Nitrate transport via NRT2.1 mediates NIN-LIKE PROTEIN-dependent suppression of root nodulation in *Lotus japonicus*

Fumika Misawa,1 Momoyo Ito,1 Shohei Nosaki,1,2 Hanna Nishida,3 Masahiro Watanabe,1 Takamasa Suzuki,4 Kenji Miura,1,2 Masayoshi Kawaguchi,5,6 and Takuya Suzuki1,2,*

1 Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan
2 Tsukuba Plant-Innovation Research Center, University of Tsukuba, Tsukuba, Ibaraki, Japan
3 Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan
4 College of Bioscience and Biotechnology, Chubu University, Kasugai, Aichi, Japan
5 National Institute for Basic Biology, Okazaki, Aichi, Japan
6 School of Life Science, Graduate University for Advanced Studies, Okazaki, Aichi, Japan

*Author for correspondence: suzaki.takuya.fn@u.tsukuba.ac.jp
†Senior author.
These authors contributed equally (F.M. and M.I.).

T.Suzuki conceived the project. F.M., M.I., H.N., M.K., and T.Suzuki designed the experiments. F.M., M.I., S.N., H.N., M.W., K.M., T.Suzuki, and T.Suzuki performed experiments and analyzed the data. T.Suzuki wrote the manuscript, which was approved by all authors.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plcell) is: Takuya Suzuki (suzaki.takuya.fn@u.tsukuba.ac.jp).

Abstract

Legumes have adaptive mechanisms that regulate nodulation in response to the amount of nitrogen in the soil. In *Lotus japonicus*, two NODULE INCEPTION (NIN)-LIKE PROTEIN (NLP) transcription factors, LjNLP4 and LjNLP1, play pivotal roles in the negative regulation of nodulation by controlling the expression of symbiotic genes in high nitrate conditions. Despite an improved understanding of the molecular basis for regulating nodulation, how nitrate plays a role in the signaling pathway to negatively regulate this process is largely unknown. Here, we show that nitrate transport via NITRATE TRANSPORTER 2.1 (LjNRT2.1) is a key step in the NLP signaling pathway to control nodulation. A mutation in the LjNRT2.1 gene attenuates the nitrate-induced control of nodulation. LjNLP1 is necessary and sufficient to induce LjNRT2.1 expression, thereby regulating nitrate uptake/transport. Our data suggest that LjNRT2.1-mediated nitrate uptake/transport is required for LjNLP4 nuclear localization and induction repression of symbiotic genes. We further show that LjNIN, a positive regulator of nodulation, counteracts the LjNLP1-dependent induction of LjNRT2.1 expression, which links to a reduction in nitrate uptake. These findings suggest a plant strategy in which nitrogen acquisition switches from obtaining nitrogen from the soil to symbiotic nitrogen fixation.

Introduction

Nitrogen acquisition is critical for plant growth. Growth of most plant species depends on water-soluble forms of nitrogen nutrients such as nitrate and ammonia in the soil (Oldroyd and Leyser, 2020). Legumes can establish endosymbiotic relationships with nitrogen-fixing bacteria called rhizobia by forming root nodules; the rhizobia in the nodules fix...
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**Background:** Through symbiosis with nitrogen-fixing bacteria, legumes can use nitrogen from the atmosphere as a nutrient. However, root nodule symbiosis requires energy and plants save energy when they can. For example, plants temporarily halt root nodule symbiosis when the soil contains an abundance of nitrogen-containing nutrients such as nitrate. In other words, plants have a mechanism to control root nodule symbiosis upon changes in the amount of nitrate. Using the model legume *Lotus japonicus*, we previously identified the NIN-LIKE PROTEINS LjNLP1 and LjNLP4 as key transcription factors that play pivotal roles in this regulation. However, how nitrate acts in the signalling pathway to control nodulation remains largely unknown.

**Question:** How do legume nitrate transporters control root nodule symbiosis in a nitrate-rich environment?

**Findings:** By analyzing mutants involved in the nitrate-mediated control of root nodule symbiosis, we showed that a defect in the nitrate transporter gene LjNRT2.1 results in the maintenance of nodulation in nitrate-rich environments. We also found that LjNLP1 directly regulated LjNRT2.1 expression. Furthermore, our data suggest that LjNRT2.1-mediated nitrate influx into the cell is relevant to nuclear localization of LjNLP4 and subsequent regulation of the expression of nodulation-related genes. Interestingly, LjNIN, a nodulation-specific transcription factor, controls nitrate uptake by interfering with LjNRT2.1 expression by LjNLP1. These findings enhance our understanding of the mechanisms of nitrogen acquisition that is unique to nodulating plants.

**Next steps:** In plant evolution, legumes that depend on nodules for their nitrogen source may have developed a unique mode of nitrogen acquisition. Comparative functional analysis of nitrate transporter genes, including NRT2.1, in various plants may be useful in testing this hypothesis.

atomic and atmospheric nitrogen, thus making it available to plants (Oldroyd et al., 2011; Roy et al., 2020). Root nodule symbiosis plays an important role in the growth and survival of symbiotic host plants in a nitrogen-deficient environment. This symbiosis, however, is not always beneficial for plants because photosynthetic products that could be used for plant growth need to be consumed as energy sources for nodule development and nitrogen fixation. To maintain a balance between gaining nitrogen and losing carbon during root nodule symbiosis, plants control root nodule symbiosis depending on nitrogen nutrient availability in the soil (Nishida and Suzuki, 2018a); in nitrate-sufficient conditions, plants negatively regulate several key processes in root nodule symbiosis, including rhizobial infection, nodule initiation and growth, and the nitrogen fixation process (Streeter and Wong, 1988; Carroll and Mathews, 1990; Nishida and Suzuki, 2018b).

Soybean (*Glycine max*) nitrate-tolerant symbiotic (nts) mutants are the first identified legume mutants that affect nitrate-mediated control of nodulation (Carroll et al., 1985). In addition to their hypernodulating phenotypes, the nts mutants are tolerant to high nitrate concentrations. The gene responsible for the nts-1 mutants encodes a shoot-acting receptor-like kinase, Nodule autoregulation receptor kinase (GmnARK; Searle et al., 2003). Defects in the GmnARK ortholog in two model legumes, *Lotus japonicus* HYPERNODULATION ABERRANT ROOT FORMATION 1 (LjHAR1) and *Medicago truncatula* SUPER NUMERIC NODULES, exhibit similar phenotypes to the Gmnark mutants (Krusell et al., 2002; Nishimura et al., 2002; Schnabel et al., 2005). In addition, the expression of CLAVATA3/ESR-related (CLE)-ROOT SIGNAL 2 (LjCLE-RS2), encoding a root-derived ligand of LjHAR1, is induced not only by rhizobial inoculation but also by nitrate treatment (Okamoto et al., 2009). In *M. truncatula*, rhizobia/nitrate-inducible MtCLE35 was recently identified as a functional counterpart of LjCLE-RS2 (Luo et al., 2021; Mens et al., 2021; Moreau et al., 2021). In nitrate-sufficient conditions, loss-of-function mutations in LjHAR1 or LjCLE-RS2 show tolerance to the nitrate-induced reduction in nodule number, not to other processes such as rhizobial infection and nodule growth (Nishida et al., 2018, 2020). These observations suggest the signaling pathway including these genes is responsible for modulating nodule number in the pleiotropic control of root nodule symbiosis by nitrate.

