Running title: Rice *NLP4* is required for nitrate-dependent growth

Title: *OsNLP4* is required for nitrate assimilation gene expressions and nitrate-dependent growth in rice

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One sentence summary: The *osnlp4* mutants exhibit abnormal nitrate response and poor growth under nitrate supply and in paddy field conditions.

Author Contributions

T.F. sponsored the research project; M.H. provided the *OsNLP4* Tos-17 insertion line; T.F., T.K., M.W. and S.T. designed the experiments; M.W. and T.H. performed experiments; Y.O. and K.Y. provided RNA-seq data; M.W. analyzed the data and M.W., T.F. wrote the paper.

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Abstract

In plants, nitrate is important nutrient and signaling molecule that modulates the expression of many genes and regulates growth. In paddy grown rice, nitrogen is mostly supplied in the form of ammonium, but nitrate also shares substantial portion of available nitrogen. A number of nitrogen transporters and nitrate assimilation enzymes have been identified and functionally characterized. However, little is known about the nitrate sensor system and regulatory mechanisms of these nitrate related genes. In recent years, NIN-like proteins (NLPs) have been described as key transcription factors of nitrogen responses in *Arabidopsis thaliana*. But the functions of OsNLPs in rice are still elusive. Here we report the characterization of *OsNLP4* to reveal its function in rice. Growths of *OsNLP4* knockout mutants were reduced under the nitrate supply, but not under ammonium supply. The mRNA accumulation of genes involved in nitrate assimilation were declined significantly and nitrate uptake rate and nitrate reductase activity were also impaired in the mutants. Using rice protoplast transient expression system, OsNLP4-GFP fusion was localized to nucleus irrespective of nitrate conditions. *OsNLP4* was also required for normal yield under paddy field conditions. We propose the *OsNLP4* is essential for regulation of genes involved in nitrate assimilation and nitrate-dependent growth in rice.

Keywords: nitrate, rice NLP family, growth, nitrate-related genes, nitrate assimilation

Introduction

Nitrogen (N) is the most important macronutrient for plant growth and development. Nitrate (NO_3^-) and ammonium (NH_4^+) are the major sources of inorganic N taken up by the roots of higher plants. Nitrate is generally present at higher concentrations than those of ammonium in the soil solution under aerobic conditions. In flooded paddy soils, ammonium is the abundant form of nitrogen than nitrate. In both cases N levels in soil fluctuates by rainfalls and addition of N sources etc. and it is necessary for plants to adapt to changing N conditions. Land plants display phenotypic plasticity and reprogram gene expression in response to nitrogen availability (Zhang and Forde

2000; Stitt 1999; Forde 2002; Sasakawa and Yamamoto 1978). In general, elongation of seminal and lateral roots of rice seedlings is inhibited by high concentrations of ammonium, but not by high nitrate levels (Hirano et al. 2008). For nitrate, it is also known to affect expression of a wide spectrum of genes, including not only genes related to nitrate transport and assimilation, but also genes involved in other metabolic pathways and plant development (Wang et al. 2003; Wang et al. 2007; Crawford 1995). For instance, *AtNIA1* and *AtNIA2* (nitrate reductase genes) are induced by nitrate and determine the levels of NO production and ABA-induced stomatal closure in *A. thaliana* (Desikan et al. 2002; Chen et al. 2016; Zhao et al. 2016). In *A. thaliana*, these responses are mediated by several factors including the nitrate transceptor NRT1.1 (Ho et al. 2009), two kinases CIPK8/23 (Ho et al. 2009; Hu et al. 2009) and the transcription factors NLP6/7/8 (Yanagisawa 2014; Yan et al. 2016; Castaings et al. 2009), LBD37-LBD39 (Rubin et al. 2009), TCP20 (Guan et al. 2017; Guan et al. 2014) and SPL9 (Krouk et al. 2010b). In rice, the functional characterization of a number of nitrate transporter genes have been reported. Such as *OsNRT2.3a*, one of the nitrate transporter genes in rice induced under nitrate supply, plays a key role in long-distance nitrate transport from root to shoot at low nitrate supply level (Tang et al. 2012). *OsNPF7.3*, a member of the nitrate transporter 1/peptide transporter family, enhances nitrogen allocation and increase grain yield in rice (Fang et al. 2017). However, the upstream molecular mechanisms controlling the wide range of nitrate response genes remain elusive.

NODULE INCEPTION (NIN) is the first transcription factor identified as essential for nodulation and the symbiotic nitrogen fixation pathway in *Lotus japonicas* (Schauser et al. 1999). NIN-like proteins (NLPs) encoded by other higher plants are proteins having homology to NIN. In total, the *A. thaliana* and rice genomes encode nine and six NLPs, respectively. Rice and *A. thaliana* NLPs are expressed in almost all organs (Chardin et al. 2014). The most conserved region in NLPs is the RWP-RK domain, possibly involved in DNA-binding and dimerization. In rice, amino acid sequences corresponding to the RWP-RK domain have diversified significantly more than in *A. thaliana* (Schauser et al. 2005). But the RWP-RK domain's activity is

unrelated to nitrate signaling, whereas the N-terminus function as a transcriptional activation domain (Konishi and Yanagisawa 2013). NLPs carry another conserved PBI domain, a protein-protein interaction domain, enabling heterodimerization between PB1 domain containing proteins at their C-terminus (Schauser et al. 2005).

