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

Simon Kelly,¹ John T. Sullivan,²
Yasuyuki Kawaharada,^{1†} Simona Radutoiu,¹
Clive W. Ronson² and Jens Stougaard^{1*}

¹Department of Molecular Biology and Genetics, Centre
for Carbohydrate Recognition and Signalling, Aarhus
University, Aarhus 8000 C, Denmark.

²Department of Microbiology and Immunology,
University of Otago, Dunedin 9054, New Zealand.

Summary

The *Lotus japonicus* symbiont *Mesorhizobium loti* R7A encodes two copies of *nodD* and here we identify striking differences in Nod factor biosynthesis gene induction by NodD1 and NodD2 both *in vitro* and *in planta*. We demonstrate that induction of Nod factor biosynthesis genes is preferentially controlled by NodD1 and NodD2 at specific stages of symbiotic infection. NodD2 is primarily responsible for induction in the rhizosphere and within nodules, while NodD1 is primarily responsible for induction within root hair infection threads. *nodD1* and *nodD2* mutants showed significant symbiotic phenotypes and competition studies establish that *nodD1* and *nodD2* mutants were severely outcompeted by wild-type R7A, indicating that both proteins are required for proficient symbiotic infection. These results suggest preferential activation of NodD1 and NodD2 by different inducing compounds produced at defined stages of symbiotic infection. We identified *Lotus* chalcone isomerase CHI4 as a root hair induced candidate involved in the biosynthesis of an inducer compound that may be preferentially recognized by NodD1 within root hair infection threads. We propose an alternative explanation for the function of multiple copies of *nodD* that provides the host plant with

another level of compatibility scrutiny at the stage of infection thread development.

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 regulator NodD proteins to regulate Nod factor (NF) gene induction (Mulligan and Long, 1985). Compatible NFs are recognized by legume NF receptors (Madsen *et al.*, 2003; Radutoiu *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 through a unique branch of this pathway (Wang, 2011). Only a few specific flavonoids are likely to act as rhizobial *nod* gene inducing for each legume species and this crucial role of flavonoid signalling in host-range determination has been recently reviewed (Liu and Murray, 2016). The symbiotically active 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 isomerases (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).

Received 26 September, 2017; revised 7 November, 2017; accepted 14 November, 2017. *For correspondence. E-mail: stougaard@mbg.au.dk; Tel. +45 87155504; Fax +45 86123178.

[†]Present address: Department of Plant Bioscience, Faculty of Agriculture, Iwate University, 3-18-8-Ueda, Morioka, Iwate, Japan.

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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 nodulation (Rodpothong *et al.*, 2009). In this study, we thoroughly examined the symbiotic phenotypes of single and double *nodD* mutant R7A strains and identified significant differences in symbiotic capacity, particularly at the stage of IT development. Disparities in NF gene induction by the mutant strains were discerned following induction with *L. japonicus* root exudates (RE) and striking differences in the induction of NF biosynthesis genes during preinfection, root hair IT progression and cortical IT penetration of nodule primordia were observed. The *Lotus* chalcone isomerase *Chi4* is symbiotically induced and our analysis of *chi4* mutants suggests that CHI4 is involved in the biosynthesis of an inducer preferentially recognized by NodD1 to promote IT formation. These results suggest that NodD1 and NodD2 preferentially perceive distinct inducing compounds produced at defined stages of symbiotic infection. We propose that this mechanism provides the host plant with an additional symbiont compatibility check during infection and may explain the presence of multiple copies of *nodD* genes in rhizobia.

Results

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

Sequencing and annotation of the ~ 500 kb *Mesorhizobium loti* R7A symbiosis island identified two copies of *nodD*, designated *nodD1* and *nodD2*. *nodD1* is located immediately downstream of the *nodL* gene in the same orientation, while *nodD2* is located directly upstream of *nodL* in divergent orientation (Sullivan *et al.*, 2002). Subsequently, the entire *M. loti* R7A genome was sequenced (Kelly *et al.*, 2014) and BLAST analysis of this confirmed that the symbiosis island copies are the only NodD-encoding genes present in the genome. Alignment of the R7A NodD1 and NodD2 amino acid sequences revealed a high degree of similarity with 84.7% amino acid identity (Fig. 1A). Interestingly, two regions of R7A NodD1 and NodD2 that showed reduced amino acid conservation are predicted to be at the entrance of the flavonoid binding cleft (Fig. 1A and B). Alignments of the NodD proteins from several rhizobial species that encode between one (*R. leguminosarum* bv. *viciae* 3841) to five (*R. tropici* CIAT899) copies of NodD showed that there is a high degree of similarity between the proteins across rhizobial species and that the predicted flavonoid binding cleft sites showed reduced conservation between NodD proteins both within and across the species investigated (Supporting Information Fig. S1).

