Overexpression of Arabidopsis NLP7 improves plant growth under both nitrogen-limiting and -sufficient conditions by enhancing nitrogen and carbon assimilation

Lin-Hui Yu, Jie Wu, Hui Tang, Yang Yuan, Shi-Mei Wang, Yu-Ping Wang, Qi-Sheng Zhu, Shi-Gui Li & Cheng-Bin Xiang

Nitrogen is essential for plant survival and growth. Excessive application of nitrogenous fertilizer has generated serious environment pollution and increased production cost in agriculture. To deal with this problem, tremendous efforts have been invested worldwide to increase the nitrogen use ability of crops. However, only limited success has been achieved to date. Here we report that NLP7 (NIN-LIKE PROTEIN 7) is a potential candidate to improve plant nitrogen use ability. When overexpressed in Arabidopsis, NLP7 increases plant biomass under both nitrogen-poor and -rich conditions with better-developed root system and reduced shoot/root ratio. NLP7–overexpressing plants show a significant increase in key nitrogen metabolites, nitrogen uptake, total nitrogen content, and expression levels of genes involved in nitrogen assimilation and signalling. More importantly, overexpression of NLP7 also enhances photosynthesis rate and carbon assimilation, whereas knockout of NLP7 impaired both nitrogen and carbon assimilation. In addition, NLP7 improves plant growth and nitrogen use in transgenic tobacco (Nicotiana tabacum). Our results demonstrate that NLP7 significantly improves plant growth under both nitrogen-poor and -rich conditions by coordinately enhancing nitrogen and carbon assimilation and sheds light on crop improvement.

Nitrogen (N) is one of the essential macronutrients for plant growth and crop productivity. To meet the increased demands for agricultural production, a vast amount of nitrogenous fertilizers were applied to soil worldwide to maximize crop yields. Global use of N fertilizer amounted to 110 million metric tons in 2007. By the year 2050, it is projected to increase to between 125 and 236 million metric tons. However, only an average of 30–50% of the applied N is taken up by the plant depending on the species and cultivar, with the remainder being lost through various pathways, leading to significant environmental pollution and ecological imbalance. Therefore, it is a great challenge to accommodate the demands of the expanding world population by developing a highly productive agriculture, whilst at the same time preserving the quality of the environment. One of the possible solutions to this dilemma is to improve the N use efficiency (NUE) of crops.

The definition of NUE has been defined in various ways, but the simplest is the amount of plant yield in terms of either total biomass or grain yield per unit of applied fertilizer N. Generally, NUE is an integration of N uptake efficiency (NUpE) and N utilization (assimilation) efficiency (NUtE). Therefore, understanding the mechanisms regulating these processes is important for crop N use improvement. Many genes have been studied for increasing the N assimilation in various plants during the past decades. Most of these genes were related to N uptake and primary assimilation, including nitrate and ammonia transporters (NRTs and AMTs), nitrate and nitrite reductase (NR and NiR), aminotransferases and dehydrogenases, glutamine synthetase (GS), and glutamate synthase (GOGAT). However, the ectopic expression of these genes have been shown to affect N uptake...
or individual enzyme activity involved in N metabolism, but few have showed phenotypic effect on NUE or other growth parameters to date. For example, overexpression of the high affinity ammonium transporter gene OsAMT1;1 in rice enhanced N uptake, but was also associated with a decrease in biomass. Overexpression of GS increased GS activity in some instances, but the effects on N assimilation and plant biomass were inconsistent. Constitutive expression of the tobacco NR-encoding genes Nia2 and Nia in different plants showed no NUE phenotype under N-limiting conditions. Overexpression of OsPTR6, a PTR/NRT1 (Peptide Transporter/Nitrate Transporter 1) family gene, in rice increased plant growth at different N conditions but decreased NUE at high ammonium supply. Although no successful commercial transgenic plants expressing these genes have been reported to date, recent research progresses in rice shed light on improving NUE of rice. Elevated expression of OsPTR9 in rice plants enhanced ammonium uptake, promoted lateral root formation and increased grain yield. Sun et al. found rice DEP1 (Dense and Erect Panicles 1) modulated N responses by interacting with the heterotrimetric G proteins. Rice plants carrying the dominant dep1-1 allele showed N insensitive vegetative growth coupled with increased N uptake and assimilation, leading to improved grain yield and harvest index at moderate levels of N fertilization. In another study, Hu et al. found a single SNP (single-nucleotide polymorphism) in rice NRT1.1B contributed to nitrate-use divergence between rice subspecies, and NRT1.1B-indica could potentially increase the NUE of japonica.

A critical coordination exists between C and N metabolism. Improving the plant NUE can be compromised unless there is sufficient C available. On the one hand, N deficiency has great impacts on chloroplasts development, content of chlorophyll and amino acids as well as proteins crucial for C assimilation, such as Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) and PEPC (phosphoenolpyruvate carboxylase). On the other hand, N assimilation requires energy, reducing power and carbon skeleton produced by C assimilation. Increasing C supply promotes N uptake and assimilation. NUE is improved in elevated CO₂ environment. Practically speaking, genetic manipulation of the crosstalk between N and C assimilation appears to be one of the most efficient attempts at improving NUE.