A recent genetic approach in *L. japonicus* identified nitrate unresponsive symbiosis 1 (nrsym1) and nrsym2 mutants that have a normal nodule number but attenuate nitrate-induced control of root nodule symbiosis (Nishida et al., 2018, 2021). NRSYM1 and NRSYM2 encode NODE INCEPTION (NIN)-LIKE PROTEIN (NLP) transcription factor, LjNLP4 and LjNLP1, respectively. NLP is paralogous to NIN, a necessary and sufficient regulator of nodulation (Schauer et al., 1999, 2005; Soyano et al., 2013; Vernié et al., 2015). In addition to its positive role in nodulation, LjNIN/MtNIN negatively regulates nodulation via induction of LjCLE-RS1/2 and MtCLE13 (Soyano et al., 2014; Laffont et al., 2020). LjNLP4 shares common DNA-binding sites on the LjCLE-RS2 promoter with LjNIN, and LjNLP4 negatively regulates nodule number by directly inducing LjCLE-RS2 expression in response to nitrate (Nishida et al., 2018). Furthermore, the expression of LjNIN target genes with positive roles in rhizobial infection and nodule organogenesis are repressed by nitrate in LjNLP4- and LjNLP1-dependent manners (Nishida et al., 2021).
LjNLP4 localizes within nuclei in response to nitrate, as do the NLPs in other plants (Marchive et al., 2013; Liu et al., 2017; Lin et al., 2018; Nishida et al., 2018). Therefore, in the presence of high nitrate levels, nuclear-localized LjNLP4 can negatively regulate nodulation by bifunctional transcriptional regulation, inducing or repressing the expression of LjNIN target genes (Nishida et al., 2021). The induced genes include LjCLE-RS2, which has a negative role in nodulation, and the repressed genes include NUCLEAR FACTOR Y-A (LjNF-YA), LjNF-YB, EXOPOLYSACCHARIDE RECEPTOR 3 (LjEPR3), and RHIZOBIAL INFECTION RECEPTOR-LIKE KINASE 1, which have positive roles in nodulation (Okamoto et al., 2009; Soyano et al., 2013; Kawaharada et al., 2015, 2017; Li et al., 2019; Shrestha et al., 2021). In M. truncatula, MtNL1 directly induces MtCLE35, a negative regulator of nodulation, and represses CYTOKININ RESPONSE 1, a positive regulator of nodulation and a direct target of MtNIN (Lin et al., 2018; Luo et al., 2021). Hence, it is likely that the mode-of-action for NLP-mediated bifunctional transcriptional regulation of symbiotic genes is conserved in L. japonicus and M. truncatula.

Generally, plants use two different nitrate transport systems depending on the nitrate concentration: a high-affinity transport system (HATS) and a low-affinity transport system (LATS) for low (<0.5 mM) and high (>0.5 mM) nitrate concentration ranges, respectively (Krapp et al., 2014). In Arabidopsis thaliana, the NITRATE TRANSPORTER 2 (NRT2) family is primarily responsible for HATS, whereas the NRT1 family is responsible for LATS (Wang et al., 1998; Filleur et al., 2001; Li et al., 2007). An exception is AtNRT1.1, which can switch between high- and low-affinity transport activities depending on the phosphorylation state of the AtNRT1.1 protein (Ho et al., 2009). Several studies on legume nitrate transporters suggest their potential roles in nodulation and nitrate-induced control of nodulation (Pellizzaro et al., 2017). In particular, the roles of some NITRATE PEPTIDE FAMILY (NPF) genes have been characterized. MtNPF1.7 positively regulates nodulation and is dispensable for nitrate-induced control of nodulation (Yendrek et al., 2010). LjNPF8.6 is involved in nitrogen fixation (Valkov et al., 2017). In addition, the MtNPF7.6 mutants have nodules of reduced size and are tolerant to nitrate, suggesting that MtNPF7.6 is required for nodule growth and nitrate-induced control of nodulation (Wang et al., 2020).

Despite the accumulating examples of the roles of legume nitrate transporters, how the nitrate transport system plays a role in the signaling pathway to control nodulation under high nitrate conditions remains mostly elusive. This study shows that a mutation in LjNRT2.1 maintains nodulation under high nitrate concentrations. Phenotypic analysis indicates that LjNRT2.1 and LjNLP1 act in the nitrate uptake/transport process. In addition, LjNLP1 is necessary and sufficient to induce LjNRT2.1 expression, which is likely to be associated with the nitrate-dependent LjNLP4 nuclear localization and induction/repression of symbiotic genes. These data indicate that nitrate transport via LjNRT2.1 is a key process in the NLP signaling pathway to control nodulation. Furthermore, we show that LjNIN can counteract LjNLP1-dependent induction of LjNRT2.1. This mechanism may reflect a plant strategy in which the acquisition of nitrogen nutrients switches from depending on nitrogen in the soil to depending on symbiotic nitrogen fixation.
Results

LjNRT2.1 is required for nitrate-induced control of nodulation

In our previous screen for *L. japonicus* ethylmethane sulfonate (EMS) mutants involved in the nitrate response during nodulation (Nishida et al., 2018), we identified two new recessive mutants, *nrsym3* and *nrsym4*. Treatment with a high nitrate concentration (10 mM) attenuated nodulation in the wild-type (WT), but the effect of nitrate was suppressed by the *nrsym3* or the *nrsym4* mutations (Figure 1A). An allelism test suggested that the nitrate-unresponsive phenotypes of *nrsym3* and *nrsym4* were caused by a mutation in an identical gene, as F1 plants obtained from a cross between the plants formed mature nodules under high nitrate conditions (Supplemental Figure S1). A genome-resequencing approach using the *nrsym3* and *nrsym4* mutants identified a point mutation that caused a missense mutation in *LjNRT2.1* (Lj3g3v3069030; Criscuolo et al., 2012; Valkov et al., 2020), a gene that encodes a protein similar to Arabidopsis NRT2 proteins, including AtNRT2.1, AtNRT2.2, and AtNRT2.4 (Figure 1, B and C).

To test whether *LjNRT2.1* could complement the *nrsym3* mutant, a 6.4-kb genomic fragment, including the promoter (3.2 kb) and the terminator (1.2 kb) regions of *LjNRT2.1*, was introduced into the *nrsym3* mutants by *Agrobacterium rhizogenes*-mediated hairy root transformation. Nodulation on the mutant roots expressing the complementation construct was inhibited by nitrate (Supplemental Figure S2, A and B). This result indicates that Lj3g3v3069030 is responsible for the *nrsym3* mutation.

Hereafter, we unified the nomenclature by re-naming *nrsym3* and *nrsym4* as *Ljnrt2.1-1* and *Ljnrt2.1-2*, respectively. The typical structure of NRT2 family proteins has 12 transmembrane regions. Mutations in the *Ljnrt2.1-1* and *Ljnrt2.1-2* mutants are located in the third (G130D) and fifth (G189E) transmembrane regions, respectively. Previous studies showed that the *L. japonicus* genome has four genes in the NRT2 family, including *LjNRT2.1*, *LjNRT2.2* (Lj3g3v3069010, Lj3g3v3069020, Lj3g3v3069040, Lj3g3v3069050), *LjNRT2.3* (Lj4g3v1085060), and *LjNRT2.4* (Lj1g3v3646440) (Criscuolo et al., 2012; Valkov et al., 2020). As the coding sequence (CDS) of *Ljnrt2.2* available from the *L. japonicus* genome database seemed to be partial and possibly inaccurate, we determined the full-length CDS of *LjNRT2.2*. We found that the structure of *Ljnrt2.2* in two WT ecotypes, MG-20 and Gifu, contains a premature stop codon, thus encoding a truncated version of protein relative to *LjNRT2.1* (Supplemental Figure S3).

Figure 2  Nitrate effects on nodulation and plant growth in the *Ljnrt2.1* mutants. A–C, Nitrate effects on nodulation in the WT and *Ljnrt2.1* mutants. A, The number of infection threads in plants growing in 0 (−) or 10 mM KNO₃ (+) at 7 dai with rhizobia that constitutively express *DsRED* (*n* = 11–12 plants). B, The number of nodules in the presence of 0 (−) or 10 mM KNO₃ (+) for 21 dai (*n* = 15 plants). C, Nodule size of the WT and *Ljnrt2.1* mutants (*n* = 19–22 nodules). The WT and *Ljnrt2.1* plants were inoculated for 7 days in nitrate-free agar plates. After the formation of nodule primordia, the plants were transferred to new plates containing 0 or 10 mM KNO₃. Individual nodule size was measured at 0, 5, 10, 15, and 20 days after the transfer. Error bars indicate standard error of the mean (SEM). *P* < 0.05 by a two-sided Welch’s *t*-test. D and E, Fresh shoot weight (D) and shoot growth (E) of the WT, *Ljnlp4*-, *Ljnlp1*, and the *Ljnrt2.1* mutants grown in 0 (−) or 10 mM KNO₃ (+) in the absence of rhizobia for 13 days (*n* = 12 plants). Error bars indicate SEM. Different letters indicate statistically significant differences (*P* < 0.05, one-way analysis of variance (ANOVA) followed by multiple comparisons). Scale bar = 1 cm.
Therefore, it is likely that LjNRT2.2 is nonfunctional in some L. japonicus ecotypes.

