

The small peptide CEP1 and the NIN-like protein NLP1 regulate *NRT2.1* to mediate root nodule formation across nitrate concentrations

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Abstract

Legumes acquire fixed nitrogen (N) from the soil and through endosymbiotic association with diazotrophic bacteria. However, establishing and maintaining N₂-fixing nodules are expensive for the host plant, relative to taking up N from the soil. Therefore, plants suppress symbiosis when N is plentiful and enhance symbiosis when N is sparse. Here, we show that the nitrate transporter *MtNRT2.1* is required for optimal nodule establishment in *Medicago truncatula* under low-nitrate conditions and the repression of nodulation under high-nitrate conditions. The NIN-like protein (NLP) *MtNLP1* is required for *MtNRT2.1* expression and regulation of nitrate uptake/transport under low- and high-nitrate conditions. Under low nitrate, the gene encoding the C-terminally encoded peptide (CEP) *MtCEP1* was more highly expressed, and the exogenous application of *MtCEP1* systemically promoted *MtNRT2.1* expression in a compact root architecture 2 (*MtCRA2*)-dependent manner. The enhancement of nodulation by *MtCEP1* and nitrate uptake were both impaired in the *Mtnrt2.1* mutant under low nitrate. Our study demonstrates that nitrate uptake by *MtNRT2.1* differentially affects nodulation at low- and high-nitrate conditions through the actions of *MtCEP1* and *MtNLP1*.

IN A NUTSHELL

Background: Nitrogen (N) is an essential nutrient and signaling molecule for plant growth and development. Legume plants can not only use N from the soil as nitrate (NO_3^-) but also obtain N through symbiotic nitrogen fixation with bacteria (rhizobia), which inhabit nodules formed on the plant roots. However, nodule formation and nitrogen fixation are energy-consuming processes for plants; therefore, plants control nodule formation depending on the concentration of available nitrate. High concentrations of nitrate inhibit nodule formation and low concentrations of nitrate promote nodule formation. However, how nitrate controls nodulation remains unclear.

Question: How do the legume nitrate transporters (NRTs) responsible for the uptake/transport of low/high nitrate control root nodule formation in *Medicago truncatula*?

Findings: Here we report that *M. truncatula* MtNRT2.1 has both high- and low-affinity nitrate uptake transport activity, and MtNRT2.1 is required for optimal nodule establishment under low nitrate and inhibiting nodulation when nitrate is sufficient. We showed that the transcription factor MtNLP1 directly activated *MtNRT2.1* expression and regulation of nitrate uptake under low and high nitrate. Under low nitrate, the gene encoding the small peptide MtCEP1 was highly expressed, and the application of this peptide systemically promoted *MtNRT2.1* expression in a MtCRA2-dependent manner. MtNRT2.1 is required for the peptide MtCEP1 to enhance nodulation and nitrate uptake. Our study demonstrates that nitrate uptake by MtNRT2.1 differentially affects nodulation under low- and high-nitrate conditions and this is controlled by MtCEP1 and MtNLP1.

Next steps: Soil N uptake and symbiotic N are important for the growth and yield of legume crops, including soybean (*Glycine max*). Evaluating the conservation of mechanisms identified in *Medicago* may provide insight on other legumes in the future.

Introduction

Nitrogen (N) is a macronutrient required for normal plant growth and can be taken up by plants in the form of nitrate (NO_3^-), which acts as a local and systemic signal in various processes of plant metabolism and development. NO_3^- uptake from soil occurs through transporters belonging to two families: nitrate transporter 2 (NRT2) and nitrate transporter/peptide transporter (NPF; Gojon et al., 2011; Krapp et al., 2014; Noguero and Lacombe, 2016). Plant roots have two types of nitrate transport systems: a low-affinity transport system (LATS, >1 mM) and a high-affinity transport system (HATS, $1 \mu\text{M}$ to ~ 1 mM), to adapt to fluctuating levels of nitrate in the soil (Crawford and Glass, 1998). In *Arabidopsis thaliana*, AtNPF6.3 (also known as CHL1/NRT1.1) was identified as a dual-affinity nitrate transporter and is thought to be the primary transporter involved in LATS (Liu et al., 1999), while AtNRT2s, which require interaction with nitrate assimilation related 2 (NAR2) family proteins for their activity, are the primary transporters involved in HATS (Li et al., 2007; Kotur et al., 2012).

In addition to using NO_3^- , most legume plants can acquire N from the atmosphere through symbiotic N fixation, which involves a mutualism between legumes and N-fixing bacteria called rhizobia (Ferguson et al., 2019; Wang et al., 2022). This interaction results in the nodule formation on the plant's roots. Within the nodule, rhizobia use the plant photosynthesis products as energy to reduce (or fix) atmospheric N into ammonia for plants' utilization.

N fixation is an energy-intensive process for plants; therefore, legume plants have evolved a systemic regulatory mechanism referred to as autoregulation of nodulation (AON) to coordinate growth and nodulation. The cost of N fixation is reflected by the inhibition of root nodule formation by legume plants when sufficient mineral N (>2 mM nitrate) is available (Streeter and Wong, 1988; Ferguson et al., 2019). Recent studies in *Medicago truncatula* and *Lotus japonicus* have identified a central role for nodule inception (NIN)-like protein (NLP) transcription factors in inhibiting nodulation under high nitrate (HN). NIN, the founding member of the NLP transcription factor family, is one of the most important nodulation genes. NIN is required for rhizobial infection, nodule organogenesis, N fixation, and AON (Liu and Bisseling, 2020; Feng et al., 2021; Jiang et al., 2021).

In contrast to NIN's positive role in infection and organogenesis, NLP1 and NLP4 are required to suppress nodulation at HN concentrations. *M. truncatula nlp1* and *L. japonicus nlp1* and *nlp4* mutants have normal nodulation in the absence of nitrate, but are tolerant to nitrate suppression of nodulation (Lin et al., 2018; Luo et al., 2021; Nishida et al., 2018, 2021). Nitrate can trigger the redistribution of MtNLP1 and LjNLP4 from the cytosol to the nucleus, and MtNLP1 and LjNLP1/4 can interact with NIN through their C-terminal PB1 domains, thus reducing the activation of symbiotic gene expression by NIN (Lin et al., 2018; Luo et al., 2021; Nishida et al., 2018, 2021). By contrast, low concentrations of nitrate (<2 mM) stimulate nodulation in several legume species (Carroll et al., 1985; Barbulova et al., 2007;

Fei and Vessey, 2009; Nanjareddy et al., 2014; Ferguson et al., 2019), but the underlying mechanism remains unknown.

A full understanding of nitrate regulation of nodulation in legumes requires identification of the specific nitrate transporters involved and their regulators. Several NPFs have been studied in legumes. Three AtNRT1.1/AtNPF6.3 orthologs were identified in *M. truncatula*, and MtNPF6.5 and MtNPF6.7 were repressed and induced by nitrate, respectively, in an MtNLP1-dependent manner. Xiao et al. (2021) found that both MtNPF6.5 and MtNPF6.7 can mediate nitrate and chloride uptake, but nodulation was not studied in this work. Morère-Le Paven et al. (2011) showed that MtNRT1.3 encodes a dual-affinity nitrate transporter that is up-regulated in the absence of nitrate, suggesting that it may be involved in the response to N-limitation, and again nodulation was not investigated. The high-affinity nitrate transporter MtNPF1.7 [also named LATD/NIP, lateral root defective/numerous infection threads (ITs), polyphenolics] positively regulates root and nodule development (Yendrek et al., 2010; Bagchi et al., 2012). The high-affinity nitrate transporter MtNPF7.6 is induced by rhizobia and nitrate and is specifically expressed in the nodule vasculature within nodule transfer cells, where it mediates nitrate uptake and affects nodulation (Wang et al., 2020). In *L. japonicus*, a series of nitrate transporters that have positive roles in nodule N fixation have been characterized, including LjNPF3.1, LjNPF8.6, and LjNRT2.4 (Valkov et al., 2017, 2020; Vittozzi et al., 2021); by contrast, LjNPF2.9 has a negative role in regulating shoot biomass and nitrate content without affecting symbiotic performance (Sol et al., 2019). Most recently, LjNRT2.1 was shown to be activated by LjNLP1 and to be required for the translocation of LjNLP4 to the nucleus to mediate nitrate inhibition of nodulation (Misawa et al., 2022).

C-terminally encoded peptides (CEPs), a family of mobile peptide hormones, play an important role in plant nitrate uptake. In Arabidopsis, they act systemically, moving from roots to shoots where they activate the leucine-rich repeat receptor-like kinase (LRR-RLK) receptors AtCEPR1 and AtCEPR2, to promote expression of the shoot-to-root systemic effectors AtCEPD1 (CEP Downstream 1) and AtCEPD2 (Tabata et al., 2014; Ohkubo et al., 2017). AtCEPD1/2 are phloem-mobile signals that relay N-deprivation information to roots to up-regulate the expression of AtNRT2.1 (Ohkubo et al., 2017). In *M. truncatula*, MtCEP genes are induced under N deprivation conditions and promote susceptibility to rhizobial infection and nodulation (Imin et al., 2013; Gautrat et al., 2020). MtNLP1 directly binds to the half-nitrate response element (NRE) in the MtCEP1 promoter and represses its expression in the presence of nitrate (Luo et al., 2022). Synthetic MtCEP1 peptides enhance nitrate uptake in *M. truncatula* under low (100 and 500 μ M) external nitrate concentrations, but had no effect at high (1 and 5 mM) concentrations (Roy et al., 2021). MtCEP1 binding to leaf vascular cells is dependent on the MtCRA2 (compact root architecture 2) receptor which is closely related to AtCEPR1/2 (Huault et al., 2014; Lee et al., 2021) and the

cra2 mutant (tr185 allele) has reduced nitrate uptake rate and N content (Bourion et al. 2014).

Considering that AtNRT2s are the major HATS transporters and that MtNRT2.1 is expressed in root hairs under HN (Pellizzaro et al., 2015; Damiani et al., 2016), we investigated whether MtNRT2.1 may be involved in the regulation of nodulation under low-nitrate (LN) and HN conditions. We show that MtNRT2.1 is required both for enhancing nodulation at LN and inhibiting nodulation at HN. We further show that, similar to Arabidopsis, MtCEP1 peptides systemically induce MtNRT2.1 expression under LN, resulting in enhanced nodulation. Furthermore, we demonstrate that MtNLP1 activates MtNRT2.1 expression under LN and HN conditions to promote or repress nodulation, respectively.

Results

MtNRT2.1 encodes a high-affinity nitrate transporter

Three NRT2 homologs were identified in *M. truncatula*, and the predicted amino acid sequences of MtNRT2.1 (MtrunA17_Chr4g0028551.1) and MtNRT2.2 (MtrunA17_Chr4g0028541.1) differ by just a single residue, but MtNRT2.2 expression is very low (Pellizzaro et al., 2015). To establish their relationship with NRT2 family members in other plant species, a phylogenetic tree is generated using NRT2s from *M. truncatula*, *L. japonicus*, and *A. thaliana*, which shows that MtNRT2.1/2.2 is closely related to LjNRT2.1 (Supplemental Figure 1). Analysis of MtNRT2.1 expression in roots using RT-qPCR reveals that MtNRT2.1 is expressed under both LN (0.5–2 mM KNO₃) and HN (5 mM KNO₃) conditions, and the expression level is higher in HN than in LN (Figure 1A). The spatial and temporal expression patterns of MtNRT2.1 during root nodulation are determined using *pMtNRT2.1::GUS* transformed *M. truncatula* hairy roots, which shows that it is expressed in root vascular tissues and the nodule meristem (Supplemental Figure 2).