Considering the complex regulation of N and C assimilation, transcription factors (TFs) might be a more efficient approach for improving N use ability, because they have the capacity to modulate expression of a set of genes coordinately. Many TFs regulated by nitrate have been identified. Ectopic expression of maize Dof1 (ZmDof1) TF in Arabidopsis and rice leads to the up-regulation of multiple genes involved in C-skeleton production and increased N assimilation and plant growth under low-N conditions. The paralogous GATA TFs GNC (GATA, nitrate-inducible, carbon-metabolism-involved) and CGA1/GNL (cytokinin-responsive GATA1/GNC-like) modulate N assimilation, chloroplast development and starch production. In Lottus japonicas, a nodule inception (NIN) TF was found necessary for symbiotic N fixation. NIT2, a homologue of the Arabidopsis NIN-like protein (NLP) genes in Chlamydomonas, was reported as a central regulator required for nitrate signaling and assimilation. Mutants of this gene in Chlamydomonas are not able to activate the expression of the genes required for nitrate assimilation, and they are unable to grow on nitrate as the sole N source. Recently, NLP TFs in Arabidopsis were reported to play a central role in nitrate signalling. One of the NLP TFs, NLP7, modulates nitrate sensing and metabolism and plays as an orchestrator of nitrate responses. Considering the complex regulation of N and C assimilation, transcription factors (TFs) might be a more efficient approach for improving N use ability, because they have the capacity to modulate expression of a set of genes coordinately. Many TFs regulated by nitrate have been identified. Ectopic expression of maize Dof1 (ZmDof1) TF in Arabidopsis and rice leads to the up-regulation of multiple genes involved in C-skeleton production and increased N assimilation and plant growth under low-N conditions. The paralogous GATA TFs GNC (GATA, nitrate-inducible, carbon-metabolism-involved) and CGA1/GNL (cytokinin-responsive GATA1/GNC-like) modulate N assimilation, chloroplast development and starch production. In Lottus japonicas, a nodule inception (NIN) TF was found necessary for symbiotic N fixation. NIT2, a homologue of the Arabidopsis NIN-like protein (NLP) genes in Chlamydomonas, was reported as a central regulator required for nitrate signaling and assimilation. Mutants of this gene in Chlamydomonas are not able to activate the expression of the genes required for nitrate assimilation, and they are unable to grow on nitrate as the sole N source. Recently, NLP TFs in Arabidopsis were reported to play a central role in nitrate signalling. One of the NLP TFs, NLP7, modulates nitrate sensing and metabolism and plays as an orchestrator of nitrate responses.

**Results**

**NLP7 increases plant biomass under both N-limiting and -sufficient conditions.** To investigate the effects of NLP7 expression level on plant growth, we generated 35S:NLP7 transgenic Arabidopsis plants and obtained a T-DNA insertion mutant nlp7-1 (SALK_26134C) (Supplementary Fig. S1). Quantitative real-time PCR (qRT-PCR) analysis showed that the transcript levels of other NLPs had no significant difference in the NLP7-overexpressing and WT plants (Supplementary Fig. S2). Plants were grown on the modified MS (Murashige and Skoog) medium in which nitrate was used as sole inorganic N source. When grown on the medium containing different concentrations of nitrate, the NLP7-overexpressing plants showed obvious growth advantages compared with wild type (WT) and nlp7-1 plants. The nlp7-1 plants showed constitutive N-deficient phenotypes on both nitrate-rich and -poor media (Fig. 1a), which agreed with the previous study. Statistical analyses indicated that overexpression of NLP7 could increase biomass of the shoot under both nitrate-rich and -poor conditions, and NLP7 knock-out impaired plant growth even under nitrate-rich conditions (Fig. 1b). We also found that the NLP7-overexpressing plants had higher chlorophyll contents than the mutant and WT plants under different nitrate conditions (Fig. 1c,d). These phenotypes were further proved by vertical growth assay as shown in Supplementary Fig. S3. Considering the influences of plant density on nitrate concentration, we grew 28 plants (7 plants in a row) per plate for further analysis. Results showed that the NLP7-overexpressing plants grew much better than the WT and nlp7-1 plants on medium containing 0.5–4 mM nitrate, especially under the higher nitrate conditions (Fig. 1e). Compared with WT and nlp7-1 plants, the NLP7-overexpressing plants had significantly increased fresh weight (FW) with the raising nitrate concentrations of the medium (Fig. 1f). Moreover, we conducted functional complementation analysis by expressing a functional pNLP7:NLP7-GFP fusion construct in the nlp7-1 plants. The results showed that nlp7-1 plants showed N-deficient phenotypes with lower FW and longer primary roots (PRs) on N-rich medium. However, these phenotypes could be restored by expressing pNLP7:NLP7-GFP construct (Supplementary Fig. S4). Taken together, these results demonstrate that the growth of nlp7-1 is relatively insensitive to N level while the NLP7-overexpressing plants improved growth and response to N availability.
Additionally, we also found that the \( nlp7-1 \) plants displayed severe N-starved phenotypes with yellow leaves, while the \( NLP7 \)-overexpressing plants still remained green after 3 days N starvation in liquid culture (Supplementary Fig. S5). Moreover, \( NLP7 \) transgenic plants grew much bigger in N-limiting soil with significantly higher rosette surface area and rosette biomass than the WT and \( nlp7-1 \) plants under short-day conditions. The mutant and WT plants showed much more severe N-deficient phenotypes with discolored rosette leaves compared with the \( NLP7 \)-overexpressing plants (Supplementary Fig. S5). Overexpression of \( NLP7 \) not only increased the shoot biomass, but also enhanced root growth with higher root biomass and longer roots, while \( nlp7-1 \) mutant had much lower shoot and root biomass and delayed flowering compared with the WT (Supplementary Fig. S5). These results demonstrate that overexpression of \( NLP7 \) in \( Arabidopsis \) enhanced the plant growth and N use as well as tolerance to N-deficiency.

**NLP7 alters root architecture and shoot/root ratio.** To verify the root architecture under different nitrate conditions, we checked the root system of the plants vertically grown on media containing different concentrations of nitrate (Fig. 2a–c). The results showed in Fig. 2a–c indicated that overexpression of \( NLP7 \) conferred higher shoot and root FW compared with WT plants under both N-rich and -limiting conditions, though not

