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SHORT COMMUNICATION

Autoregulation of nodulation pathway is dispensable for nitrate-induced control of rhizobial infection

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ABSTRACT

Legumes possess the autoregulation of nodulation (AON) pathway which is responsible for maintaining optimal root nodule number. In *Lotus japonicus*, AON comprises the CLE-HAR1-TML module, which plays an essential role in transmitting signals via root-to-shoot-to-root long-distance signaling. In addition to AON's principal role of negatively regulating nodule number, a recent study revealed another in the systemic control of rhizobial infection. Nitrate also negatively regulates the pleiotropic phases of legume-*Rhizobium* symbioses, including rhizobial infection and nodule number. Nitrate signaling has recently been shown to use AON components such as CLE-RS2 and HAR1 to control nodule number. Here we consider the role of a loss-of-function mutation in *CLE-RS1, -RS2* and *TML* in rhizobial infection in relation to nitrate. Our results agree with previous findings and support the hypothesis that AON is required for the control of rhizobial infection but not for its nitrate-induced control. Furthermore, we confirm that the *tml* mutants exhibit nitrate sensitivity that differs from that of *cle-rs2* and *har1*. Hence, while the nitrate-induced control mechanism of nodule number uses AON components, an unknown pathway specific to nitrate may exist downstream of HAR1, acting in parallel with the HAR1> TML pathway.

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Text

Nodulation is a host specific symbiosis between legumes and rhizobia in which symbiotic organs called nodules are formed on the host roots. The host plant benefits by gaining access to atmospheric nitrogen but nodule production and rhizobial nitrogen fixation is biosynthetically costly.[1,2] A mechanism called autoregulation of nodulation (AON) acts as a negative regulatory system in this symbiosis.[3,4] It enables the host plant to strictly control the number of nodules and in turn control energy consumption. AON involves long-distance signaling between the root and shoot systems. The production of CLE peptides plays an important role in the initiation of AON. In *Lotus japonicus*, three CLE peptides (CLE-RS1/2/3) have been shown to be produced by rhizobia in the roots of host plants.[5,6] The induction timing of corresponding *CLE* genes is different after rhizobial infection but constitutive expression of each gene strongly reduces nodule number in a shoot-acting HAR1 receptor-like kinase dependent-manner.[5–7] Hence, the three root-derived CLE peptides function systemically to negatively regulate nodule number. Indeed, a mature form of CLE-RS2 has been detected in xylem sap which can physically interact with HAR1.[8] As a result of this CLE-HAR1 interaction, cytokinin is produced in shoots by the upregulation of a cytokinin synthesis gene, *IPT3*. The resulting cytokinin is translocated from shoot to root, where it inhibits nodulation.[9] A root-acting putative F-box protein, TML, is required for the action of shoot-derived cytokinin.[9–11] In addition, miR2111 which is downstream of HAR1 can be translocated from shoot to root where it targets TML. Unlike shoot-derived cytokinin, miR2111 stimulates nodulation and the miR2111-TML module appears to contribute to the inhibitory effect of AON on nodulation.[12]

While the formation of an increased number of nodules is the most striking phenotype of AON loss-of-function mutants,[10,13,14] these mutants also exhibit hyperinfected phenotypes which are identifiable by an increased number of infection threads (ITs). ITs are plant-derived structures in which the rhizobia move from the root surface to the interior of the root.[15] More recently, CLE-RS1 and -RS2 have been demonstrated to systemically inhibit ITs formation in the unique *nin* mutant, *daphne*, which loses the ability to form nodules yet forms excessive numbers of ITs.[16,17] HAR1 is a prerequisite for the action of these peptides, suggesting that the CLE-HAR1 pathway plays a role in negatively regulating rhizobial infection. Importantly, this is a potentially novel role of the AON pathway. Nevertheless, the detailed mechanism remains elusive. For example, the loss-of-function effects of the *CLE* genes on rhizobial infection are unknown.

