.

According to the previous studies, nucleocytosolic shuttling of AtNLP7 is specific for nitrate rather than ammonium (Marchive et al., 2013), so did ZmNLP6 and ZmNLP8 in maize (Cao et al., 2017). Paddy rice has long been considered to prefer ammonium over nitrate as the N source due to the anaerobic soil conditions in flooded fields (Sawaki et al., 2013). However, rice has evolved with the ability to utilize both nitrate and ammonium at high rates. The aerenchyma cells in rice adventitious roots can transport oxygen to the rhizosphere, where nitrification of ammonium to nitrite can occur (Li et al., 2008). Actually, it has been estimated that up to 25–40% of total rice N derives from nitrate in the waterlogged paddy rhizosphere (Kirk and Kronzucker, 2005). Besides responding to nitrate, our data showed that OsNLP4 also modulates many N-responsive genes expression in response to ammonium (Fig. S7). It is worthy of note that OsNLP4 also regulates the expression of ammonium assimilation and uptake-related genes (Figures 3C, 4F and Fig. S7). Positive regulation of OsAMT1.1 and OsAMT1.2, which are key ammonium transporter genes (Lee et al., 2020; Li et al., 2016), indicates
that OsNLP4 may also modulate ammonium acquisition in rice. Consistently, osnlp4 mutants showed growth retardation while the OsNLP4-overexpressing lines showed growth advantages in medium with ammonium as the sole N source (Fig. S3). Further analysis found that resupply of ammonium after N starvation increased the OsNLP4-GFP protein levels and promoted its subcellular localization in nucleus under our experimental conditions, although in a much slower and weaker manner compared with nitrate resupply (Figure 6 and Fig. S11). These results suggest that the ammonium may also be a signalling molecule that regulates the OsNLP4-GFP protein synthesis and its nucleus and cytoplasm shuttle. However, why the OsNLP4-GFP protein responds so slowly to ammonium compared to nitrate, and whether the ammonium is the direct signal molecule that causes the nucleocytoplasmic shuttle of OsNLP4-GFP protein remains to be resolved.

In conclusion, our results demonstrate that OsNLP4 acts as a pivotal regulator of NUE by coordinating N uptake, assimilation and signalling, can significantly improve the grain yield and NUE under different N conditions, and is a promising candidate gene for improving yield and NUE of the crops.

**Methods**

**Plant materials and growth conditions**

The rice (Oryza sativa L.) varieties Zhonghua 11 (ZH11) and Dongjin (DJ) were obtained by Hangzhou Biogle Co., LTD. (Hangzhou, China) (http://www.biogle.cn), using CRISPR/Cas9 technique (Lu et al., 2017). Mutants were selected by screening for resistance to hygromycin B. The homozygous osnlp4-3 T-DNA insertion mutant (PFG_18-04333_L, DJ background) was ordered from the Korea Rice Mutant Center (Pohang, Korea) (Jeong et al., 2002). ACTIN1:OsNLP4 expression construct was made by inserting the coding region of OsNLP4 into pCB2006 via GATEWAY cloning system (Lei et al., 2007). The binary vector was transferred into Agrobacterium tumefaciens (EHA105) for rice transformation. Homozygous lines (T3 generation) were selected using glufosinate and expression was confirmed by RT-PCR and quantitative RT-PCR, then propagated to obtain the next generation of seeds (T4 generation) for experimental analysis.

The Arabidopsis thaliana ecotype Columbia (Col-0) was used in this study. The nlp7-1 (SALK_26134C) mutant was obtained from Arabidopsis Biological Resource Center (ABRC). 3SS:OsNLP4 overexpression construct was made by inserting the coding region of OsNLP4 into pCB2004 via GATEWAY cloning system. The OsNLP4-overtransgenic Arabidopsis were obtained by Agrobacterium-mediated floral-dip method.

For hydroponic culture, rice seedlings were grown in modified Kimura B solution in an artificial climate chamber with a 16-h light/8-h dark photoperiod. For evaluation the phenotype of soil-grown plants, seeds were germinated and grew in soil at 22 °C under 16-h light/8-h dark photoperiod.