In order to determine the function of MtNRT2.1 in root nodule formation, we isolated a *Tnt1* insertion mutant line, NF2226, which has a *Tnt1* insertion in the first exon of MtNRT2.1 (Supplemental Figure 3A). A primer set consisting of one primer common to both NRT2.1 and NRT2.2 and a primer specific to the 3' UTR of MtNRT2.1 is used for genotyping (Supplemental Figure 3B). Further RT-qPCR analysis confirms that MtNRT2.1 expression is dramatically reduced in *Mtnrt2.1* roots (Supplemental Figure 3C). RT-PCR using NRT2.2-specific primers show that the NRT2.2 cDNA is barely detectable in the roots of wild type and the *nrt2.1* mutant (Supplemental Figure 3D), consistent with a previous study (Pellizzaro et al., 2015). Thus, we focused our study on MtNRT2.1.

MtNRT2.1 homologs in *A. thaliana* and rice (*Oryza sativa*) were shown to be HATS components (Filleur et al., 2001; Orsel et al., 2004; Feng et al., 2011), and NAR2 was required for their activity (Okamoto et al., 2006; Orsel et al., 2006). The nitrate transport activity of MtNRT2.1 was assessed by

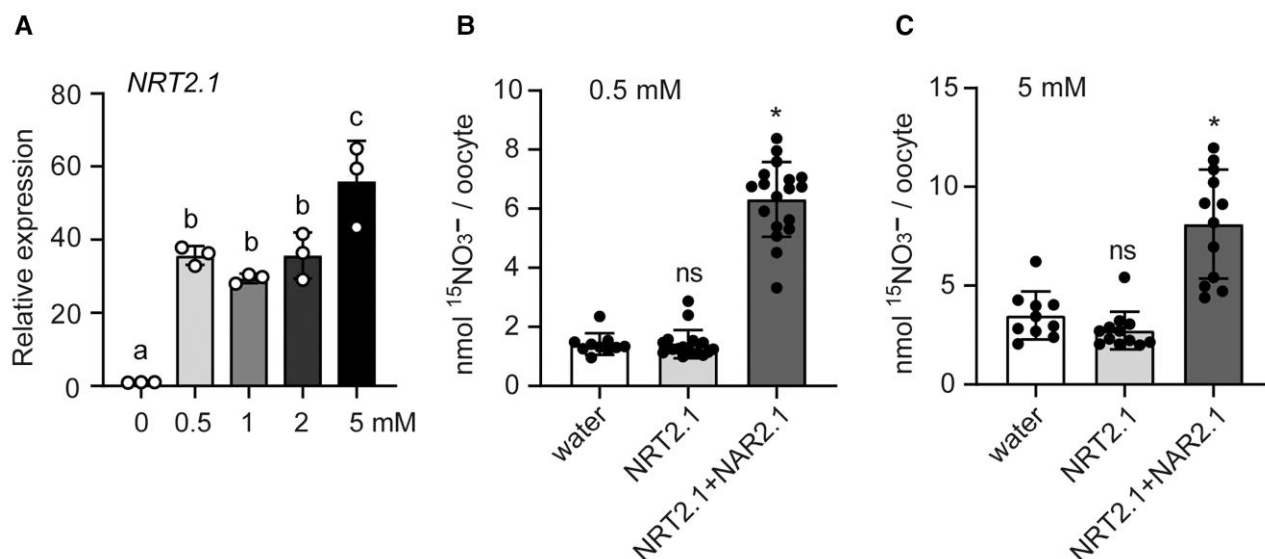


Figure 1 MtNRT2.1 was induced by nitrate and has nitrate transport activity in *X. laevis* oocytes. A, Relative expression of *MtNRT2.1* in roots of *M. truncatula* seedlings grown on 0, 0.5, 1, 2, and 5 mM KNO_3 . Plants were grown on a nitrogen-free FP medium for 5 days and then transferred to new plates containing different concentrations of KNO_3 for an additional 3 days ($n=3$, each made up of 8–12 plants). Different letters represent statistically significant differences ($P<0.05$, one-way ANOVA with Tukey's multiple comparisons). B and C, Uptake of 0.5 mM (B) and 5 mM (C). K^{15}NO_3 into oocytes injected with water (control), single *MtNRT2.1* cRNA or *MtNRT2.1* and *MtNAR2.1* mixtures. Oocytes were incubated with the indicated concentrations of K^{15}NO_3 at pH 5.5 for 3 h, and ^{15}N contents were determined. Values are means $\pm$ SD ($n=10$ –18 oocytes). Asterisks indicate significant differences compared with the H_2O -injected oocytes (two-tailed *t* test, * $P<0.05$; ns, not significant).

measuring $^{15}\text{NO}_3^-$ uptake activity in *MtNRT2.1*/*MtNAR2.1*-cRNA injected *Xenopus laevis* oocytes. The injected oocytes were incubated with 0.5 or 5 mM ^{15}N nitrate at pH 5.5, then their $^{15}\text{NO}_3^-$ content was determined. The measurement of ^{15}N enrichment in oocytes shows that only the combination of injecting with *MtNRT2.1* and *MtNAR2.1* results in a significant uptake when compared with water and *MtNRT2.1*-injected oocytes, at both 0.5- and 5-mM nitrate (Figure 1, B and C). This indicates that *MtNRT2.1* has both high- and low-affinity nitrate uptake activity which requires *NAR2.1*.

MtNRT2.1 mediates nitrate uptake

To determine the role of *MtNRT2.1* in nitrate uptake in *M. truncatula*, we assessed the nitrate content of wild-type (WT) and *Mtnrt2.1* roots using high-performance liquid chromatography (HPLC) 6- or 12-h after treatment with LN (0.5 mM KNO_3) or HN (5 mM KNO_3). The nitrate content of WT is higher at 12 h after treatment with LN or HN than at 6 h, but remains low in *Mtnrt2.1* roots (Figure 2, A and D). The nitrate uptake ability of WT, *Mtnrt2.1*, and *Mtnlp1* roots under LN and HN was further examined using $^{15}\text{NO}_3^-$. In this assay, the $^{15}\text{NO}_3^-$ content was assessed after 30 min of incubation. The results show that the root and shoot $^{15}\text{NO}_3^-$ contents of both *Mtnrt2.1* and *Mtnlp1* are significantly lower than WT under LN and HN conditions (Figure 2, B, C, E, and F).

To verify the relationship between nitrate uptake/transport and gene expression, the expression of three nitrate-inducible genes, *MtCLE35*, *Nitrate Reductase 1*

(*NIA1*), and *Nitrite Reductase 1* (*NIR1*), was analyzed in non-inoculated roots by RT-qPCR. These genes show no induction at ≤ 0.5 mM nitrate, are weakly induced at 1.0 or 2.0 mM nitrate, and are highly induced at 5 mM nitrate; moreover, the induction level is significantly attenuated in *Mtnrt2.1* mutants (Figure 2, G–I). These data indicate that *MtNRT2.1* is responsible for the majority of nitrate taken up by *M. truncatula* in the 0.5–5 mM range and that it is required for nitrate-induced gene expression.

MtNRT2.1 promotes nodulation at LN

A low concentration of nitrate can stimulate nodulation in several legume species (Nanjareddy et al., 2014; Ferguson et al., 2019). Given that *MtNRT2.1* has high-affinity nitrate transport activity, we speculated that it may function in the stimulation of nodulation under LN. First, we analyzed the temporal expression pattern of *MtNRT2.1* after LN treatment. RT-qPCR analysis shows that it is induced after one day of LN treatment and the induction level increases over time (Supplemental Figure 4A). The expression of three nitrate-induced genes, *MtCLE35*, *MtNIA1*, and *MtNIR1*, was analyzed at 1, 3, and 5 days after transfer to LN in WT and *Mtnrt2.1*. This reveals that after 5 days of LN treatment, the expression of these genes is weakly induced by LN and that this induction is abolished in *Mtnrt2.1* (Supplemental Figure 4, B–D).

We then analyzed the nodulation phenotype of the *Mtnrt2.1* mutants in the absence of nitrate (zero nitrate, ZN) and under LN conditions. Consistent with previous

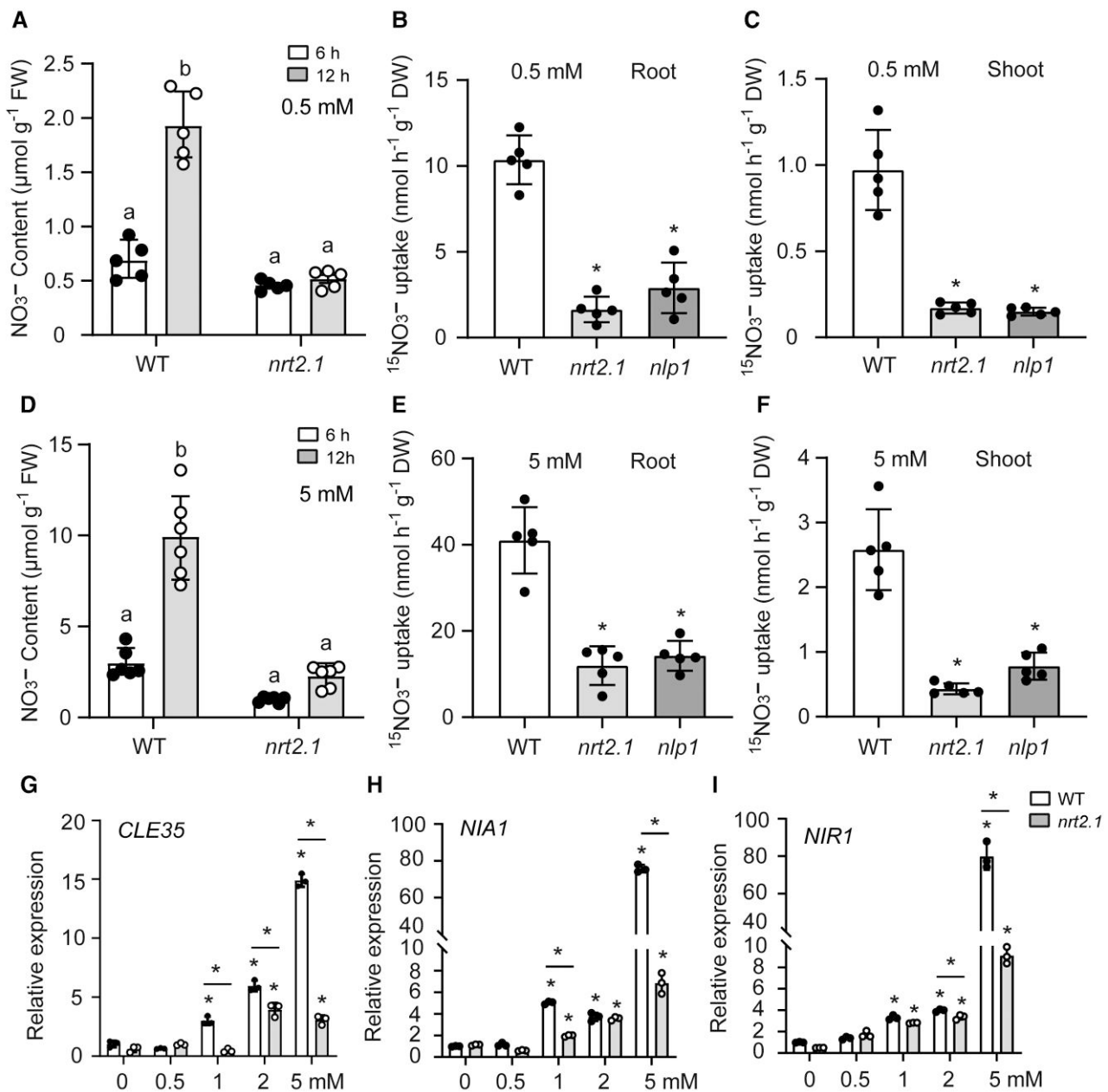


Figure 2 MtNRT2.1 mediates nitrate uptake in *M. truncatula*. A and D, Nitrate content in WT and *Mtnrt2.1* mutants after treatment with 0.5 mM KNO_3 (A) and 5 mM KNO_3 (D) as determined by HPLC. $n = 5-6$ with each data point representing a pool of roots from 3 to 5 plants. Plants were grown on a nitrogen-free FP medium for 12 days and then supplied with 0.5 or 5 mM KNO_3 for 6 h or 12 h. Different letters represent statistically significant differences ($P < 0.05$, two-way ANOVA with Tukey's test for multiple comparisons). B and C, Nitrate uptake by roots (B) and shoots (C) of WT and *Mtnrt2.1* in 0.5 mM KNO_3 . E and F, Nitrate uptake in roots (E) and shoots (F) of WT and *Mtnrt2.1* in 5 mM KNO_3 . Plants were grown on a nitrogen-free FP medium for 7 days, pretreated with 0.5 mM or 5 mM KNO_3 for 4 h, then transferred to FP hydroponics medium with 0.5 or 5 mM K^{15}NO_3 for 30 min. ^{15}N content of shoots and roots was analyzed. * $P < 0.05$ by a two-sided Student's *t* test. G–I, Relative gene expression of *MtCLE35* (G), *MtNIA1* (H), and *MtNIR1* (I) in WT and *Mtnrt2.1* roots after treatment with 0, 0.5, 1, 2, and 5 mM KNO_3 . Plants were grown on FP medium for 5 days and then transferred to new plates containing 0, 0.5, 1, 2, and 5 mM KNO_3 for additional 3 days ($n = 3$, each made up of 9–12 plants). * $P < 0.05$ indicate significant differences compared with WT with ZN by a two-sided Student's *t* test. Error bars show SD.