Nitrate has pleiotropic effects on key events of nodulation, including rhizobial infection, nodule initiation, and nodule growth (Streeter and Wong, 1988; Carroll and Mathews, 1990; Nishida and Suzuki, 2018b). We, therefore, investigated whether the Ljnrt2.1 mutation influenced these nitrate-affected nodulation processes. In the WT, the formation of infection threads, an indicator of rhizobial infection foci (Murray, 2011), was attenuated by nitrate; however, the nitrate-induced reduction in infection thread number was not observed in the Ljnrt2.1 mutants (Figure 2A). In addition, the number of nodules formed on Ljnrt2.1 roots in the presence of nitrate was comparable to that in the absence of nitrate (Figure 2B). Next, to focus on nodule growth, the WT and Ljnrt2.1 plants were inoculated for 7 days in nitrate-free agar plates. After the formation of nodule primordia, the plants were transferred to new plates containing nitrate, and the successive changes in nodule size were measured. WT nodule growth was halted by nitrate, but the nitrate effect was largely abolished in the Ljnrt2.1 mutants (Figure 2C). Therefore, these results indicate that LjNRT2.1 is required for nitrate-mediated control of rhizobial infection, nodule initiation, and growth.

The nodulation phenotypes of the Ljnlp4 and the Ljnlp1 mutants under high nitrate conditions are similar to those of the Ljnrt2.1 mutants (Nishida et al., 2018, 2021). We then created multiple mutants and investigated the potential genetic relationships between the corresponding genes. Single, double, and triple mutants formed similar numbers of mature nodules that were indistinguishable from each other under high nitrate conditions (Supplemental Figure S4), suggesting that Ljnlp4, Ljnlp1, and LjNRT2.1 act in the same genetic pathway at least in the nitrate-induced control of nodulation.

We next tested the effect of nitrate on plant growth in the absence of rhizobia. Promotion of shoot growth by nitrate was diminished in each mutant relative to the WT, and the severity of the phenotype was strongest in Ljnlp1, weakest in Ljnlp4, and intermediate in Ljnrt2.1 (Figure 2, D and E). The defect in nitrate-promoted shoot growth in the Ljnrt2.1 plants was rescued by the introduction of a complementation construct (Supplemental Figure S2C), suggesting that LjNRT2.1 is responsible for the phenomenon.

LjNRT2.1 and LjNLP1 mediate nitrate uptake/transport

In WT, nitrate-induced inhibition of nodulation occurred in a dose-dependent manner: treatment with a high nitrate concentration (5 mM) reduced nodulation, whereas treatment with a low nitrate concentration (0.2 mM) did not affect nodulation (Figure 3, A and B; Nishida et al., 2018). The effect of nitrate (5 mM) was suppressed by the Ljnrt2.1 mutations. As LjNRT2.1 belongs to the NRT2 family and AtNRT2 genes are primarily responsible for HATS (Wang et al., 1998; Filleur et al., 2001; Li et al., 2007), we suspected that LjNRT2.1 might be involved in nitrate uptake/transport. To examine this possibility, the WT and Ljnrt2.1 plants were treated with 0.2- or 5-mM nitrate solution that included a $^{15}$N stable isotope in the presence of rhizobia, and the amount of $^{15}$N in planta was measured. The nitrate uptake capacity in the root and shoot of the Ljnrt2.1 mutants was significantly reduced compared with WT regardless of the exogenous nitrate concentration (Figure 3, C and D). In terms of nitrate uptake, noninoculated roots of the Ljnrt2.1 mutants had a similar defect: the Ljnrt2.1 mutations attenuated nitrate uptake in both low and high nitrate conditions (Supplemental Figure S5, A and B). These results suggest that LjNRT2.1 is required for nitrate uptake/transport.

We next analyzed the temporal changes in nitrate contents after nitrate treatment in noninoculated roots. Although the nitrate contents at relatively shorter time points (1 and 3 h) was unaffected in the Ljnrt2.1 mutants, the reduction in nitrate contents become evident 6 h after nitrate treatment (10 mM; Figure 3E). Retarded shoot growth of the Ljnlp4 and Ljnlp1 mutants in the presence of nitrate implied that the mutants might be impaired in nitrate transport and/or utilization (Figure 2, D and E). We then investigated the nitrate contents 24 h after nitrate treatment (10 mM) in single, double, and triple mutants of Ljnlp4, Ljnlp1, and Ljnrt2.1. The nitrate contents were unaffected in the Ljnlp4 mutants but were reduced in the Ljnlp1 mutants to the same extent as in the Ljnrt2.1 mutants (Figure 3F). The defect in nitrate uptake in the Ljnlp1 mutants was also observed in the nitrate uptake assay using $^{15}$N stable isotope (Supplemental Figure S5B). Therefore, these results suggest that Ljnlp1 and Ljnrt2.1 have roles in nitrate uptake/transport. Ljnlp1 and Ljnrt2.1 likely regulate nitrate uptake/transport in the same genetic pathway, as the nitrate contents in the Ljnlp1 Ljnrt2.1 double mutants were equivalent to that of the Ljnlp1 or the Ljnrt2.1 single mutants. The relatively stronger defects in shoot growth in the Ljnlp1 and the Ljnrt2.1 mutants compared to the Ljnlp4 mutants (Figure 2, D and E) were consistent with the defects in nitrate uptake in these mutants.

To verify the relationship between nitrate uptake/transport and gene expression, the expression of two nitrate-inducible genes, NITRATE REDUCTASE (LjNIA) and LjCLE- RS2, was analyzed in noninoculated roots by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Whereas the expression of LjNIA was rapidly (<0.5 h) induced, it took 6 h for the expression of LjCLE-RS2 to be induced after nitrate treatment in the WT (Supplemental Figure S5, C and D). Transcript abundance of both genes was significantly reduced at later time points (>6 h) in the Ljnrt2.1 mutants (Supplemental Figure S5, C and D), a result consistent with the observation that the Ljnrt2.1 mutation attenuated nitrate uptake by 6 h and later (Figure 3E).

LjNLP1 is necessary and sufficient to induce Ljnrt2.1 expression in response to nitrate

We next analyzed the temporal expression pattern of Ljnrt2.1 in inoculated roots after nitrate treatment (10 mM) and the potential implications of LjnLP4 and
LjNLP1 in LjNRT2.1 expression. RT-qPCR analysis showed that, in the timescale tested, LjNRT2.1 expression was inducible by nitrate treatment, and the expression level was highest at 3 h in the WT (Figure 4A). LjNRT2.1 expression in the Ljnlp4 mutants was largely comparable to that of the WT at least earlier time points (<3 h). At later time points (>24 h), the LjNRT2.1 expression in the Ljnlp4 mutants was constantly higher than WT. Of note, the LjNRT2.1 expression

Figure 3 The capacity for nitrate uptake in the Ljnrt2.1 mutants. A and B, Nodule number and nodulation phenotypes of the WT and Ljnrt2.1-1 mutants in the presence of 0, 0.2, or 5 mM KNO₃ at 21 dai (n = 11–12 plants). Scale bars = 1 mm. C and D, The capacities for nitrate uptake by the WT and Ljnrt2.1-1 mutants root and shoot in 0.2 (C) and 5 mM (D) KNO₃ in the presence of rhizobia. The plants were grown with 0.2 or 5 mM KNO₃ for 12 days in the presence of rhizobia. They were then treated with same concentration of K¹⁵NO₃ for 24 h (n = 3 independent pools of roots derived from 12 plants). E, Temporal changes in the nitrate contents of noninoculated roots of the WT and Ljnrt2.1-1 mutants after treatment with 10 mM KNO₃ (n = 3 independent pools of roots derived from seven plants). F, The nitrate contents of noninoculated roots of the WT and seven respective mutants 24 h after treatment with 10 mM KNO₃ (n = 3 independent pools of roots derived from seven plants). Error bars indicate SEM. *P < 0.05 by a two-sided Welch’s t test (C–E). Different letters indicate statistically significant differences (P < 0.05, one-way ANOVA followed by multiple comparisons) (A) and (F).
was abolished in the Ljnlp1 mutants at every time point. The LjnRT2.1 expression pattern in the Ljnlp4 Ljnlp1 double mutants was indistinguishable from that of Ljnlp1 (Figure 4A).

We next investigated whether LjnLP1 was sufficient to induce LjnRT2.1 expression by a transactivation assay using mesophyll protoplasts of L. japonicus. Although LjnLP4 or LjnIN did not affect LjnRT2.1 expression, LjnLP1 did induce LjnRT2.1 expression in a nitrate-dependent manner (Figure 4B). The 3.2-kb LjnRT2.1 pro region used in the transactivation assay had an LjnIN-binding sequence (NBS; Soyano et al., 2015). Given the similarity of the DNA-binding sites between NLP and NIN (Soyano et al., 2015; Nishida et al., 2021), we assumed that the NBS in the LjnRT2.1 pro might be relevant to LjnLP1-mediated LjnRT2.1 expression. LjnLP1 failed to express the GUS reporter gene under the control of a modified LjnRT2.1 pro in which the NBS was specifically deleted (Figure 4B; Supplemental Figure S6A). Taken together, these results suggest that LjnLP1 is a crucial factor for LjnRT2.1 expression.
together with the observation of abolished expression of LjNRT2.1 in the Ljnlp1 mutants (Figure 4A), the results from the transactivation assay indicate that LjNLP1 is necessary and sufficient to induce LjNRT2.1 expression in response to nitrate.