Nitrogen sources, such as nitrate, act as another regulatory factor in legume-*Rhizobium* symbioses. Nodule development and rhizobial nitrogen fixation are energy-consuming processes, thus it would be advantageous to the plant to cease the symbiosis when there is sufficient available nitrogen in the soil to allow growth. Host plants respond to nitrate by negatively regulating

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multiple phases of the root nodule symbiosis, including rhizobial infection, nodule number, nodule growth and nitrogen fixation.[2,18–20] We previously identified a *nitrate unresponsive symbiosis 1* (*nrsym1*) mutant in *L. japonicus*. Phenotypic analysis provides evidence that NRSYM1 plays an essential role in the control of nitrate-induced pleiotropic regulation of symbiosis.[21] *NRSYM1* encodes an NLP transcription factor and regulates nitrate-inducible gene expression both in symbiotic and non-symbiotic conditions. In the presence of nitrate, NRSYM1 uses *CLE-RS2* as a direct target gene for the control of nodule number. Nitrate concentration does not affect the excessive nodule production exhibited by *har1* mutants but does influence other elements of the symbiosis such as rhizobial infection, nodule growth and nitrogen fixation. These observations suggest that in NRSYM1-mediated pleiotropic nitrate regulation, the NRSYM1> CLE-RS2> HAR1 pathway predominantly has a role in the control of nodule number.[21]

In order to determine the precise role of *CLE* genes in the control of rhizobial infection, we investigated the infection phenotype of the *cle-rs1, -rs2* and double mutants in conditions with and without nitrate. The *cle-rs* mutants were generated in a previous study using the CRISPR-Cas9 genome editing system.[21] The nodulation phenotypes suggest that *CLE-RS1* and *-RS2* have a redundant function in the negative regulation of nodule number.[21] Nodule number in the *cle-rs2* mutant was unaffected by nitrate concentration suggesting that CLE-RS2 plays a role in controlling nodule production in response to nitrate. In the present study, a *Mesorhizobium loti* strain constitutively expressing *DsRED* was inoculated onto host plants and subsequent IT production was then assessed. In the absence of nitrate, each *cle-rs* single mutant produced a normal number of ITs at 7 days after inoculation (dai) (Figure 1a). By contrast, the number of ITs observed in the *cle-rs1 -rs2* double mutants was significantly greater, compared to other genotypes. This result suggests that the two *CLE* genes possess a redundant function in negatively regulating rhizobial infection. The hyperinfected phenotype of the *cle-rs1 -rs2* double mutant is consistent with those of other AON mutants.[10,13,21] In both the *cle-rs* and double mutants, exposure to a high nitrate concentration (10 mM) resulted in a reduced number of ITs to a level similar to that observed in wild-type (WT) plants (Figure 1a). Therefore, the two *CLE* genes are dispensable for nitrate-induced control of rhizobial infection.

In addition to its predominant role for positively regulating nodulation, NIN plays a role for negatively regulating nodulation by directly activating *CLE-RS1* and *-RS2*.[4,22] We investigated if *NIN* is required for nitrate response in nodulation. The excessive ITs formation in *daphne* was inhibited by high nitrate treatment (Figure 1b). Thus, NIN is unlikely to be involved in nitrate-induced control of rhizobial infection.

As previously mentioned, the CLE-HAR1 module is responsible for the nitrate-induced control of nodule number. In order to determine whether this is via the downstream AON factor of HAR1, we reassessed the *tml* mutant phenotypes in the presence of nitrate. It has previously been demonstrated that the *tml* mutant is more sensitive to nitrate than *har1* and our findings broadly agreed with this finding;[10] the number of nodules at 21 dai were significantly reduced by nitrate in *tml*, although they were still greater than in the WT control (Figure 2a). We next generated the double mutant *nrsym1 tml* and showed that it maintained increased nodule number in the presence of nitrate (Figure 2a). This result suggests that NRSYM1 is implicated in the observed nitrate-induced reduction of nodule number in *tml* single mutants. We next investigated the effect of nitrate on rhizobial infection in the *tml* mutants. The increased ITs formation was inhibited by high nitrate in an NRSYM1-dependent manner (Figure 2b), suggesting that TML is not required for nitrate-induced control of rhizobial infection.