reports (Fei and Vessey, 2009), we found that WT plants develop ~60% more nodules at LN than in ZN conditions at 1, 2, and 3 weeks post inoculation (wpi; Figure 3, A and B). By contrast, the nodule number of *Mtnrt2.1* does not increase

compared with ZN and LN at all time points examined (Figure 3B). The number of rhizobial ITs at 1 wpi increases in WT at LN, but does not change in *Mtnrt2.1*, in line with the nodule number (Figure 3C). Acetylene reduction activity

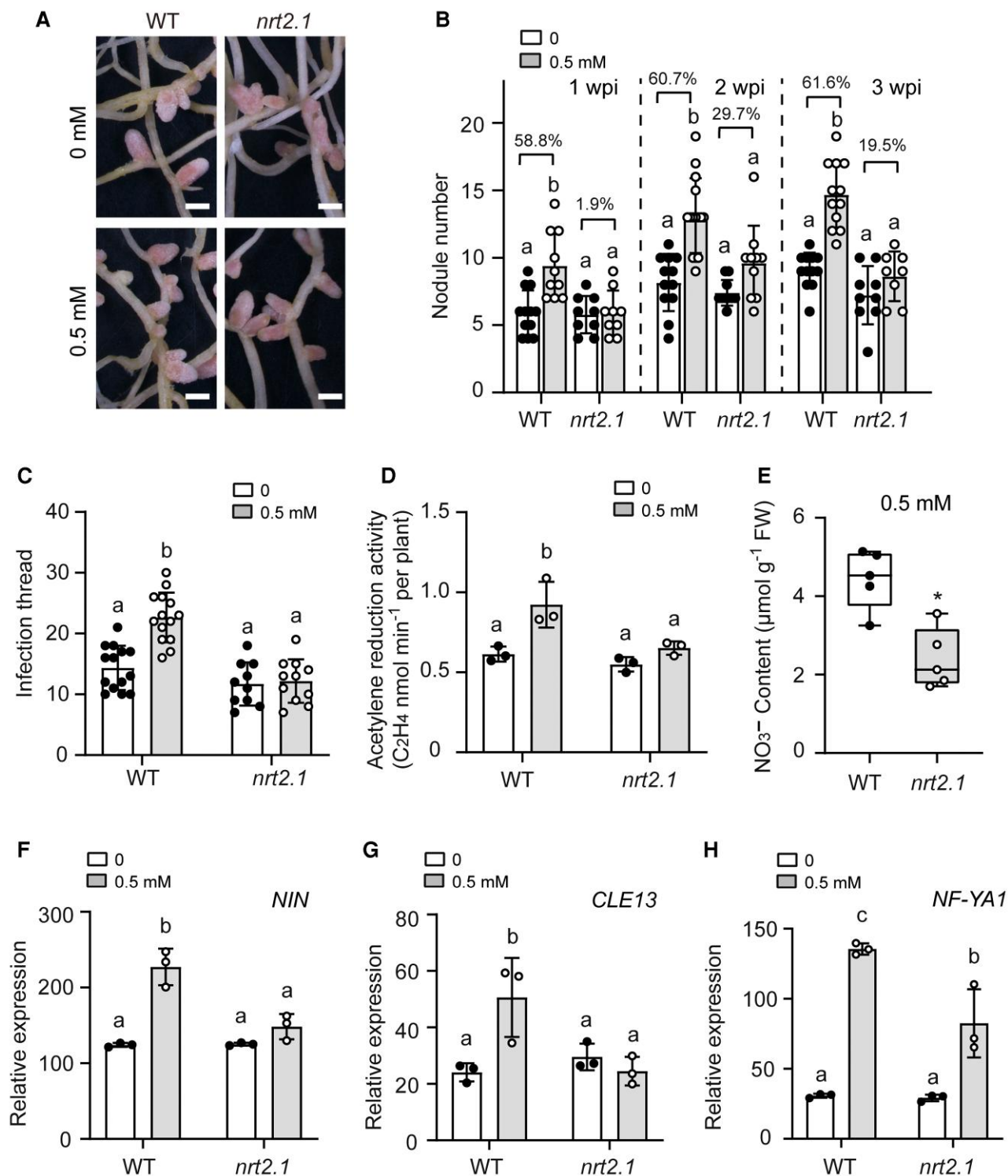


Figure 3 *Mtnrt2.1* nodulation and nitrate accumulation phenotypes under LN. A–E, Nodule phenotype (A), nodule numbers (B), IT numbers (C), nitrogenase activity (D), and nitrate content (E) of WT and *Mtnrt2.1* after continuous treatment with LN (0.5 mM KNO₃) 1–3 (B), 1 (C), or 2 (A, D, and E) wpi with *S. meliloti* ($n = 10$ –15 plants). $n = 3$ in (D), $n = 5$ (E), each data point represents a pool of roots from 2 to 3 plants. F–H, Relative gene expression of *MtNIN* (F), *MtCLE13* (G), and *MtNF-YA1* (H) in WT and *Mtnrt2.1* roots after treatment with 0.5 mM KNO₃ at 7 dpi ($n = 3$, each made up of 9–12 plants). Expression levels were normalized relative to the roots without inoculation. Different letters represent statistically significant differences ($P < 0.05$, two-way ANOVA with Tukey's multiple comparisons test). Asterisk (change figure to one asterisk) indicates significant differences ($* P < 0.05$, two-tailed t test). Error bars show SD.

(ARA) was used to estimate nodule nitrogenase activity at 2 wpi. This shows that ARA is higher in WT at LN compared to ZN and that this effect is lost in *Mtnrt2.1* (Figure 3D). Together, these analyses suggest that the enhancement of symbiosis at LN relative to ZN is dependent on MtNRT2.1. This is consistent with the lower nitrate accumulation in *Mtnrt2.1* roots under LN (Figure 3E).

To further validate the role of MtNRT2.1 in nodulation under LN, the expression of three symbiotic genes, *MtNIN*, *MtCLE13*, and *MtNF-YA1* (Nuclear Factor YA1), was examined in WT and *Mtnrt2.1* at 1 wpi under ZN and LN. The results show that these symbiotic genes are relatively more highly expressed in WT at LN, but that this increase is abolished or reduced in *Mtnrt2.1* (Figure 3, F–H). The shoot and root weight of WT is higher at LN compared with ZN, and this increase is reduced in the *Mtnrt2.1* mutant (Supplemental Figure 5). The nodulation and growth phenotypes and nitrate content of *Mtnrt2.1* are complemented by the transgenic expression of *MtNRT2.1*, driven by its native promoter (Supplemental Figure 6). Together, these results demonstrate that MtNRT2.1 is required for LN stimulation of root nodulation and plant growth.

MtNRT2.1 is required for CEP1-induced nodulation

MtCEP1 is up-regulated by LN and the overexpression of MtCEP1 or application of synthetic MtCEP1 peptide to roots can enhance nodule number in *M. truncatula* (Imin et al., 2013). We used RT-qPCR to test whether MtNRT2.1 is required for MtCEP1 expression at different nitrate concentrations. We found that in WT roots, CEP1 expression is higher at LN (0.5 mM KNO₃) and is lower at 1-, 2-, or 5-mM nitrate treatments, compared with ZN (Figure 4A). However, the effects of different nitrate concentrations on MtCEP1 expression are mostly lost in *Mtnrt2.1* roots, with only a slight increase observed at LN (0.5 mM KNO₃) and no changes seen at the other concentrations tested, relative to ZN (Figure 4A). We then analyzed changes in MtCEP1 expression over time after LN treatment. Our results reveal that MtCEP1 expression shows MtNRT2.1- and NLP1-dependent increase at 3 and 5 days after LN treatment (Figure 4B).

In Arabidopsis, CEP1 peptide can systemically induce AtNRT2.1 to regulate N acquisition (Ohkubo et al., 2017). To examine whether similar regulation occurs in *M. truncatula*, we checked MtNRT2.1 expression levels in WT, *Mtcra2*, and *Mtnlp1* roots after treatment with synthetic MtCEP1 peptide. This reveals that MtNRT2.1 is induced by MtCEP1 peptide in WT and *Mtnlp1*, but not in *Mtcra2* roots (Figure 4C). This suggests that CEP1's effect on nodulation may depend on MtNRT2.1-mediated nitrate uptake. To address this possibility, we assessed the nitrate uptake ability of the *Mtcra2* mutant using K¹⁵NO₃ at 0.5 mM (LN) or 5 mM (HN). This shows that the nitrate uptake ability of the mutant is reduced compared with WT both at LN and HN (Figure 4, D–G). These findings are consistent with observations made for the Arabidopsis *cepr1 cepr2* mutant (Tabata

et al., 2014) and the *M. truncatula cra2* mutant (Bourion et al., 2014).

We then used a split-root system to investigate whether this effect was systemic. The split roots are grown under ZN conditions, with the media containing CEP1 peptide on just one side (R; Figure 4H). RT-qPCR analysis reveals that MtNRT2.1 expression is higher and essentially equal at both sides of the split root when one side is treated with MtCEP1 (Figure 4I). This suggests that, as in Arabidopsis (Ohkubo et al., 2017), MtCEP1 induces MtNRT2.1 expression in a systemic manner. We then tested nitrate uptake in WT and *Mtnrt2.1* roots under LN conditions after MtCEP1 peptide treatment. We found that treatment with MtCEP1 enhances nitrate uptake in WT roots at LN (Figure 4J), consistent with an earlier report (Roy et al., 2021). However, MtCEP1's effect on nitrate uptake is totally abolished in *Mtnrt2.1* roots (Figure 4K). This suggests that MtNRT2.1 is required for CEP1 promotion of nitrate uptake.

MtCEP1 peptide can increase nodule number in LN conditions (Imin et al., 2013). We examined nodulation in WT and *Mtnrt2.1* after MtCEP1 treatment at LN. We found that MtCEP1 induces an increase in nodule number and ARA at LN, and that these effects are reduced in *Mtnrt2.1* (Figure 4, L and M). In addition, MtCEP1 treatment results in higher nitrate accumulation, and increases shoot and root weight in WT at LN, and these increases are abolished in *Mtnrt2.1* (Supplemental Figure 7). Taken together, our data suggest that the positive effects of MtCEP1 on plant growth and nodule number at LN partly require MtNRT2.1.