Figure 1. **NLP7 improves plant growth under both nitrate-rich and -poor conditions.** (a) The phenotypes of the 16-day-old WT, \( nlp7-1 \) and \( NLP7 \)-overexpressing plants grown on medium plates containing different concentrations of nitrate with a density of 80 plants per plate. Diameter of the plate is 14.5 cm. Bar = 1.7 cm. (b) Shoot fresh weight of the 16-day-old plants grown under different nitrate conditions. Values are the mean ± standard deviation (SD) of three independent replications each containing 20 plants per genotype (*P < 0.05, **P < 0.01). (c–d) The chlorophyll a (c) and b (d) contents of the 16-day-old plants. Values are the mean ± SD of three independent replications (*P < 0.05, **P < 0.01). (e) The phenotypes of the 10-day-old plants grown with a density of 28 plants per plate under different nitrate conditions. Bar = 0.5 cm. (f) Fresh weight of the 10-day-old plants under different nitrate conditions. Values are the mean ± SD of three independent replications each containing 20 plants per genotype (*P < 0.05, **P < 0.01, ***P < 0.001).
so significantly under 1 mM nitrate condition. The nlp7-1 plants showed reduced shoot FW compared with WT plants under different nitrate conditions, with more significantly reduced under higher nitrate conditions (Fig. 2b). However, there was no significant difference of root FW between the nlp7-1 and WT plants. On the contrary, root FW of the nlp7-1 mutant had slightly increased under 10 mM nitrate condition (Fig. 2c). Consequently, the shoot to FW weight ratio, an important parameter for nutrient starvation, was significantly higher for NLP7-overexpressing plants and much lower for the nlp7-1 plants under different nitrate conditions, especially under N-rich conditions (Fig. 2d). More detailed analysis of the root showed that the NLP7-overexpressing plants significantly increased PR length and lateral root (LR) density under both nitrate-rich and -poor conditions. However, the nlp7-1 plants developed longer PRs and increased LR density under 3 mM and 10 mM nitrate conditions compared with WT plants (Fig. 2e,f). These results were confirmed by the data of time-course analysis of root development as shown in Supplementary Fig. S6, where NLP7-overexpressing plants had better-developed root system with increased LR number and slightly longer PRs compared with WT under both low and high nitrate conditions. However, the nlp7-1 plants developed slightly more LRs and longer PRs compared with WT under 10 mM nitrate condition. In contrast, its LR number had a slight reduction with no obvious difference in PR length under 1 mM nitrate condition. The phenotypes of nlp7-1 plants agree with the previous results that NLP7 knockout confers constitutive N-starved phenotypes under different N conditions. These data indicate that NLP7 may be involved in root development and overexpression of NLP7 promotes root development.

Enhanced N uptake and assimilation in NLP7-overexpressing plants. To gain molecular insights for the improved growth of the NLP7 transgenic plants under different N conditions, we measured several metabolite markers for N assimilation. Contents of glutamine (Gln) and glutamate (Glu), markers for N utilization, were measured respectively. Interestingly, we found that both the NLP7-overexpressing and mutant plants had higher Glu contents than WT plants under 1 mM and 3 mM nitrate conditions. Under 10 mM nitrate condition, Glu content increased in the WT plant, but decreased dramatically in NLP7-overexpressing plants (Fig. 3a). However, under this condition, Gln content was significantly higher in the NLP7-overexpressing plants, whereas was significantly lower in the nlp7-1 mutant (Fig. 3b). These data implies that more Glu is converted to Gln in the NLP7-overexpressing plants than in the mutant plants under nitrate-rich conditions. Glu and Gln contents and their ratio are controlled by GS/GOGAT cycle, which is affected by many factors, such as GS/GOGAT enzyme activities, ammonium assimilation, 2-oxoglutarate (2OG) production and photorespiration. In fact, we found the GS enzyme activities were much higher in the NLP7-overexpressing plants while significantly lower in the mutant (Fig. 3c), which might partly explain the Glu and Gln contents in the plants. Total protein
content increased markedly in the NLP7-overexpressing plants under 1 mM and 3 mM nitrate conditions but not under 10 mM nitrate conditions compared with WT plants, while in nlp7-1 mutant, the total protein content was significantly lower at 3 and 10 mM nitrate but not at 1 mM nitrate compared with the WT plants (Fig. 3d).

Interestingly, we found that nitrate accumulated considerably in the nlp7-1 mutant under different nitrate conditions, especially under high nitrate conditions. Conversely, nitrate content in the NLP7-overexpressing plants decreased significantly (Fig. 3e). These results led us to test the enzymatic activities of NR. Figure 3f shows that NR activities increased markedly in the NLP7-overexpressing plants in contrast to the dramatic decrease in the nlp7-1 mutant, suggesting higher nitrate assimilation efficiency in the NLP7-overexpressing plants. Moreover, the NLP7-overexpressing plants exhibited a much higher nitrate uptake activity at 5 mM 15NO3− external concentration (Fig. 3g).

Overexpression of NLP7 broadly up-regulates the expression of genes involved in N assimilation and signalling. To further assess the role of NLP7 in N assimilation in plant, we performed qRT-PCR to investigate the expression of some genes related with nitrate assimilation and signalling in 3 days N-starved seedlings and seedlings resupplied with nitrate for 0.5 h and 1 h, respectively. As showed in Fig. 4, after 3 days N-starved, most of the tested nitrate-responsive genes had similar expression levels in the WT, nlp7-1 and NLP7-overexpressing plants, except NRT1.1, NLA, ANR1 and AFB3, which had significant higher expression levels in the NLP7-overexpressing plants. However, after nitrate resupplied, almost all the genes were highly induced in WT and NLP7-overexpressing plants, but the induction levels were much lower in the nlp7-1. When resupplied with nitrate for 1 h, all these genes displayed considerable higher expression levels in the NLP7-overexpressing plants compared with in WT and nlp7-1 plants. These results indicate that NLP7 plays a vital role in nitrate assimilation and signalling, which is consistent with the recent reports. In addition, we checked the expression levels of all these genes in 7-day old plants grown on MS medium and observed that 11 of the 13 tested genes, except NRT1.1 and NIR1, had much lower expression levels in the nlp7-1 mutant. On the other hand, 10 out of the 13 genes showed prominent higher expression levels in the NLP7-overexpressing plants (Supplementary Fig. S7).
NLP7 affects photosynthesis rate and C assimilation. N metabolism is known to coordinate with photosynthesis and C metabolism. In this study, we found nlp7-1 plants displayed N-starved phenotypes with a much smaller and pale rosette when grown in soil (Fig. 5a). Further analysis found chlorophyll content was remarkably reduced in the leaves of nlp7-1 plants, whereas increased in the NLP7-overexpressing plants (Fig. 5b). Photosynthesis rate was enhanced in the NLP7-overexpressing plants and decreased in the nlp7-1 plants (Fig. 5c). Furthermore, total C content displayed no significant difference between NLP7-overexpressing, nlp7-1 and WT plants under 1 mM nitrate condition. However, compared with WT, total C content reduced by 2.8% in nlp7-1, while significantly increased by 4.7% in NLP7-overexpressing plants under 10 mM nitrate condition (Fig. 5d). Total N content was found decreased in nlp7-1 while markedly increased in the NLP7-overexpressing plants compared with WT under both nitrate-rich and -deficient conditions (Fig. 5e). As a result, C/N ratio decreased significantly in the NLP7-overexpressing plants under low nitrate conditions, but not so significantly changed under high nitrate conditions compared with the WT plants (Fig. 5f).