To verify the protein–DNA interaction in more detail, we carried out an electrophoretic mobility shift assay (EMSA). The recombinant LjNLP1 and LjNIN proteins, consisting of an RWP-RK and a PB1 domain, were incubated with the FAM-labeled DNA probe (Supplemental Data Set 1). Blue, green, and red nucleotides indicate conserved motifs among the DNA fragments. Brackets and asterisks, respectively, indicate the position of shifted bands showing protein–DNA interaction and of free probes that did not interact with proteins. Transactivation of LjCLE-RS2pro:GUS in L. japonicus mesophyll protoplasts transformed with respective constructs (n = 3 independent pools of protoplasts). GFP, LjNLP4, and LjNLP1 used as effectors. Two types of LjCLE-RS2pro:GUS reporter constructs were used, one with an intact LjCLE-RS2 promoter and one with a modified LjCLE-RS2 promoter in which the NRE/NBS (Figure 5C; Nishida et al., 2021) was specifically deleted (ΔNRE/NBS) (Supplemental Figure S6). Transformed protoplasts were incubated with 0 (−) or 10 mM (+) KNO3. GUS activity was measured relative to 35Spro:LUC activity. Transactivation data were normalized to the condition in which GFP was expressed in the absence of KNO3. Error bars indicate SEM. Different letters indicate statistically significant differences (P < 0.05, one-way ANOVA followed by multiple comparisons).

Transactivation assay showed that LjNLP1, as well as LjNLP4, could activate LjCLE-RS2 expression through direct binding to the cis-element in a nitrate-dependent manner (Figure 5D; Supplemental Figure S6B).

LjNRT2.1, LjNLP4, and LjNLP1 have overlapping expression patterns during nodulation

To determine spatial expression pattern of LjNRT2.1 and its relevance to those of LjNLP4 and LjNLP1, we conducted promoter-GUS reporter analysis. We first identified the functional promoter that could rescue the corresponding mutants to the same extent as constructs using the LjUBQ promoter, when used to express each gene (Supplemental Figure S7, A–C). The promoter fragments LjNRT2.1pro (3.2 kb), LjNLP4pro (2.3 kb) and LjNLP1pro (4.0 kb), harbored same regions as genomic fragments used in complementation assays (Supplemental Figure S2; Nishida et al., 2018, 2021). At initial developmental stages of nodulation, the
The epidermal LjNRT2.1 was predominantly expressed in the epidermis (Supplemental Figure S7E). The epidermal LjNRT2.1 expression might be insufficient to ensure the full LjNRT2.1 function, as the epidermis-specific expression of LjNRT2.1 using the Epi promoter (Hayashi et al., 2014) failed to rescue the Ljnrt2.1 phenotype (Supplemental Figure S7A).

Our data have shown that LjNLP1–LjNRT2.1 pathway has a role in nitrate-induced control of nodulation. We then asked if the expression of LjNRT2.1 could compensate for LjNLP1 function. Constitutive LjNRT2.1 expression from the LjUBQ promoter could not rescue the Ljnlp1 mutation (Supplemental Figure S7C). Therefore, the expression of LjNRT2.1 alone might be insufficient for nitrate-induced control of nodulation in the Ljnlp1 mutants.

LjNRT2.1 function is linked to the nitrate-dependent regulation of LjNLPI–LjNRT2.1 nuclear localization and control of symbiotic gene expression

One of the features of LjNLPI is that its nuclear localization is regulated by nitrate (Nishida et al., 2018). Given the involvement of LjNRT2.1 and LjNLPI in the nitrate-induced control of nodulation and the nitrate uptake/transport process, we verified that these proteins might be involved in regulating the subcellular localization of LjNLPI. In agreement with a previous study (Nishida et al., 2018), immunohistochemistry using an LjNLPI–myc fusion protein showed that LjNLPI was localized within nuclei in response to nitrate in the WT (Figure 7A; Supplemental Figure S8A). In contrast, a strong signal for LjNLPI was not evident in the nuclei of the Ljnlp1 and the Ljnrt2.1 mutants even in the presence of nitrate (Figure 7, B and C; Supplemental Figure S8A). Therefore, our observations suggest that LjNLPI and LjNRT2.1 act upstream of the nitrate-dependent LjNLPI nuclear localization; LjNLPI-mediated activation of LjNRT2.1 expression and subsequent nitrate transport may be required for LjNLPI nuclear localization.

To further characterize the involvement of LjNRT2.1 in the nitrate-induced control of nodulation, we focused on the expression of symbiotic genes whose expression was affected by nitrate. Whereas nitrate upregulates the expression of LjCLE-RS2, nitrate downregulates the expression of LjNF-YA, LjNF-YB, and LjEPR3 in an LjNLPI- and LjNLPI-dependent manner (Nishida et al., 2021). For RT-qPCR analysis, the WT and Ljnrt2.1 plants were pretreated with nitrate for 24 h before rhizobial inoculation. Roots were collected at 3 days after inoculation (dai) with continuous nitrate treatment. Consequently, we found that the nitrate-induced level of LjCLE-RS2 expression was lower in the Ljnrt2.1 mutants than the WT (Figure 8A). In contrast, nitrate-repressed level of LjNF-YA, LjNF-YB, and LjEPR3 was lower in the Ljnrt2.1 mutants relative to the WT (Figure 8, B–D). These nitrate-induced/repressed expression patterns of the symbiotic genes in the Ljnrt2.1 mutants resembled those in the Ljnlp1 and the Ljnlp2 mutants (Nishida et al., 2021). Furthermore, we found that the LjNIN expression was nitrate-repressible in WT and the nitrate-mediated reduction in LjNIN expression was abolished in the Ljnrt2.1 mutants (Figure 8E).

LjNLPI can substitute for LjNLPI function

In addition to the phenotypic similarity of the mutants, our previous RNA-seq analysis indicated that LjNLPI and LjNLPI had mostly similar downstream genes (Nishida et al., 2021).
Indeed, LjCLE-RS2 was identified as a common direct target of LjNLP4 and LjNLP1 (Figure 5). These findings led us to postulate that the two NLPs might have overlapping functions. To examine the functional relationships of LjNLP4 and LjNLP1, we expressed one gene in mutants of the other gene. The nodulation phenotype of the Ljnlp4 mutants in the presence of nitrate was rescued by constitutive expression of LjNLP1 (Figure 9A). In contrast, nodulation of the Ljnlp1 mutants was unaffected by LjNLP4 expression (Figure 9B). These results suggest that LjNLP1 can functionally substitute for LjNLP4.

LjNIN counteracts LjNLP1 function

A previous study showed that LjNIN inhibits the nitrate-inducible expression of LjNRT2.1 and LjNIR1 (Soyano et al., 2015). We sought to elucidate a more detailed mechanism underlying this LjNIN-mediated repression of nitrate-inducible gene expression. In transgenic hairy roots, LjNIN overexpression reduced the nitrate-induced levels of LjNRT2.1 and LjNIR1 (Figure 10, A–C), confirming a previous finding (Soyano et al., 2015). Since LjNLP1 has now been identified as a transcription factor that induces LjNRT2.1 expression, and the NBS within the LjNRT2.1pro was required for the LjNLP1-dependent LjNRT2.1 expression (Figures 4, B and 5, A), we reasoned that LjNIN might interfere with LjNLP1 function via protein–DNA interaction, thereby downregulating LjNRT2.1 expression. To test this hypothesis, we first co-expressed LjNLP1 and LjNIN in L. japonicus protoplasts and measured the expression of LjNRT2.1. The co-expression of LjNLP1 and LjNIN significantly reduced the relative expression level of LjNRT2.1 compared to the case in which LjNLP1 alone was expressed (Figure 10D). In addition, EMSA showed LjNLP1 and LjNIN bound competitively to the cis-element of LjNRT2.1 promoter (Figure 10E).