MtNRT2.1 is required for HN inhibition of nodulation

LjNRT2.1 was shown to have a central role in nitrate-mediated inhibition of nodulation (Misawa et al., 2022). As MtNRT2.1 is similarly induced by HN (Figure 1A), we further analyzed the *Mtnrt2.1* nodulation phenotype in the presence of 5 or 10 mM KNO₃ 2 wpi with *Sinorhizobium meliloti*. As expected, the total number of nodules and the number of pink nodules in WT are suppressed at both 5 and 10 mM KNO₃, and the effect is stronger at higher concentrations (Figure 5, A–C). In *Mtnrt2.1*, the total nodule number is not suppressed at 5 mM, and is only partly suppressed at 10 mM KNO₃ (Figure 5B). In addition, *Mtnrt2.1* produces more pink nodules and has higher ARA than WT at both KNO₃ concentrations, but still shows some nitrate suppression (Figure 5, A, C, and D). The nitrate content and shoot and root fresh weight of *Mtnrt2.1* are lower than WT at HN (Figure 5E, Supplemental Figure 5).

To further investigate MtNRT2.1's potential role in HN inhibition of nodulation, the expression of three symbiotic genes, *MtNIN*, *MtCLE13*, and *MtNF-YA1*, was analyzed. Their expression is reduced in WT at HN and this repression is lost in *Mtnrt2.1* (Figure 5, F–H). The *Mtnrt2.1* nitrate tolerant nodulation phenotype, nitrate content, and root growth defect at HN are rescued by complementation with MtNRT2.1 (Supplemental Figure 8). The data demonstrate that

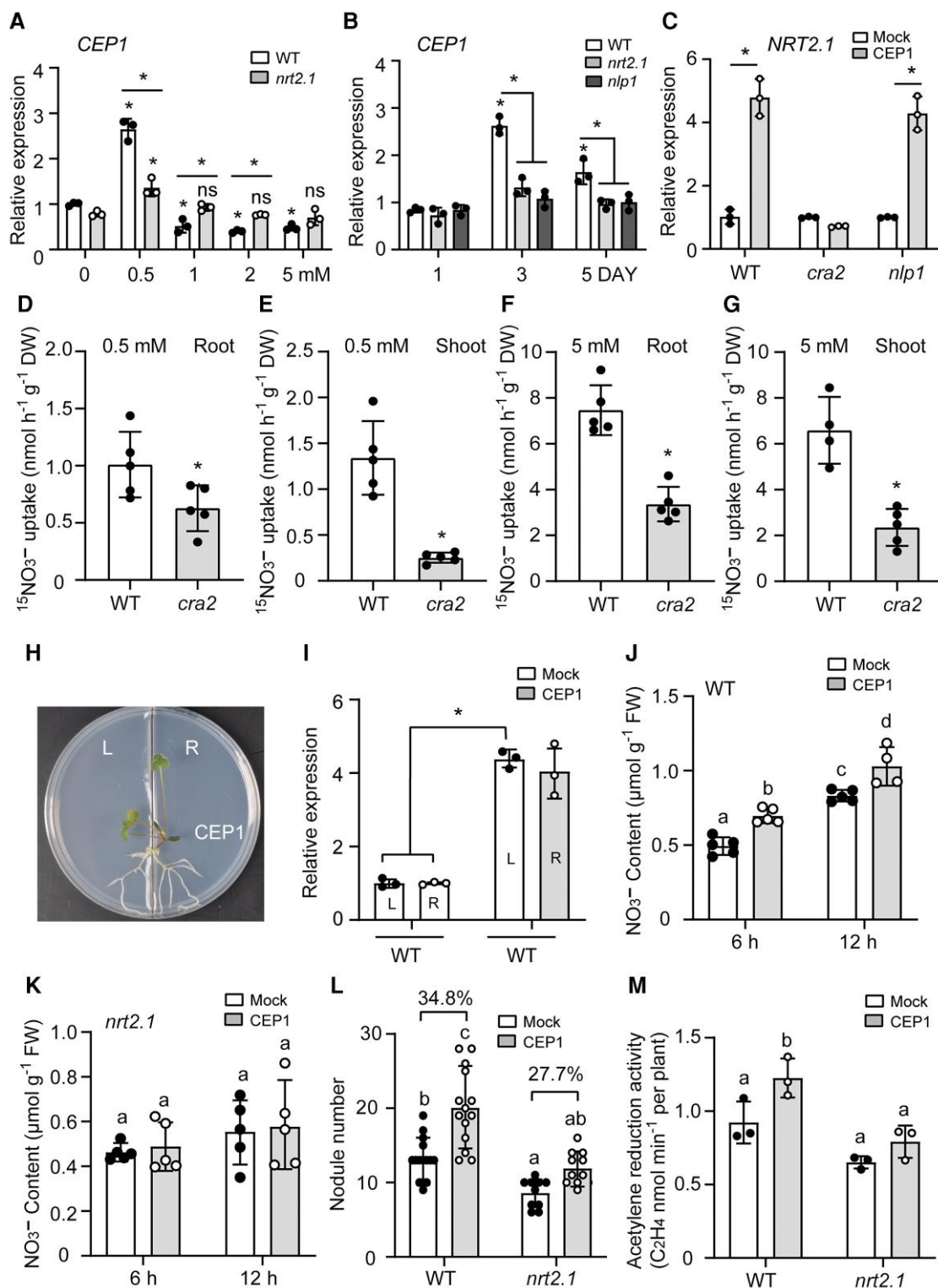


Figure 4 MtCEP1 enhances nodulation via MtNRT2.1. A, Relative expression of MtCEP1 in WT and the *Mtnrt2.1* mutant with 0, 0.5, 1, 2, and 5 mM KNO₃. B, Relative expression of MtCEP1 in WT and *Mtnrt2.1* mutant after treatment with 0.5 mM KNO₃ at 1, 3, and 5 days. Asterisks indicate significant differences compared with the plants with ZN (**P* < 0.05, ns, not significant by a two-tailed *t* test). C, Relative expression of MtNRT2.1 after treatment with 1 μM MtCEP1. Asterisks indicate significant differences at **P* < 0.05 compared with the plants' treatment with water (Mock) by a two-tailed *t* test. Plants were grown on FP plates for 5 days and then transferred to a new plate containing indicated concentrations of KNO₃ (A, B) or 1 μM MtCEP1 peptide (C) for another 3 days. (*n* = 3, each comprising 8–10 plants). D–G, Nitrate uptake by roots (D, F) and shoots (E, G) of WT and *Mtcra2* in 0.5 mM

(continued)

MtNRT2.1 plays an important role in the nitrate inhibition of nodulation.

MtNLP1 activates MtNRT2.1 in response to nitrate

MtNRT2.1 is expressed under LN, HN, and ZN conditions, and expression is lower in *Mtnlp1* roots than in WT roots (Lin et al., 2018). We first confirmed that the loss of MtNLP1 results in lower expression of MtNRT2.1 and showed, using a time course, that LN does not promote MtNRT2.1 expression in *nlp1* (Figure 6A). We then examined whether MtNLP1 is required for LN stimulation of nodulation and found that nodulation stimulation under LN is abolished in the *Mtnlp1* mutant at 2 wpi with *S. meliloti* (Figure 6B). Overexpression of MtNLP1 (*p35S-NLP1*) results in increased induction of MtNRT2.1 in *M. truncatula* hairy roots, and the increase is higher at HN compared with LN (Figure 6C).

We further investigated the nature of this activation by analyzing the MtNRT2.1 promoter sequence. This reveals a putative NRE motif in the MtNRT2.1 promoter region at –248 bp upstream of the start codon (Figure 6D). Then we investigated whether MtNLP1 can directly bind to the MtNRT2.1 promoter to activate its expression using the *pUb-MtNLP1-GR* inducible system (Luo et al., 2021). The expression of MtNRT2.1 in *M. truncatula pUb-MtNLP1-GR* transformed hairy roots treated with dexamethasone (DEX) or DEX and cycloheximide (CHX) was investigated. RT-qPCR analysis revealed that the expression MtNRT2.1 was induced at 4 and 20 h by treatment with DEX. The addition of CHX does not repress the DEX-inducible expression and even enhances it at 4 h (Figure 6E), demonstrating that de novo protein synthesis is not required for the activation of MtNRT2.1 expression. The potential binding of MtNLP1 to the MtNRT2.1 promoter region was then examined using a yeast one-hybrid assay. The results show that MtNLP1 can bind to the MtNRT2.1 promoter region containing the NRE motif, however, this binding does not occur when this NRE motif is deleted (Δ NRE; Figure 6F). We then used a dual-luciferase reporter assay in which *pMtNRT2.1:LUC* or *pMtNRT2.1 Δ NRE:LUC* was co-expressed with *p35S-MtNLP1* or empty vector (EV) in *N. benthamiana* leaves to analyze whether MtNLP1 affects MtNRT2.1 transcriptional activity. This reveals that MtNLP1 can slightly enhance *pMtNRT2.1:LUC* expression at LN, and can highly activate *pMtNRT2.1:LUC* expression at HN, but that the MtNLP1

activation of MtNRT2.1 expression is absent when the NRE motif is removed (*pMtNRT2.1 Δ NRE:LUC*; Figure 6G). These results suggest that MtNLP1 directly binds to the MtNRT2.1 promoter to activate its expression.

Nitrate can trigger MtNLP1 protein shuttling from the cytosol to the nucleus (Lin et al., 2018). We then tested whether this phenomenon also occurs at LN. A construct containing *p35S:GFP-NLP1* and *pLjUb-nls-DsRed* was expressed in *M. truncatula* WT hairy roots, and localization of the GFP-fusion protein was analyzed 10–20 min after nitrate treatment. In agreement with our previous study (Lin et al., 2018), confocal microscopy observations showed that the MtNLP1 localized in the cytosol in the absence of nitrate and translocated to the nucleus in WT roots after HN treatment. After LN treatment, some MtNLP1 translocation to the nucleus is detectable, but is much lower compared to that observed at HN (Supplemental Figure 9). Taken together, our results demonstrate that MtNLP1 directly targets MtNRT2.1 to activate its expression, including a slight activation of MtNRT2.1 expression at LN, and that MtNLP1 protein translocation to the nucleus may be proportional to the level of nitrate present.

Overexpression of MtNRT2.1 can partially rescue the *Mtnlp1* phenotype

To further investigate MtNRT2.1 function in nitrate regulation of root nodule formation, we overexpressed MtNRT2.1 from the *Lotus Ubiquitin* promoter (*pUb-MtNRT2.1*) in WT and *Mtnlp1-1*. The transgenic roots were watered with LN or HN, and the phenotype was characterized 2 wpi with *S. meliloti*. The WT plants transformed with *pUb-MtNRT2.1* develop more total nodules and pink nodules at LN, but less of both types of nodules at HN compared with the EV control (Figure 7, A and B; Supplemental Figure 10). This result suggested that overexpressing MtNRT2.1 in WT can enhance nodulation at LN, but confers hypersensitivity to HN suppression of nodulation. However, the overexpression of MtNRT2.1 can enhance nitrate accumulation, as well as root weight at LN and HN conditions (Figure 7C; Supplemental Figure 10, A, C, E, and F). The overexpression of MtNRT2.1 in *Mtnlp1* similarly enhances the total nodule number and the number of pink nodules at LN, but decreases nodule formation at HN compared with the EV control (Figure 7, A and B; Supplemental Figure 10B), while the

Figure 4 (Continued)

KNO₃ (D, E) and 5 mM KNO₃ (F, G). Plants were grown on a nitrogen-free FP medium for 7 days, pretreated with 0.5 mM or 5 mM KNO₃ for 4 h, then transferred to FP hydroponics medium with 0.5 mM or 5 mM K¹⁵NO₃ for 30 min. ¹⁵N content of shoots and roots was analyzed. H, Split-root system in which the roots of a plant were separated into left (L) and right (R) parts, and right (R) parts which were supplied with either mock (deionized water) or 1 μ M MtCEP1 peptide for 3 days. I, Relative expression of MtNRT2.1 on each side of the split roots of WT ($n = 3$ independent pools of each part of roots from 4 to 6 plants). J, K Nitrate content of the WT (J) and the *Mtnrt2.1* mutant (K) treated with 0.5 mM KNO₃ and supplied with 1 μ M MtCEP1. The roots were harvested and then nitrate content was measured by HPLC ($n = 4–5$, with each biological consisting of 3–5 pooled roots). L, M Nodule numbers (L), nitrogenase activity (M) of WT and the *Mtnrt2.1* mutant in the presence of 0.5 mM KNO₃ supplied with 1 μ M MtCEP1 at 2 wpi with *S. meliloti* ($n = 10–15$ plants). $n = 3$ with each data point in (M) representing a pool of roots from 2 to 3 plants. Different letters represent statistically significant differences ($P < 0.05$, two-way ANOVA with Tukey's multiple comparisons test). Asterisk indicates significant differences (* $P < 0.05$, two-tailed t test). Error bars show SD. The % increase between mock and MtCEP1 treatments is indicated in (L).