We also measured some C metabolism-related compounds. Table 1 showed that there were more sucrose, fructose, and glucose accumulation in NLP7 transgenic plants under 10 mM nitrate condition, and less sucrose and glucose under 1 mM nitrate condition. Conversely, nlp7-1 plants accumulated more soluble sugars under 1 mM nitrate condition and less sucrose and fructose under 10 mM nitrate condition. Moreover, starch content, which is known to correlate negatively with biomass, was found much lower in the NLP7-overexpressing plants under 1 mM nitrate condition. These data suggest that NLP7 may affect C metabolism.

In addition, we found that NLP7 affected the expression level of AtPPC genes, which encode the PEPC. NLP7 transgenic plants showed higher transcript levels of AtPPC1 in response to nitrate re-addition (Fig. 5g). Expression of AtPPC2 and AtPPC3 were induced by nitrate in both the WT and NLP7-overexpressing plants, but more dramatically in the NLP7-overexpressing plants. However, expression of these two genes did not change much in the nlp7-1 mutant after nitrate re-addition (Fig. 5h,i). These data indicate NLP7 may affect PEPC activities. Indeed, further analyses showed that PEPC activities increased noticeably in the NLP7-overexpressing plants while...
reduced in the nlp7-1 plants, especially under N-limiting conditions (Fig. 5). Meanwhile, NLP7-overexpressing plants had higher expression levels of genes encoding cytosolic isocitrate dehydrogenase (ICDH), mitochondrial ICDH and peroxisomal ICDH after 3 days N-starvation and 0.5 h of N re-addition, with only increased expression

Table 1. Carbohydrate contents of 16-day-old WT, nlp7-1 mutant and NLP7-overexpressing plants grown under different nitrate conditions. Data are means ± SD of three independent replications (*P < 0.05, **P < 0.01, ***P < 0.001).
of cytosolic ICDH gene after 1 h of nitrate re-addition (Supplementary Fig. S8). These results suggest that NLP7 expression level influenced not only N assimilation but also C assimilation.

**NLP7 enhanced N assimilation and growth of the transgenic tobacco.** In order to investigate whether NLP7 can similarly modulate N assimilation in different plant species, we generated NLP7-overexpressing transgenic tobacco plants. Figure 6a–c showed that the transgenic tobacco plants exhibited growth advantages with increased FW compared with WT under different nitrate conditions. The NLP7-overexpressing tobacco plants also had much longer PRs (Fig. 6d). In addition, we assayed the N use level of the transgenic tobacco using hydroponic culture method. The results showed that the NLP7-overexpressing plants grew much better with higher shoot and root biomass and higher shoot/root ratio compared to the WT plants (Fig. 6e–n). These results suggest that the strategy with NLP7 may be applicable to improve N use in other plant species.
**Discussion**

Decreasing fertilizer N inputs by improving plant NUE is an important strategic goal for global agriculture. However, traditional breeding strategies to improve NUE in some crop plants have experienced a plateau. Although genetic engineering for improving N use has been broadly studied in recent decades, the success has been limited because of focusing on single gene manipulation. Most of these studies attempted to improve plant N use by individually constitutive overexpression of the genes involved in N uptake or metabolism. Unfortunately, most of these transgenic plants did not show increased NUE, and some even showed negative pleiotropic effects, indicating the notions of single-point rate-limiting regulation being oversimplified. Probably, the effects of alterations in the amount and/or activity of any single enzyme in the N metabolic or uptake pathway may be masked by concurrent mechanisms that are activated to maintain homeostasis, including post-transcriptional, translational and/or feedback regulation. In addition, unnecessary accumulation of metabolic intermediates may affect the plant development. Therefore, it should be much better to engineer the plant metabolism by enhancing a few steps cooperatively instead of one step in a metabolic pathway to avoid these weaknesses. The introduction of the ZmDof1 gene into *Arabidopsis* and rice highlighted the great utility of TFs in engineering N and C metabolisms in plants. In this study, we found another TF, NLP7, could simultaneously coordinate many processes in N utilization and signaling pathway as well as C fixation and metabolism pathway, resulting in improved N use and plant growth.

Recently, NLPs had proved to play a central role in nitrate signaling in *Arabidopsis*. One mechanism of NLPs modulates nitrate-induced gene expression probably through post-translational regulation. The underlying mechanisms for how inactive form of NLPs been converted into active form by nitrate signalling are not clear. However, according to the ATH1 chip data by Scheible et al. 7 NLP genes of the N-depleted seedlings responded quickly and transiently to nitrate re-addition. Based on data from GENEVESTIGATOR database, Chardin et al. 27 found the expression of NLP8 responds to a large number of treatments, including several N nutrient treatments, whereas NLP4 and NLP9 are responsive to specific stimuli only, such as heat stress and N treatments, respectively. Transcription of OsNLP4 is repressed by several abiotic stress treatments and induced by low phosphate availability. Konishi and Yanagisawa 27 found all the NLPs were not significantly induced by nitrate re-addition for one hour. However, this result can not eliminate the possibility that the expression of different NLPs being regulated by nitrate at other time points. Moreover, constitutive overexpression of NLP6-VP16 using the 35S promoter up-regulated the expression of nitrate-inducible genes *NIR1* and *NIA2* in both the N-starved seedlings and seedlings resupplied with nitrate. All these results imply that not only post-translational but also transcriptional regulation of NLPs probably plays roles in the regulation of N signalling and assimilation. Based on this hypothesis, it is possible for us to improve plant N use by change the transcriptional levels of NLPs.