In several previous studies in *A. thaliana*, NLPs are identified as nitrate-responsive *cis*-element (NRE)-binding proteins with transcriptional activation roles and function in the nitrate-regulated expression of a number of genes. For example, suppression of *AtNLP6* down-regulates nitrate-inducible genes, including nitrate transporter genes (*NRT1.1* and *NRT2.1*), nitrate assimilation-related enzyme genes (*NIA1*, *NIA2*, *NIR1*, and *UPM1*) and transcription factor genes (a GARP-type transcription factor gene and *LBD39*) (Konishi and Yanagisawa 2013). Overexpression of *AtNLP7* improves plant growth under both nitrogen-poor and nitrogen-rich conditions by coordinately enhancing nitrogen and carbon assimilation (Yu et al. 2016). *AtNLP8* is essential for nitrate-promoted seed germination, reducing abscisic acid levels in a nitrate-dependent manner (Yan et al. 2016). On the other side, the NRE region (a 43 bp-long palindromic sequence) does not exist in genes other than *NIA1* and *NIR1* in *A. thaliana*. In contrast, in maize the NRE region is found to be over-represented in the promoter of genes coding nitrate transporters and ammonium transporters (Liseron-Monfils et al. 2013). In rice, NRE-like sequence comprises two half-sites, 5'-AAG(A/G)GCC-3' and 5'-GCCCTTT-3' separated by 10 nucleotides (Schauser et al. 2005).

It has also been demonstrated that no *NLP* gene is significantly induced by nitrate treatment in *A. thaliana* (Konishi and Yanagisawa 2013). The subcellular localizations of NLPs have also been reported in *A. thaliana*. *AtNLP8* localizes to nuclei with and without nitrate treatment (Yan et al. 2016) but a rapid accumulation of *AtNLP7* in the nucleus is induced by nitrate (Marchive et al. 2013). Furthermore, *AtNLP6* is reported to serve as a partially redundant activator with *AtNLP7*. The teosinte branched1/cycloidea/proliferating cell factor1-20 (TCP20) can interact with *AtNLP6&7* to construct TCP20- NLP6&7 heterodimers functioning as the active complexes to control plant responses to nitrate availability (Guan et al. 2017). *AtNLPs*

are also found to be activated post-transnationally. Upon nitrate supply, an inactive form of AtNLPs is converted into an active form (Konishi and Yanagisawa 2013). A uniquely conserved serine (Ser205) is identified in AtNLP7 as an important phosphorylation site for converting to an active form and nitrate-triggered nuclear retention. The subgroup III Ca^{2+} -sensor protein kinases (CPK10, CPK30, and CPK32) are master regulators in primary nitrate signaling and phosphorylate the nitrate-responsive domain of AtNLP7 *in vivo* in the presence of nitrate (Liu et al. 2017a).

Nevertheless, little is known about the role of OsNLP family in rice, except that OsNRT1;1 affect the subcellular localization of OsNLP4 (Wang et al. 2018). In the present study, we investigated *OsNLP4* is an important gene on growth and nitrate accumulation under a condition which nitrate was used as the sole nitrogen source. Our results revealed the high mRNA expression level of *OsNLP4* under no supplemental nitrogen condition and down-regulated expression of genes related to nitrate assimilation in the *osnlp4* mutant lines. In addition, our research also suggested that unlike AtNLP7, OsNLP4 did not show nitrate-promoted nucleocytosolic shuttling mechanism. The paddy field data showed *OsNLP4* has a vital effect on rice production. The role and mechanism of OsNLP4-mediated regulation of nitrate-promoted growth were discussed.

142 Results

143 ***OsNLP4* is essential for nitrate-dependent rice growth**

144 To investigate the function of the rice NLP family, we obtained an *OsNLP4* Tos-17
 145 insertion line (*osnlp4-1*) which has a Tos-17 insertion in the second exon (in the front
 146 of ¹⁰⁴⁴A) in the Nipponbare background. A homozygous mutant line in terms of
 147 Tos-17 insertion in *OsNLP4* were established and they were grown under the 2 mM
 148 ammonium or 2 mM nitrate conditions for two weeks. When grown on the modified
 149 Kimura B solution in which nitrate was used as sole inorganic N source, the mutant
 150 line showed growth defects compared with that of wild type (Figure 1A). The
 151 *osnlp4-1* plants grown hydroponically on 2 mM nitrate showed shorter shoot length
 152 and root length, almost 70% of wild type (Figure 1B, C). In addition, the shoot dry
 153 weight and root dry weight were reduced in the *osnlp4-1* by 58% and 53%,
 154 respectively (Figure 1D, E). Such reduction in growth was not observed when plants
 155 were grown with ammonium (Figure 1B, C, D, E). These results suggested that
 156 *OsNLP4* plays an important role in nitrate dependent growth in rice.

157 To further verify if the phenotype of *osnlp4-1* is due to the disruption of the
 158 *OsNLP4* gene, we constructed two *OsNLP4* CRISPR/Cas9 lines, *osnlp4-2* and *osnlp4-3*.
 159 The *osnlp4-2* has 2 bp deletion in the second exon, while *osnlp4-3* has 1 bp insertion
 160 in the fourth exon of gene *OsNLP4* (Figure 2A). The *osnlp4-2* and *osnlp4-3* reduced
 161 shoot length, root length, shoot dry weight and root dry weight only under nitrate
 162 condition (Figure 2B, C, D, E). The fact that all the three independently obtained
 163 *osnlp4* mutants exhibited impaired growth under nitrate condition, but not under
 164 ammonium condition, established that mutations in *OsNLP4* are responsible for
 165 these phenotypes.