**RNA extraction and RT-qPCR**

Total RNA isolated using Trizol reagent (Invitrogen, Carlsbad, California) were used for reverse transcription. RT-qPCR was performed with a StepOne Plus Real Time PCR System by using a Takara SYBR Premix Ex Taq II reagent kit. Rice Actin1 was used as the internal reference. All the primers used are shown in Table S4.

**Promoter-GUS analyses**

A 3.0-kb promoter region of OsNLP4 was amplified from ZH11 and cloned into pcB308R (Lei et al., 2007; Xiang et al., 1999) to generate OsNLP4:promoter:GUS, and the resulting vector was transformed into ZH11. For GUS staining, seedlings of OsNLP4:promoter:GUS transgenic rice under different nitrogen conditions were sampled for histochemical detection of GUS expression. After staining for 12 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 HIROX MX5040RZ digital optical microscope (Questar China Limited) and photographed using a digital camera (Nikon D700).

**Time-course analyses of OsNLP4 expression in response to N availability**

Rice seedlings (ZH11) were cultured in modified Kimura B solution with 0.5 mM KNO3 as the sole N source for 10 days. Then, seedlings were cultivated in modified Kimura B solution without nitrogen for 8 h and transferred to 5 mM KNO3 or NH4Cl (5 mM KCl used as the control) in modified Kimura B solution for nitrogen induction. The whole plants were collected at 0, 0.5, 1, 2, 4, 6 and 8 h, and the expression of OsNLP4 was determined using RT-qPCR. Growth chamber conditions were 16-h light (30 °C)/8-h dark (28 °C) photoperiod, ~300 μmol/m²/s photon density and ~60% humidity.

**Subcellular localization of OsNLP4-GFP**

To investigate the subcellular localization of OsNLP4, the ACTIN1: OsNLP4-GFP fusion constructs were produced by inserting the full length CDS of OsNLP4 into the pCB2006-ACT:GFP vector, and the resulting vector was transformed into ZH11. To investigate the nuclear-cytoplasmic shuttling of OsNLP4, rice seedlings were germinated and grown on the modified Kimura B solution without N for 10 days, then treated with 50 mM KNO3 for 60 min, and returned to the N-free medium. Laser scanning confocal imaging was performed using the Zeiss 710 microscope.
equipped with an argon laser (488 nm for green fluorescent protein (GFP) excitation).

**Western blot analysis**

Proteins were extracted from the roots of the sample using the RIPA lysis buffer (strong) (Beyotime, China). For Western blot analysis, proteins were electroblotted from 10% acrylamide gel to nitrocellulose membrane (Immobilon-P, MILLIPORE Corporation, Bedford, MA, USA) after the separation of SDS-PAGE. Antibodies used in Western blot were as follows: anti-GFP antibody (M20004, Mouse mAb, Abmart, Shanghai, China), 1 : 1000 for Western blot; anti-ACTIN antibody (M20009, Mouse mAb, Abmart, Shanghai, China), 1 : 1000 for Western blot and goat anti-mouse IgG-HRP (M21001, Abmart, Shanghai, China), 1 : 5000 for Western blot. Image Quant LAS 4000 (GE, USA), as the CCD camera system, was used for the band intensity quantification with Super Signal West Femto Trial Kit (Thermo, Rockford, IL, USA).

**Metabolite analyses**

The metabolite analyses were performed on the seedlings of 16-day-old plants grown hydroponically with different concentrations of nitrate. The per cent total N content in oven-dried plant material was measured with an NC analyser (Vario EL III model, Elementar, Hanau, Germany) according to the manufacturer’s instructions. Nitrate was extracted in 50 mM HEPES-KOH (pH 7.4), and measured by the method based on the previous report (Cataldo et al., 1975). The maximum in vitro activity of NR was assayed as described previously (Sylvie et al., 1998). NiR, GS, GOGAT enzyme activities, glutamine and glutamate contents were measured using assay kits (Su Zhou Keming Bioengineer Company, China) following to the manufacturer’s instructions. All the materials analysed were whole plants.