Lastly, we investigated if rhizobial inoculation could influence nitrate uptake. To test this possibility, we measured the nitrate contents using inoculated and noninoculated WT plants grown without nitrate and then fed nitrate. The nitrate content in inoculated roots (7 dai) 24 h after nitrate treatment (10 mM) was lower than noninoculated roots of
the same (Figure 10F). Of note, the rhizobia-dependent reduction in nitrate uptake was not observed in the Ljnlin mutants (Figure 10F). Therefore, these data suggest that LjNIN has a role in modulating nitrate uptake by counteracting the LjNLP1 function that activates LjNRT2.1 expression.

Discussion

The role of nitrate transporter genes in the nitrate-induced control of nodulation has been largely unknown. A recently identified gene, MtNPF7.6, is expressed in nodule transfer cells and mediates nitrate transport in nodules; Mtnpf7.6 mutations affect the nitrate-induced control of nodulation (Wang et al., 2020). In our study, we showed that the Ljnrt2.1 mutants maintained nodulation in high nitrate conditions. Although the MtNPF7.6 function appears to be restricted to nodules, LjNRT2.1 has a more general role in nitrate transport since it is involved in nitrate transport even in the absence of rhizobia.

In L. japonicus, LjNLP4 and LjNLP1 are known to have pivotal roles in transcriptional regulation in response to nitrate (Nishida et al., 2021); however, details about the genetic relationship of LjNLP4 and LjNLP1 have been incomplete. Here, we identified a critical functional difference between LjNLP4 and LjNLP1; LjNLP1 has an ability to induce LjNRT2.1 expression perhaps because LjNLP1 can bind to a cis-element of the LjNRT2.1 promoter more strongly than LjNLP4. MtNRT2.1 expression was compromised by the mutation of MtNLP1, which is orthologous to LjNLP1 (Lin et al., 2018). Therefore, the NLP1-NRT2.1 regulatory module may be conserved between L. japonicus and M. truncatula.

The nitrate uptake/transport process was attenuated not only by the Ljnrt2.1 mutations but also by the Ljnlp1 mutations. Nitrate-promoted shoot growth was more severely
affected in the *Ljnlp1* or the *Ljnrt2.1* mutants than the *Ljnlp4* mutants, an observation that may result from defects in nitrate uptake/transport. Phenotypic analysis using multiple mutants suggested that *LjnRT2.1* acts in the same genetic pathway as *LjnLP4* and *LjnLP1* in the nitrate-induced control of nodulation. Furthermore, the nuclear localization of nitrate-dependent *LjnLP4*, a feature related to NLPs activity, was diminished by either the *Ljnlp1* or the *Ljnrt2.1* mutations.

Based on these observations and previous findings, we propose a signaling pathway model that acts in the nitrate-induced control of nodulation (Figure 11). In the model, *LjnLP1* induces *LjnRT2.1* expression in the presence of exogenous nitrate. The activated *LjnRT2.1* may enhance nitrate transport, which ultimately triggers *LjnLP4* nuclear localization. Then, the expression of symbiotic genes is induced or repressed by *LjnLP4* depending on the nature of their regulation; *LjnLP4* acts synergistically to inhibit nodulation. Since the expression of *LjnRT2.1* was insufficient to rescue the *Ljnlp1* mutations, *LjnLP1* likely has different downstream pathway from that includes *LjnRT2.1* to control nodulation. Indeed, we demonstrated that *LjnLP1* can directly induce *LjnCLE-RS2* expression in response to nitrate. The NLP1-CLE regulatory module is also identified in *Medicago truncatula* (Luo et al., 2021). Considering *LjnLP1*'s specific role in response to nitrate, *LjnLP1* must be activated in some way by nitrate. Generally, there are two types of nitrate transporters in terms of gene expression pattern: whose expression is either constitutively expressed or is affected by nitrate (Cerezo et al., 2001; Krapp et al., 2014). An unknown constitutively expressed nitrate transporter independent of *LjnRT2.1* may mediate the first step of nitrate uptake/transport to activate *LjnLP1*. Such a transporter may function similarly to *AtNRT1.1*, which is thought to act upstream of *AtNLP6/7*-mediated nitrate signaling (Liu et al., 2017). Our results also show that the expression of *LjnLP1* substituted for the *LjnLP4* function. Although the results need to be carefully interpreted, as a constitutive promoter (*LjUBQpro*) was used

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**Figure 9** Reciprocal complementation assays of *LjnLP4* and *LjnLP1*. A and B, Nodule number and nodulation phenotypes of transgenic hairy roots of the *Ljnlp4-1* (A) and *Ljnlp1* (B) mutants transformed with either the *LjUBQpro-GUS*, *LjUBQpro-LjnLP4*, or *LjUBQpro-LjnLP1* constructs in the presence of 0 (–) or 10 mM KNO₃ (+) at 21 dai (*n* = 6–11 plants). Transgenic roots were identified by GFP fluorescence. Error bars indicate SEM. *P* < 0.05 by a two-sided Welch’s *t* test. Scale bars = 1 mm.

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The function and regulation of NRT2.1 in *Lotus japonicus* THE PLANT CELL 2022: 34; 1844–1862 | 1855
in the assay, it is possible that LjNLP1 per se can regulate the expression of symbiotic genes in the same way as LjNLP4. Meanwhile, LjNLP4 was insufficient to replace LjNLP1’s function, which is likely to reflect the fact that LjNLP4 could not induce LjNRT2.1 expression. Elucidating the details of the functional overlap between LjNLP4 and LjNLP1 is a future challenge.

The temporal expression pattern of LjNRT2.1 after nitrate treatment showed that nitrate-induced LjNRT2.1 activation is transient, suggesting there is a feedback mechanism to control LjNRT2.1 expression. Notably, AtNRT2.1 expression is downregulated by nitrate assimilation products (Lejay et al., 1999). In the Ljnp4 mutants, the expression of LjNIA and LjNIR1 genes was reduced (Nishida et al., 2018). Therefore, the higher levels of LjNRT2.1 expression at later time points in the Ljnp4 mutants relative to the WT may be due to failed feedback regulation of LjNRT2.1 by nitrate assimilation products. Although expression of the nitrate assimilation genes was also low in the Ljnlp1 mutants (Nishida et al., 2021), LjNRT2.1 expression was also compromised. Our
interpretation of these results is that the defect in the induction of LjNRT2.1 expression in the ljnlp1 mutants precedes that responsible for its feedback regulation by nitrate assimilation products.

How physiological processes in plants are regulated during root nodule symbiosis is poorly understood at the molecular level. Here, we provide a dataset that suggests that the nitrate uptake process can be modulated during nodulation. LjNIN, whose expression is induced specifically by rhizobial infection (Schauser et al., 1999; Suzuki et al., 2013), counteracts LjNLP1-dependent induction of LjNRT2.1 expression (Figure 11). LjNIN and LjNLP1 have common cis-elements on the LjNRT2.1 promoter. Importantly, the LjNIN–DNA interaction does not result in gene expression; instead, the interaction seems to block LjNRT2.1 expression by LjNLP1. A previous study that supports our notion indicated that the expression of LjNRT2.1 is downregulated by rhizobial inoculation (Criscuolo et al., 2012). Indeed, the negative regulation of LjNRT2.1 by LjNIN is associated with a reduction in nitrate uptake. The physiological significance of this mechanism needs to be elucidated in the future; however, an attractive hypothesis is that this mechanism may be relevant to a switch in the plant’s strategy concerning nitrogen acquisition. As nodule development progresses, legumes may switch from depending on nitrogen levels in the soil to symbiotic nitrogen fixation, thereby reducing soil nitrate uptake.

In Arabidopsis, the AtNRT2 family proteins are thought to play exclusive roles in HATS (Kiba et al., 2012; Krapp et al., 2014). AtNRT2.1 is responsible for 72% of HATS but is not involved in LATS (Wang et al., 1998; Filleur et al., 2001; Li et al., 2007). In L. japonicus, the effect of the ljnrt2.1 mutation was pronounced in the presence of high nitrate (10 mM). As far as we examined, WT and ljnrt2.1 mutants showed no obvious nodulation phenotypes at low nitrate concentrations. In addition, LjNRT2.1 is required for nitrate transport irrelevant to the exogenous nitrate concentration. Hence, it is possible that the biochemical function of LjNRT2.1 differs from that of AtNRT2.1.