166

167 ***OsNLP4* mRNA accumulation was increased significantly under nitrogen free** 168 **condition**

169 All six members of rice NLP gene family (*OsNLPs*) encompassed RWP-RK domain and
 170 PBI domain. *OsNLPs* can be classified into three clades (*OsNLP1* and *OsNLP4*, *OsNLP2*
 171 and *OsNLP5*, *OsNLP3* and *OsNLP6*) (Supplemental Figure S1).

Here we determined mRNA accumulation of *OsNLP* genes in rice. We performed RNA-seq analyses using RNA extracted from roots of 15-day wild type plants grown under 2 mM KNO₃ (nitrate), 2 mM NH₄Cl (ammonium), 1 mM KNO₃ + 1 mM NH₄Cl (normal) or 2 mM KCl (nitrogen free). Compared with the normal condition, the mRNA accumulations of a number of genes were up-regulated or down-regulated under the other three conditions. Under the nitrogen free condition, 2062 genes were up-regulated specifically (Figure 3A). *OsNLPs* were among these genes except *OsNLP3* and *OsNLP6*. Especially, *OsNLP1* and *OsNLP4* had significantly higher mRNA expression levels (Figure 3B). Expression of *OsNLPs* was further analyzed by quantitative real-time PCR (qRT-PCR) to detect their mRNA expression levels under the nitrate and the nitrogen free conditions. The result was consistent with RNA-seq data. *OsNLP4* was increased significantly only under the nitrogen free condition in both shoots and roots (Figure 3C). This implies possible involvement of *OsNLP4* under the nitrogen free condition, but the *osnlp4* mutant lines did not show apparent difference in physiological phenotype with wild type under the nitrogen free condition (Supplemental Figure S2A, B, C, D, E).

Taken together, our results suggested that the accumulation of *OsNLP4* transcript in rice is induced by nitrogen starvation.

Suppression of *OsNLP4* broadly down-regulates the expression of genes involved in nitrate assimilation.

To further determine the origin of the mutant's N-starved like phenotypes and assess the role of *OsNLP4* in nitrate signaling, RNA-seq experiment was performed using RNA extracted from wild type and the *osnlp4-1* roots grown for 15 days on the nitrate condition. RNA-seq data showed there were 580 genes up-regulated and 622 genes down-regulated for the *osnlp4-1* compared with wild type. Most genes are related to catalytic activity, transporter activity and binding in both parts (Supplemental Figure S3A, B). Genes related to nitrate assimilation (*NIA*, nitrate reductase gene; *NIR*, nitrite reductase gene) were among those down-regulated genes (Supplemental Figure S3C). In addition, the expression levels of *IRT1* (encoding

iron transporter) and *MOT1* (encoding molybdenum transporter) were also impaired in the *osnlp4-1* (Supplemental Figure S3C).

Expression of some selective genes was further analyzed by qRT-PCR to examine their mRNA expression levels. Result indicated that in roots, mRNA accumulations of *NIA1* and *NIR1* were declined in the *osnlp4* mutants compared to the wild type (Figure 4). On the other hand, genes related to nitrate transport, i.e., *NRT2.1*, high-affinity nitrate transporter; *NRT1.5a*, root-to-shoot transporter, had similar expression levels between wild type and the *osnlp4* mutants (Figure 4). In shoots, the accumulations of *NIA2* and *NRT2.1* transcripts were increased in the *osnlp4* mutants while *NIA1* was reduced (Figure 4). These results indicated that mRNA accumulation of genes related to nitrate assimilation but not those related to nitrate transport are impaired in the *osnlp4* mutants.

Overall, our analysis suggest that OsNLP4 plays a role in nitrate regulation of gene expression including genes involved in nitrate metabolism.

OsNLP4 is involved in nitrate uptake and assimilation

To examine physiological defects of *osnlp4* mutants, we determined the nitrate absorption rate of wild type and the *osnlp4* mutants under continuous nitrate and one-day nitrate starvation conditions. It was found that nitrate uptake rate of the *osnlp4* mutants were lower than that of the wild type in both conditions (Figure 5A, B). In particular, after one-day nitrate starvation treatment, wild type showed high nitrate absorption rate at almost 1.5 NO_3^- $\mu\text{mol}/\text{root fresh weight (g)}/\text{h}$ while the *osnlp4* mutants displayed less than half of that of wild type (Figure 5B).

In the aforementioned RNA-seq result, *MOT1* and *IRT1* mRNA accumulations were reduced in the *osnlp4-1* (Supplemental Figure S3C). Molybdenum and iron have been reported to be essential for the activity of nitrate reductase through covalently bounding to specific domains of the enzyme (Liu et al., 2017b, Ford et al., 2016). We checked the mRNA expression levels of *MOT1*, *IRT1* and *IRT2* in root via qRT-PCR. The expressions of *IRT1* and *IRT2* were strongly impaired in the *osnlp4* mutants (Figure 5C). And the total content of iron and molybdenum was declined in the *osnlp4*

mutants (Figure 5D). It is possible that NR activity is affected by OsNLP4. We compared the nitrate reductase (NR) activity of the *osnlp4* mutants with WT grown under nitrate condition and discovered that NR activity was lower in the *osnlp4* mutants, almost only 30% of WT, establishing that OsNLP4 is required to maintain NR activity (Figure 5E).

We next analyzed shoot nitrogen content to verify that OsNLP4 is important for rice to assimilate nitrate by CN coder. The C/N ratio (carbon-to-nitrogen ratio) in shoot was nearly two-fold higher in the *osnlp4* mutants under nitrate condition which means the nitrogen limitation existed in the *osnlp4* mutant lines (Figure 5F).

These results collectively suggested that OsNLP4 is indispensable for the expression of genes involved in then nitrate transduction pathway both in nitrate uptake and assimilation. OsNLP4 also affects nitrate use efficiency, probably through the regulation of nitrate uptake and assimilation.