**Uptake of 15N-nitrate and 15N-ammonium**

15N-accumulation assay after 15N-nitrate labelling was performed with 15N-labelled KNO3 (99 atom % 15N, Sigma-Aldrich, no. 335134) or 15N-labelled NH4Cl (98 atom % 15N, Sigma-Aldrich, no. 299251), respectively. For 15N-nitrate accumulation assay, rice seedlings were cultured in the Kimura B solution for 10 days. Next, the seedlings were pre-treated with the Kimura B solution for 2 h and then transferred to modified Kimura B solution containing 5 mM 15N-KNO3 for 24 h. At the end of labelling, the roots were washed for 1 min in 0.1 mM CaSO4. Seedlings (whole plants) were then dried at 60 °C in an oven for measurements of grain yield per plant. All grains in a single plot were collected and treated as described above for measurements of actual yield.

**Field tests of rice**

To investigate the application potential of OsNLP4, field tests for rice using OsNLP4-OE plants and nlp4 mutants in the ZH11 background (nlp4-3 was DJ background) were carried out in the paddy field under natural growth conditions during 2017–2019 at two experimental stations: Lingshui and Chengdu. For the field test in Chengdu in 2019 (April to September), urea was used as the N source with 80 kg N/ha for low N, 200 kg N/ha for normal N and 500 kg N/ha for high N. The plants were transplanted in 10 rows × 20 plants for each plot (8 m²), and four replicates were used for each N condition. For field test in Lingshui (December 2017 to April 2018), urea was used as the N source, and the planting density was 8 rows × 10 plants for each plot (3.4 m²) with four replicates under normal N application condition (100 kg N/ha). 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.

**Analysis of yield and NUE**

All the agronomic traits were analysed as described previously (Hu et al., 2015). All filled grains from a single plant were collected and dried at 60 °C in an oven for measurements of grain yield per plant. All grains in a single plot were collected and treated as described above for measurements of actual yield. NUE was defined as the total amount of yield in the form of grain or biomass achieved per unit of available N (actual yield per plot/total N application per plot) (Hawkesford, 2014).

**RNA-sequencing analysis**

Each strain has about 100 seedlings (ZH11 background) of every treatment was grown hydroponically in a growth chamber with the condition described above. The seedlings were cultured in modified Kimura B solution after germination with different nitrate concentrations (0.02/2 mM). 16-day-old seedlings (whole plants) were sampled for RNA-sequencing. For each treatment, 20 seedlings were collected as a sample, and three independent biological replicates were conducted. RNA library construction and sequence analysis were conducted as described previously (Khan et al., 2016).

**Chromatin immunoprecipitation**

Seeds were grown in modified Kimura B solution (0.02 mM or 2 mM KNO3) for 10 days. The ChIP assay was performed as reported previously (Cai et al., 2014). ACTI1/NLP4-HA transgenic plants (whole seedlings), anti-HA antibodies (Abmart) and salmon sperm DNA/protein A agarose beads (Millipore) were used for ChIP experiment. DNA was purified using phenol/ chloroform (1:1, v/v) and precipitated. The enrichments of DNA fragments were quantified by qPCR using specific primers (Table S4). Enriched values were normalized with the level of input DNA.

**Yeast one-hybrid assay (Y1H)**

A cDNA fragment encoding OsNLPI was amplified and inserted into plasmid pAD-GAL4-2.1 (AD) to get AD/OsNLP4. The putative NRE from the flanking sequences of N uptake and assimilation genes were ligated into the pHS2 (BD) vector. The constructed vectors were co-transferred into yeast Y187 competent cells and grew the cells on SD-Trp-Leu medium for 3 days at 30 °C, then transferred to SD-Trp-Leu-His medium with 10 mM 3-
aminotriazole (3-AT, sigma) at different dilutions. The yeasts were incubated at 30 °C for 5 days and the extent of yeast growth was determined. The AD and BD empty vector were used for negative control.

Statistical analysis
Statistically significant differences were computed based on the one-way ANOVA.

Acknowledgements
This work was supported by grants from the National Key R & D Program of China (grant no. 2016YFD0100701), National Natural Science Foundation of China (grant no. 3157110003 and 31572183), China Postdoctoral Science Foundation (grant no. 2015M580544), Special Fund of China Postdoctoral Science Foundation (2016T90577), Fundamental Research Funds for the Central Universities (WK6030000122), and Ministry of Science and Technology of China (grant no. 2018ZX08009-11B, 2016ZX08005-004-003 and 2016ZX08001003). The authors thank the Rice T-DNA Insertion Sequence Database (RISD) for providing T-DNA insertion lines used in this study.