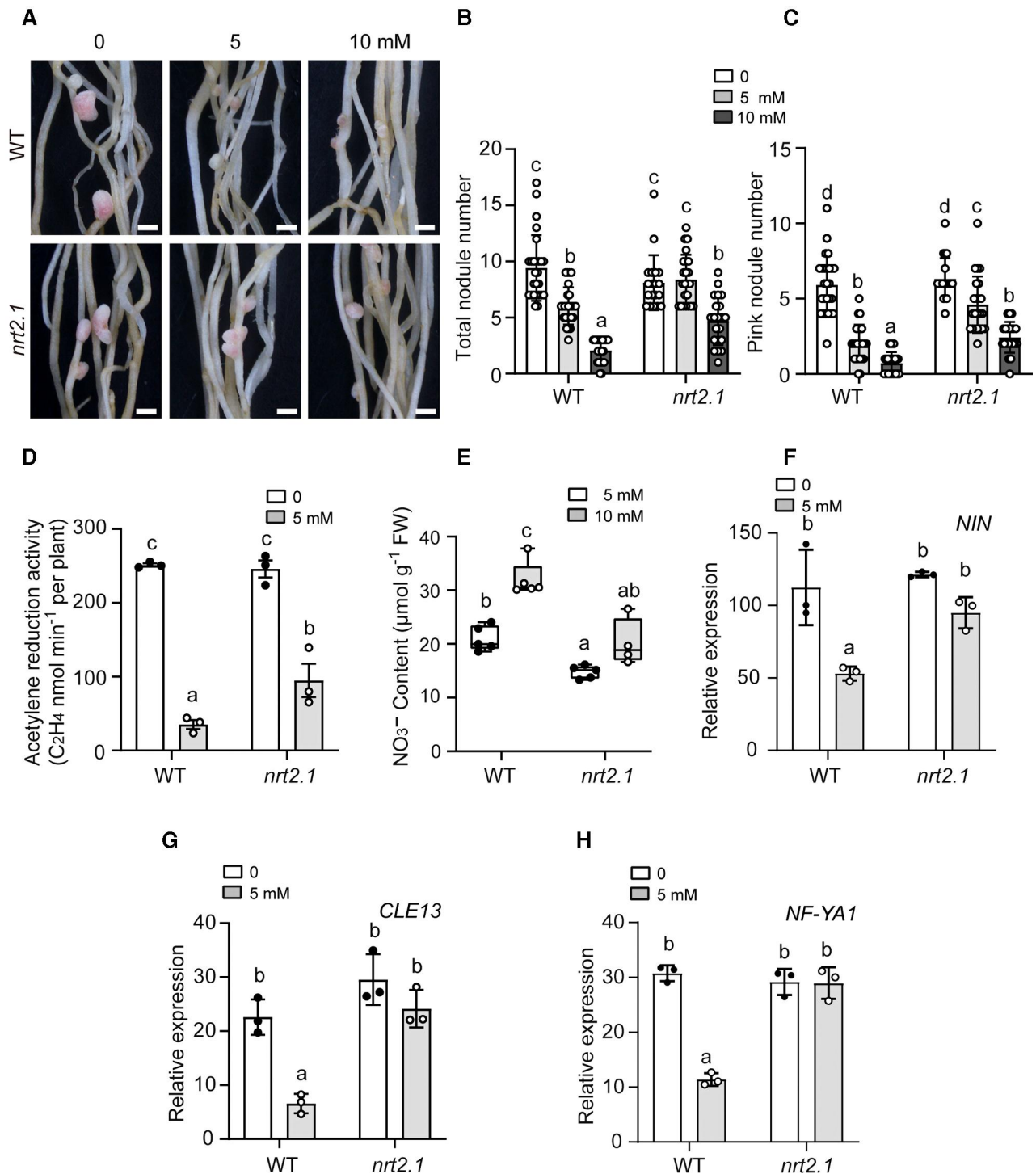


Figure 5 *Mtnrt2.1* nodulation phenotype at HN. A–E, Nodule phenotype (A), total nodule number (B), pink nodule number (C), nitrogenase activity (D), and nitrate content (E) of WT and the *Mtnrt2.1* mutant in the presence of HN at 2 wpi with *S. meliloti* ($n = 19$ –26 plants). $n = 3$ in (D), $n = 4$ –5 in (E), each data point representing a pool of roots from 2 to 3 plants. F–H, Relative expression of *MtNIN* (F), *MtCLE13* (G), and *MtNF-YA1* (H) in WT and *Mtnrt2.1* roots after treatment with 5 mM KNO₃ at 7 dpi. ($n = 3$, each made up of 9–12 plants). Expression levels were normalized relative to the roots without inoculation. Different letters represent significant differences ($P < 0.05$, two-way ANOVA with Tukey's multiple comparisons). Scale bars = 1 mm in (A). Error bars show SD.

nitrate uptake ability of *Mtnlp1* is enhanced under both LN and HN conditions (Figure 7C). Moreover, the

overexpression of *MtNRT2.1* in *Mtnlp1* enhances root weight at HN (Supplemental Figure 10, B, D, E, and G). These results

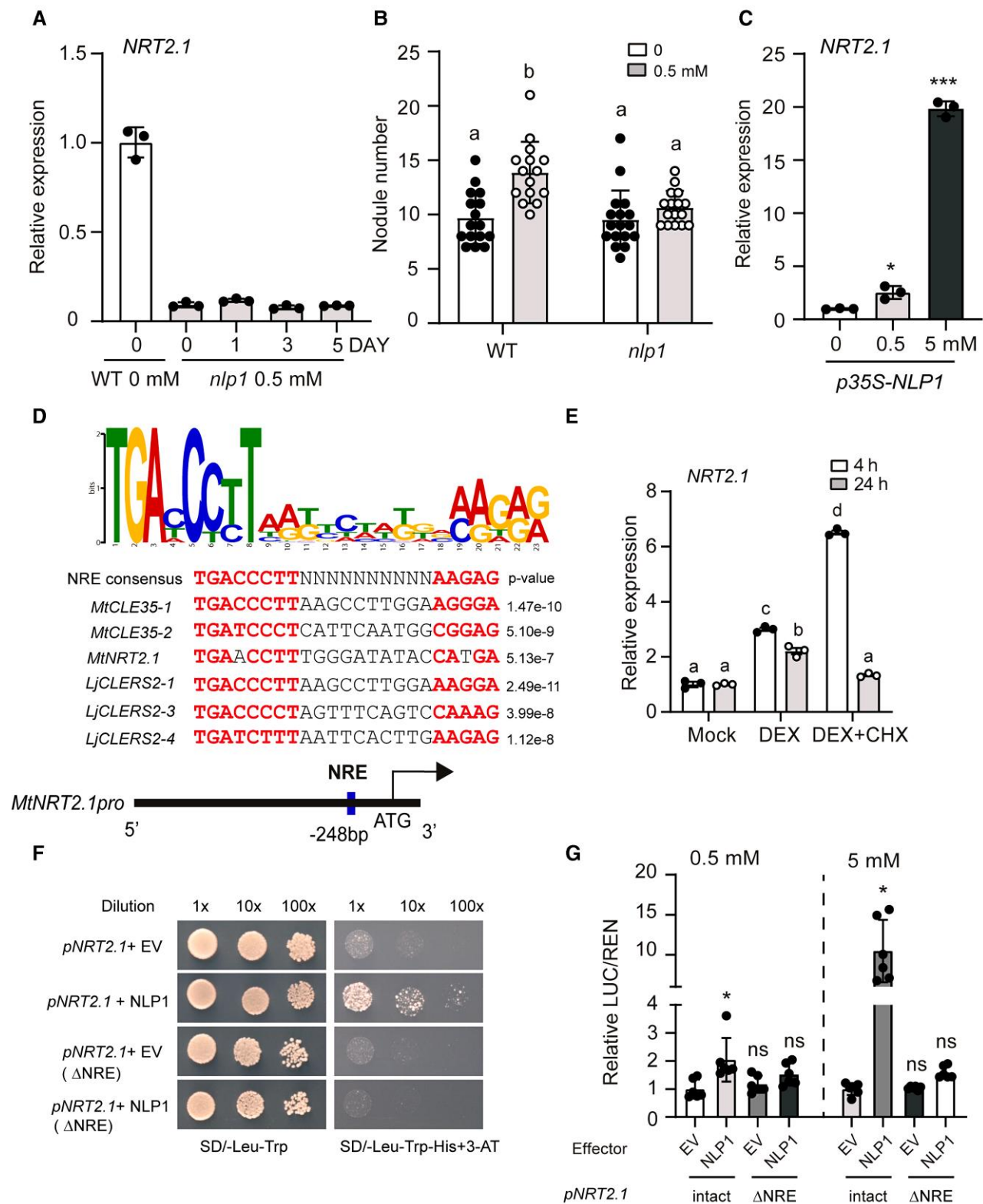


Figure 6 MtNLP1 activates *MtNRT2.1* expression. A, Relative expression of *MtCEP1* in WT and *Mtnlp1* roots after treatment with 0.5 mM KNO_3 for 1, 3, and 5 days. Plants were grown on a nitrogen-free FP medium for 5 days and then transferred to new plates containing 0 or 0.5 mM KNO_3 for an additional 1, 3, or 5 days ($n = 3$, each made up of 8–12 plants). B, Nodule number of WT and the *Mtnlp1* in the presence of 0.5 mM KNO_3 at 2 wpi with *S. meliloti* ($n = 19$ –26 plants). C, Relative expression of *MtNRT2.1* in transgenic hairy roots of WT transformed with EV control and NLP1-overexpression (*p35S-GFP-MtNLP1*) constructs in the presence of 0.5 and 5 mM KNO_3 ($n = 3$, each made up of 7–11 plants). Asterisks indicate

(continued)

suggested that overexpression of *MtNRT2.1* can partially restore *Mtnlp1*'s defects in nitrate uptake, growth, and nitrate inhibition of root nodule formation, and further demonstrates that *MtNRT2.1* promotes nodulation at LN but inhibits nodulation at HN.

To further test whether NLP1 and NRT2.1 function in the same genetic pathway, a construct designed to target *MtNLP1* using RNAi (Lin et al., 2018) was introduced into *Mtnrt2.1* using hairy root transformation and the nodulation phenotype was analyzed in the presence of LN and HN. The results show that compared to EV control roots, knockdown of *MtNLP1* in *Mtnrt2.1* roots does not affect nodule number under either LN or HN conditions (Supplemental Figure 11). This suggests that *MtNLP1* and *MtNRT2.1* function in the same genetic pathway to regulate nodulation in response to nitrate.