OsNLP4 is localized in nucleus

In *A. thaliana*, AtNLP6 and AtNLP7 are retained in the nucleus in the presence of nitrate, but AtNLP8 are not (Yan et al., 2016, Yu et al., 2016, Guan et al., 2017) and these changes in subcellular localization is implied as an important step for nitrate dependent gene expression, we tested whether the subcellular localization of OsNLP4 is under the control of nitrate conditions. Rice protoplasts were transiently transformed with 35S::*OsNLP4::GFP*. After 18h incubation with or without nitrate, OsNLP4-GFP signal was detected mainly in the nucleus with minor signals and these pattern of subcellular localization does not seem to be affected by the nitrate condition (Figure 6). These results indicated that OsNLP4 is localized to nucleus irrespective of nitrate conditions. This is in contrast to the case of AtNLP7, implying that OsNLP4 in rice is regulated by a different mechanism.

The *osnlp4-1* line has low rice yield in paddy field

Rice is the main cereal crop in substantial portions of the world and nitrogen fertilizers are frequently used with the aim to achieve high yields. In order to check

262 agronomic traits of our *OsNLP4* Tos-17 insertion line, we planted WT (Nipponbare)
263 and the *osnlp4-1* in nitrogen-supply (+N) and no-nitrogen supply(-N) plots in the
264 Kashimadai paddy field of Tohoku University (Osaki-city, Japan) in 2017, 2018, and
265 2019 (Figure 7A, B, C). Honestly, these three years data are not consistent with each
266 other very well because of many uncontrollable factors, like temperature, typhoon
267 etc. Although there was no significant difference of tiller number between WT and
268 the *osnlp4-1*, the *osnlp4-1* line showed the trend of decreased productive panicle
269 weight and straw weight (Figure 7A, B, C). Significant difference on grain yield was
270 also observed between WT and the *osnlp4-1* line in the no-nitrogen supply plot
271 (Figure 7A, C). It is possible that residual nitrogen existed in no-nitrogen supply plot.
272 These results indicated the importance of *OsNLP4* in yields in paddy fields. We
273 believe that the study of *OsNLP4* has a potential to increase rice yield in paddy fields.
274
275

276 Discussion

277 Rice accumulates *OsNLP4* at RNA level under no nitrogen condition

278 Most of the genes involved in nitrate signaling are induced by nitrate itself, although
 279 its mode of action is poorly understood (Hoff et al. 1994; Krouk et al. 2010a;
 280 Gutierrez 2012; Krapp et al. 2014; Konishi and Yanagisawa 2014). Recently, NLPs had
 281 proved to work as key transcription factors responsible for nitrate-inducible
 282 expression of a number of genes including nitrate transporter, nitrate reductase,
 283 nitrite transporter and nitrite reductase in response to nitrate in *A. thaliana* (Yu et al.
 284 2016; Castaings et al. 2009; Yanagisawa 2014). AtNLPs can directly bind to the NRE
 285 domain within some nitrate-induced genes probably through post-translational
 286 activation (Konishi and Yanagisawa 2013). Our present study identified *OsNLP4* as an
 287 important gene for regulating growth under nitrate condition in rice. The *osnlp4*
 288 mutants displayed prominent nutrient deficiency symptom when nitrate was used as
 289 sole nitrogen source (Figure 1A, B, C, D, E & Figure 2B, C, D, E). Notably, qRT-PCR
 290 results showed that mRNA expression level of *OsNLP4* was increased significantly
 291 under no nitrogen condition (Figure 3B, C). In *A. thaliana*, no *AtNLP* was induced by N
 292 source or N starvation (Konishi and Yanagisawa 2013), but *AtNLP8* and *AtNLP9* were
 293 highly induced in imbibed seeds (Yan et al. 2016). One possibility is that *OsNLP4* is
 294 inactive when no nitrate is supplied. We speculate that rice accumulates a high level
 295 of *OsNLP4* in order to react to nitrate quickly, converting the inactive form of *OsNLP4*
 296 into active form when nitrate signaling is sensed.

297 Among the *OsNLP* family, in addition to *OsNLP4*, it seemed like that rice also
 298 accumulate *OsNLP1* and *OsNLP2* especially in shoots and roots under nitrate
 299 starvation while *OsNLP6* was the only repressed one (Figure 3B, C). *OsNLP6* has a
 300 conserved N-terminal part of the RWP-RK domain, but lacks the downstream half
 301 (Schauser et al. 2005). This protein might have lost putative ancestral DNA-binding
 302 function and have acquired a divergent function. Our results support this hypothesis
 303 that NLPs for a specific functional role is different among plant species.

305 *OsNLP4* triggers a number of nitrate specific responses

We found 622 down-regulated genes in the *osnlp4* mutants dependent of nitrate application, among which most were genes related to catalytic activity (Supplemental Figure S3B). Transcript accumulations of *NIA1*, *NIA2*, *NIR1*, *IRT1*, *MOT1*, and *NAS1* in the *osnlp4* mutants were lower than WT in root (Supplemental Figure S3C & Figure 5C). Nitrate reductase requires iron and molybdenum to consist co-factors to catalyze the reduction of nitrate-to-nitrite in cytosol. And the reduction needs NAD(P)H as energy (Hoff et al. 1994). As expected, the NR activity was impaired in the *osnlp4* mutants (Figure 5E). Reduced NR activity further led to low total nitrogen content and negatively affected growth in *osnlp4* mutants under nitrate-sufficient condition (Figure 5F & Figure 1A). On the other hand, no obvious difference was detected of *NRT2.1* and *NRT1.5a* mRNA levels between WT and the *osnlp4* mutants in roots. In shoots, the mRNA expression levels of *NIA2* and *NRT2.1* were even higher than WT in the *osnlp4* mutants (Figure 4). As reported, both nitrate assimilation and nitrate transport were impaired in *atnlp7* mutants (Castaings et al. 2009). On the basis of our results, we propose that OsNLP4 mainly regulates the expression of nitrate assimilation genes but not nitrate transporter genes in root, acting as a nitrate sensor to trigger secondary responses. It is possible that OsNLP4 displays different requirements for NRE-like motif recognition and has different functions in shoots and roots.

