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Regulation of Nod factor biosynthesis by alternative NodD proteins at distinct stages of symbiosis provides additional compatibility scrutiny

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Introduction

The exchange of signal molecules between rhizobia and compatible legume hosts is essential for establishment of a successful nitrogen-fixing symbiotic interaction (Oldroyd et al., 2011). This communication is initiated by legume flavonoid compounds secreted into the rhizosphere that act as chemoattractants and are perceived by compatible rhizobia through constitutively expressed LysR-type transcriptional regulators (Madsen et al., 2003; Bek et al., 2010; Broghammer et al., 2012), initiating the symbiotic signalling cascades that promote infection thread (IT) formation and nodule organogenesis (Madsen et al., 2010; Kelly et al., 2017). Flavonoids represent a large variety of plant secondary metabolites synthesized from the phenylpropanoid pathway. Legumes produce a distinct class of flavonoids known as isoflavonoids though a unique branch of the flavonoid(s) produced by the model legume Lotus japonicus that are responsible for inducing NF production by its symbiotic partner Mesorhizobium loti have not been determined. However, components of flavonoid biosynthesis pathways have been identified, including a genomic cluster of four chalcone isomerases (Chi) that encode one type I (general) and three type II (legume-specific) chalcone isomerase (Shimada et al., 2003). The isomerization of 6′-deoxychalcone to 5-deoxyflavanone by Type II chalcone isomerases is essential for the production of isoflavone and flavone derivatives that have been shown to play important roles in legume symbiosis with rhizobia (Kimura et al., 2001).

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Rhizobia perceive flavonoids through uptake of the compounds that bind to NodD proteins. Crystal structures of NodD, with or without bound flavonoids, remain to be resolved; however, mutation studies and modelling based on known structures of other LysR-type transcriptional regulators has provided insight into important residues for transcriptional activity as well as potential inducer-binding sites (Kostiuk et al., 2013; Peck et al., 2013). NodD proteins bind to conserved regulatory DNA sequences, termed nod boxes, which are found in the promoters of NF biosynthesis genes and some secretion system operons. Flavonoid binding is not necessarily required for NodD to bind DNA but it is proposed that flavonoid binding enhances DNA bending by NodD resulting in transcriptional activation (Chen et al., 2005; Peck et al., 2006). Generally, rhizobial genomes contain multiple copies of nodD genes, with the number encoded not necessarily reflective of host-range. The complex interaction of multiple NodD proteins was recently illustrated in R. tropici CIAT 899 which contains five nodD genes, the most identified in rhizobia to date. The symbiotic proficiency of individual nodD mutants varied depending on the particular host species with one to four NodD proteins required for efficient nodulation of different hosts (del Cerro et al., 2015). NodD proteins may also act as repressors of nod gene activation (Garcia et al., 1996; Fellay et al., 1998) and, therefore, the various nodD genes encoded by some rhizobia may be involved in maintaining tight regulation of NF synthesis.

The NodD1 and NodD2 proteins of the L. japonicus symbiont M. loti R7A are functionally redundant for nodulation, with nodD1 mutants exhibiting a slight delay in induction of NF biosynthesis genes, the most identified in rhizobia to date. The symbiotic proficiency of individual nodD mutants varied depending on the particular host species with one to four NodD proteins required for efficient nodulation of different hosts (del Cerro et al., 2015). NodD proteins may also act as repressors of nod gene activation (Garcia et al., 1996; Fellay et al., 1998) and, therefore, the various nodD genes encoded by some rhizobia may be involved in maintaining tight regulation of NF synthesis.

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Results

M. loti R7A requires both NodD1 and NodD2 for proficient symbiotic infection of Lotus

Sequencing and annotation of the ~ 500 kb Meso...
**Fig. 1.** Alignment of *M. loti* R7A NodD1 and NodD2 and predicted protein structure.

A. Alignment of *M. loti* R7A NodD1 and NodD2 proteins reveals the high degree of similarity between the two proteins. (*) indicates a conserved residue, (.) indicates weakly similar properties. Red lines indicate the amino acid positions highlighted by the red circles in B.