Conflict of interests
The authors declare that they have no conflict of interests.

Author’s contribution
J.W., L.-H.Y. and C.-B.X. designed the experiments. J.W. performed experiments and data analysis, and wrote the manuscript. Z.-S.Z., L.-H.Y., J.-Q.X., A.A., Y.S., Y.-J.H., G.-Y.W., L.-Q.S., H.T. and Y.L. contributed to performing part of the experiments. S.-M.W., Q.-S.Z., P.Q., Y.-P.W., S.-G.L., C.-Z.M., G.-Q.Z.., Z.-S.Z., L.-H.Y., J.-Q.X., A.A., Y.S., Y.-J.H., G.-Y.W., L.-Q.S., H.T. performed experiments and data analysis, and wrote the manuscript.

References
Alfaiith, A., Wu, J., Zhang, Z.S., Xia, J.Q., Jan, S.U., Yu, L.H. and Xiang, C.B. (2020) Rice NIN-LIKE PROTEIN 1 rapidly responds to nitrogen deficiency and improves yield and nitrogen use efficiency. J. Exp. Botany, eraa292. https://doi.org/10.1093/jxb/eraa292
Alvarez, J.M., Riveras, E., Vidal, E.A., Gras, D.E., Contreras-Lopez, O., Tamayo, K.P., Aceituno, F. et al. (2014) Systems approach identifies TGA1 and TGA4 transcription factors as important regulatory components of the nitrate response of Arabidopsis thaliana roots. Plant J. 80, 1–13.
Alvarez, J.M., Schinke, A.L., Brooks, M.D., Pasquina, A., Leonelli, L., Varala, K., Safi, A. et al. (2020) Transient genome-wide interactions of the master transcription factor NLP7 initiate a rapid nitrogen-response cascade. Nat. Commun. 11, 1157.
Andrews, M., Lea, P.J., Raven, J.A. and Lindsey, K. (2004) Can genetic manipulation of plant nitrogen assimilation enzymes result in increased crop yield and greater N-use efficiency? An assessment. Annals Appl. Biol. 145, 25–40.
Bi, Y.M., Kant, S., Clarke, J., Gidda, S., Ming, F., Xu, J., Rochon, A. et al. (2009) Increased nitrogen-use efficiency in transgenic rice plants over-expressing a nitrogen-responsive early nodulin gene identified from rice expression profiling. Plant Cell Environ. 32, 1749–1760.
Borisov, A.Y., Madsen, L.H., Tsyganov, V.E., Umehara, Y., Voroshilova, V.A., Batagov, A.O., Sandal, N. et al. (2003) The Sym35 Gene Required for Root Nodule Development in Pea Is an Ortholog of Nin from Lotus japonicus. Plant Physiol. 131, 1009–1017.
Cai, X.T., Xu, P., Zhao, P.X., Liu, R., Yu, L.H. and Xiang, C.B. (2014) Arabidopsis ERF109 mediates cross-talk between jasmonic acid and auxin biosynthesis during lateral root formation. Nat. Commun. 5, 5833.
Camargo, A., Llamas, A., Schnell, R.A., Higuera, J.J., Gonzalez-Ballester, D., Lefebvre, P.A., Fernandez, E. and et al. (2007) Nitrate signaling by the regulatory gene NIT2 in Chlamydomonas. Plant Cell, 19, 3491–3503.
Cao, H.R., Qi, S.D., Sun, M.W., Li, Z.H., Yang, R., Crawford, N.M. and Wang, Y. (2017) Overexpression of the Maize ZmNLP4 and ZmNLP8 can complement the arabidopsis nitrate regulatory mutant nlp7 by restoring nitrate signaling and assimilation. Front. Plant Sci. 8, 1703.
Caiaiangi, L., Camargo, A., Pocholle, D., Gaudon, V., Texier, Y., Boutet-Mercey, S., Taconnat, L. et al. (2009) The nodule infection-like protein 7 modulates nitrate sensing and metabolism in Arabidopsis. Plant J. 57, 426–435.
Cataldo, D.A., Maroon, M., Schrader, L.E. and Youngs, V.L. (1975) Rapid colorimetric determination of nitrate in plant tissue by nitrification of salicylic acid. Commun. Soil Sci. Plant Anal. 6, 71–80.