325

OsNLP4 does not have nitrate-promoted nucleocytoplasmic shuttling mechanism

The localization or retention of AtNLP7 in nuclei is controlled by nitrate signaling and the phosphorylation of a conserved serine residue is essential for the nitrate-simulated rapid nuclear translocation (Marchive et al. 2013; Liu et al. 2017a). However, OsNLP4-GFP was localized to the nucleus independent of nitrate and did not show nitrate-promoted nucleocytoplasmic shuttling mechanism in our experimental condition (Figure 6). Wang et al. (2018) demonstrated possible shuttling mechanisms of OsNLP3 and OsNLP4 in rice upon exposure to 10 mM nitrate. In our experiments we used 2 mM nitrate which is the condition required to observe growth phenotype of *osnlp4* mutants. It is possible that shuttling of OsNLP4 between cytoplasm and

nucleus does not occur at 2 mM but occurs at 10 mM and the role of OsNLP4 maybe different between 2 mM and 10 mM nitrate conditions (Wang et al. 2018). It is known that except nitrate multiple signals coordinately interact with each other through NLPs in plant to modulate downstream events, such as Ca^{2+} signaling, phytohormones and drought (Guan 2017; Almeida et al. 2017; Liu et al. 2017a). It is possible that multiple mechanisms that activate NLPs functions exist in different plant species.

A schematic model of proposed OsNLP4 action in rice

We propose a working model to illustrate how OsNLP4 might react to nitrate signaling to regulate the nitrate assimilation pathway in rice. Nitrate is perceived as signal by some unknown sensors, then transmitted via a signal transduction pathway towards OsNLP4. OsNLP4 controls the expression of a wide range of genes related to nitrate assimilation that have an impact on NR activity and other developmental processes (Figure 8). In addition, the expression of nitrate-assimilation related genes is also regulated by various environmental factors. Probably, crosstalk may occur between OsNLPs and other transcription factors to coordinate the nitrate assimilation pathway.

Recently, it is reported that in *A. thaliana* NLPs also take a role in developmentally programmed processes independent of nitrate. AtNLP7 can regulate root cap cell release likely through regulation of *CELLULOSE5* (Karve and Iyer-Pascuzzi 2018). So further studies are clearly required to understand how OsNLP4 reacts to nitrate signaling with changing from inactive form to active one and the functions of other OsNLPs in rice. NLPs might take a part in abundant developmental and metabolic processes as a transcription factor in plant. Identification and characterization of NLP family probably provide clues for understanding nitrogen or other metabolic pathway.

OsNLP4 have potential effect on sustainable agriculture

Furthermore, nitrogen use efficiency (NUE) is an important indicator for the

development of sustainable agriculture (Xu et al. 2012). In order to increase grain yield, farmers use high amount of chemical fertilizers, especially nitrogen fertilizers, but with considerable negative impacts on the environment (Han et al. 2015). Our paddy field data indicated that the *osnlp4* impaired grain yield (Figure 7). The elevated gene expression in *OsNLP4* might enhance NUE in rice paddy field. Comprehension of the function of *OsNLP4* involved in nitrate signaling may increase crop growth and productivity under nitrogen constraints.

Materials and Methods

Plant materials and growth conditions

Oryza sativa L. cv. Nipponbare was used as the wild type. The *OsNLP4* Tos-17 insertion line (*osnlp4-1*), which has an insertion in second exon, was obtained from the collection of Tos-17 mutant panel by National Institute of Agro Biological Resources, Tsukuba, Japan. Homozygous Tos-17 line was identified using *OsNLP4* gene-specific primers and Tos-17 left-border primer. To generate *OsNLP4* CRISPR/Cas9 lines (*osnlp4-2* and *osnlp4-3*), sgRNAs designed on CRISPR-P (<http://crispr.hzau.edu.cn/CRISPR2/>) were annealed into pU6gRNA vector (Shan et al. 2013) and subsequently cloned into the modified binary vector pZDgRNA_Cas9 ver.2_HPT (Endo et al. 2015). All constructs were sequence verified and transformed into Nipponbare using the *Agrobacterium*-mediated rice transformation method. The *osnlp4-2* line has a ⁸⁶⁷GA deletion in second exon and the *osnlp4-3* line has a ¹⁹⁵⁹A insertion in fourth exon. The primers used are listed in the Supplementary Table 1.

Wild type and the *osnlp4* lines were surface sterilized with 10% (v/v) bleach for 30 min and then rinsed thoroughly with deionized water. The sterilized seeds were germinated on plastic plate containing moist filter paper for 5 days. Uniform seedlings were selected and then transferred to a tank containing 3 L modified Kimura B solutions (Uraguchi et al. 2009) supplemented with one of the following as the sole N source: 2 mM KNO₃; 2 mM NH₄Cl; 1 mM KNO₃ + 1 mM NH₄Cl and 2 mM KCl. All the plants were grown in a chamber with a 16-h-light/ 8-h-dark photoperiod, and the temperature was controlled at 30 °C. The solution was refreshed every 7

days. Samples were harvested at 15 days for phenotypic analysis, RNA isolation and protein extraction. For nitrate uptake speed analysis, 14-day old plants grown on 2 mM KNO₃ were transferred to 2 mM KCl and removed plants to 2 mM KNO₃ again after one day. For the preparation of rice protoplasts, Nipponbare were grown on 1/2MS medium at 30 °C for 10 days under continuous light.