To investigate the symbiotic role of R7A NodD1 and NodD2, phenotypes of *nodD1* (NodD1⁻/NodD2⁺) and *nodD2* (NodD1⁺/NodD2⁻) single mutants, together with the double mutant *nodD1/nodD2* (NodD1⁻/NodD2⁻) were examined on *Lotus japonicus* ecotype Gifu. Both single mutant strains formed nitrogen-fixing (pink) nodules. However, *nodD1* (D1⁻/D2⁺) induced significantly reduced nodule numbers compared to R7A, while the nodule number in *nodD2* (D1⁺/D2⁻) was unaffected (Fig. 2A). Both *nodD1* (D1⁻/D2⁺) and *nodD2* (D1⁺/D2⁻) inoculated plants harboured uninfected (white) nodules at later time points compared to R7A-inoculated plants. The *nodD1/nodD2* (D1⁻/D2⁻) double mutant formed no nodules. To examine the diversity in symbiotic phenotypes of the *nodD* mutants, nodulation phenotypes were investigated on a collection of different *Lotus* species. Comparable phenotypes to those identified on Gifu were observed with *nodD1* (D1⁻/D2⁺) generally inducing reduced numbers of nitrogen-fixing nodules compared to R7A and *nodD2* (D1⁺/D2⁻) largely unaffected, except on *L. japonicus* ecotype MG-20 where significantly increased nodulation was observed with *nodD2* (D1⁺/D2⁻) (Supporting Information Fig. S2). Investigation of root hair IT formation by the mutants revealed contrasting phenotypes for *nodD1* (D1⁻/D2⁺) and *nodD2* (D1⁺/D2⁻). *nodD1* (D1⁻/D2⁺) formed significantly fewer root hair ITs than R7A, whereas *nodD2* (D1⁺/D2⁻) formed

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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 flavonoid binding cleft. Red circles indicate regions of the amino acid sequence showing reduced conservation, as highlighted by red lines in A.

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 coinoculation 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 coinoculated 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 coinoculated 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 coinoculation 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, on control plants coinoculated with 1:1 R7A-DsRED and R7A-GFP no competitive advantage was observed with 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* have 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, and examine 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

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Fig. 2. *nodD1* and *nodD2* symbiotic phenotypes.

A. Nodulation assay on Gifu inoculated with 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 42 days.

B. Infection thread counts were performed on plants inoculated with DsRed-tagged strains at 10 dpi. Error bars represent SEM and statistical comparisons between inoculations for pink nodule formation and infection threads are shown using ANOVA and Tukey *post hoc* testing with *p* values (< 0.05 for A) and (< 0.01 for B) as indicated by different letters.

(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

Fig. 3. Infection thread and nodule colonization competition assays. Gifu plants were coinoculated with 1:1 ratios of the indicated strains.

A. Infection thread counts at 10 dpi. Error bars represent SEM and statistical comparisons between total numbers of infection threads are shown using ANOVA and Tukey *post hoc* testing with *p* values (< 0.05), as indicated by different letters.

B. Percent of 10 dpi infection threads formed by each strain(s) based on fluorescence.

C. Nodule colonizing strain(s) were determined based on colony fluorescence of nodule isolates (recovered at 21 dpi).

analysis performed on bacterial cells isolated from the rhizosphere of Gifu roots 3 and 7 dpi with R7A, *nodD1* ($D1^{-}/D2^{+}$), *nodD2* ($D1^{+}/D2^{-}$) or *nodD1/nodD2* ($D1^{-}/D2^{-}$) (Fig. 5B). Compared to R7A, a low level of *nod* gene induction was detected in the *nodD2* ($D1^{+}/D2^{-}$) mutant background, while in *nodD1* ($D1^{-}/D2^{+}$) the induction was greatly increased over that in R7A. The *nodD1/nodD2* ($D1^{-}/D2^{-}$)

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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 of the type IV secretion system (T4SS). A cascade 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*

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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.