B. Ribbon model of predicted R7A NodD1 protein structure showing the predicted flavinoid binding cleft. Red circles indicate regions of the amino acid sequence showing reduced conservation, as highlighted by red lines in A.

NodD1 and NodD2 control of Nod factor biosynthesis

significantly more (Fig. 2B). Root hair ITs formed by the mutants were indistinguishable in appearance to those formed by R7A.

The symbiotic competitiveness of the nodD mutants with R7A was examined through co-inoculation of Gifu plants with a 1:1 ratio of R7A-GFP, nodD1–DsRED (D1*/D2*), and nodD2–DsRed (D1*/D2*) fluorescently labelled strains (Fig. 3). IT counts at 10 dpi revealed a significant reduction in the total number of root hair ITs on plants co-inoculated with nodD1 (D1*/D2*), indicating that the negative effect causing reduced IT formation by nodD1 (D1*/D2*) (Fig. 2B) also impaired root hair IT formation by R7A on the co-inoculated plants (Fig. 3A). Both nodD1 (D1*/D2*) and nodD2 (D1*/D2*) were outcompeted by R7A in terms of the percentage of ITs formed by the mutants compared to R7A, with nodD2 (D1*/D2*) being more severely outcompeted (Fig. 3B).

At 21 dpi, 20 nodules were harvested from plants corresponding to each co-inoculation condition, surface sterilized and the inhabiting rhizobia were plated out. The particular strain(s) present within each nodule were determined based on their fluorescence. As expected, 9/20 nodules contained only GFP expressing colonies, 8/20 only DsRED expressing colonies and 3/20 contained both GFP and DsRED strains. However, R7A strongly out competed nodD1 (D1*/D2*) with only 1/20 nodules harbouring nodD1 (D1*/D2*) alone and 3/20 containing both R7A and nodD1 (D1*/D2*), nodD2 (D1*/D2*) was completely outcompeted by R7A with all 20 nodules examined containing only R7A. These phenotypic results indicate that NodD1 has a primary function during root hair IT formation that cannot be completely complemented by NodD2, which appears to play a negative role in the early stages of infection.

R7A NodD1 and NodD2 show striking differences in nod gene induction in vitro

Due to the differing symbiotic phenotypes of nodD1 (D1*/D2*) and nodD2 (D1*/D2*), the induction of nod genes responsible for NF biosynthesis in the mutant strains was investigated. To date, the inducer of *M. loti* NodD produced by *Lotus* has not been identified. Therefore, crude Gifu RE were collected and used to induce cultures of R7A, nodD1 (D1*/D2*) and nodD2 (D1*/D2*). Striking differences in nod gene induction were observed between nodD1 (D1*/D2*) and nodD2 (D1*/D2*) (Fig. 4). qRT-PCR of strains induced for 1 h with RE indicated that both R7A and nodD1 (D1*/D2*) induced core (nodA) and accessory (nodZ) NF biosynthesis genes to comparable levels, while in the nodD2 (D1*/D2*) background no induction was observed (Fig. 4A). To further investigate this observation, the induction kinetics, plate reader assays were performed on strains carrying a PnodA–gfp construct, that transcribes gfp under control of the R7A nodA promoter. Induction of the PnodA–gfp construct, measured as GFP fluorescence, was observed in R7A and nodD1 (D1*/D2*) with maximal levels reached at 30 h post-induction.
(hpi) and no further increase/decrease over the 72 h period was observed (Fig. 4B). R7A and nodD1 (D1\(^{-}\)/D2\(^{-}\)) induced PnodA-gfp expression at comparable rates up to 16 hpi, at which point the expression level in nodD1 (D1\(^{-}\)/D2\(^{-}\)) increased appreciably compared to R7A before plateauing at 30 hpi. No discernible induction of PnodA-gfp expression was observed in nodD2 (D1\(^{-}\)/D2\(^{-}\)) over the entire 72 h assay (Fig. 4B). These results indicate that nodD2 rather than nodD1 is activated by the inducer present in RE at the preinfection stage and that nodD1 may antagonize the nodD2-mediated activity from the nodA promoter over time.