NLP7 was reported to orchestrate the early response to nitrate. However, the expression of NLP7 is not induced by the N source or nitrate, but the localization of NLP7 protein is regulated by nitrate via a nuclear retention mechanism. Therefore, it is reasonable to speculate that if we overexpressed NLP7 in plant, more NLP7 protein would be produced and accumulated in the nucleus in response to nitrate availability. This hypothesis could be proved by the results of Marchive et al. 28 (see supplementary Fig. S4 of the paper). In this figure, less NLP7-GFP protein was accumulated in both the cytosol and the nucleus of root cells of N-starved pNLP7:NLP7-GFP plantlets compared with that of N-starved p35S:NLP7-GFP plantlets. Once nitrate was re-supplied, within minutes, noticeably more NLP7-GFP protein was accumulated in the nucleus of root cells in p35S:NLP7-GFP plants compared with that of pNLP7:NLP7-GFP plantlets. As a result, the more NLP7 protein accumulated in nuclear would enhance the expression of N assimilation genes and thus improve the N use ability of the NLP7-overexpressing plants.

Indeed, our results support this hypothesis that the overexpression of NLP7 significantly enhanced the plant N use by enhancing N assimilation efficiency. Overexpression of NLP7 in *Arabidopsis* led to higher shoot and root biomass under both N-rich and -deficient conditions (Figs 1 and 2, Supplementary Figs S3 and S5), with remarkable rise in multiple N metabolites and N content, an elevation in enzyme activities of N metabolism (Figs 3 and 5). On the contrary, the nlp7-1 mutant had impaired N use ability and showed more severe N-deficient phenotypes, even under rich N conditions (Figs 1 and 2). In sharp contrast to the nlp7-1, overexpression of NLP7 resulted in up-regulation of a range of genes involved in nitrate transport (NRT1.1, NRT2.1), N assimilation (GS1, NIA1, NIA2, NIR1), and N signaling (LBD37, LBD38, LBD39, ANR1, AFB3) (Fig. 4, Supplementary Fig. S7). Increased transcript levels of these genes would lead to enhanced N uptake and metabolism as revealed in Fig. 3. On the other hand, the up-regulated TFs would produce a broad range of regulatory outcomes. These coordinated regulations by NLP7 enable plants to rapidly adapt to N availability and maintain plant N homeostasis. Notably, transcript level of NLA, a positive regulator of plant adaptation to N limitation, was also found up-regulated in NLP7-overexpressing plants (Fig. 4h, Supplementary Fig. S7), contributing to better performances of the transgenic plants under N-deficient conditions. Moreover, ectopic expression of *Arabidopsis* NLP7 in tobacco also had similar effects as in *Arabidopsis* (Fig. 6), suggesting that this might be a conserved mechanism in plants. All these data indicate that overexpression of NLP7 can improve plant N use ability by coordinately regulating N metabolism, transport and signalling pathways.

Root is the most important organ for sensing N availability and morphological adaptation to N supply. Agreed with the previous study, our results also found that NLP7 was highly expressed in leaves, central cylinder of roots and LR primordia at different stages (Supplementary Fig. S9), implying a possible function of this gene in root development. In fact, our results revealed that overexpression of NLP7 conferred increased LR density and PR length under both high and low nitrate conditions by vertical growth assay (Fig. 2 and Supplementary Fig. S6). The expression of many genes involved in root development in response to N availability, such as *NRT1.1*, *NRT2.1*, *ANR1* and *AFB3*, changed significantly in the NLP7-overexpressing and knockout plants (Fig. 4). The up-regulation of these genes in the NLP7-overexpressing plants probably contributes to the root architecture.
changes to some extent. Overall, these morphological changes in the root system of NLP7-overexpressing plant enhanced its N acquisition ability to match its high-efficient N metabolism.

Plants have the ability to optimize biomass partitioning to maximize whole-plant growth rate according to the external environment. According to Thornley's model, under N deficiency stress, the greater part of the N taken up was used for root growth, thus decreasing shoot/root ratio. Therefore, shoot/root ratio can reflect the N use level and adaptation ability to N deficiency. Our results showed that the nlp7-1 displayed typical N-starved phenotypes with decreased shoot/root ratios, irrespective of N supply. However, the NLP7-overexpressing plants had much higher shoot/root ratios, particularly under nitrate-rich conditions (Figs 2d and 6h). These results implied that NLP7-overexpressing plant has higher N use ability, thus leading to more tolerance to the N deficiency and more N allocated to the shoot to maximize its relative growth rate.

N and C assimilation processes are closely linked and tightly co-regulated. NUE is not only dependent on N assimilation, and manipulating C metabolism was useful in some cases in improving NUE. Therefore, it is reasonable to improve both the C and N utilization efficiencies simultaneously to optimize plant growth and yield. Interestingly, we found such cooperation between N and C assimilations in the NLP7-overexpressing plants. In addition to the increased N content (Fig. 5e), NLP7-overexpressing plants also had significantly increased C content compared with that of the nlp7-1 mutant under N-rich condition (Fig. 5d). Overexpression of NLP7 conferred significant higher photosynthesis rate (Fig. 5c). The changed chlorophyll contents of the NLP7-overexpressing and mutant plants (Figs 1c,d and 5b) may have direct influences on C fixation (Fig. 5c). Moreover, expression levels of PEPC genes and ICDH genes were found up-regulated in the NLP7-overexpressing plants after nitrate induction (Fig. 5g-i, Supplementary Fig. S8). Consequently, the NLP7-overexpressing plants had much higher PEPC activities under both N-rich and -deficient conditions (Fig. 5j). The CHIP-chip data by Marchive et al., one of our tested PEPC genes (cytosolic ICDH) are bound by NLP7 in the presence of nitrate (see Supplementary Data 1 and 8 of the paper), suggesting that NLP7 may directly regulate the transcription of these PEPC and ICDH genes. It has been well documented that PEPC, a key enzyme in photosynthesis, also acts as a key player in N storage and C fixation and as a crosstalk point between C and N metabolisms. ICDH genes encode the key enzymes to provide 2OG necessary for ammonium assimilation. Therefore, up-regulation of these genes might positively regulate C assimilation as well as N assimilation, thus affecting the C/N balance under N-rich conditions (Fig. 5f). Balanced C and N nutrient provisions are beneficial to ensure maximal N use and to maintain an appropriate shoot/root ratio for plant development and growth.