In this study, we determined the role of CLE-RS1 and -RS2 in the control of rhizobial infection. In the absence of nitrate, the CLE peptides have a redundant function of negatively regulating rhizobial infection. This observation provides further evidence in support of a model in which AON plays a role in regulating rhizobial infection.[17] However, it is reasonable to conclude that the AON pathway is not required for nitrate-induced control of rhizobial infection because mutants of key components of AON, *cle-rs1/2, har1* and *tml*, exhibit nitrate dependent IT formation. In addition, based on the

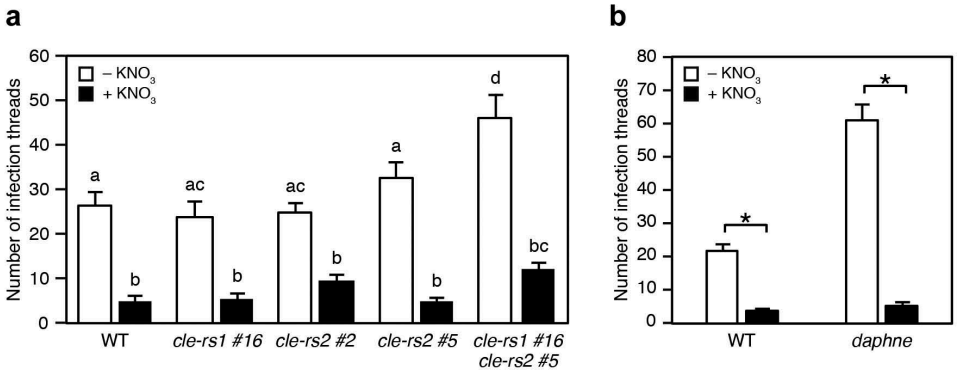

Figure 1. (a) The number of infection threads observed in the WT, *cle-rs1* and *cle-rs2* mutants and in the *cle-rs1 cle-rs2* double mutant with 0 or 10 mM KNO_3 at 7 days after inoculation (dai) with *Mesorhizobium loti* MAFF303099 constitutively expressing *DsRed* (n = 10–12 plants). Stable *cle-rs* mutants were previously created by the CRISPR-Cas9 genome editing system.[21] Columns with the same lower-case letter indicate no significant difference (Tukey's test, $P < .05$). (b) The number of infection threads in the WT and *daphne* mutants with 0 or 10 mM KNO_3 at 5 dai with *Mesorhizobium loti* MAFF303099 constitutively expressing *DsRed* (n = 10–12 plants). *$P < .05$ by Student's *t*-test. Error bars indicate SE.