R7A nodD1 and nodD2 induce nod gene induction at distinct stages of symbiotic infection

The marked differences in nodA and nodZ gene induction in the nodD mutant backgrounds in vitro prompted us to investigate expression in planta during progression of symbiotic infection. Gifu plants inoculated with R7A, nodD1 (D1\(^{+}\)/D2\(^{+}\)) and nodD2 (D1\(^{+}\)/D2\(^{+}\)) strains carrying the PnodA-gfp reporter construct were examined by microscopy. Clear differences in induction of the PnodA-gfp reporter between nodD1 (D1\(^{+}\)/D2\(^{+}\)) and nodD2 (D1\(^{+}\)/D2\(^{+}\)) were apparent. GFP-expressing bacteria were readily observed on and around the root surface, including micro-colonies formed within curled root hairs, for both R7A and nodD1 (D1\(^{+}\)/D2\(^{+}\)), while no fluorescent cells were observed for nodD2 (D1\(^{-}\)/D2\(^{-}\)) (Fig. 5A). In general, a higher level of fluorescence of nodD1 (D1\(^{+}\)/D2\(^{+}\)) compared to R7A was observed. These results are consistent with the in vitro assays where only nodD2 responded to RE and higher levels of expression were detected in nodD1 (D1\(^{+}\)/D2\(^{+}\)) compared to R7A in the in vitro plate reader assays.

One of the earliest responses of legumes to the perception of rhizobial NF are root hair deformations (Heidstra et al., 1994). All inoculated plants, including those inoculated with nodD2 (D1\(^{-}\)/D2\(^{-}\)), displayed root hair deformations, suggesting that nodD2 (D1\(^{-}\)/D2\(^{-}\)) was producing some NF despite the lack of fluorescent cells observed (Fig. 5A). This was confirmed by qRT-PCR...
NodD1 and NodD2 control of Nod factor biosynthesis

Double mutant showed no NF gene induction. This in planta data is consistent with the in vitro assays in suggesting that NodD2 is primarily activated by the plant inducer at the preinfection stage. Expression of the nodD genes themselves was also investigated in the bacteria recovered from the rhizosphere (Supporting Information Fig. S3). The expected absence of nodD1 and nodD2 expression in the respective single mutants and the double mutant was observed. Expression of nodD1 in the nodD2 (D1″/D2″) mutant was reduced at 3 dpi compared to R7A controls but a comparable level of expression was observed at 7 dpi. Expression of nodD2 in the nodD1 (D1″/D2″) mutant was comparable to R7A at both time points. These results indicate that the differences between nodD1 (D1″/D2″) and nodD2 (D1″/D2″) cannot be explained by a significant upregulation of the remaining copy of nodD in the single mutant strains.

At the stage of root hair IT development, a dramatic reversal in Pnoda-gfp induction by nodD1 (D1″/D2″) and nodD2 (D1″/D2″) was observed. Pnoda-gfp induction was clearly observed within ITs formed on R7A and nodD2 (D1″/D2″) inoculated plants. nodD1 (D1″/D2″) inoculated plants continued to show high levels of fluorescence on the root surface, but fluorescence within ITs was greatly reduced compared to R7A and nodD2 (D1″/D2″) (Fig. 6A). The frequency of ITs counted based on GFP fluorescence from the Pnoda-gfp construct quantifies this substantial difference in nod gene induction within ITs containing the nodD mutant strains (Fig. 6B). Remarkably, a further switch in the activity of the NodD1 and NodD2 proteins was observed within nodules. The induction of Pnoda-gfp within nodules mirrored that observed on the root surface, with R7A and nodD1 (D1″/D2″) showing strong induction and nodD2 (D1″/D2″) showing no detectable induction (Fig. 6A). These in planta results demonstrate that NodD1 is primarily responsible for NF gene induction within root hair ITs and NodD2 at the root surface and within nodules.