In conclusion, we have demonstrated that NLP7 is potentially a promising candidate for improving plant N use ability. The localization of NLP7 is controlled by nitrate via a nuclear retention mechanism. Amount of NLP7 protein in the cytosol and nucleus may maintain a dynamic balance in response to the nitrate availability. Constitutively overexpression of NLP7 in the plant might break the normal balance of NLP7 localization between the cytosol and nucleus and promoted NLP7 protein relocation to the nucleus, especially under N-rich condition. Being activated by nitrate signalling, the nuclear accumulated NLP7 would enhance N assimilation by cooperatively modulating a number of genes related to N metabolism, transport and signalling. Consequently, the overexpression of NLP7 conferred better growth under both N-deficient and -sufficient conditions. Moreover, overexpression of NLP7 also improved C assimilation simultaneously. Our results also imply that NLP7-mediated nitrate regulation is not only through post-translational mechanisms, probably also through translational levels. It is conceivable that NLP7 can be used to enhance N use ability and increase crop yield.

Material and Methods

**Plant material.** The nlp7-1 (SALK_26134C) mutant was obtained from Arabidopsis Biological Resource Center (ABRC). Homozygous mutant plants were confirmed by RT-PCR using the primers NLP7 RT-PCR LP and RP. 35S:NLP7 overexpression construct was made by inserting the coding region of NLP7 amplified by PCR using NLP7-attp-LP and NLP7-attp-RP into pCB2004 via GATEWAY cloning system. For nlp7-1:NLP7-GFP construct, a fragment containing NLP7 promoter and coding region amplified by genomic PCR with primers NLP7-attp-LP and NLP7-attp-RP2 was cloned into pMD110 to fuse with GFP. The NLP7-overexpressing transgenic Arabidopsis were obtained by Agrobacterium-mediated floral-dip method and identified by qRT-PCR with specific primers NLP7-qPCR LP and RP. The transgenic tobacco plants were generated as previously described and identified by RT-PCR with the primers NLP7 RT-PCR LP and RP. All the primers used are listed in Table S1.

**Plant growth conditions.** Seeds were sterilized with 15% bleach for 12 min, and then washed five times with sterile water. Sterilized seeds stratified at 4 °C for 2 days, and plated on solid medium containing 1% (w/v) sucrose and 0.6% (w/v) agar. Nitrate-less medium was modified on MS medium with KNO3 as sole N source: 10 mM nitrate medium (similar to MS except 20 mM KNO3, and 20 mM NH4NO3 was replaced with 10 mM KCl and 10 mM KNO3), 3 mM nitrate medium (similar to MS except 20 mM KNO3, and 20 mM NH4NO3 was replaced with 7 mM KCl and 3 mM KNO3), 1 mM nitrate medium (similar to MS except 20 mM KNO3, and 20 mM NH4NO3, was replaced with 9 mM KCl and 1 mM KNO3).

To investigate different growth rates under different N conditions, seeds were germination and grew on medium containing different concentrations of nitrate at 22 °C under 10-h light/14-h dark photoperiod. For evaluation the phenotype of soil-grown plants, seeds were germinated and grew in N-limited soil without N fertilizer applying during the whole growth stage at 22 °C under 10-h light/14-h dark photoperiod. N-limited soil was the soil had been used once for Arabidopsis growth.

**RNA extraction and qRT-PCR.** 1 μg of total RNA isolated using Trizol reagent (Invitrogen, Carlsbad, California, USA) were used for reverse transcription. qRT-PCR was performed with a StepOne Plus Real Time PCR System by using a TaKaRa SYBR Premix Ex Taq II reagent kit. All the primers used are shown in Table S1.
Metabolite analyses. The metabolite analyses were performed on the seedlings of 16-day-old plants grown on agar medium with different concentrations of nitrate. Total soluble protein was measured using the Bradford Protein Assay Kit (Sangon Biotech, Shanghai, China) and total amino acid according to Rosen. Concentrations of glutamate and glutamine were determined with the glutamate/glutamine determination kit (GLN-1; Sigma-Aldrich). Sucrose, fructose, and glucose content were measured using assay kits (Su Zhou Keming Bioengineer Company, China) following to the manufacturer’s instructions. Then starch was extracted by incubating the pellets with 35% HClO4 overnight and the glucose liberated was analyzed by anthrone-H2SO4 method. Chlorophyll a and b were extracted entirely from the aerial parts by using 80% acetone, and measured by spectrophotometric method as Arnon. Nitrate was extracted in 50 mM HEPES–KOH (pH 7.4), and measured by the method according to Cataldo et al. The percent total N and C content in oven-dried plant material was measured with an NC analyzer (Vario EL III model, Elementar, Hanau, Germany) according to the manufacturer’s instructions.

Enzyme activity assays. Enzymes were extracted from 16-day-old plants grown on agar medium with different concentrations of nitrate. The maximum in vitro activities of NR was assayed as described previously. GS enzyme activities was measured according to Cai et al. PEPC activities were measured by an enzyme-coupled spectrophotometric method using assay kit (Su Zhou Keming Bioengineer Company, China) following to the manufacturer’s instructions.

Photosynthesis rate measurement. Photosynthesis rates were measured using a portable photosynthesis system (LI-COR LI-6400XT) in the morning (9:30 to 11:30 AM) under constant light in the greenhouse as described by Yu et al. All of the measurements were taken at a constant air flow rate of 500 μmol s⁻¹. The CO2 concentration was set as 400 μmol mol⁻¹ using the system's CO2 injector (model 6400-01, LI-COR).

Hydroponic culture. Tobacco seeds were sterilized with 15% bleach for 15 min and germination on MS agar medium at 22 °C under 16-h light/8-h dark photoperiod. 20-day-old seedlings were used for hydroponic culture. The roots of the tobacco seedling were wrapped in sponge and then grown on a support made of thick polystyrene foam board with holes to allow the root systems of the plants to grow into the hydroponic solution. The floating body floated on hydroponic solution with 1 mM nitrate (similar to MS nutrient solution except 20 mM KNO3 and 20 mM NH4NO3 was replaced with 9 mM KCl and 1 mM KNO3). Plants were cultured at 22 °C under 16-h light/8-h dark photoperiod. Nutrient solution was changed every 5 days.

