Lotus japonicus NLP1 and NLP4 transcription factors have different roles in the regulation of nitrate transporter family gene expression

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Root nodule symbiosis is promoted in nitrogen-deficient environments, whereas host plants cease the symbiosis if they can obtain enough nitrogen from their surrounding soil. In *Lotus japonicus*, recent reports indicate that two NODULE INCEPTION (NIN)-LIKE PROTEIN (NLP) transcription factors, LjNLP1 and LjNLP4, play important roles in the regulation of gene expression and nodulation in response to nitrate. To characterize the redundant and unique roles of LjNLP1 and LjNLP4 in more detail, we reanalyzed our previous transcriptome data using *Ljnlp1* and *Ljnlp4* mutants. Although downstream genes of LjNLP1 and LjNLP4 mostly overlapped, we found that nitrate-induced expression of *NITRATE TRANSPORTER 2* (*LjNRT2*) family genes was specifically regulated by LjNLP1. In contrast, *LjNRT1* gene family expression was regulated by both LjNLP1 and LjNLP4. Therefore, it is likely that the two NLPs play distinct roles in the regulation of nitrate transport.

Key words: nitrate, NLP transcription factor, nitrate transporter, root nodule symbiosis

Nitrogen is one of the most important inorganic nutrients for plant growth. While nitrate and ammonium in the soil are the main sources of nitrogen for land plants, their abundance in the environment is not stable, suggesting that nitrogen is a critical limiting element for the growth of most plants. Root nodule symbiosis is an important strategy adopted mainly by legumes to enhance nitrogen acquisition; in response to a signal from rhizobia, legumes form specialized organs called root nodules. Symbiotic nitrogen fixation in root nodules containing rhizobia enables legumes to thrive in nitrogen-deficient soil (Suzaki et al., 2015). In contrast, host plants are known to cease the root nodule symbiosis if they can obtain enough nitrogen from their surrounding soil. Various processes of root nodule symbiosis, such as rhizobial infection, nodule initiation, nodule development and nitrogen fixation, are negatively regulated by high nitrate (Nishida and Suzaki, 2018a). Host plants need to invest in photosynthetic products as an energy source for nodule development and nitrogen fixation. Therefore, when plants can obtain nitrogen nutrients directly from the soil, reducing the energy expended on symbiosis is thought to be a strategy to fulfill the nitrogen demands of plants without unnecessary loss of carbon (Nishida and Suzaki, 2018b).

In *Lotus japonicus*, we have recently reported that two NODULE INCEPTION (NIN)-LIKE PROTEIN (NLP) transcription factors, LjNLP1 and LjNLP4, play essential roles in the nitrate-induced pleiotropic regulation of root nodule symbiosis (Nishida et al., 2018, 2021). Loss-of-function mutations in *LjNLP1* or *LjNLP4* cause normal nodulation even in the presence of high nitrate concentration. NLPs are considered to be master regulators

of nitrate signaling in Arabidopsis; AtNLP6 and AtNLP7 are activated in a nitrate-dependent manner and regulate the expression of many nitrate-responsive genes through direct binding to the nitrate-responsive *cis*-element on the promoters (Vidal et al., 2020). Transcriptome analysis of *L. japonicus* roots showed that the expression of almost all nitrate-inducible genes was suppressed in *Ljnlp1*

Ljnlp4 double mutants. Thus, LjNLP1 and LjNLP4 have key roles to regulate the nitrate response (Nishida et al., 2021). Nevertheless, little is known about redundant and unique roles of LjNLP1 and LjNLP4 in regulating nitrate-inducible gene expression.