The type IV secretion system does not contribute to the nodD1 and nodD2 symbiotic phenotypes

Previous studies of R7A revealed a role for NodD in regulation initially through NodD induction of virA and then through the VirA/VirG regulatory system is responsible for expression of the T4SS machinery and effectors (Hubber et al., 2004; Hubber et al., 2007). Given the importance of secretion systems in the establishment of symbiosis, in planta expression of components of the T4SS (virA and virB1) were investigated in the nodD mutant backgrounds from bacteria recovered from the rhizosphere (Supporting Information Fig. S3). The level of vir gene expression was considerably higher in nodD1 (D1″/D2″) than in nodD2.
Fig. 4. In vitro assays of Nod factor biosynthesis gene induction.
A. qRT-PCR of Nod factor biosynthesis genes in cultures induced for 1 h with Gifu RE compared to controls.
B. Plate reader assays of strains carrying the PnodA-gfp reporter construct induced with Gifu RE over 72 h. The presence/absence of NodD1 and NodD2 in mutant strains is indicated. The results of both experiments represent the averages of three biological replicates with error bars indicating SEM.

Fig. 5. In planta assays of Nod factor gene induction during early stages of symbiosis.
A. Microscopy of Gifu roots inoculated with R7A, nodD1 and nodD2 carrying the PnodA-gfp reporter construct at 4 dpi, the presence/absence of NodD1 and NodD2 in mutant strains is indicated. Ten roots were examined for each strain with results comparable to those shown observed on all. Scale bars are 200 μM.
B. NF gene expression measured by qRT-PCR of bacteria recovered from the rhizosphere at 3 and 7 dpi. The results represent the averages of three biological replicates with error bars indicating SEM.
(D1⁻/D2⁻) with R7A displaying an intermediate level of expression, suggesting that NodD1 may inhibit vir gene expression in the rhizosphere. Low level expression of both vir genes, particularly at 7dpi, was observed in the nodD1/nodD2 (D1⁻/D2⁻) double mutant, suggesting that induction of the T4SS may not be completely dependent on NodD activity. To investigate if effects of the nodD mutants on regulation of the T4SS influenced the observed nodD1 (D1⁻/D2⁻) and nodD2 (D1⁺/D2⁻) symbiotic phenotypes, the nodulation kinetics of nodDvirG double mutants were investigated. No significant differences were observed in the nodDvirG double mutants compared to the respective single nodD mutants (Supporting Information Fig. S4).

Lotus chalcone isomerase CHI4 involvement in biosynthesis of a root hair NodD1 activator

The NodD-activating compound(s) and genes responsible for their production have not been determined in any Lotus species; however, components of the legume-specific iso-flavonoid pathway have been identified in L. japonicus including a family of chalcone isomerases (Shimada et al., 2003). The type II chalcone isomerase CHI4 is strongly involved in biosynthesis of a root hair NodD1 activator.
induced in Gifu roots on *M. loti* inoculation or NF treatment (Shimada *et al.*, 2003; Kawaharada *et al.*, 2017a,b). To investigate the potential role of CHI4 in the biosynthesis of R7A nodD1 and nodD2, the presence/absence of NodD1 and NodD2 in mutant strains is indicated. Both nitrogen-fixing (pink) and uninfected (white) nodules were counted periodically over 35 days. Error bars show SEM and *t* test *p* values indicate *t* test significant differences between Gifu and chi4-1 mutant inoculated with each strain (*p* < 0.05, **p** < 0.01).

B. Infection thread counts were performed on Gifu and two independent chi4 mutant alleles inoculated with DsRed-tagged strains at 7 dpi. Error bars represent SEM and statistical comparisons between genotypes and inoculations are shown using ANOVA and Tukey post hoc testing with *p* values < 0.05 indicated by different letters.