Nitrate uptake assay using 15NO3⁻. The nitrate uptake activity was assayed using 15NO3⁻ as described previously. 10-day-old seedlings were transferred first to 0.1 mM CaSO4 for 1 min, then to modified MS nutrient solution with 5 mM K15NO3 (99% atom) as sole N source for 30 min and finally to 0.1 mM CaSO4 for 1 min. Seedlings were then dried at 70 °C to a constant weight and grinded. 15N content was analyzed using a continuous-flow isotope ratio mass spectrometer (Thermo-MAT253) coupled with a elemental analyzer (Flash 2000 HT, Thermo Fisher Scientific, Inc., USA).

Statistical analysis. Statistically significant differences were computed based on the Student’s t-tests.

References
1. Nosengo, N. Fertilized to death. Nature 425, 894–895 (2003).
2. Good, A. G. & Beatty, P. H. Biotechnological Approaches to Improving Nitrogen Use Efficiency in Plants: Alanine Aminotransferase as a Case Study in The Molecular and Physiological Basis of Nutrient Use Efficiency in Crops (eds Hawkesford M. J. & Barraclough P.) Ch. 9, 165–191 (Wiley-Blackwell, 2011).
3. Garnett, T., Conn, V. & Kaiser, B. N. Root based approaches to improving nitrogen use efficiency in plants. Plant Cell Environ 32, 1272–1283 (2009).
4. Xu, G. H., Fan, X. R. & Miller, A. J. Plant Nitrogen Assimilation and Use Efficiency. Annual Review of Plant Biology, Vol 63, 153–182 (2012).
5. Good, A. G., Shrawat, A. K. & Mueng, D. G. Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? Trends in Plant Science 9, 597–605 (2004).
6. McAllister, C. H., Beatty, P. H. & Good, A. G. Engineering nitrogen use efficient crop plants: the current status. Plant Biotechnol J 10, 1011–1025 (2012).
7. Kumar, A., Kaiser, B. N., Siddiqi, M. Y. & Glass, A. D. M. Functional characterisation of OsAMT1.1 overexpression lines of rice, Orzaya sativa. Funct Plant Biol 33, 339–346 (2006).
8. Hoque, M. S., Masle, J., Udvardi, M. K., Ryan, P. R. & Upadhyaya, N. M. Over-expression of the rice OsAMT1-1 gene increases ammonium uptake and content, but impairs growth and development of plants under high ammonium nutrition. Funct Plant Biol 33, 153–163 (2006).
9. Curtis, L. S., Power, J. B., de Laat, A. M. M., Coboche, M. & Davey, M. R. Expression of a chimeric nitrate reductase gene in transgenic lettuce reduces nitrate in leaves. Plant Cell Rep 18, 889–896 (1999).
10. Djennane, S., Chauvin, J. E., Quillere, I., Meyer, C. & Chupeau, Y. Introduction and expression of a deregulated tobacco nitrate reductase gene in potato lead to highly reduced nitrate levels in transgenic tubers. Transgenic Res 11, 175–184 (2002).
11. Fan, X. R. et al. Over-expression of OsPTR6 in rice increased plant growth at different nitrogen supplies but decreased nitrogen use efficiency at high ammonium supply. Plant Sci 227, 1–11 (2014).
12. Fang, Z. M. et al. Altered expression of the PTR/NRT1 homologue OsPTR9 affects nitrogen utilization efficiency, growth and grain yield in rice. Plant Biotechnol 11, 446–458 (2013).
13. Sun, H. Y. et al. Heterotrimeric G proteins regulate nitrogen-use efficiency in rice. Nature Genetics 46, 652–656 (2014).
14. Hu, B. et al. Variation in NRT1.1B contributes to nitrate-use divergence between rice subspecies. Nat Genet 47, 834–838 (2015).
15. Kant, S. et al. Improving yield potential in crops under elevated CO2: Integrating the photosynthetic and nitrogen utilization efficiencies. Front Plant Sci 3, 162 (2012).
16. Makino, A., Mae, T. & Ohira, K. Photosynthesis and Ribulose 1,5-Bisphosphate Carboxylase in Rice Leaves—Changes in Photosynthesis and Enzymes Involved in Carbon Assimilation from Leaf Development through Senescence. Plant Physiology 73, 1002–1007 (1983).
17. Laza, R. C., Bergman, B. & Vergara, B. S. Cultivar Differences in Growth and Chloroplast Ultrastructure in Rice as Affected by Nitrogen. Journal of Experimental Botany 44, 1643–1648 (1993).
18. Coruzzi, G. & Bush, D. R. Nitrogen and carbon nutrient and metabolite signaling in plants. Plant Physiology 125, 61–64 (2001).
19. Stitt, M. & Krapp, A. The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. Plant Cell Environ 22, 383–621 (1999).
20. Scheible, W. R., et al. Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of Arabidopsis in response to nitrogen. Plant Physiol 136, 2483–2499 (2004).
21. Yanagisawa, S., Akiyama, A., Kisaka, H., Uchimiya, H. & Miwa, T. Metabolic engineering with DoF1 transcription factor in plants: Improved nitrogen assimilation and growth under low-nitrogen conditions. P Natl Acad Sci USA 101, 7833–7838 (2004).
22. Kura, T. et al. Introduction of the ZmDoF1 gene into rice enhances carbon and nitrogen assimilation under low-nitrogen conditions. Plant Biotechnol J 9, 826–837 (2011).
23. Hudson, D. et al. GNC and CGA1 modulate chlorophyll biosynthesis and glutamate synthase (GLU1/Fd-GOGAT) expression in Arabidopsis. Plos One 6, e26765, doi: 10.1371/journal.pone.0026765 (2011).
24. Schauer, L., Roussis, A., Stiller, J. & Stougard, I. A plant regulator controlling development of symbiotic root nodules. Nature 402, 191–195 (1999).
25. Galvan, A. & Fernandez, E. Eucahyrtic nitrate and nitrate transporters. Cell Mol Life Sci 58, 225–233 (2001).
26. Camargo, A., et al. Nitrate signaling by the regulatory gene NIT2 in Chlamydomonas. Plant Cell 19, 3491–3503 (2007).
27. Konishi, M. & Yanagisawa, S. Arabidopsis NIN-like transcription factors have a central role in nitrate signalling. Nat Commun 4, 1617, doi: 10.1038/ncomms2621 (2013).