Discussion

Legume N fixation is a highly energy-consuming process, and the inhibition of nodulation by HN availability ensures its efficient regulation. In recent years, several studies have demonstrated that NLPs play important roles in HN inhibition of nodulation (Lin et al., 2018; Nishida et al., 2018, 2021). However, compared to N-starvation, low concentrations of nitrate and ammonium, through unknown mechanisms, stimulate nodulation and N_2 fixation in several types of legumes (Barbulova et al., 2007; Fei and Vessey, 2009; Nanjareddy et al., 2014; Ferguson et al., 2019). Notably, the effective nitrate concentration stimulating nodulation differed across these studies. This discrepancy could result from many factors, including the species used, the size of the plant, the growth conditions used, and the type and frequency of N provided. Of the nitrate concentrations (0, 0.5, 1, 2, and 5 mM KNO_3) used in this study, only 0.5 mM was effective in promoting the nodulation of *M. truncatula*. This correlated closely with *CEP1* expression, supporting the role of *CEP1* in this phenomenon. We found that the promotion of nodulation under LN occurs through the nitrate transporter *MtNRT2.1*. Hence, *MtNRT2.1* plays a dual role in nitrate regulation of nodulation, by promoting nodulation at LN, which requires both NLP1 and *CEP1/CRA2*, and inhibiting nodulation at HN, which requires *MtNLP1*.

Although legume plants can fix N, the uptake of inorganic N is still important, especially during early seedling growth when N_2 fixation has not yet been well developed. Harper (1974) reported that soybean plants that depend solely on N fixation show poor growth and low seed yield, suggesting that both soil N and symbiotic N are required for optimum soybean production. Several nitrate transporters have been identified in *L. japonicus* and *M. truncatula*, but none of them have been reported to be involved in LN stimulation of nodulation. Here, we found that *MtNRT2.1* is expressed in root vascular tissues and the apex of nodules, implying that *MtNRT2.1* might be involved in root nodulation. Indeed, the *Mtnrt2.1* mutant develops less nodules and has lower ARA, and overexpression of *MtNRT2.1* can enhance nodulation at LN, suggesting that *MtNRT2.1* is essential for nitrate uptake and the stimulation of nodulation at LN. In *A. thaliana*, *AtNRT2.1* interacts with *AtNAR2.1* to form functional HATS (Orsel et al., 2006). Similarly, we found that *MtNRT2.1*'s ability to uptake nitrate in *X. laevis* oocytes requires *MtNAR2*, suggesting that *MtNRT2.1* is a typical HATS transporter. However, we also noticed that *MtNRT2.1* functions at HN, and thus appears to also operate as a LATS transporter. Moreover, nitrate uptake ability was significantly impaired in *Mtnrt2.1* and *Mtnlp1* at both LN and HN, suggesting that they contribute to nitrate uptake across a broad range of nitrate concentrations. *Mtnrt2.1* shoot and root biomass were smaller than the wild type at both LN and HN. This differs from *Atnrt2.1* (Orsel et al., 2006), but is similar to *Ljnrt2.1*, which has reduced shoot biomass at HN (Misawa et al., 2022). These observations suggest that legume NRT2.1s may function differently than their orthologs in nonsymbiotic species.

MtNRT2.1 is up-regulated by LN and HN in a *MtNLP1*-dependent manner (Lin et al., 2018). *MtNLP1* needs to translocate to the nucleus to function as a transcription factor, and this translocation was minimal after LN treatment. Nonetheless, *MtNRT2.1* expression was very low in *Mtnlp1* mutants even in the absence of external nitrate, and overexpression of *MtNLP1* can slightly activate *MtNRT2.1* expression under LN. This suggests that *MtNLP1* activation of *MtNRT2.1* is proportional to the external nitrate concentration, and that our inability to detect *MtNLP1* in the nucleus in the absence of external nitrate may reflect the

Figure 6 (Continued)

significant differences compared with the transformed roots with ZN (* $P < 0.05$, *** $P < 0.001$, one-way ANOVA with Dunnett's multiple comparisons test). D, Alignment of NRE motifs previously identified in *MtCLE35*, *LjCLE-RS2*, and *MtNRT2.1* promoters, performed using the MEME algorithm (<http://meme-suite.org/index.html>). Red letters represent conserved nucleotides. E, Relative expression of *MtNRT2.1* in *pUbi-MtNLP1-GR* transgenic hairy roots supplemented with mock (DMSO), dexamethasone (DEX), or DEX + cycloheximide (CHX) for 4 or 24 h ($n = 3$, each consisting of pooled roots from 6 to 8 plants). Different letters represent statistically significant differences ($P < 0.05$, two-way ANOVA with Tukey's multiple comparisons test). F, Yeast one-hybrid assay of *MtNLP1* binding to the NRE in the *MtNRT2.1* promoter. Binding assays were performed with *MtNLP1* against the synthetic NRE, or against the *MtNRT2.1* with a deleted NRE region. Medium lacking leucine and tryptophan (-Leu -Trp) or leucine, tryptophane, and histidine (-Leu -Trp -His) was supplemented with 100 mg mL⁻¹ 3-AT. Yeast cells were diluted in a 10× dilution series. G, The luciferase activity of *N. benthamiana* leaves co-transformed with LUX driven by the *MtNRT2.1* promoter and p35S-*MtNLP1* in the presence of 0.5 and 5 mM KNO_3 ($n = 6$). Asterisks indicate significant differences compared with the EV control (* $P < 0.05$, one-way ANOVA with Dunnett's multiple comparisons test). Error bars show SD.

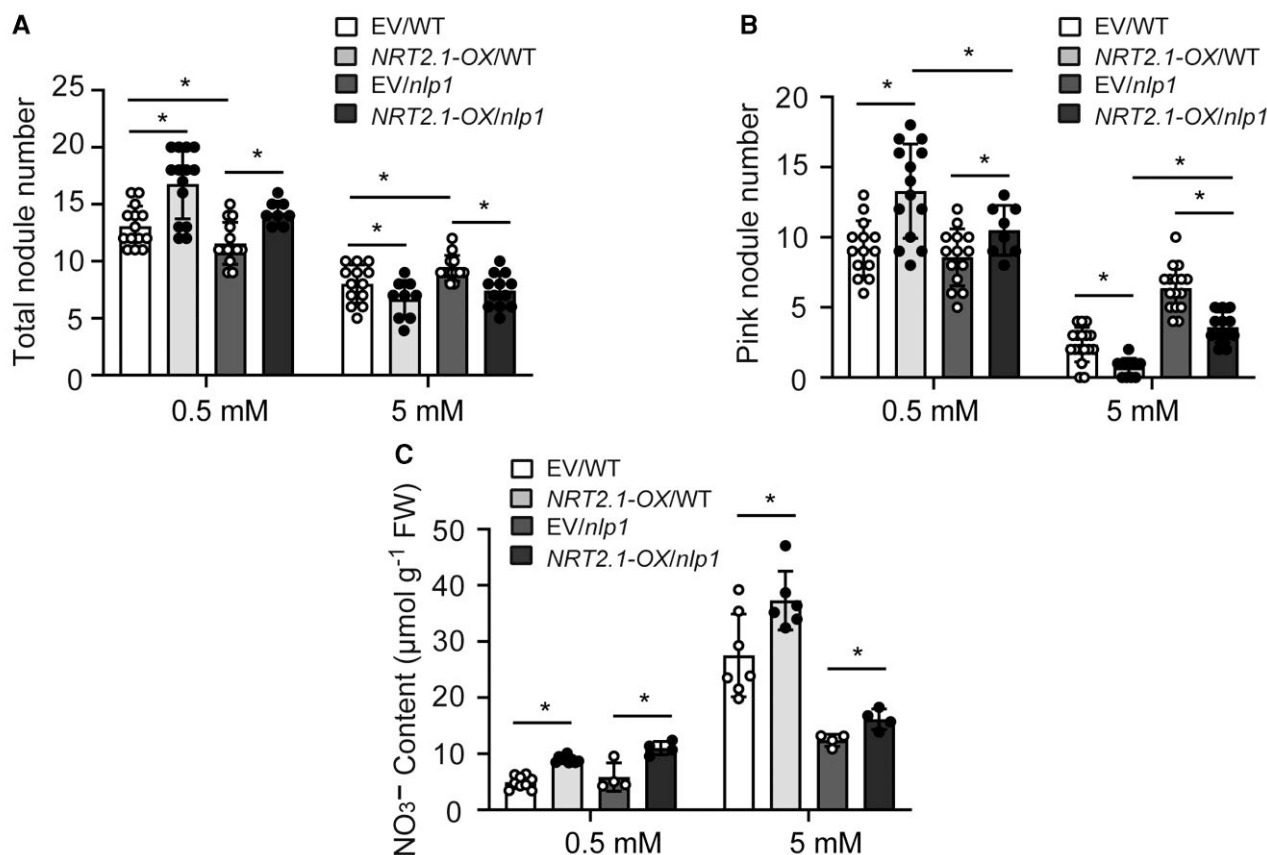


Figure 7 The effects of overexpression *MtNRT2.1* in WT or *Mtnlp1* hairy roots on nodule number and nitrate content. A–C, Total nodule number (A), number of pink nodules (B), and nitrate content (C) in the hairy roots of WT and *Mtnlp1* overexpressing *MtNRT2.1*. The phenotype was scored at 2 wpi with *S. meliloti* with continuous treatment with 0.5 or 5 mM KNO₃ ($n = 8–16$ plants). Each data point representing a pool of roots from 1 to 3 plants in (C). Asterisk indicates significant differences (* $P < 0.05$, two-tailed t test). Error bars show SD.

sensitivity of detection. A similar proportional effect of nitrate levels on gene expression was reported for the nodule-expressed NLP2 (Jiang et al., 2021).

The AtCEP1 peptide is recognized as a “hunger signal” by the plant, and systemically up-regulates the expression of *AtNRT2.1* depending on the CEP1 receptors AtCEPR1 and AtCEPR2 (Ohkubo et al., 2017). A previous study found that *MtCEP1* expression is higher in nitrate-starved plants compared to those fed with mixed N (Imin et al., 2013), while our data indicate that *CEP1* expression is higher in plants under 0.5 mM KNO₃ than in nitrate-starved plants. This apparent discrepancy can be explained by the strong negative impact of ammonia on *NRT2.1* expression (Muños et al., 2004). We thus conclude that *MtCEP1* expression is higher under 0.5 mM nitrate than under nitrate starvation. This is consistent with a recent study that shows that synthetic *MtCEP1* peptide can increase nitrate content at 0.5 mM KNO₃, but not at higher or lower concentrations (Roy et al., 2021). Similar to findings reported in *Arabidopsis*, we found that *MtCEP1* can induce *MtNRT2.1* expression in a systemic manner, and that *MtNRT2.1* is required for *MtCEP1* induction by LN. However, *MtCEP1* expression is repressed by *MtNLP1* at HN (Luo

et al., 2022). Here, we found that LN promotes *MtCEP1* expression in an *MtNLP1* and *MtNRT2.1*-dependent manner. We speculate that under LN conditions, a limited amount of *MtNLP1* can enter the nucleus, where it directly induces low-level *MtNRT2.1* expression and activates *MtCEP1* expression through an unknown mechanism. This suggests that plants dynamically adjust the expression of CEPs to respond to environmental fluctuations in nitrate availability to appropriately regulate growth. Moreover, the enhancement of nodulation by *MtCEP1* peptide was impaired in *Mtnrt2.1* at LN, suggesting that *MtCEP1* peptide and *MtNRT2.1* form a regulatory loop for plant growth and nodulation under LN. *MtCEP1* belongs to a large gene family, some of which are similarly regulated and have activities in nodulation. *MtCEP1*, 2, and 12 were shown to redundantly regulate lateral root number and nodulation (Zhu et al., 2021). Recently we showed that *MtCEP1* and *MtCEP2* are highly expressed in *Mtcr2* roots in the presence or absence of nitrate (Luo et al., 2021). Moreover, *MtCEP7*, which is transcriptionally activated by *MtNIN*, similarly promotes nodulation through CRA2 (Laffont et al., 2020). Thus, it appears likely that CEP1 acts together with other CEPs to promote nodulation under LN