Although the number of ITs formed on chi4 mutants by nodD2 (D1*/D2*) was significantly reduced compared to Gifu, expression of the PnodA-gfp construct within ITs could still be observed on the mutant plants. These symbiotic phenotypes suggest that CHI4 is involved in the production of an inducing compound preferentially recognized by NodD1 to induce NF biosynthesis within ITs. Expression of CHI4 during the symbiotic process was investigated on Gifu hairy roots transformed with a CHI4 promoter-reporter construct (Fig. 8). Consistent with the previously reported qRT-PCR studies (Shimada *et al.*, 2003; Kawaharada *et al.*, 2017a,b), CHI4 expression was not detected in uninoculated control plants. Following inoculation with R7A, CHI4 promoter activity was observed.
within root hairs but was absent from nodules. The expression pattern of Chi4 strongly correlates with the observed PnodA-gfp expression pattern in the nodD2(D1+/D2-)/nodD2(D11/D2-) background (Fig. 6), supporting the notion that CHI4 is responsible for the production of an inducer preferentially recognized by NodD1 within root hair ITs.

Discussion

Transcription of NF biosynthesis genes is controlled by NodD regulators and perception of NF by compatible host legumes triggers the symbiotic signalling cascade promoting rhizobial infection and nodule organogenesis (Madsen et al., 2010; Oldroyd et al., 2011; Kelly et al., 2017). The detailed analysis of nodD1(D1+/D2-) and nodD2(D1+/D2-) symbiotic phenotypes presented here confirmed a degree of functional redundancy between the two NodD proteins (Rodpothong et al., 2009), but also revealed significant differences in the ability of the single nodD mutants to promote IT development and nodule colonization. Furthermore, nodD1(D1+/D2-) and nodD2(D1+/D2-) were severely outcompeted by R7A in infection assays, demonstrating that both NodD proteins are required for proficient symbiotic infection. The severely impaired IT phenotype of nodD1(D1+/D2-) was not overcome by R7A in coinoculation assays. This may be due to overproduction of NF by nodD1(D1+/D2-), evidenced from in vitro plate assays and observation of PnodA-gfp reporter strains in planta, inducing IT-inhibiting effects. Recently, R7A NF has been shown to induce ethylene production on L. japonicus Gifu (Dugald Reid, personal communication) and ethylene is known to inhibit symbiotic signalling and IT formation in legumes (Pennmetsa and Cook, 1997; Oldroyd et al., 2001). In support of this, nodD2(D1+/D2-) produced very little NF on the root surface and showed a hyper-IT phenotype, while coinoculation of nodD2(D1+/D2-) with R7A reduced IT numbers to a wild-type level.
R7A NodD1 and NodD2 show very high amino acid sequence identity, but with notably reduced conservation in two stretches corresponding to the entrance of the predicted inducer-binding cleft in predicted protein structure models. NodD-inducer docking simulations predict this region to be important for inducer specificity (Kostiuik et al., 2013). R7A NodD1 and NodD2 may, therefore, preferentially bind different inducer compounds or have altered binding affinities for a common inducer. We observed striking differences in the NF gene induction capacity of nodD1 (D1′/D2′) and nodD2 (D1′/D2′). In addition, both in vitro and in planta monitoring of nod gene induction revealed a higher level of induction in the nodD1 (D1′/D2′) background compared to R7A or nodD2 (D1′/D2′). These results suggest that the inducing activity of NodD2 at the preinfection stage is negatively affected by the presence of NodD1. R. leguminosarum NodD has been shown to bind DNA as a tetramer, consisting of a dimer of dimers (Feng et al., 2003). It seems possible that R7A NodD1 and NodD2 may form a hetero-multimer complex with the level of transcription modulated by their inducer-bound states.

nodD1 (D1′/D2′) strongly induced NF biosynthesis in the rhizosphere and within nodules, while nodD2 (D1′/D2′) appeared to specifically induce NF biosynthesis within root hair ITs. These results suggest that NodD2 binds an inducer present within the rhizosphere to promote NF biosynthesis, and subsequent induction of the symbiotic signalling cascade induces the symbiotic infection process and production of an additional inducer within ITs that is preferentially recognized by NodD1 (Fig. 9).