Many current research efforts to learn about plant adaptive strategies in nitrogen-deficient environments are primarily focused on Arabidopsis, a nonnodulating plant. Arabidopsis seems to have developed HATS to acquire low concentrations of soil nitrogen successfully (Kiba and Krapp, 2016; Oldroyd and Leyser, 2020). In contrast, it is enigmatic how nodulating plants such as legumes have adapted to nitrogen-deficient environments when they do not produce nodules. Given that coexistence with rhizobia is a prerequisite in nature, we propose that legumes have always depended on symbiotic nitrogen fixation and might not have needed to develop HATS. An observation potentially related to this hypothesis is that the number of NRT2 family genes in legumes is lower than in Arabidopsis; there are three NRT2 family genes in L. japonicus and M. truncatula, five members in soybean, and seven NRT2 family genes in Arabidopsis (Valkov et al., 2020). A comparative functional analysis of NRT2 family genes in the future may provide clues for elucidating the conserved and diverse roles of nitrate transport systems in nodulating and nonnodulating plants.

**Materials and methods**

**Plant materials and growth conditions**

The Miyakojima MG-20 ecotype of L. japonicus was used as the WT plant (Kawaguchi, 2000). The nrsym3 and nrsym4 mutants were isolated in a previous screening for EMS...
mutants involved in the nitrate response during nodulation (Nishida et al., 2018). The Ljnt2.1-1 mutants were backcrossed with WT once and descendent Ljnt2.1-1 mutants were used for all analyses in this study. A description of Ljnlp4-1, Ljnlp1, and Ljnln-9 plants was published previously (Yoro et al., 2014; Nishida et al., 2018, 2021). Plants were grown with or without Mesorhizobium loti MAFF 303099 in autoclaved vermiculite or on 1% agar plates with Broughton and Dilworth (B&D; Broughton and Dilworth, 1971) solution under a 16-h light/8-h dark cycle at 24°C.

Genome-resequencing of the nrsym3 and nrsym4 mutants

The nrsym3 and nrsym4 mutants were crossed with MG-52, and F2 progeny displaying nitrate-tolerant phenotype were screened. Genomic DNA was extracted from pools of leaves derived from 20 plants using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Libraries were constructed using a TruSeq DNA Sample Prep Kit (Illumina, San Diego, CA, USA) and sequenced using a NextSeq 500 (Illumina) instrument with an 86-bp single-end sequencing protocol. Reads were mapped against the L. japonicus genome version 3.0 (http://www.kazusa.or.jp/lotus/) by Bowtie-0.12.9 (Langmead et al., 2009). Single-nucleotide polymorphism candidates were identified using the Msutsc program (Suzuki et al., 2018).

Phylogenetic analysis

Phylogenetic analysis was conducted in MEGA X. The phylogenetic tree was built using the Neighbor-Joining method. The optimal tree with the sum of branch length = 2.47369594 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and to infer the phylogenetic tree.
Measurement of nitrate uptake and contents
For the analysis using an $^{15}$N stable isotope, samples were prepared based on the method described previously (Tabata et al., 2014). In inoculated conditions, plants were grown with 0.2 or 5mM KNO$_3$ in the presence of rhizobia for 12 days, and plants were then treated with 0.2 or 5mM K$^{15}$NO$_3$ for 24 h. In noninoculated conditions, 12-day-old noninoculated seedlings grown on B&D medium without KNO$_3$ were transferred to new B&D medium with 0.2 or 10mM KNO$_3$ for 12 h. Then, they were washed by 0.1mM CaSO$_4$ for 1 min and transferred to B&D medium with 0.2 or 10mM K$^{15}$NO$_3$ for 5 min. At the end of the $^{15}$N labeling, roots were washed for 1 min in 0.1mM CaSO$_4$ and were separated from shoots. Each sample was dried for 2 days at 75°C and analyzed for total N and $^{15}$N contents by elemental analysis–isotope ratio mass spectrometry (Flash 2000-DELTA plus Advantage ConFlo III System; Thermo Fisher Scientific, Waltham, MA, USA).

Nitrate contents were measured based on the method previously described (Hachiya and Okamoto, 2017). Seedlings grown under each experimental condition were treated with 10mM KNO$_3$. Then, they were washed by sterilized water and frozen by liquid nitrogen. Each sample was crushed using a TissueLyser II (Qiagen), and 10 μL of sterilized water at 80°C was added per 1 mg of sample weight, and vortexing was performed every 5 min for 20 min at 100°C. The sample was then chilled on ice and spun down. The supernatant was stored as extraction solution at −80°C. About 20 μL of 0.05% salicylic acid in sulfuric acid or sulfuric acid was added to 5 μL of extraction solution in a tube, which was vortexed, spun down, and left at room temperature for 20 min. Then, 500 μL of 8% NaOH in sterilized water was added and the mixture was vortexed until it became clear. Nitrate content in the solution was determined by absorbance at 410 nm using a Multiskan GO (Thermo Fisher Scientific) or a Synergy LX (Biotek, Winooski, VT, USA).

EMSA
Recombinant proteins were prepared based on the method previously described (Nishida et al., 2021). For preparing the probes, DNA fragments (Supplemental Data Set 1) were labeled with carboxyfluorescein (FAM). The labeled DNA fragments were purified on the Superdex 200 10/300 GL column (GE Healthcare, Chicago, IL, USA). The purified DNA fragments (0.25 μM) and poly (dl-dC) (50 ng/μL) were mixed with the purified proteins in buffer D (10mM Tris–HCl pH 7.5, 100mM KCl, 100mM NaCl, 1mM DTT, 2.5% glycerol, and 5mM MgCl$_2$), and incubated at 25°C for 30 min. The mixtures were loaded on a 10% polyacrylamide gel, and fluorescence was detected using LuminoGraph III WSE-6300 (ATTO, Tokyo, Japan).

Gene expression analysis
The primers used for PCR are shown in Supplemental Data Set 1. Total RNA was isolated from whole roots using the PureLink Plant RNA Reagent (Invitrogen) or the Plant Total RNA Mini Kit (Favorgen Biotech, Ping-Tung, Taiwan). First-strand cDNA was prepared using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). RT-qPCR was performed using a 7900HT Real-Time PCR system (Applied Biosystems Waltham, MA, USA) with a THUNDERBIRD SYBR qPCR Mix (Toyobo) following the manufacturer’s instructions.

Immunohistochemistry
Immunohistochemistry was conducted based on the method (Nishida et al., 2018). A monoclonal anti-myc antibody and an antibody conjugated to Alexa Fluor 488 Plus anti-mouse IgG-Alexa Fluor Plus 488 (Invitrogen) were used for detecting the signal derived from LjNLP4-myc. Before observing the signal, the roots were stained with 5μg mL$^{-1}$ 4', 6-diamidino-2-phenylindole (DAPI; Dojindo, Kumamoto, Japan) for 15 min. Fluorescent images were obtained using an LSM700 confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany) equipped with ZEN software (Carl Zeiss). The obtained images were analyzed using ImageJ; first, the threshold of the green signals derived from LjNLP4-myc was set equally among the images, and then the ratio of the number of the nuclei with green signals was quantified against the number of the total, namely DAPI-stained, nuclei in every image.

Transactivation using L. japonicus protoplasts
The isolation of L. japonicus mesophyll protoplasts and the transactivation assay were conducted based on the method (Nishida et al., 2021). To exclude the effect of endogenous nitrate on gene expression, plants were grown without nitrate but with 10mM NH$_4$Cl for 16 days. Fluorescence and luminescence were measured using a Synergy LX (Biotek). For transformation of protoplasts, equal amount of DNA (10 μg each) of effector, reporter and internal control plasmids were used. 35SproLUC was used as the internal control plasmid.