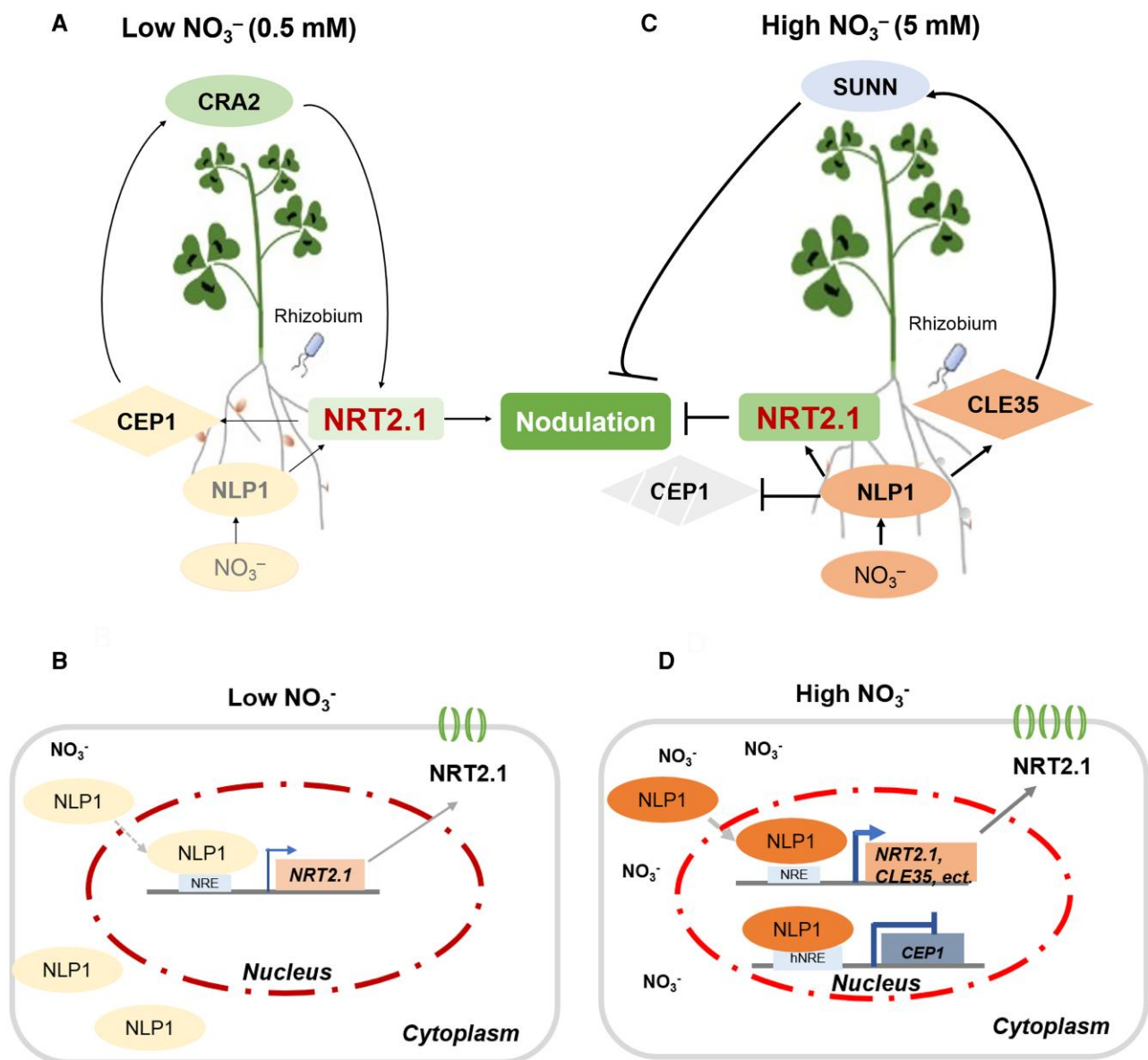


Figure 8 Proposed a model for LN and HN regulation of nodulation in *M. truncatula*. A, In the presence of low NO_3^- (0.5 mM), *MtCEP1* is expressed and the encoded peptides systemically induce *MtNRT2.1* expression through *MtCRA2* in the shoot. *MtNRT2.1* mediated nitrate uptake further enhances nodulation and *MtCEP1* expression. B, Under low NO_3^- , limited nucleus localization of *MtNLP1* activates low-level *MtNRT2.1* expression. C and D, In the presence of high NO_3^- (5 mM), nitrate triggers *MtNLP1* shuttling to the nucleus, then *MtNLP1* activates *MtCLE35* and represses *MtCEP1* expressions which further systemically regulate nodulation. Meanwhile, *MtNLP1* activates *MtNRT2.1* expression and promote nitrate uptake which further represses nodulation.

conditions. In the study of *LjNRT2.1*, 0.2 mM nitrate failed to stimulate nodulation in wild-type plants (Misawa et al., 2022). This might be due to the different nitrate concentrations used. Moreover, there are so far no reports on *CEP* function in legumes that form determinate nodules, so whether the mechanisms identified here in *Medicago* are present in other legumes remains to be determined.

The stimulation of nodulation and nitrate content by LN and *MtCEP1*, although attenuated, was intact in

Mtnrt2.1, suggesting that other nitrate transporters, such as *MtNRT2.2* may be involved. However, the expression of *MtNRT2.2* is very low (Pellizzaro et al., 2015), implying that it may not play an important role in the promotion of nodulation under LN. Knockout of another high-affinity nitrate transporter, *MtNPF7.6*, reduced nitrate responsiveness both at LN and HN, but its involvement in LN promotion of nodulation was not studied (Wang et al., 2020).

Based on our findings and the findings of others (Roy et al., 2021; Luo et al., 2022; Misawa et al., 2022), we propose that LN induces *MtCEP1* expression, which systemically up-regulates *MtNRT2.1* expression in a *MtCRA2*-dependent manner. Meanwhile, under LN, limited nuclear localization of *MtNLP1* activates low-level *MtNRT2.1* expression, thus enhancing nitrate uptake to improve plant growth and nodulation (Figure 8, A and B). However, HN triggers a major migration of *MtNLP1* to the nucleus, where it activates *CLE35* expression, which negatively regulates nodulation through *SUNN* in the shoot. In addition, *MtNLP1* activates *MtNRT2.1* expression to promote nitrate uptake, and further inhibit nodulation (Figure 8, C and D), and suppresses *MtCEP1* expression which would otherwise positively regulate nodulation through *MtCRA2* in the shoot (Luo et al., 2021, 2022).

Materials and methods

Plant materials and growth conditions

In this study, the R108 ecotype of *M. truncatula* was used as the WT. The *Mtnrt2.1* mutant was obtained from the *Tnt1* retrotransposon-tagged mutants of *M. truncatula* as described below. The *Mtcra2-2* mutant was previously described by Huault et al. (2014). The *Mtnlp1-1* mutant was previously described by Lin et al. (2018). The *Agrobacterium rhizogenes* strain AR1193 was used for *M. truncatula* root transformation, and the *Agrobacterium tumefaciens* EHA105 or GV3101 strains were used for *N. benthamiana* transient expression. *Sinorhizobium meliloti* 2011 was used in nodulation assays. *M. truncatula* seeds were scarified using sandpaper, then immersed in 10% sodium hypochlorite for 3–5 min, and washed with sterilized water. The sterilized seeds were imbibed overnight in sterilized water, and then transferred to 0.8% agar plates for germination. The germinated seedlings were grown on a mixture of perlite and vermiculite (1:1) or 1.2% N-free FP agar plates under a 16 h/8 h light (250 $\mu\text{mol m}^{-2} \text{s}^{-1}$)/dark photoperiod at 22°C.

Mtnrt2.1 mutant genotyping

The *Mtnrt2.1 Tnt1* insertion mutant (line NF6636) was identified from the *Tnt1*-mutant collection at Oklahoma State University (<https://medicago-mutant.dasnr.okstate.edu/mutant/index.php>). The *MtNRT2.1* specific reverse primer and *Tnt-R1* primer were used for genotyping. The insertion site was identified by sequencing the PCR-amplification products using the 2×HiEff PCR Master Mix (Cat No.10102ES60; Yeasen, Shanghai, China). The relevant primers are shown in Supplemental Table 1.

MtNRT2.1 overexpression and *Mtnrt2.1* mutant complementation

Full-length *MtNRT2.1* coding region was obtained by PCR from *M. truncatula* A17 genomic DNA and cloned into pDONR207, then transferred into pUb-GW-GFP by LR recombination reaction to generate pUb-*MtNRT2.1*. To

generate the *MtNRT2.1pro-MtNRT2.1* construct, the *Lotus Ubiquitin* promoter was replaced by a 2-kb *MtNRT2.1* promoter using *MssI/XbaI* restriction sites using the ClonExpress II One-Step Cloning kit (Vazyme Biotech co., Ltd). The relevant primers are listed in Supplemental Table 1. The constructs were introduced into WT or the *Mtnrt2.1* mutant by *A. rhizogenes* mediated hairy root transformation. The GFP reporter gene was used to identify transgenic roots using a fluorescence lamp (LUYOR-3415RG, LUYOR). Three weeks after transformation, the transgenic plants were transferred to a vermiculite and perlite (1:1) mixture and acclimatized for 3–5 days prior to inoculation with Sm2011. The plants were watered with the indicated concentrations of KNO_3 twice a week. The nodule number was scored 2 weeks after inoculation with rhizobia. These experiments were repeated 3 times.

K^{15}NO_3 uptake in *X. laevis* oocytes

Oocyte preparation, cRNA injection, and $^{15}\text{NO}_3^-$ uptake measurements were conducted as previously described (He et al., 2017). Briefly, # the coding sequence of *MtNRT2.1* or *MtNAR2.1* was amplified by PCR from *M. truncatula* A17 cDNA and was cloned into the oocyte expression vector pOO2 using *BamHI/SpeI* restriction sites. cRNA was synthesized using the Ambion mMessage mMachine kit according to the manufacturer's protocol. Oocytes were injected with 50 ng indicated cRNA. Water-injected oocytes were used as the negative control. Oocytes were incubated in ND-96 Ringer solution for 2 days as described (Li et al., 2010). Then the oocytes were incubated with 0.5 and 5 mM K^{15}NO_3 at pH 5.5 for 3 h. Each oocyte was dried for 2 days at 70°C and the ^{15}N contents were determined by a continuous-flow isotope ratio mass spectrometer coupled to a carbon–nitrogen elemental analyzer (Vario EL III/Isoprime; Elementar).

Analysis of nitrate uptake using $^{15}\text{NO}_3^-$

Nitrate uptake was assessed by the $^{15}\text{NO}_3^-$ stable isotope as described (Zhang et al., 2014; Misawa et al., 2022). 7-day-old seedlings grown on N-free FP medium were then transferred to new FP liquid medium with 0.5 mM or 5 mM KNO_3 for 4 h. Then, they were washed with 0.1 mM CaSO_4 for 1 min, and placed in FP hydroponics medium with 0.5 mM or 5 mM K^{15}NO_3 with 99% atom excess of ^{15}N for 30 min. Afterward, plants were washed for 1 min in 0.1 mM CaSO_4 and again washed at least three times by ultrapure water; then, roots were separated from shoots. Each sample was dried for 2 days at 65°C and analyzed for ^{15}N contents by a continuous-flow isotope ratio mass spectrometer coupled to a carbon–nitrogen elemental analyzer (Vario EL III/Isoprime; Elementar).

Analysis of nitrate content using HPLC

Nitrate content was measured by HPLC (Agilent 1200 series) as described (Meng et al., 2016). Wild-type and *Mtnrt2.1* mutant plants were grown in FP agar medium without nitrate for 12 days and then pretreated with 1 μM *MtCEP1* peptide

or ultrapure water control for 48 h as described by Roy et al. (2021). Next, 5 mL of the indicated concentration of nitrate solution in ultrapure water was added to each square plate (grown 8–10 seedlings). Roots were sampled and washed three times with ultrapure water. Three to five roots were pooled together to make a biological repeat: 5–6 biological repeats each time. This experiment was repeated three times.