It remains to be determined whether the phenotypes exhibited by nodD1 (D1′/D2′) and nodD2 (D1′/D2′) are due to differences in the amount of NF produced at particular stages of symbiosis or if NodD1 and NodD2 are responsible for the biosynthesis of particular species of NF. In addition to the regulation of NF biosynthesis, NodD proteins are also known to impact the production of rhizobial surface polysaccharides including exopolysaccharide (Swanson et al., 1993; Barnett and Long, 2015; Acosta-Jurado et al., 2016) and lipopolysaccharide (Kobayashi et al., 2004) that are known to be important in the symbiotic process (Frayssie et al., 2003; Kelly et al., 2013; Kawaharada et al., 2015).

Symbiotically induced root hair expression of components of isoflavonoid biosynthesis has previously been reported in the Medicago – Sinorhizobium meliloti symbiosis. Several chalcone O-methyl transferases that are involved in converting isoflavanogen to the potent S. meliloti nod gene inducer methoxychalcone were found to be induced in root hairs following inoculation (Maxwell et al., 1989; Breakspear et al., 2014). The identification of Lotus inducers of M. loti nod gene expression remains a challenge. Investigation of inducing compounds from L. corniculatus seed and RE identified a potential compound with low molecular weight and high polarity but further characterization was unsuccessful (Kojima et al., 2012).

Numerous Lotus mutant screens performed over the years have not identified any symbiotically impaired isolates affected in potential inducer biosynthesis, suggesting genetic redundancy in the biosynthetic pathway(s). This is
also reflected in the limited number of studies to date that have directly shown that silencing of flavonoid biosynthesis pathways severely inhibits rhizobia-legume symbiosis (Subramanian et al., 2006; Wasson et al., 2006; Zhang et al., 2009). Genes involved in flavonoid biosynthesis have been identified in Lotus, including a family of four chalcone isomerases (Shimada et al., 2003). We have demonstrated that mutation of the symbiotically induced Chi4 from this family resulted in reduced nodulation by R7A and nodD2 (D1′/D2′) and a drastic reduction in IT formation by nodD2 (D1′/D2′). Analysis of Chi4 promoter activity indicated that expression of Chi4 is largely restricted to within root hair tips and is absent from nodules. The expression pattern of Chi4 together with the observed induction of nod genes during symbiotic infection in nodD2 (D1′/D2′) suggests that Chi4 is involved in the biosynthesis of a root hair inducer that is preferentially recognized by NodD1 (Fig. 9).

Lotus CH1, CHI3 and CHI4 share a high level of amino acid sequence identity (Shimada et al., 2003), indicating potential functional redundancy. It may be that the other members of this family are primarily involved in inducer biosynthesis at different stages of the symbiotic process. However, the lack of mutant alleles in all family members and the fact that the chalcone isomerase genes form a tandem cluster within 15 kb on the genome make the investigation of multiple mutants challenging.

We propose that the preferential activation of R7A NodD proteins by differing inducers provides the plant with a further checkpoint for symbiotic compatibility during infection. An inducer present in RE is required to initiate symbiotic signalling in the rhizosphere and is recognized by NodD2. The subsequent induction of biosynthesis of an alternative inducer that is recognized by an additional NodD protein, NodD1, within the IT provides another level of compatibility scrutiny. This system may represent a common process in other rhizobia-legume symbioses and may provide an explanation in addition to expanded host range for why the majority of rhizobia encode multiple copies of nodD.