RNA isolation and Quantitative real-time PCR

Total RNA was extracted from shoots and roots using NucleoSpin RNA Kit (MACHEREY-NAGEL). 500 ng total RNA was reverse-transcribed using PrimeScript RT Master Mix (Perfect Real Time) (TaKaRa). Quantitative real-time PCR was performed using SYBR Premix Ex Taq II (Tli RNaseH Plus, TaKaRa) and monitored with the Thermal Cycler Dice Real Time System II (TaKaRa). The relative gene expression was normalized to the expression of *OsACT*. Triplicate biological replicates were analyzed. The primers used for qRT-PCR are listed in Supplementary Table 2.

RNA-seq analyses

Total RNA was used for preparing the library with BGI (<https://www.bgi.com/us/>) (twofold biological samples). Purification of the Poly-A mRNA was performed using TruSeq RNA Library Preparation (Illumina, <https://www.illumina.com/>) and paired-end sequencing identified 100-bp sequences at both ends of the cDNA fragments. Fastq files, downloaded from the main facility, were used for data analysis. Reads in the fastq file were mapped to the rice genome, *Oryza sativa* Japonica-IRGSP 1.0.23 (Ensembl Plants <http://plants.ensembl.org/index.html>) using TopHat2.0.9 (<http://ccb.jhu.edu/software/tophat/index.shtml>) Finally, Cufflinks2.1.1 (<http://cole-trapnell-lab.github.io/cufflinks/>) was used to determine differential gene expression and obtain FPKM value.

Nitrate uptake rate assay

Three 15-day-old seedlings were used for a measurement of nitrate uptake. Three plants were transferred to a 50 ml tube containing 50 ml Kimura B solution with 2

426 mM KNO₃ and their roots were rinsed thoroughly with distilled H₂O. After 3 h and 6 h,
427 sucked 500 µl solution from the tube to detect the concentrations of remaining
428 nitrate using High Performance Capillary Electrophoresis System (Agilent
429 Technologies). Then measured the root fresh weights and calculated the nitrate
430 uptake speeds (NO₃⁻ (µmol)/root fresh weight (g)/h). Three biological replicates of
431 WT and *osnlp4* mutant lines were analyzed for each treatment.

432

433 **Measurement of C/N ratio**

434 The total nitrogen content was investigated using CN coder Vario MAX cube
435 (Elementar). The 15-day shoot samples were dried in a 70 °C oven for 3 days. Then
436 removed samples into assorted metal containers. The machine burned samples and
437 detected total contents of carbon and nitrogen automatically.

438

439 **Analysis of NR activity**

440 The nitrate reductase activity was measured by the modified method described by
441 Maeda et al. and Pinto et al. (Pinto et al. 2014). The roots and shoots (1.0 g fresh
442 weight) were separated and ground in a chilled mortar containing 4 ml extraction
443 buffer (50 mM HEPES-KOH pH 8.0, 5 mM EDTA, 10 mM β-mercaptoethanol, 1 mM
444 DTT, 0.5 mM PMSF, 5% (w/v) PVPP). The resulting homogenate was then centrifuged
445 at 13,000 rpm for 30 min at 4 °C. Samples of the supernatant were used for the
446 determination of protein content (NanoDrop ND-1000) and the assays of *in vitro* NR
447 activity (300 µl). The reaction mixture (450 µl) of the latter consisted of 50 mM
448 HEPES-KOH pH 8.0, 1 mM DTT, 2 mM KNO₃. After incubation at 30 °C for 1 min, the
449 reaction was started by adding 150 µl 0.6 mM NADH. After incubation at 30 °C for 1h,
450 the reaction was ended by the addition of 100 µl cold water. After 1h the
451 NO₂⁻ produced was colorimetrically measured at 543 nm (SHIMADZU UV-1850) after
452 the addition of 1% (w/v) sulphanilamide dissolved in 2 M HCl and 0.02% (w/v)
453 N-1-naphthylamine. NR activity was expressed as µg NO₂⁻ mg⁻¹ protein h⁻¹.

454

455 **Protoplast transient assay**

To understand the subcellular localization of OsNLP4, p35S-OsNLP4-GFP and p35S-GFP modified from pMDC83 vector (Curtis and Grossniklaus 2003) were transformed into rice protoplasts. The rice protoplast preparation and transfection followed previously described procedures with some modifications (Bart et al. 2006). Rice protoplasts were isolated from stem of rice seedlings after sowing for 10 days. Briefly, 100 µl of protoplast suspension was transfected with DNA for various constructs using PEG-mediated method. After transfection, cells were cultured with 0.25 ml inoculation solution containing 2 mM KCl or KNO₃ for 18 h at 25 °C. Cells were collected, then GFP signal in the cells was analyzed using a confocal laser scanning microscope (OLYMPUS FV10-MCPSU).