Chardin, C., Girin, T., Roudier, F., Meyer, C. and Krapp, A. (2014) The plant RWP-RK transcription factors: key regulators of nitrate responses and of gametophyte development. J. Exp. Botany, 65, 5577–5587.
Chardin, F., Noel, V. and Nasclaux-Daubresse, C. (2012) Exploring NUE in crops and in Arabidopsis ideotypes to improve yield and seed quality. J. Exp. Botany, 63, 3401–3412.
Crawford, N.M. and Forde, B.G. (2002) Molecular and developmental biology of inorganic nitrogen nutrition. Arabidopsis Book, 1, e0011.
Gao, Z.Y., Wang, Y.F., Chen, G., Zhang, A.P., Yang, S.L., Shang, L.G., Wang, D.Y. et al. (2019) The indica nitrate reductase gene OsNR2 allele enhances rice yield potential and nitrogen use efficiency. Nat. Commun. 10, 5207.
Garnett, T., Conn, V. and Kaiser, B.N. (2009) Root based approaches to improving nitrogen use efficiency in plants. Plant Cell Environ. 32, 1272–1283.
Ge, M., Wang, Y., Liu, Y., Jiang, L., He, B., Ning, L., Du, H. et al. (2020) The NIN-like protein 5 (ZmNLP5) transcription factor is involved in modulating the nitrogen response in maize. Plant J. 102, 353–368.
Han, M., Okamoto, M., Beatty, P.H., Rothstein, S.J. and Good, A.G. (2015) The genetics of nitrogen use efficiency in crop plants. Annual Rev. Genet. 49, 269–289.
Hawkesford, M.J. (2014) Reducing the reliance on nitrogen fertilizer for wheat production. J. Cereal Sci. 59, 276–283.
Hirel, B., Têtu, T., Lea, P.J. and Dubois, F. (2011) Improving nitrogen use efficiency in crops for sustainable agriculture. Sustainability, 3, 1452–1485.
Ho, C.H. and Tsay, Y.F. (2010) Nitrate, ammonium, and potassium sensing and signaling. Curr. Opin. Plant Biol. 13, 604–610.
Hoque, M.S., Masle, J., Udvardi, M.K., Ryan, P.R. and Upadhyaya, N.M. (2006) Over-expression of the rice OsAMT1-1 gene increases ammonium uptake and content, but impedes growth and development of plants under high ammonium nutrition. Functional Plant Biol. 33, 153.
Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., Widmaier, P. et al. (2008) Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. Adv. Bioinform. 2008, 5.
Hu, B., Jiang, Z., Wang, W., Qin, Y., Zhang, Z., Liu, Y., Li, A. et al. (2019) Nitrate-NRT1.1B-SPX4 cascade integrates nitrogen and phosphorus signalling networks in plants. Nat. Plants. 5, 401–413.
Hu, B., Wang, W., Ou, S., Tang, J., Li, H., Che, R., Zhang, Z. et al. (2015) Variation in NRT1.1B contributes to nitrate-use divergence between rice subspecies. Nat. Genet. 47, 834–838.
Hu, H.C., Wang, Y.Y. and Tsay, Y.F. (2009) AICPK8, a CBL-interacting protein kinase, regulates the low-affinity phase of the primary nitrate response. Plant J. 57, 264–278.
Huang, X., Qian, Q., Liu, Z., Sun, H., He, S., Luo, D., Xia, G. et al. (2009) Natural variation at the DEP1 locus enhances grain yield in rice. Nat. Genet. 41, 494–497.
Jagadehsan, B., Sathee, L., Meena, H.S., Jha, S.K., Chinnumsay, V., Kumar, A. and Kumar, S. (2020) Genome wide analysis of NLP transcription factors reveals their role in nitrogen stress tolerance of rice. Scientific Rep. 10, 9368.
Jeong, D.H., An, S., Kang, H.G., Moon, S., Han, J.J., Park, S., Lee, H.S. et al. (2002) T-DNA insertional mutagenesis for activation tagging in rice. Plant Physiol. 130, 1636–1644.