Immunoblot analysis
The plants with transgenic hairy roots were treated with 10mM KNO$_3$ for 1 h, and their roots (150 mg), which were derived from five plants, were ground by bead beating after freezing with liquid nitrogen. Lysis buffer (50mM Tris–HCl, pH 8.0, 120mM NaCl, 0.2mM sodium orthovanadate, 100mM NaF, 10% glycerol, 0.2% Triton X-100, 5mM DTT, 5mM EDTA, 1mM PMSF, and 1× protein inhibitor cocktail [Nacalai Tesque, Kyoto, Japan]) were added. The ground roots and buffer were thoroughly mixed by bead beating, and then sonicated on ice. The suspension was centrifuged at 20,000 g for 20 min at 4°C. The supernatant was collected and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4%–15% Mini-PROTEAN TG Precast Protein Gels (Bio-Rad, Hercules, CA, USA). The separated proteins were electrically transferred onto a PVDF membrane (Amersham Hybond P PVDF; GE Healthcare). A primary antibody against c-Myc (Santa Crux Biotechnology, Dallas, TX, USA; catalog no. c-789) diluted in blocking buffer (1% bovine standard albumin and 1% polyvinylpyrrolidone
in TBST buffer [Tris-buffered saline, 0.1% Tween-20]) was applied to the membrane for overnight incubation at 4°C. After extensive washing, a Horseradish Peroxidase (HRP)-conjugated secondary antibody (Rockland; catalog no. 18-8816-31) diluted in the same blocking buffer was applied for 1 h at room temperature. Immobilon Forte Western HRP substrate (Merck, Kenilworth, NJ, USA) chemiluminescence reagent was used to detect antibody binding. Signals were detected using LuminoGraph III WSE-6300 (ATTO). For antibody stripping, the membrane was incubated in stripping buffer (6.4mM Tris–HCl, pH 6.8, 2% SDS, and 10mM DTT) for 45 min at 50°C. After being washed by the TBST buffer, the membrane was incubated with a primary antibody against GFP (Invitrogen; catalog no. A11122) diluted in blocking buffer. The antibody binding was detected in the same way as above.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA). Normality was checked using the Shapiro-Wilk test and $P > 0.05$ was considered as normal distribution. The F-test was used to test if the variances of two populations were equal or not. Appropriate methods were chosen according to the nature of the data. The criterion of $P < 0.05$ means statistically significant difference in this study. The results of the statistical analyses are shown in Supplemental Data Set 2.

**Accession number**

Data from the short reads from genome-resequencing of nrsym3 and nrsym4 were deposited in the DNA Data Bank of Japan Sequence Read Archive under the accession number DRA011845.

**Supplemental data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Allelism test of the nrsym3 and the nrsym4 mutants (Supports Figure 1).

**Supplemental Figure S2.** Complementation of the nrsym3 phenotypes (Supports Figure 1).

**Supplemental Figure S3.** Comparison of LjNRT2.1 and LjNRT2.2 (Supports Figure 1).

**Supplemental Figure S4.** Nitrate effects on nodulation of multiple combinations of mutants among Ljnlp4, Ljnlp1, and Ljnt2.1 (Supports Figure 2).

**Supplemental Figure S5.** Effects of the Ljnt2.1 mutations on nitrate uptake and gene expression (Supports Figure 3).

**Supplemental Figure S6.** A schematic diagram of the promoter-GUS constructs used in transactivation assay (Supports Figures 4 and 5).

**Supplemental Figure S7.** Complementation of the Ljnt2.1, Ljnlp4, and Ljnlp1 phenotypes and LjNRT2.1pro-GUS (Supports Figure 6).

**Supplemental Figure S8.** The ratio of LjNLP4-accumulating nuclei in immunohistochemistry (Supports Figure 7).

**Supplemental Data Set 1.** Primers used in this study.

**Supplemental Data Set 2.** Statistical analysis data.

**Supplemental File S1.** Sequence alignment of NRT2s in fasta format.

**Supplemental File S2.** Phylogenet tree in newick format.

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**Conflict of interest statement.** The authors declare no conflict of interests.

**References**

Broughton WJ, Dilworth MJ (1971) Control of leghaemoglobin synthesis in snake beans. Biochem J 125: 1075–1080

Carroll BJ, Mathews A (1990) Nitrate inhibition of nodulation in legumes. In PM Cresshof, ed, The Molecular Biology of Symbiotic Nitrogen Fixation, CRC Press, Boca Raton, FL, pp 159–180

Carroll BJ, McNeil DL, Gresshoff PM (1985) A supernodulation and nitrate-tolerant symbiotic (nts) soybean mutant. Plant Physiol 78: 34–40

Cerezo M, Tillard P, Filleur S, Munos SP, Daniel-Vedele FO, Gojon A (2001) Major alterations of the regulation of root NO uptake are associated with the mutation of Nrt2.1 and Nrt2.2 genes in Arabidopsis. Plant Physiol 127: 262–271

Cricuolo G, Valkov VT, Parlati A, Alves LM, Chiurazzi M (2012) Molecular characterization of the Lotus japonicus NRT1(PTR) and NRT2 families. Plant Cell Environ 35: 1567–1581

Filleur S, Dorbe MF, Cerezo M, Orsel M, Granier F, Gojon A, Daniel-Vedele F (2001) An Arabidopsis T-DNA mutant affected in Nrt2 genes is impaired in nitrate uptake. FEBS Lett 489: 220–224

Hachiya T, Okamoto Y (2017) Simple spectroscopic determination of nitrate, nitrite, and ammonium in Arabidopsis thaliana. Bio-protocol 7: e2280

Hayashi T, Shimoya Y, Sato T, Tabata S, Imaizumi-Anraku H, Hayashi M (2014) Rhizobial infection does not require cortical expression of upstream common symbiosis genes responsible for the induction of Ca2+ spiking. Plant J 77: 146–159

Ho CH, Lin SH, Hu HC, Tsay YF (2009) CHL1 functions as a nitrate sensor in plants. Cell 138: 1184–1194

Kawaguchi M (2000) Lotus japonicus ‘Miyakoijima’ MG-20: an early-flowering accession suitable for indoor handling. J Plant Res 113: 507–509

Kawaharada Y, Kelly S, Nielsen MW, Hjuler CT, Gysel K, Muszynska A, Carlson RW, Thygesen MB, Sandal N, Asmussen MH, et al. (2015) Receptor-mediated exopolysaccharide perception controls bacterial infection. Nature 523: 308–312
Kawaharada Y, Nielsen MW, Kelly S, James EK, Andersen KR, Rasmussen SR, Fuchtbauer W, Madsen LH, Heckmann AB, Radutoiu S, et al. (2017) Differential regulation of the Epr3 receptor coordinates membrane-restricted rhizobial colonization of root nodule primordia. Nat Commun 8: 14534

Kiba T, Feria-Bourrellier AB, Lafouge F, Lezhneva L, Boutilier Mercey S, Orsel M, Bréhaut V, Miller A, Daniel-Vedele F, Sakakibara H, et al. (2012) The Arabidopsis nitrate transporter NRT2.4 plays a double role in roots and shoots of nitrogen-starved plants. Plant Cell 24: 245–258

Kiba T, Krapp A (2016) Plant nitrogen acquisition under low availability: regulation of uptake and root architecture. Plant Cell Physiol 57: 707–714

Krapp A, David LC, Chardin C, Girin T, Marmagne A, Leprince AS, Chaillou S, Ferrario-Mery S, Meyer C, Daniel-Vedele F (2016) Nitrate transport and signalling in Arabidopsis. J Exp Bot 65: 789–798

Krusell L, Madsen LH, SATA S, Aubert G, Genua A, Szczyglowski K, Duc G, Kaneko T, Tabata S, de Bruijn F, et al. (2002) Shoot control of root development and nodulation is mediated by a receptor-like kinase. Nature 420: 422–426

Laffont C, Ivanovic A, Gautrait P, Braut M, Djordjevic MA, Krapp A, David LC, Chardin C, Girin T, Marmagne A, Leprince AS, Chaillou S, Ferrario-Mery S, Meyer C, Daniel-Vedele F (2016) Nitrate transport and signalling in Arabidopsis. J Exp Bot 65: 789–798

Lejay L, Tillard P, Lepetit M, Olive FD, Filleur S, Daniel-Vedele F, Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 10: R25

Liu BH, Niu Y, Konishi M, Wu Y, Du H, Sun Ch, Li L, Bououdsocq M, McCormack M, Maekawa T, Ishida S, Zhang C, et al. (2017) Discovery of nitrate–CKP–NLP signalling in central nutrient–growth networks. Nature 545: 311–316

Luo Z, Lin JS, Zhu Y, Fu M, Li X, Xie F (2021) NLP1 reciprocally regulates nitrate inhibition of nodulation through SUNN-CRA2 signalling in Medicago truncatula. Plant Commun 2: 100183

Maekawa T, Kusakabe M, Shimoda Y, SATA S, Tabata S, Murooka Y, Hayashi M (2008) Polyubiquitin promoter-based binary vectors for overexpression and gene silencing in Lotus japonicus. Mol Plant-Microbe Interact 21: 375–382

Marchive C, Roudier F, Castaing L, Bréhaut V, Blondet E, Colot V, Meyer C, Krapp A (2013) Nuclear retention of the transcription factor NLP7 orchestrates the early response to nitrate in plants. Nat Commun 4: 1713

Mens C, Hastwell AH, Su H, Gresshoff PM, Mathiesius U, Ferguson BJ (2021) Characterisation of Medicago truncatula CLE34 and CLE35 in nitrate and rhizobia regulation of nodulation. New Phytol 229: 2525–2534