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Fig. 6. R7A NodD1 NodD2 show contrasting induction of *nodA* at distinct stages of symbiotic infection.

A. Microscopy of Gifu roots inoculated with R7A, *nodD1* and *nodD2* carrying the *PnodA-gfp* reporter construct, the presence/absence of NodD1 and NodD2 in mutant strains is indicated. ITs were investigated at 10 dpi and white arrows indicate GFP expressing bacteria within ITs. Scale bars are 100 μM. Whole nodules and nodule sections at 19 dpi with yellow arrows indicating examples of cortical infection threads. Scale bars are 1 mm for whole nodules and 100 μM for nodule sections.

B. ITs counted based on *PnodA-gfp* expression at 10 dpi. Error bars represent SEM and statistical comparisons between inoculations are shown using ANOVA and Tukey *post hoc* testing with *p* values < 0.01 indicated by different letters.

(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 *nodD/virG* double mutants were investigated. No significant differences were observed in the *nodD/virG* 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 isoflavonoid 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

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Fig. 7. *chi4* mutants exhibit reduced symbiotic efficiencies with R7A and *nodD2*.

A. Nodulation assay on Gifu and a *chi4* mutant inoculated with 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 (* = < 0.05, ** = < 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.

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 NodD inducers, two *chi4* LORE1 insertion mutant alleles (Małolepszy *et al.*, 2016) were isolated and their symbiotic phenotypes with R7A, *nodD1* (D1⁺/D2⁺) and *nodD2* (D1⁺/D2⁺) examined. Wild-type Gifu and *chi4* mutant plants formed comparable numbers of nodules when inoculated with *nodD1* (D1⁺/D2⁺); however, *chi4* mutant plants inoculated with R7A or *nodD2* (D1⁺/D2⁺) formed significantly fewer nodules than wild-type plants (Fig. 7A). IT formation by *nodD2* (D1⁺/D2⁺) was severely impaired on the *chi4* mutants, while IT formation by R7A or *nodD1* (D1⁺/D2⁺) was not significantly affected (Fig. 7B).

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

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Fig. 8. *Chi4* is induced by rhizobial inoculation specifically within root hairs. *Chi4* promoter activity was monitored using a *Pchi4*-GUS construct in transformed Gifu roots. A and B. No promoter activity was detected in control H₂O-inoculated plants. C and D. 10 dpi with R7A *Chi4* promoter activity was detected within root hairs. E and F. No promoter activity was observed within nodules. Images are representative of all plants examined. $n = 5$ for H₂O-inoculated plants and $n = 7$ for R7A-inoculated plants. Scale bars are 200 μ M.

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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* ($D1^+/D2^-$) 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 (Penmettsa 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.

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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 (Kostiuk *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 (Frayssé *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 isoliquiritigenin 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

Fig. 9. R7A NodD1 and NodD2 are activated by different inducers to control expression of NF biosynthesis genes at specific stages of symbiotic infection. An inducer (inducer 1) is secreted into the rhizosphere and is preferentially recognized by NodD2 to induce NF biosynthesis in the rhizosphere. NF-receptor perception of NF initiates symbiotic signalling and induces the expression of *Chi4* within root hairs. *CHI4* is involved in the biosynthesis of a root-hair specific inducer (inducer 2) that is preferentially recognized by NodD1 to induce NF biosynthesis within infection threads. Within nodule primordia, activation of NodD2 by inducer 1 is responsible for NF production within the nodule.

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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 hairs 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 CHI1, 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 YMB or G/RDM medium (Ronson *et al.*, 1987). *nodD/virG* double mutant strains were constructed by in-frame markerless deletion, as previously described (Rodpohong *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.

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 AR1193 (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:hydrogen peroxide (35%). Nodules were then washed with 5× changes of autoclaved water. Individual nodules were then placed in microtiter 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 d 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 (Moscatiello *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

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grown 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 using 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 OD₆₀₀ = 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 OD₆₀₀ = 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 H₂O 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 pellets 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 P*Chi4*-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 meta confocal 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 Axioplan 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 EMBOSS Needle (http://www.ebi.ac.uk/Tools/psa/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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