OsNLP4 controls nitrogen use efficiency 459
Khan, A.U.H., Rathore, M.G., Alende-Vega, N., Vo, D.N., Belkhaled, S., Orecchioni, S., Talarico, G. et al. (2016) Human Leukemic Cells performing Oxidative Phosphorylation (OXPHOS) generate an antioxidant response independently of Reactive Oxygen species (ROS) Production. *ElibioMedicine*, 3, 43–53.

Kirk, G.J. and Kronzucker, H.J. (2005) The potential for nitrification and nitrate uptake in the rhizosphere of wetland plants: a modelling study. *Annals Botany*, 96, 639–646.

Koishi, M. and Yanagisawa, S. (2013) Arabidopsis NIN-like transcription factors have a central role in nitrate signalling. *Nat. Commun.*, 4, 1617.

Koishi, M. and Yanagisawa, S. (2019) The role of protein-protein interactions mediated by the PB1 domain of NLP transcription factors in nitrate-inducible gene expression. *BMC Plant Biol.* 19, 90.

Krapp, A., David, L.C., Chardin, C., Girin, T., Marmagne, A., Leprince, A.S., Konishi, M. and Yanagisawa, S. (2013) Arabidopsis NIN-like transcription factors have a central role in nitrate signaling. *Nature* 402, 191–195.

Sonoda, Y., Ikeda, A., Saiki, S., Wiresn, N., Yamaya, T. and Yamaguchi, J. (2003) Distinct expression and function of three ammonium transporter genes (OsAMT1;1–1;3) in rice. *Plant Cell Physiol.* 44, 726–734.

Stitt, M., Müller, C., Matt, P., Gibson, Y., Carillo, P., Morcuende, R., Scheible, W.R. and et al. (2002) Steps towards an integrated view of nitrogen metabolism. *Exp. Botany*, 53, 959–970.

Sun, H., Qian, Q., Wu, K., Luo, J., Wang, S., Zhang, C., Ma, Y. et al. (2014) Heterotrimetric G proteins regulate nitrogen-use efficiency in rice. *Nat. Genet.* 46, 652–656.

Sylvie, F.M., Valadier, M.H. and Foyer, C. (1998) Overexpression of nitrate reductase in tobacco delays drought-induced decreases in nitrate reductase activity and mRNA. *Plant Physiol.* 117, 293–302.

Tang, W., Ye, J., Yao, X., Zhao, P., Xuan, W., Tian, Y., Zhang, Y. et al. (2019) Genome-wide associated study identifies NAC42-activated nitrate transporter conferring high nitrogen use efficiency in rice. *Nat. Commun.* 10, 5279.

Uemura, H.A., Alvarez, J.M. and Gutierrez, R.A. (2014) Nitrate regulation of AFB3 and NAC4 gene expression in Arabidopsis roots depends on NRT1.1 nitrate transport function. *Plant Signal. Behav.* 9, e28501.

Vidal, E.A., Araus, L.J., Vitousek, P.M., Naylor, R., Crews, T., David, M.B., Drinkwater, L.E., Holland, E., Johnes, P.J. et al. (2009) Nutrient imbalances in agricultural development. *Science* 324, 1519–1520.

Wang, W., Hu, B., Yuan, D., Liu, Y., Che, R., Hu, Y., Ou, S. et al. (2018) Expression of the nitrate transporter gene OsNRT1.1A/OsNPF6.3 confers high yield and early maturation in rice. *Plant Cell*, 30, 638–651.

Wang, R., Xing, X., Wang, Y., Tran, A. and Crawford, N.M. (2009) A genetic screen for nitrate regulatory mutants captures the nitrate transporter gene NRT1.1. *Plant Physiol.* 151, 472–478.

Wei, J., Jiang, Y., Feng, H., Hu, Q., Fan, X., Yamaji, N., Ma, J.F. et al. (2018) OsNRT2.4 encodes a dual-affinity nitrate transporter and functions in nitrate-regulated root growth and nitrate distribution in rice. *J. Exp. Botany*, 69, 1095–1107.

Wilkinson, J.I. and Crawford, N.M. (1993) Identification and characterization of a chlorate-resistant mutant of Arabidopsis thaliana with mutations in both nitrate reductase structural genes NIA1 and NIA2. *Mol. Gen. Genet.* 240, 289–297.

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.