28. Marchive, C. et al. Nuclear retention of the transcription factor NLP7 orchestrates the early response to nitrate in plants. Nat Commun 4, 1713, doi: 10.1038/ncomms2650 (2013).
29. Castaings, L. et al. The nodule inception-like protein 7 modulates nitrate sensing and metabolism in Arabidopsis. Plant J 57, 426–435 (2009).
30. Xu, N. et al. The Arabidopsis NRG2 Protein Mediates Nitrate Signaling and Interacts with and Regulates Key Nitrate Regulators. Plant Cell 28, 485–504 (2016).
31. Lawler, D. W., Lemaire, G. & Gastal, F. Nitrogen, plant growth and crop yield in Plant nitrogen (eds Lea, P. J. & Morot-Gaudry, I. F.) 343–367 (Springer-Verlag, 2001).
32. Lancien, M., Gadal, P. & Hodges, M. Enzyme redundancy and the importance of 2-oxoglutarate in higher plant ammonium assimilation. Plant Physiol 123, 817–824 (2000).
33. Novitskaya, L., Trevanion, S. J., Driscoll, S., Foyer, C. H. & Noctor, G. How does photosynthesis modulate leaf amino acid contents? A dual approach through modelling and metabolite analysis. Plant Cell Environ 25, 821–835 (2002).
34. Stitt, M. et al. Steps towards an integrated view of nitrogen metabolism. Journal of Experimental Botany 53, 959–970 (2002).
35. Sulpice, R. et al. Starch as a major integrator in the regulation of plant growth. Proc Natl Acad Sci USA 106, 10348–10353 (2009).
36. Andrews, M., Lea, P. J., Raven, J. A. & Forde, B. G. Signal transduction during nitrogen assimilation. Proc Natl Acad Sci USA 102, 13693–13698 (2005).
37. Vidal, E. A. et al. Nitrate-responsive miR393/AF83 regulatory module controls root system architecture in Arabidopsis thaliana. P Natl Acad Sci USA 107, 4477–4482 (2010).
38. Krook, G. et al. Nitrate-Regulated Auxin Transporter by NRT1.1 Defines a Mechanism for Nutrient Sensing in Plants. Developmental Cell 18, 927–937 (2010).
39. Zhang, H. & Forde, B. G. An Arabidopsis MAD5 box gene that controls nutrient-induced changes in root architecture. Science 279, 407–409 (1998).
40. Hilbert, D. W. Optimization of Plant-Root-Shoot Ratios and Internal Nitrogen Concentration. Ann Bot-London 66, 91–99 (1990).
41. Thorley, J. H. M. A balanced quantitative model for root: shoot ratios in vegetative plants. Ann Bot-London 56, 433–449 (1986).
42. Chardon, F., Noel, V. & Masclaux-Daubresse, C. Exploring NUE in crops and in Arabidopsis ideotypes to improve yield and seed quality. Journal of Experimental Botany 63, 3401–3412 (2012).
43. Cheung, N. et al. The putative high-affinity nitrate transporter NRT2.1 represses lateral root initiation in response to nutritional cues. Proc Natl Acad Sci USA 102, 13693–13698 (2005).
44. Patrick, W. R. et al. The Arabidopsis NRT1.1 protein modulates plant growth and development. Plant Physiol 152, 1369–1380 (2010).
45. Anaya, D. et al. High-throughput binary vectors for plant gene function analysis. Journal of Integrative Plant Biology 49, 556–567 (2007).
46. Curtis, M. D. & Grossniklaus, U. A gateway cloning vector set for high-throughput functional analysis of genes in plants. Plant Physiol 133, 462–469 (2003).
47. Clough, S. J. & Bent, A. F. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16, 735–743 (1998).
48. Horsch, R. B. et al. Analysis of Agrobacterium tumefaciens virulence mutants in leaf discs. Proc Natl Acad Sci USA 83, 2571–2575 (1986).
49. Rosen, H. A Modified Ninhydrin Colorimetric Analysis for Amino Acids. Arch Biochem Biophys 67, 10–15 (1957).
50. Hansen, J. & Møller, I. Percolation of Starch and Soluble Carbohydrates from Plant-Tissue for Quantitative-Determination with Anthrone. Anal Biochem 68, 87–94 (1975).
51. Arnon, D. I. Copper Enzymes in Isolated Chloroplasts-Polyphenoloxidase in Beta-Vulgaris. Plant Physiology 24, 1–15 (1949).
52. Cataldo, D. A., Haroon, M., Schrader, L. E. & Youngs, V. L. Rapid Colorimetric Determination of Nitrate in Plant-Tissue by Nitration of Salicylic Acid. Commun Soil Sci Plant Anal 6, 71–80 (1975).
53. Ferrario-Mery, S., Valadier, M. H. & Foyer, C. H. Overexpression of nitrate reductase in tobacco delays drought-induced decreases in nitrate reductase activity and mRNA. Plant Physiology 117, 293–302 (1998).
54. Cai, H. et al. Overexpressed glutamine synthetase gene modifies nitrogen metabolism and abiotic stress responses in rice. Plant Cell Rep 28, 527–537 (2009).
55. Yu, L. et al. Arabidopsis enhanced drought tolerance1/HOMEODOMAIN GLABROUS1 confers drought tolerance in transgenic rice without yield penalty. Plant Physiol 162, 1378–1391 (2013).
56. Lin, S. H. et al. Mutation of the Arabidopsis NRT1.1 nitrate transporter causes defective root-to-shoot nitrate transport. Plant Cell 20, 2514–2528 (2008).
57. Schmidt, G. W. & Delaney, S. K. Stable internal reference genes for normalization of real-time RT-PCR in tobacco (Nicotiana tabacum) during development and abiotic stress. Molecular Genetics and Genomics 283, 233–241 (2010).
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Author Contributions
C.-B.X. and L.-H.Y. designed the experiments. L.-H.Y. and J.W. performed most of the experiments and data analyses. H.T., Y.Y., S.-M.W., Y.-P.W., Q.-S.Z. and S.-G.L. prepared samples and participated in metabolite and enzyme activity analyses. L.-H.Y. wrote the manuscript. C.-B.X. supervised the project and revised the manuscript.

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