Rice plant sampling and analysis

To measure the rice grain yield, WT (Nipponbare) and the *osnlp4-1* line were planted in nitrogen-supply area (+N) and no-nitrogen supply area (-N) of Kashimadai paddy field of Tohoku University (Osaki-city, Japan) in May, 2017/2018/2019. Tree frames (4 lines × 4 lines) were harvested in each area in October, 2017/2018/2019. In the no-nitrogen supply paddy field, phosphorus and potassium were supplied as normal. The panicle weight, straw weight and tiller number were measured from 20 lines sampled from the three harvested frames.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers: AP014959 (*OsNLP1*), AP014960 (*OsNLP2*), AP014957 (*OsNLP3*), AP014965 (*OsNLP4*), AP014967 (*OsNLP5*), AP014958 (*OsNLP6*).

Supplemental Data

Supplemental Figure S1. Phylogenic relationship between AtNLPs and OsNLPs.

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Acknowledgments

The authors acknowledge the China Scholarship Council (CSC), Japan Society for the Promotion of Science (JSPS, Nos. 18H05490 and 19H05637) supporting this work.

Figure Legends

Figure 1. Morphological and physiological phenotype of *OsNLP4* Tos-17 insertion line.

A, Plants grown in hydroponic culture for 15 days on 2 mM ammonium (NH_4Cl) or 2 mM nitrate (KNO_3) modified KimuraB solution with of a density of 8 seeds/L. Scale bars, 10 cm. The shoot length (**B**), root length (**C**), shoot dry weight (**D**) and root dry weight (**E**) of the *osnlp4-1* were compared to wild type for plants grown as in **A**. Error bars represent the standard deviation for 6 plants. Differences are statistically significant (t-test, $**p < 0.01$).

Figure 2. Morphological and physiological phenotype of *OsNLP4* CRISPR/Cas9 lines.

A, The CRISPR/Cas9 lines of *OsNLP4*. The *osnlp4-2* has 2 bp deletion in the second exon, while *osnlp4-3* has 1 bp insertion in the fourth exon. Lines represent introns, whereas boxes represent exons. The shoot length (**B**), root length (**C**), shoot dry weight (**D**) and root dry weight (**E**) of *osnlp4-2* and *osnlp4-3* compared to wild type for plants grown in 2 mM KNO_3 . Error bars represent the standard deviation for 6 plants. Differences are statistically significant (Dunnett's test, $*p < 0.05$, $**p < 0.01$).

Figure 3. The mRNA expression levels of *OsNLPs* in wild type under a range of nitrogen conditions.

Total RNA was extracted from 15-day-old plants and was subjected to RNA-seq and qRT-PCR. **A**, Crossing pin charts were used to present the comparison and overlaps between up-regulated and down-regulated genes defined under different nitrogen conditions (Nitrate, 2 mM KNO_3 ; Ammonium, 2 mM NH_4Cl ; Nitrogen free, 2 mM KCl) compared with normal condition (1 mM KNO_3 + 1 mM NH_4Cl) according to RNA-seq data. **B**, RNA-seq data showed transcript accumulations of six *OsNLP* family members in wild type under four different nitrogen conditions. **C**, Relative expression of *OsNLPs* was analyzed by qRT-PCR and normalized to the expression of *ACT*. Each

gene had three biological replicates and the assays were repeated two times. Differences are statistically significant (t-test, * $p < 0.05$, ** $p < 0.01$).

Figure 4. The mRNA expression levels of nitrate reductase genes and nitrate transporter genes in the *osnlp4* mutants under nitrate condition.

The steady-state transcript amounts for the nitrate reductase genes *NIA1* and *NIA2*, nitrite reductase gene *NIR1*, high-affinity nitrate transporter genes *NRT2.1* and root-to-shoot nitrate transporter gene *NRT1.5a* were determined by qRT-PCR. The results given are a percentage of the level of mRNA for the *ACT* gene. Error bars, s.d, n=3 biological replicates (Dunnett's test, * $p < 0.05$, ** $p < 0.01$).

Figure 5. The *osnlp4* mutants impair nitrate uptake rate and nitrate assimilation.

A, Nitrate absorb rate of WT and *osnlp4* mutants under continuous nitrate condition. **B**, The 14-day old plants grown on 2 mM KNO_3 were transferred to 2 mM KCl. After one day, removed plants to 2 mM KNO_3 again and detected nitrate absorb rate. **C**, The mRNA expression levels of molybdenum transporter gene *MOT1* and iron transporter genes *IRT1*, *IRT2* in root were determined by qRT-PCR. The mRNA accumulations were normalized by that of *ACT*. **D**, Iron and molybdenum concentrations in root. **E**, Nitrate reductase activity in root. **F**, The carbon to nitrogen ratio of the *osnlp4* mutants. Error bars, s.d (Dunnett's test, * $p < 0.05$, ** $p < 0.01$).

Figure 6. Subcellular localization of OsNLP4 :: GFP in the KCl- or KNO_3 - treated rice protoplasts.

The pMDC83 vector was used as a negative control and 35S :: GFP was used as a positive control of GFP signal. After PEG-mediated transformation, added KCl or KNO_3 to the protoplast incubation medium at 2 mM final concentration. Chl : chloroplasts autofluorescence. A bar indicates 10 μm . Images are representative of 10 protoplasts.

Figure 7. Agronomic traits of the *osnlp4* Tos-17 insertion line in paddy field.

Tiller number, panicle weight and straw weight per plant were determined for the *osnlp4-1* in comparison to WT in a field trial conducted in Sendai, Japan in 2017 (A), 2018 (B), 2019 (C) (+ N, Nitrogen-supply paddy field; -N, No-Nitrogen supply paddy field). Error bars represent s.d, n = 20, 40, 68 (t-test, * $p < 0.05$, ** $p < 0.01$).