To examine the function of LjNLP1 and LjNLP4 for the expression of downstream genes, we reanalyzed our
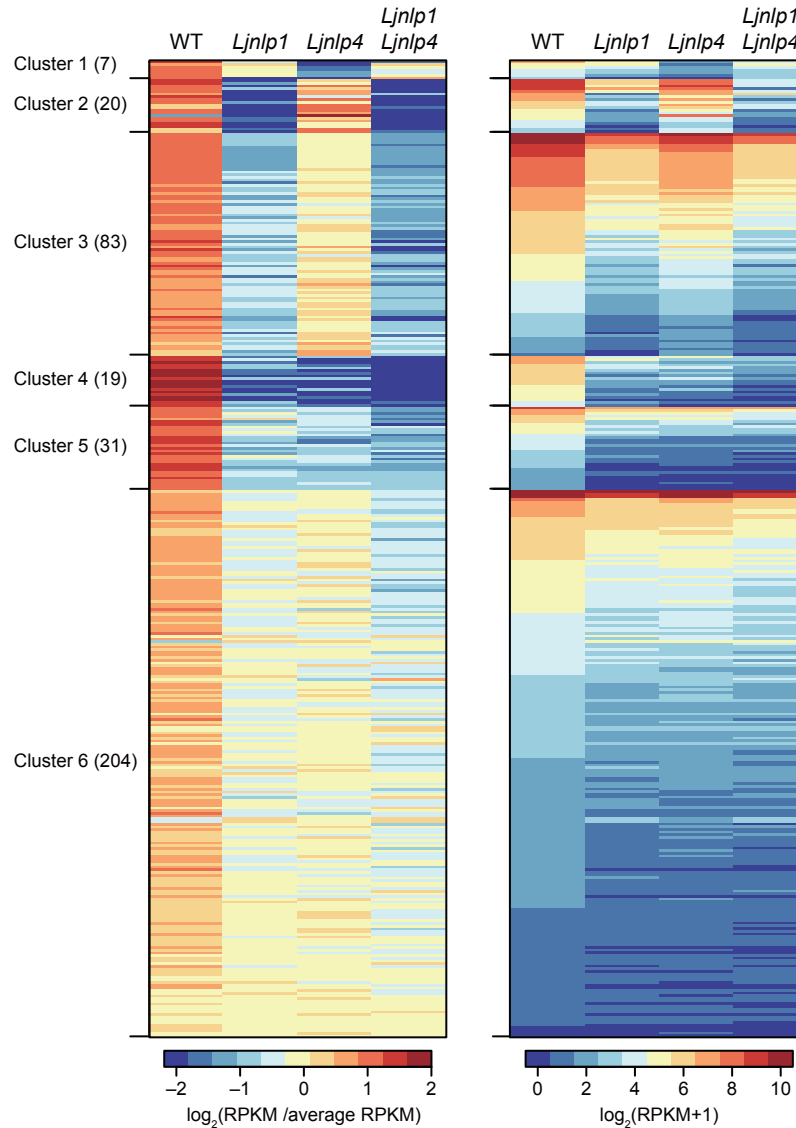

Fig. 1. Heat map analysis of 364 genes that are upregulated by nitrate application in the WT. Plants were grown with 0 or 10 mM KNO_3 for 24 h and then inoculated with *Mesorhizobium loti* MAFF 303099. RNA was extracted for RNA-seq three days after the roots were inoculated (n = 3 independent pools of roots derived from 10 plants). The raw sequence data were deposited in the DNA Data Bank of Japan Sequence Read Archive under the accession number DRA010705. Genes with higher expression in nitrate-treated WT than in nitrate-free WT (log_2 fold changes > 1 and false discovery rate < 0.05) were selected as nitrate-inducible. Heatmaps show the relative expression levels (left) and the expression levels (right) when treated with nitrate for 364 nitrate-inducible genes in WT, *Ljnlp1*, *Ljnlp4* and *Ljnlp1 Ljnlp4* double mutants. The relative expression levels mean the expression levels of each sample divided by the average expression levels of all samples. Gene expression levels were normalized to gene size and library size by counting the reads per kilobase of exon per million mapped reads (RPKM). Nitrate-inducible genes were grouped into six clusters based on relative expression levels of each plant with nitrate, using the R *k-means* function. Genes were ordered by expression levels in WT within each cluster.