Xiang, C.B., Han, P., Lutziger, I., Wang, K. and Oliver, D.J. (1999) A mini binary vector series for plant transformation. *Plant Mol. Biol.* 40, 711–717.

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

Xu, N., Wang, R., Zhao, L., Zhang, C., Li, Z., Lei, Z., Liu, F. et al. (2016) The Arabidopsis NRG2 protein mediates nitrate signaling and interacts with and regulates key nitrate regulators. *Plant Cell.*, 28, 485–504.

Yam, D., Easaarwan, V., Chau, V., Okamoto, M., Ierullo, M., Kimura, M., Endo, A. et al. (2016) NIN-like protein B is a master regulator of nitrate-promoted seed germination in Arabidopsis. *Nat. Commun.* 7, 13179.

Yan, M., Fan, X., Feng, H., Miller, A.J., Shen, Q. and Xu, G. (2011) Rice OSA2.1 interacts with OsNRT2.1, OsNRT2.2 and OsNRT2.3a nitrate transporters. *Mol. Plant* 4, 1012–1025.

McAllister, C.H., Beatty, P.H. and Good, A.G. (2012) Engineering nitrogen use efficient crop plants: the current status. *Plant Biotechnol.* 10, 1011–1025.

Mukerji, C.H., Beatty, P.H. and Good, A.G. (2012) Engineering nitrogen use efficient crop plants: the current status. *Plant Biotechnol.* 10, 1011–1025.

Mukerji, C.H., Beatty, P.H. and Good, A.G. (2012) Engineering nitrogen use efficient crop plants: the current status. *Plant Biotechnol.* 10, 1011–1025.

Mukerji, C.H., Beatty, P.H. and Good, A.G. (2012) Engineering nitrogen use efficient crop plants: the current status. *Plant Biotechnol.* 10, 1011–1025.
transporters to provide uptake over high and low concentration ranges. Plant Cell Environ. 34, 1360–1372.
Yu, L.H., Wu, J., Tang, H., Yuan, Y., Wang, S.M., Wang, Y.P., Zhu, Q.S. et al. (2016) Overexpression of Arabidopsis NLP7 improves plant growth under both nitrogen-limiting and -sufficient conditions by enhancing nitrogen and carbon assimilation. Scientific Rep. 6, 27795.
Zhang, H. (1998) An arabidopsis MADS box gene that controls nutrient-induced changes in root architecture. Science 279, 407–409.
Zhang, Z., Hu, B. and Chu, C. (2020) Towards understanding the hierarchical nitrogen signalling network in plants. Curr. Opin. Plant Biol. 55, 60–65.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.
Table S1. The FPKM values of the genes involved in N metabolism.
Table S2. The FPKM values of the genes involved in C metabolism.
Table S3. The NRE motifs found in the N-related genes.
Table S4. Primers used for PCR.

Figure S1. Identification of OsNLP4 knockout mutants and overexpression lines.
Figure S2. Loss-of-function of OsNLP4 results in severe nitrogen deficiency phenotype in DJ background.

Figure S3. OsNLP4 influences plant growth under different ammonium conditions.
Figure S4. OsNLP4 overexpression plants exhibit increased grain yields in the field.
Figure S5. Differentially expressed genes (DEGs) in the WT, ko and OE under low N (LN) and high N (HN) conditions.
Figure S6. OsNLP4 regulates the expression of multiple N metabolism genes in response to nitrate.
Figure S7. OsNLP4 regulates the expression of multiple N metabolism genes in response to ammonium.
Figure S8. Loss-of-function of OsNLP4 significantly affects N metabolism in DJ background.
Figure S9. OsNLP4 regulates ammonium uptake and assimilation.
Figure S10. Expression pattern of OsNLP4 revealed by rice lines expressing OsNLP4 promoter-GUS reporter.
Figure S11. Subcellular localization and protein levels of OsNLP4 in responses to ammonium.
Figure S12. OsNLP4 restores the N deficiency phenotype of Arabidopsis nlp7-1 mutant.
Figure S13. OsNLP4 broadly regulates the genes related to N utilization and signalling in Arabidopsis nlp7-1 mutant.
Figure S14. OsNLP4 does not affect the expression of OsNLP1 in response to nitrate and ammonium.