557 **Figure 8. A schematic model of proposed OsNLP4-regulated nitrate assimilation in**
558 **response to nitrate provision in rice.**

559 Nitrate is perceived as signal and transmitted via unknown pathway towards
560 transcription factor OsNLP4. Then OsNLP4 promotes the expression of nitrate
561 assimilation-related genes and other unknown regulatory genes. High active nitrate
562 reductase catalyzes nitrate reduction to trigger nitrate specific growth responses.

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Supplemental Figure S1. Phylogenetic relationship between AtNLPs and OsNLPs.

Phylogenetic tree presented evolutionary relationships between rice and *A. thaliana*. The scale bar represents the evolutionary distance. Six OsNLP family members were classified into three clades. OsNLPs sequences and AtNLPs sequences were obtained from RAP-DB (<http://rapdb.dna.affrc.go.jp/>) and TAIR (<https://www.arabidopsis.org/>) relatively. Phylogenetic tree computed by the MEGA5 software.

Supplemental Figure S2. Physiological phenotype of WT and the *osnlp4* mutant lines under nitrogen free condition.

A, Plants were grown in hydroponic culture without nitrogen for 15 days. Scale bars, 10 cm. The shoot length (**B**), root length (**C**), shoot dry weight (**D**) and root dry weight (**E**) of the *osnlp4* mutants were compared to wild type. Error bars represent the standard deviation for 6 plants. There was no statistical differences between wild type and mutants (Dunnett's test, $*p < 0.05$).

A

- [antioxidant activity \(GO:0016209\)](#)
- [binding \(GO:0005488\)](#)
- [catalytic activity \(GO:0003824\)](#)
- [structural molecule activity \(GO:0005198\)](#)
- [transporter activity \(GO:0005215\)](#)

B

- [antioxidant activity \(GO:0016209\)](#)
- [binding \(GO:0005488\)](#)
- [catalytic activity \(GO:0003824\)](#)
- [signal transducer activity \(GO:0004871\)](#)
- [structural molecule activity \(GO:0005198\)](#)
- [translation regulator activity \(GO:0045182\)](#)
- [transporter activity \(GO:0005215\)](#)

C

Down-regulated Genes

Gene ID	Gene Name	Gene Description
Os08g0468100	<i>NIA1</i>	Nitrate Reductase
Os02g0770800	<i>NIA2</i>	Nitrate Reductase
Os01g0357100	<i>NIR1</i>	Nitrite Reductase
Os03g0667500	<i>IRT1</i>	Iron Transporter
Os08g0101500	<i>MOT1</i>	Molybdenum Transporter
Os03g0307300	<i>NAS1</i>	Nicotianamine Synthase
Os02g0649900	<i>YSL2</i>	Nicotianamine Transporter

Supplemental Figure S3. GO analysis of the *osnlp4-1* according to RNA-seq data.

GO analysis showed the molecular function categories of 580 up-regulated genes (**A**) and 622 down-regulated genes (**B**) in the *osnlp4-1* under nitrate condition. **C**, A short list of down-regulated genes in the *osnlp4-1*.

Mutant Line	Primer Name	Sequence (5'-3')
<i>osnlp4-2</i>	<i>OsNLP4_CRISPR_Guide20_F</i>	GTTGAGCCATTCACTCTCGATCAG
	<i>OsNLP4_CRISPR_Guide20_R</i>	AAACCTGATCGAGAGTGAATGGCT
<i>osnlp4-3</i>	<i>OsNLP4_CRISPR_Guide4_F</i>	GTTGGTGACCCAGGTCTGAGCTAA
	<i>OsNLP4_CRISPR_Guide4_R</i>	AAACTTAGCTCAGACCTGGGTCAC

Supplementary Table 1. Primer pairs used for constructing *OsNLP4* CRISPR/Cas9 lines.

Gene	Forward (5'-3')	Reverse (5'-3')
<i>OsNLP1</i>	TCCGTCTTCAGCCTAGTTGG	AGCAGGTCTGCATCACAGGT
<i>OsNLP2</i>	CTTGCCAACTGGACACACAG	TGCATGTCCTATGGAAGTAGGC
<i>OsNLP3</i>	GCACTGAGATATTGGACCATCC	AGGAAGCTTGTGCTCTTCACA
<i>OsNLP4</i>	TCCTTCGGCAGTACTTTGCT	CAGCGGTTTATGCCATGCT
<i>OsNLP5</i>	ATCTGGGCTCCAATCGAAGC	AACTCTTCCTCAGCACCCA
<i>OsNLP6</i>	GAGGAGGAGGTTGAAGTCGC	CCATCGATTCTGGGTCGCATA
<i>NIA1</i>	ACTGGTGCTGGTGCTTCTGG	CGGCTGGGTGTTGAGGGACT
<i>NIA2</i>	CATGCAGCATTTTCGTTTCT	CCAATTCTTTCATCGTGTTCT
<i>NIR1</i>	GTGACAGTACAAGCGTGCCA	TTGCTTGCTCCGGTGATCTC
<i>NRT2.1</i>	GCGACCGAGACCAGCAATAC	TTCATCACCGTTTGCAACAAG
<i>NRT1.5a</i>	AGACCTGCGCCATATTCCAG	CCTGATCAGGTACAGCCGC
<i>MOT1</i>	CAAGCAGGAGTCGTTCGTCA	CTCAGGCGTAGCAACAGGTA
<i>IRT1</i>	AGGTCGGTGCTCGTCTTCT	TGTCCCTGTACACCCTGGTC
<i>IRT2</i>	TGCATGATGTATAGGTGAAGGTG	CGGCAGAAGCTGGTCTTTATTA

Supplementary Table 2. Primer pairs used for qRT-PCR

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