Experimental procedures
Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Supporting Information Table S1. Wild-type Mesorhizobium loti R7A (Sullivan et al., 2002; Kelly et al., 2014) and mutant strains were cultured at 28°C in YM or G/RDM medium (Ronson et al., 1987). nodDvirG double mutant strains were constructed by in-frame markerless deletion, as previously described (Rodpethong et al., 2009). Strains expressing fluorescent reporters were constructed by the introduction of pSKDSRED or pSKGFP (Kelly et al., 2013). Antibiotics were added to media as required at the following concentrations: tetracycline, 2 μg ml⁻¹; rifampicin, 100 μg ml⁻¹; spectinomycin, 100 μg ml⁻¹.

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Plant material

Lotus japonicus ecotype Gifu (Handberg and Stougaard, 1992) was used as the wild-type plant. chi4 mutant plants were identified and ordered through the Lotus Base website (Mun et al., 2016) with homozygous mutant plants identified as previously described (Urbański et al., 2012; Małolepszy et al., 2016). Seed sterilization and plant-growth setups for nodulation and infection thread assays were as previously described (Kawaharada et al., 2015). Plants were grown at 21°C with day and night cycles of 16 and 8 h respectively. Agrobacterium strain A1193 (Stougaard et al., 1987) was used for hairy-root transformation experiments, carried out as described previously (Petit et al., 1987). Plant growth plates, each containing 10 plants, were inoculated with 750 μl of OD₆₀₀ = 0.02 bacterial suspensions, as required.

Competition assays

For infection thread and nodule occupancy competition assays, plant plates (each containing 10 plants) were inoculated with 750 μl of OD₆₀₀ = 0.02 bacterial suspensions. For co-inoculated plants, equal volumes of bacterial suspensions were mixed and 750 μl of this used for plant inoculation. Infection threads were counted at 10 dpi based on GFP and/or DsRED fluorescence. Nodules of 21 dpi plants were harvested and surface sterilized by shaking for 1 min in 70% ethanol followed by 1 min in a 1:1 mix of 96% ethanol and hydrogen peroxide (35%). Nodules were then washed with 5× changes of autoclaved water. Individual nodules were then placed in microtitre plate wells containing 50 μl autoclaved water and crushed with sterile pestles. Nodule exudate (30 μl) was then streaked onto YMB agar plates or used to make a dilution series prior to plating on YMB agar. Following 5 days growth at 28°C, colony growth was examined using a Leica M165 FC epifluorescence stereomicroscope with GFP (excitation 500/20 nm; barrier 535/30 nm) and DsRED (excitation 545/30 nm; barrier 620/60 nm) filters. All colony growth from streaked and dilution plating’s was examined for GFP or DsRED fluorescence to determine the occupying strain(s). The number of colony forming units isolated from nodules for which dilutions were plated is provided in Supporting Information Table S2.

Collection of root exudates

Root exudates were collected using a method similar to that described by (Moscatelli et al., 2009). Approximately 50 germinated seedlings were transferred to a mesh grid that allowed the roots to protrude into 50 ml H₂O within a magenta container. After 3 weeks growth, the liquid containing crude RE was filter-sterilized (0.45 μm, Millipore) and lyophilized.

Plate reader assays

Lyophilized RE was re-suspended in 4 ml G/RDM and filter-sterilized (0.45 μm, Millipore). Ninety μl of the G/RDM + RE (G/RDM for uninduced controls) was added to microtitre plate (Corning) wells. OD₆₀₀ = 0.1 suspensions were prepared from 5-ml G/RDM cultures of PnodA-gfp strains that had been
grew for 72 h at 28°C. Ten μl of this was added to appropriate wells. GFP fluorescence was measured over 72 h at 28°C with a FLUOstar Omega plate reader (BMG Labtech) with excitation/emission wavelengths of 485/520 nm.