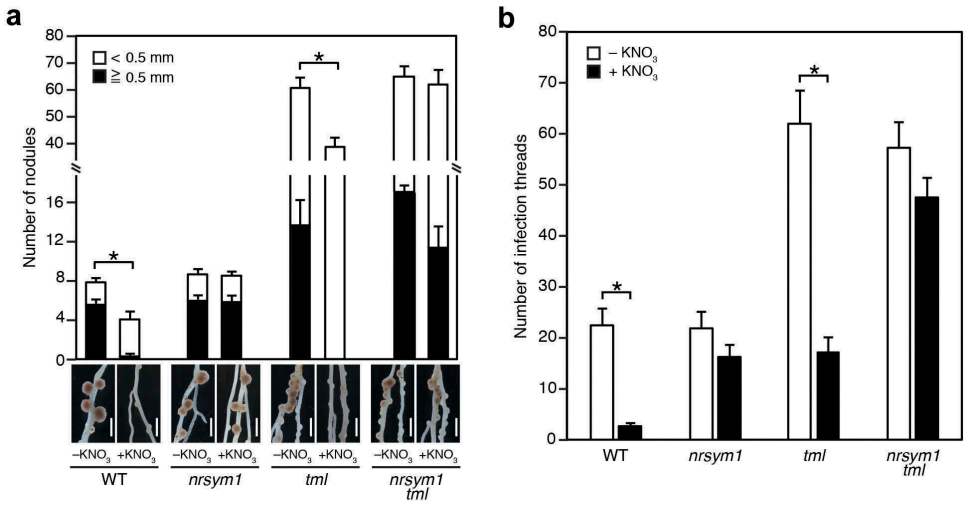

Figure 2. (a) Nodulation and nodule numbers in the WT, *nrsym1-1* and *tml-4* mutants, and the *nrsym1-1 tml-4* double mutant grown in the presence of 0 or 10 mM KNO_3 at 21 dai with *Mesorhizobium loti* MAFF303099 (n = 10–12 plants). Mature (black bars) and immature (white bars) nodules were separately counted. *$P < .05$ by Student's *t*-test (comparison of total nodule number). Scale bars: 2 mm. (b) The number of infection threads in the WT, *nrsym1-1* and *tml-4* mutants, and the *nrsym1-1 tml-4* double mutants with 0 or 10 mM KNO_3 at 5 dai with *Mesorhizobium loti* MAFF303099 constitutively expressing *DsRed* (n = 9–12 plants). *$P < .05$ by Student's *t*-test. Error bars indicate SE.

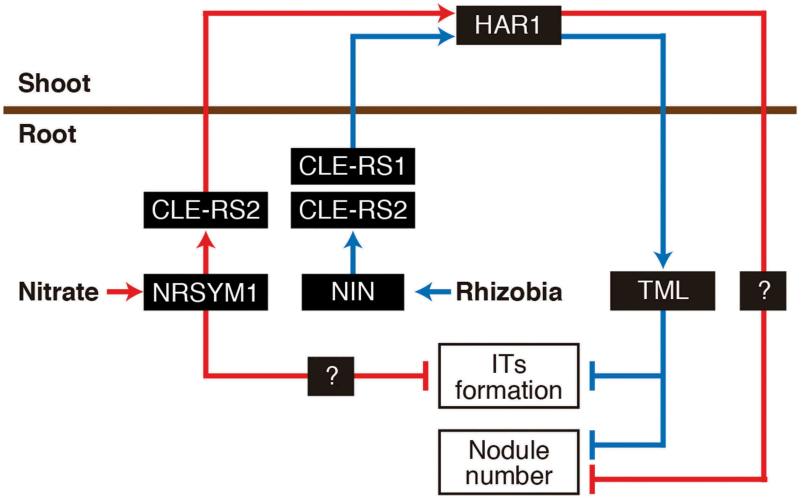

Figure 3. Model for the negative regulation of rhizobial infection and nodule number in *L. japonicus*. In response to rhizobial infection, NIN activates the CLE-RS1/2> HAR1> TML signaling pathway[22] to suppress infection threads (ITs) formation and nodule number (blue lines). The nitrate-induced NRSYM1> CLE-RS2> HAR1 signaling pathway could negatively regulate nodule number by using unknown downstream factors other than TML. In the presence of nitrate NRSYM1 also controls IT formation locally in the root through a mechanism independent of AON pathway (red lines).

nitrate-sensitive phenotype of *daphne*, NIN, an activator of *CLE-RS1/2*, may not be required for nitrate-induced control of rhizobial infection. The mechanism by which host plants inhibit rhizobial infection in response to nitrate is currently unclear. Given that NRSYM1 has the ability to control the nitrate-affected pleiotropic phases of root nodule symbiosis (including rhizobial infection) it could involve the targeting of unknown genes which act during rhizobial infection. Indeed, in *Medicago truncatula*, MtNLP1 could inhibit nodulation by targeting *MtCRE1* which encodes a cytokinin receptor required for rhizobial infection and nodule organogenesis.[23] We also reassessed the involvement of TML, a root-acting factor in AON, in the nitrate-induced control of nodule formation. Our results agreed with previous findings in that *tml* was much more sensitive to nitrate than other AON mutants.[10] Therefore, in terms of the nitrate-induced control of nodule number, it is likely that NRSYM1 uses CLE-RS2 and HAR1 but not TML as downstream factors. Since nodule number in *har1* is unaffected by nitrate, there might be a nitrate specific pathway downstream of HAR1, which acts in parallel with the HAR1> TML pathway (Figure 3). Future identification of such a pathway would help complete our understanding of the mechanism of nitrate-induced control of nodule number.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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