Nitrate content measurement was performed using the salicylic acid method (Xu et al., 2016; Hachiya and Okamoto, 2017). Briefly, root samples (50 or 100 mg) were frozen in liquid N and crushed into powder using a SM-48R Freeze Grinder (Shanmi, China). Then, 1 mL of ultrapure water (preheated at 80°C) was added into a 2 mL tube followed by incubating at 100°C for 20 min. The tubes were centrifuged at 20,400g for 10 min. Next, 10 µL of the supernatant was transferred into a 1.5 mL tube and 40 µL of 0.5% (w/v) salicylic acid in sulfuric acid was added, and then, the samples were vortexed well. The reactions were incubated at room temperature for 20 min; then 1 mL of 8% NaOH solution was added gently and vortexed. The OD 410 nm absorbance values of the samples were determined by a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific). The nitrate content was calculated using the formula: [true nitrate concentration (mM)] × [extracted volume (mL)]/[fresh weight (g)]. A standard curve was made with KNO₃ dilution series (0, 0.125, 0.25, 0.5, 1, 2, 4, and 8 mM). This experiment was repeated 3 times.

ARA assays

ARA experiments were conducted according to the previous description (Luo et al., 2021). The plants were harvested at 2 wpi, roots were removed from the plant and placed in 10 mL sealed glass vials (three plants per sample), and then, 0.5 mL acetylene was injected into each vial and incubated for 30 min at 28°C. Acetylene and ethylene concentrations were measured using a gas chromatograph (GC9310, Shanghai Chromatographic Instruments Co., Ltd., Shanghai, China). These experiments were repeated 3 times: 3–5 biological repeats each time.

CEP1 peptides treatment and rhizobium inoculation

MtCEP1 domain 1 peptide with hydroxyprolines (HyP) 4- and HyP11-modification were synthesized by GL Biochem Pty Ltd (Shanghai, China; Mohd-Radzman et al., 2015). WT and *Mtnrt2.1* mutant germinated seedlings were transferred to the vermiculite and perlite mixture pots grown for 7 days and pretreated with 0.5 mM KNO₃, then treated twice with 1 mM CEP1 peptide. One week later, the 14 days old plants were inoculated with Sm2011 (OD₆₀₀ = 0.02). Then, the plants were continuously watered with the 0.5 mM KNO₃ supplement with 1 mM CEP1 twice a week. The nodule number, ARA, growth phenotype, and nitrate accumulation were scored at 2 wpi. This experiment was repeated 2 times: 10–15 plants for each biological repeat.

Split-root experiments

To generate split roots, we used *A. rhizogenes* AR1193-induced adventitious roots as described in the *M. truncatula* Handbook, and the roots were allowed to grow in N-free FP agar plates for additional 7 days. Then, the two-part roots of each plant were transferred into a 90 mm petri dish with two compartments (Leiboer, China) containing a FP medium on one side and a FP medium supplemented with 1 µM MtCEP1 peptide on the other side. Three days later, the two root parts were separately harvested to analyze the gene expression. This experiment was repeated 3 times.

Gene expression analysis

Total RNA was extracted from *M. truncatula* roots using the TRIzol Reagent (Invitrogen). RNA quantification was measured by using a Nanodrop 2000 (Thermo Fisher). First-strand cDNA was prepared from 1 µg total RNA using the TransScript One-Step gDNA Removal kit and the cDNA Synthesis SuperMix (Transgen, China). RT-qPCR was performed using a Step-one Plus PCR system (Thermo Fisher, USA) with a TB Green Premix Ex Taq (Takara, China). *MtUbiquitin10* (*MtUBQ10*) was used as a reference gene for normalization. The relative levels of gene expression were calculated using the $2^{-\Delta\Delta C_t}$ method. The primers used in RT-qPCR are listed in Supplemental Table 1.

Promoter:GUS analysis

The upstream 1499 bp promoter region of *MtNRT2.1* was amplified by PCR using *M. truncatula* A17 genomic DNA as a template. The PCR product was inserted into the pCambia1381 vector via *Bam*HI/*Nco*I restriction sites and confirmed by sequencing. The relevant primers are shown in Supplemental Table 1. The *pMtNRT2.1:GUS* construct was then introduced into *M. truncatula* by *A. rhizogenes* mediated transformation to generate transgenic roots. These transformed plants were then transferred into vermiculite/perlite (1:1) pots and inoculated with Sm2011. Transgenic roots were harvested at indicated time points and then incubated in a GUS staining solution at 37°C for 30–60 min.

MtNLP1 overexpression and dexamethasone/cycloheximide treatments

MtNLP1 overexpression and dexamethasone induction of gene expression were performed as described previously (Luo et al., 2021). A previously reported 35S-GFP-MtNLP1 construct (Lin et al., 2018) was used for overexpression. MtNLP1-overexpressing composite plants were supplemented with 0, 0.5, and 5 mM KNO₃. A previously created *pUb-NLP1-GR* plasmid (Luo et al., 2021) was used for the DEX induction of gene expression, and the transformed roots of plants were incubated with 10 µM dexamethasone (DEX) or with cycloheximide (CHX) + DEX combination (10 µM each) for 4 h or 24 h. The transgenic roots were then harvested for RNA extraction and analyzing gene expression.

Dual-luciferase (LUC) reporter assays

The upstream 2-kb promoter regions of *MtNRT2.1* was amplified from *M. truncatula* A17 genomic DNA, and cloned into the pGreenII-0800 vector to generate *MtNRT2.1pro::LUC* reporter construct. *MtNRT2.1pro::ΔNRE::LUC* was amplified by PCR using the *MtNRT2.1pro::LUC* vector as a template and recombination by the ClonExpress II One-Step Cloning kit. Primers used are listed in [Supplemental Table 1](#). A previously reported 35S-*MtNLP1* was used as an effector construct (Luo et al., 2021). These constructs were then introduced into *N. benthamiana* leaves by *A. tumefaciens* GV3101-mediated transient transformation. The dual-luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's protocol. This experiment was repeated 3 times.

Yeast one-hybrid assay

About 300 bp upstream of the initiation codon of *MtNRT2.1* promoter was amplified using the *MtNRT2.1pro::LUC* vector as a template and the *MtNRT2.1pro::ΔNRE* was generated by PCR using the *MtNRT2.1pro::ΔNRE::LUC* vector as a template, then inserted into the *pHIS2* vector through *EcoRI* and *MluI* restriction sites. Primers used are listed in [Supplemental Table 1](#). The pGADT7-NLP1 construct was previously described (Luo et al., 2021). These different vectors were co-transformed into the Y187 yeast strain using the Matchmaker One-Hybrid Library Screening System (Clontech). Transformed yeasts were selected on SD/-Leu-Trp and SD/-Leu-Trp-His medium supplemented with the 100 mM 3-AT antibiotic, and yeast cells were diluted in a 10× dilution series.

NLP1 knockdown by RNAi

A previously reported *pUb-MtNLP1-RNAi* construct (Lin et al., 2018) was introduced into WT or the *Mtnrt2.1* mutant by *A. rhizogenes* mediated hairy root transformation. The *pUb-GWS-GFP* EV was used as a control. The GFP reporter gene was used to identify transgenic roots. Three weeks after transformation, the transgenic plants were transferred to a vermiculite and perlite (1:1) mixture and acclimatized for 3–5 days prior to inoculation with Sm2011. The plants were watered with the indicated concentrations of KNO₃ twice a week. The nodule number was scored at 2 wpi.

NLP1 subcellular location

The *pDONR207-MtNLP1* (Lin et al., 2018) was recombined into a modified pK7WGF2 (a *pUb-nls-DsRed* cassette was inserted using *XbaI* and *BspTI* restriction sites) via LR reaction. This construct was expressed in WT hairy roots and the *nls-DsRed* reporter gene, which was used as a nuclear localization marker. The transgenic plants were grown on N-free FP medium for 10–14 days, and were then incubated with 0, 0.5, and 5 mM KNO₃ for 10–20 min before observation using a confocal microscope (TCS SP8 STED, Leica). This experiment was repeated 3 times, and 5–10 plants were used each time.

Phylogenetic analysis

The protein sequences were obtained from Phytozome (<https://phytozome-next.jgi.doe.gov/>), and were aligned using ClustalW. Phylogenetic analysis was performed in MEGA11 software using the neighbor-joining method with the bootstrapping value set at 1,000 replications. The percentage of replicate trees in which the associated taxa clustered is shown below 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. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Sequence alignment and machine-readable tree files are provided as [Supplemental Files 1 and 2](#), respectively.

Statistical analyses

Statistically significant differences were calculated using GraphPad Prism version 8 (GraphPad software). Statistical analyses were conducted using the two-tailed *t* test or ANOVA test. One-way or two-way ANOVAs were performed with Tukey's or Dunnett's multiple comparisons test at a significant level of *P* < 0.05. For each boxplot, the center line in the box shows the median; the box limits are the upper and lower quartiles; and the whiskers represent the maximum and minimum values. Statistical data are provided in [Supplemental Data Set 1](#).

Accession numbers

MtNRT2.1, MtrunA17_Chr4g0028551; *MtNRT2.2*, MtrunA17_Chr4g0028541; *MtCEP1*, MtrunA17_Chr8g0374821; *MtNIA1*, MtrunA17_Chr5g0424491; *MtNIR1*, MtrunA17_Chr4g0044831; *MtCLE13*, MtrunA17_Chr4g0040951; *MtCLE35*, MtrunA17_Chr2g0325381; *MNIN*, MtrunA17_Chr5g0448621; *MtNF-YA1*, MtrunA17_Chr1g0177091; *MtNLP1*, MtrunA17_Chr3g0143921 from the *M. truncatula* genome v5.0 (<https://medicago.toulouse.inra.fr/MtrunA17r5.0-ANR/>); *MtNRT2.1*, MtrunR108HiC_017743; *MtNRT2.2*, MtrunR108HiC_017742 were retrieved from the *M. truncatula* R108 genome v1.0 (https://medicago.toulouse.inrae.fr/MtrunR108_HiC/).

Supplemental data

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

Supplemental Figure 1. Phylogenetic tree of the NRT2 family from *M. truncatula*, *L. japonicus*, and *A. thaliana*. (Supports Figure 1.)

Supplemental Figure 2. *MtNRT2.1* expression pattern in *M. truncatula* root nodule formation. (Supports Figure 1.)

Supplemental Figure 3. Identification of the *Mtnrt2.1* mutant. (Supports Figure 1.)

Supplemental Figure 4. Time course of *MtNRT2.1*, *MtCLE35*, *MtNIA1*, and *MtNIR1* gene expression after LN treatment. (Supports Figure 3.)

Supplemental Figure 5. The growth phenotypes of *Mtnrt2.1* in the presence of low- and high- nitrate. (Supports Figure 3.)

Supplemental Figure 6. Genetic complementation of *Mtnrt2.1* at low nitrate. (Supports Figure 3.)

Supplemental Figure 7. MtCEP1 peptide promotes nodulation at LN in a *MtNRT2.1* dependent way. (Supports Figure 4.)

Supplemental Figure 9. MtNLP1 accumulates in the nuclei in response to low- and high-nitrate. (Supports Figure 6.)

Supplemental Figure 10. Phenotype of overexpression *MtNRT2.1* in WT and *Mtnlp1*. (Supports Figure 7.)

Supplemental Figure 11. Nodulation phenotype in *MtNLP1* RNAi in *Mtnrt2.1*. (Supports Figure 7.)

Supplemental Table 1. Primer sequences used in this study.

Supplemental Data Set 1. Statistical analysis data.

Supplemental File 1. The NRT2s protein sequence alignment for phylogenetic analysis.

Supplemental File 2. Machine-readable tree file for phylogenetic analysis.

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