qRT-PCR analysis of gene expression

For qRT-PCR of induced cultures, cells were pelleted from 1 ml aliquots of OD_{600} = 0.1 G/RDM broth cultures of PnodA-gfp strains by centrifugation. Cell pellets were re-suspended in 1 ml G/RDM (uninduced) or 1 ml G/RDM + RE and grown for 1 h at 28°C. RNA was stabilized by adding 2 ml of RNAprotect Bacteria reagent (QIAGEN) according to the manufacturer’s instructions. For gene expression within rhizosphere bacteria, plates containing 10 plants each were inoculated with 1 ml of OD_{600} = 0.1 suspensions of R7A, nodD1, nodD2 or nodD1/nodD2 at the appropriate time so that they would be 3 or 7 dpi after 12 d growth on the plates. For each sample, consisting of 30 plants, a 15 ml Falcon tube containing 3.75 ml RNAProtect Bacteria reagent (QIAGEN) and 1.875 ml autoclaved H_2O was prepared. The roots of 30 plants were added to each tube and vortexed for 1 min, roots were removed and the cells pelleted by centrifugation at 4, 248 × g for 30 min at 4°C.

Total RNA was isolated from bacterial cells using a NucleoSpin RNA kit (Macherey-Nagel) with a second in-solution rDNase treatment performed. cDNA synthesis was performed using RevertAid reverse transcriptase (Thermo Scientific) with a random hexamer primer according to the manufacturer’s instructions. qRT-PCR was performed using a Lightcycler480 instrument and Lightcycler480 SYBR Green I master mix (Roche Diagnostics GmbH). 16S rRNA was used as a reference gene. The primers used for detecting expression levels of the various gene targets are listed in Supporting Information Table S3.

Plasmid construction

Plasmid PnodA-gfp was constructed using overlap extension PCR to form a translational fusion between the nodA promoter region and gfp at the ATG start site. The primers used are listed in Supporting Information Table S3. The overlap extension PCR product was then cloned into pFAJ1700 as a BamHI/HindIII restriction digest fragment forming PnodA-gfp.

A putative 2 kb Chi4 promoter was amplified from Gifu genomic DNA as a series of overlapping PCR products to remove BsaI sites and cloned into Golden Gate compatible vectors. Primer details are provided in Supporting Information Table S3. Assembly of PChi4-GUS for use in hairy-root transformation of Gifu was carried out as previously described (Reid et al., 2017).

Microscopy

Infection thread counts were performed on strains harbouring fluorescent reporter constructs pSKDSRED, pSKGFP or PnodA-gfp using a Zeiss Axioplan 2 image fluorescence microscope. Confocal microscopy of PnodA-gfp fluorescence in the various strain backgrounds during the symbiotic infection process was performed using a Zeiss LSM780 metaconfocal microscope. Objective lenses were Zeiss Plan-Neofluar 10×/0.3 and 20×/0.5 with laser excitation at 488 nm and emission filters at 505–550 nm. Images were processed using Zen software (Zeiss).

For analysis of Chi4 promoter-GUS activity, roots were stained as previously described (Kawaharada et al., 2017a,b). Light microscopy was performed with a Zeiss Axioskop 2 microscope using a Zeiss Plan-Neofluar 10×/0.3 objective lens. Nodule were embedded in 3% agarose and 70–80 μM sections were prepared using a Leica VT 1000 S vibratome.

Bioinformatics

NodD protein sequences were obtained from the Integrated Microbial Genomes (IMG) resource (Markowitz et al., 2012) https://img.jgi.doe.gov/cgi-bin/m/main.cgi. Alignments between NodD protein sequences were made using EMBASSY Needle (http://www.ebi.ac.uk/Tools/pfa/emboss_needle/) and CLC Main Workbench 7 (QIAGEN). Predicted protein structures of R7A NodD1 and NodD2 were generated using Phyre2 (Kelley et al., 2015) and viewed in CLC Main Workbench 7 (QIAGEN).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Alignment of rhizobial NodD proteins.

Fig. S2. Nodulation of diverse *Lotus* species by *nodD1* and *nodD2*.

Fig. S3. qRT-PCR of *nod* and *vir* genes in bacteria recovered from the rhizosphere.

Fig. S4. NodD/T4SS double mutant nodulation assay.

Table S1. Bacterial strains and plasmids.

Table S2. Colony counts from nodule competition assays.

Table S3. Primer details.

References