previous RNA-seq data (Nishida et al., 2021) from rhizobia-inoculated roots of wild-type (WT), *Ljnlp1*, *Ljnlp4* and *Ljnlp1 Ljnlp4* double mutants grown in the presence of 0 or 10 mM KNO$_3$. We focused on 364 genes upregulated by nitrate application in the WT. These 364 nitrate-inducible genes were grouped into six clusters based on their relative expression levels in each plant with nitrate, using the *k-means* method (Fig. 1). The expression of 254 genes in cluster 4, cluster 5 and cluster 6 was decreased in both *Ljnlp1* and *Ljnlp4* compared with the WT (Fig. 1). Many nitrate-inducible genes were included in these clusters, raising the possibility that the two NLPs have overlapping functions. By contrast, 103 nitrate-inducible genes in clusters 2 and 3 were suppressed in *Ljnlp1* but not *Ljnlp4*, suggesting that they are LjNLP1-specific downstream genes (Fig. 1).

Notably, several genes encoding nitrate transporters were included in the 364 nitrate-inducible genes. Nitrate is taken up by roots mainly by the NITRATE TRANS-PORTER 1 (NRT1) and NRT2 family (Vidal et al., 2020). Plants have two uptake systems depending on the nitrate concentration, namely, a high-affinity transport system (HATS) in the low concentration range (< 1 mM), and a low-affinity transport system (LATS) in the high concentration range (> 1 mM: Vidal et al., 2020). In Arabidopsis, LATS and HATS are thought to be regulated by different transporters, the NRT1 and NRT2 family, respectively (Vidal et al., 2020). *LjNRT2.1* (Lj3g3v3069030) and *LjNRT2.2* (Lj3g3v3069040, Lj3g3v3069010) were included in clusters 2 or 3 (Table 1). This result is consistent with a recent report that the nitrate-dependent activation of *LjNRT2.1* requires LjNLP1 but not LjNLP4 (Misawa

et al., 2022). Additionally, Lj4g3v1415270, included in cluster 2 (Table 1), shows similarity with AtNRT3.1, which regulates HATS with AtNRT2.1 (Okamoto et al., 2006). These results suggest that nitrate transport via the LjNRT2 family is specifically regulated by LjNLP1. In contrast, because LjNRT1 family members (Lj2g3v2002200, Lj4g3v1273870, Lj2g3v2002190, Lj4g3v1273860, Lj1g3v4082070) were included in cluster 4 or 6 (Table 1), LjNLP1 and LjNLP4 probably function redundantly in LjNRT1-mediated nitrate transport. The four nitrate-inducible *LjNRT1* genes in cluster 4 belong to the same NRT1 subgroup I that contains AtNRT1.1 (Criscuolo et al., 2012). AtNRT1.1 is the only known dual-affinity transporter, which functions in both LATS and HATS, in the AtNRT1 family (Vidal et al., 2020).

Seven nitrate-inducible genes in cluster 1 were LjNLP4-specific downstream genes (Fig. 1). We noticed that cluster 1 contained genes encoding phosphate transporters (Table 1). Phosphorus is another important nutrient for plants, and maintaining a balance between nitrogen and phosphorus utilization is critical for proper plant growth (Maeda et al., 2018). LjNLP4 may have a function in nitrate–phosphate signaling crosstalk.