Moreau C, Gautrait P, Frugier F (2021) Nitrate-induced CLE35 signaling peptides inhibit nodulation through the SUNN receptor and miR2111 repression. Plant Physiol 185: 1216–1228

Murray JD (2011) Invasion by invitation: rhizobial infection in legumes. Mol Plant-Microbe Interact 24: 631–639

Nishida H, Handa Y, Tanaka S, Suzuki T, Kagawah M (2016) Expression of the CLE-RS3 gene suppresses root nodulation in Lotus japonicus. J Plant Res 129: 909–919

Nishida H, Ito M, Miura K, Kagawah M, Suzuki T (2020) Autoregulation of nodulation pathway is dispensable for nitrate-induced control of rhizobial infection. Plant Signal Behav 15: 1733814

Nishida H, Nosaki S, Suzuki T, Ito M, Miyakawa T, Nomoto M, Tada Y, Miura K, Tanokura M, Kagawah M, et al. (2021) Different DNA-binding specificities of NLP and NIN transcription factors underlie nitrate-induced control of root nodulation. Plant Cell 23: 2340–2359

Nishida H, Suzuki T (2018a) Two negative regulatory systems of root nodule symbiosis: how are symbiotic benefits and costs balanced? Plant Cell Physiol 59: 1733–1738

Nishida H, Suzuki T (2018b) Nitrate-mediated control of root nodule symbiosis. Curr Opin Plant Biol 44: 129–136

Nishida H, Tanaka S, Handa Y, Ito M, Sakamoto Y, Matsunaga S, Betsuyaku S, Miura K, Sotano Y, Kagawah M, et al. (2018) A NIN-LIKE PROTEIN mediates nitrate-induced control of root nodule symbiosis in Lotus japonicus. Nat Commun 9: 499

Nishimura R, Hayashi M, Wu GJ, Kouchi H, Imaiuzumi-Anraku H, Murakami Y, Kawanishi S, Akao S, Ohmori M, Nagasawa M, et al. (2002) HAR1 mediates systemic regulation of symbiotic organ development. Nature 420: 426–429

Okamoto S, Ohnishi E, SATA S, Takahashi H, Nakazono M, Tabata K, Kagawah M (2009) Nod factor/nitrate-induced CLE genes that drive HAR1-mediated systemic regulation of nodulation. Plant Cell Physiol 50: 67–77

Okamoto S, Yoro E, Suzuki T, Kagawah M (2013) Hairy root transformation in Lotus japonicus. Bio-protocol 3: e795

Oldroyd GE, Murray JD, Poole PS, Downie JA (2011) The rules of engagement in the legume-rhizobial symbiosis. Annu Rev Genet 45: 119–144

Oldroyd GE, Leyser O (2020) A plant’s diet, surviving in a variable nutrient environment. Science 368: eaab0196

Pellizzaro A, Alibert B, Planchet E, Limami AM, Meroué-Le Paven MC (2017) Nitrate transporters: an overview in legumes. Planta 246: 585–595

Roy S, Liu W, Nandetty RS, Crook A, Mysore KS, Pilirialui C, Frugoli J, Dickstein R, Udvardi MK (2020) Celebrating 20 years of genetic discoveries in legume nodulation and symbiotic nitrogen fixation. Plant Cell 32: 15–41

Schauer L, Roussis A, Stillier J, Stougard J (1999) A plant regulator controlling development of symbiotic root nodules. Nature 402: 191–195

Schauer L, Wieloch W, Stougard J (2005) Evolution of NIN-like proteins in Arabidopsis, rice, and Lotus japonicus. J Mol Evol 60: 229–237

Schnabel E, Journet EP, de Carvalho-Niebel F, Duc G, Frugoli J (2005) The Medicago truncatula SUNN gene encodes a CLV1-like leucine-rich repeat receptor kinase that regulates nodule number and root length. Plant Mol Biol 58: 809–822

Searle IR, Men AE, Laniya TS, Buzas DM, Iturbe-Ormaetxe I, Carroll BJ, Gresshoff PM (2003) Long-distance signaling in nodulation directed by a CLAVATA1-like receptor kinase. Science 299: 109–112

Shrestha A, Zhong S, Therrien J, Huebert T, SATA S, Mun T, Andersen SU, Stougard J, Lepage A, Niebel A, et al. (2021) Lotus japonicus Nuclear Factor YA1, a nodule emergence stage-specific regulator of auxin signalling. New Phytol 229: 1535–1552

Soyano T, Hirakawa H, SATA S, Hayashi M, Kagawah M (2014) NODULE INCEPTION creates a long-distance negative feedback loop involved in homeostatic regulation of nodule organ production. Proc Natl Acad Sci USA 111: 14607–14612

Soyano T, Kouchi H, Hirota A, Hayashi M (2013) NODULE INCEPTION directly targets NF-Y subunit genes to regulate
essential processes of root nodule development in *Lotus japonicus*. PLoS Genet 9: e1003352

Soyano T, Shimoda Y, Hayashi M (2015) NODULE INCEPTION antagonistically regulates gene expression with nitrate in *Lotus japonicus*. Plant Cell Physiol 56: 368–376

Streeter J, Wong PP (1988) Inhibition of legume nodule formation and N₂ fixation by nitrate. Crit Rev Plant Sci 7: 1–23

Suzuki T, Kim CS, Takeda N, Szczyglowski K, Kawaguchi M (2013) TRICOT encodes an AMP1-related carboxypeptidase that regulates root nodule development and shoot apical meristem maintenance in *Lotus japonicus*. Development 140: 353–361

Suzuki T, Takeda N, Nishida H, Hoshino M, Ito M, Misawa F, Handa Y, Miura K, Kawaguchi M (2019) LACK OF SYMBIONT ACCOMMODATION controls intracellular symbiont accommodation in root nodule and arbuscular mycorrhizal symbiosis in *Lotus japonicus*. PLoS Genet 15: e1007865

Suzuki T, Yano K, Ito M, Umehara Y, Suganuma N, Kawaguchi M (2012) Positive and negative regulation of cortical cell division during root nodule development in *Lotus japonicus* is accompanied by auxin response. Development 139: 3997–4006

Suzuki T, Kawai T, Takemura S, Nishiwaki M, Suzuki T, Nakamura K, Ishiguro S, Higashiyama T (2018) Development of the Mitsucal computer system to identify causal mutation with a high-throughput sequencer. Plant Reprod 31: 117–128

Tabata R, Sumida K, Yoshii T, Ohyama K, Shinohara H, Matsubayashi Y (2014) Perception of root-derived peptides by shoot LRR-RKs mediates systemic N-demand signaling. Science 346: 343–346

Valkov VT, Rogato A, Alves LM, Sol S, Noguero M, Lérán S, Lacombe B, Chiurazzi M (2017) The nitrate transporter family protein LjNPF8.6 controls the N-fixing nodule activity. Plant Physiol 175: 1269–1282

Valkov VT, Sol S, Rogato A, Chiurazzi M (2020) The functional characterization of LjNRT2.4 indicates a novel, positive role of nitrate for an efficient nodule N₂-fixation activity. New Phytol 228: 682–696

Vernié T, Kim J, Frances L, Ding Y, Sun J, Guan D, Niebel A, Gifford ML, de Carvalho-Niebel F, Oldroyd GED (2015) The NIN transcription factor coordinates diverse nodulation programs in different tissues of the *Medicago truncatula* root. Plant Cell 27: 3410–3424

Wang Q, Huang Y, Ren Z, Zhang X, Ren J, Su J, Zhang C, Tian J, Yu Y, Gao GF, et al. (2020) Transfer cells mediate nitrate uptake to control root nodule symbiosis. Nat Plants 6: 800–808

Wang R, Liu D, Crawford NM (1998) The *Arabidopsis* CHL1 protein plays a major role in high-affinity nitrate uptake. Proc Natl Acad Sci USA 95: 15134–15139

Yendrek CR, Lee YC, Morris V, Liang Y, Pislariu CI, Burkart G, Meckfessel MH, Salehin M, Kessler H, Wessler H, et al. (2010) A putative transporter is essential for integrating nutrient and hormone signaling with lateral root growth and nodule development in *Medicago truncatula*. Plant J 62: 100–112

Yoro E, Suzaki T, Toyokura K, Miyazawa H, Fukaki H, Kawaguchi M (2014) A positive regulator of nodule organogenesis, NODULE INCEPTION, acts as a negative regulator of rhizobial infection in *Lotus japonicus*. Plant Physiol 165: 747–758