In this study, we compared the downstream genes of LjNLP1 and LjNLP4 and showed that LjNLP1 and LjNLP4 have partly distinct functions in the regulation of the nitrate and phosphate transporter genes. Our previous study comprehensively identified LjNLP4 target gene candidates using DNA affinity purification (DAP)-seq in combination with RNA-seq (Nishida et al., 2021). In contrast, only a few LjNLP1 target genes have been reported so far, including *LjNRT2.1* (Misawa et al., 2022). To reveal the detailed function of these two transcription

Table 1. List of representative nitrate-inducible genes in each cluster

Cluster	Gene ID (*L. japonicus* genome v3.0)	RPKM				vs Araport11 pep (BLASTP)
		WT	*Ljnlp1*	*Ljnlp4*	*Ljnlp1 Ljnlp4*	
1	Lj6g3v0030000	24.702	11.361	2.993	10.828	PHT1;7 | phosphate transporter 1;7
1	Lj6g3v0030010	17.162	8.902	2.572	6.074	PHT5,PHT1;5 | phosphate transporter 1;5
2	Lj4g3v1415270	672.902	74.505	348.118	19.249	NRT3.1,ATNRT3.1 | NITRATE TRANSPORTER 3.1
2	Lj3g3v3069030	553.216	26.102	558.187	11.193	NRT2.4,ATNRT2.4 | nitrate transporter 2.4
2	Lj3g3v3069020	105.807	3.070	111.910	1.556	NRT2:1,ATNRT2:1 | NITRATE TRANSPORTER 2.1
2	Lj3g3v3069040	32.364	1.005	35.459	0.593	NRT2:1,ATNRT2:1 | NITRATE TRANSPORTER 2.1
3	Lj3g3v3069010	52.990	16.338	57.444	8.053	NRT2:1,ATNRT2:1 | NITRATE TRANSPORTER 2.1
4	Lj2g3v2002200	45.883	2.929	2.879	0.531	NRT1.1,CHL1 | nitrate transporter 1.1
4	Lj4g3v1273870	45.761	2.929	2.879	0.531	NRT1.1,CHL1 | nitrate transporter 1.1
4	Lj2g3v2002190	29.410	1.393	1.486	0.336	NRT1.1,CHL1 | nitrate transporter 1.1
4	Lj4g3v1273860	27.379	1.393	1.486	0.336	NRT1.1,CHL1 | nitrate transporter 1.1
6	Lj1g3v4082070	20.261	13.637	12.765	14.664	NPF3.1,AtNPF3.1 | NRT1/ PTR family 3.1

Gene expression levels (RPKM) in inoculated roots at 3 days after inoculation under high-nitrate conditions are shown. A BLASTP search against the Arabidopsis Araport11 pep database was performed.

factors in the regulation of gene expression, further analysis of the direct targets of LjNLP1 will be an important task. NLPs are conserved among diverse plant species and multiple genes encoding NLPs have been identified in the genome of each plant (Schauser et al., 2005; Chardin et al., 2014; Lin et al., 2018). In Arabidopsis, the most studied plant species in relation to nitrate response, AtNLP6 and AtNLP7 redundantly regulate the expression of genes involved in nitrate utilization, including nitrate transporter genes (*AtNRT2.1* and *AtNRT2.2*: Marchive et al., 2013; Guan et al., 2017). AtNLP8 has a specific function in the promotion of seed germination by nitrate (Yan et al., 2016). Additionally, a recent report indicates that AtNLP4 and AtNLP5 play a central role in the rhizobia-induced changes in the root system of Arabidopsis under high-nitrate conditions (Hernández-Reyes et al., 2022). In *Medicago truncatula*, MtNLP1, like LjNLP4, regulates the suppression of nodulation in response to nitrate (Lin et al., 2018). Furthermore, MtNLP2 directly regulates the expression of leghemoglobins, essential factors for symbiotic nitrogen fixation, regardless of nitrate (Jiang et al., 2021). This latter report raises the possibility that NLPs have other important functions besides the nitrate response. Although the *L. japonicus* genome contains five NLPs, the functions of only LjNLP4 and LjNLP1 have been reported to date (Nishida et al., 2018, 2021; Misawa et al., 2022). Biochemical and genetic characterization of the remaining three NLPs in the future will contribute to a deeper understanding of the nitrate response and of root nodule symbiosis.

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