Nodulation Gene Mutants of *Mesorhizobium loti* R7A—*nodZ* and *nolL* Mutants Have Host-Specific Phenotypes on *Lotus* spp.

Patsarin Rodpothong,^{1,2} John T. Sullivan,^{1,2} Kriangsak Songsrirote,³ David Sumpton,³ Kenneth W. J.-T. Cheung,³ Jane Thomas-Oates,³ Simona Radutoiu,^{2,4} Jens Stougaard,^{2,4} and Clive W. Ronson^{1,2}

¹Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand; ²Centre for Carbohydrate Recognition and Signaling, University of Aarhus, Aarhus, Denmark; ³Department of Chemistry, University of York, York, United Kingdom; and ⁴Department of Molecular Biology, University of Aarhus, Aarhus, Denmark

Submitted 1 June 2009. Accepted 14 July 2009.

Rhizobial Nod factors induce plant responses and facilitate bacterial infection, leading to the development of nitrogen-fixing root nodules on host legumes. Nodule initiation is highly dependent on Nod-factor structure and, hence, on at least some of the nodulation genes that encode Nod-factor production. Here, we report the effects of mutations in *Mesorhizobium loti* R7A nodulation genes on nodulation of four *Lotus* spp. and on Nod-factor structure. Most mutants, including a *ΔnodSΔnolO* double mutant that produced Nod factors lacking the carbamoyl and possibly *N*-methyl groups on the nonreducing terminal residue, were unaffected for nodulation. R7A*ΔnodZ* and R7A*ΔnolL* mutants that produced Nod factors without the (acetyl)fucose on the reducing terminal residue had a host-specific phenotype, forming mainly uninfected nodule primordia on *Lotus filicaulis* and *L. corniculatus* and effective nodules with a delay on *L. japonicus*. The mutants also showed significantly reduced infection thread formation and *Nin* gene induction. In planta complementation experiments further suggested that the acetylfucose was important for balanced signaling in response to Nod factor by the *L. japonicus* NFR1/NFR5 receptors. Overall the results reveal differences in the sensitivity of plant perception with respect to signaling leading to root hair deformation and nodule primordium development versus infection thread formation and rhizobial entry.

The establishment of a successful *Rhizobium*–legume symbiosis requires an intricate molecular communication involving recognition of signaling molecules produced by both partners. The process of nodule organogenesis is initiated by compatible rhizobia that produce lipochito-oligosaccharide signaling molecules known as Nod factors (NF). Host-specificity in the symbiosis is manifested both by specific flavonoids released by the host legume to induce NF production in compatible rhizobia and by the specific NF produced that are recognized by the compatible host. Early NF-induced responses include ion fluxes, calcium spiking, root hair deformation, cortical cell di-

vision, and induction of early nodulin genes. NF are also required for the formation of infection threads (Spaink 2000; Geurts et al. 2005; Oldroyd and Downie 2008). The plant NF receptor is possibly a multisubunit complex comprising at least two receptor-like kinases with extracellular LysM domains (NFR1 and NFR5 in *Lotus japonicus*; NFP, LYK3, and possibly other LYK proteins in *Medicago truncatula*), because mutants defective in NFR1, NFR5, and NFP do not exhibit any NF responses (Amor et al. 2003; Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003). It has been suggested that there may be two distinct NF-induced events, a low-affinity “signaling” event required for root hair curling and primordium induction and a high-stringency “entry” event (Ardourel et al. 1994; Riely et al. 2006). It is not currently known if these events are mediated by the same or different NF receptor complexes. However, recent evidence suggests that, in *M. truncatula*, LYK3 in conjunction with NFP acts as the entry receptor while NFP in conjunction with another LYK protein acts as the signaling receptor (Smit et al. 2007).

Mesorhizobium loti is the natural microsymbiont of *Lotus* spp. The genes required for nodule formation and nitrogen fixation in *M. loti* R7A are located on a 502-kb chromosomally located transferable element known as a symbiosis island, which was recently renamed ICEM/Sym^{R7A} (Sullivan et al. 1995; Sullivan and Ronson 1998; Ramsay et al. 2006). Sequence analysis of ICEM/Sym^{R7A} revealed that the genes necessary to produce the complete NF are arranged in seven operons, each preceded by a conserved DNA motif known as a *nod* box (Sullivan et al. 2002). An eighth *nod* box is found in front of a regulatory gene required for the expression of a type IV secretion system (Sullivan et al. 2002; Hubber et al. 2004). Expression of the nodulation genes is induced through the binding of the transcriptional activator NodD to the *nod* box (Rostas et al. 1986; Fisher and Long 1993). ICEM/Sym^{R7A} carries two copies of the *nodD* gene, *nodD1* and *nodD2*.

The structures of NF produced by several *M. loti* strains, including NZP2238, the parental strain of R7A, have been determined. The “complete” NF produced by both strains is an *N*-acetylglucosamine pentasaccharide. The nonreducing residue is *N*-methylated and *N*-acylated with *cis*-vaccenic acid (C18:1) or stearic acid (C18:0) and carries a carbamoyl group, and the reducing terminal residue carries a 4-*O*-acetylfucosyl residue (López-Lara et al. 1995; Niwa et al. 2001) (Supplementary Fig. S1). The importance of the terminal decorations for nodulation of various host *Lotus* spp. has not been widely studied.

Corresponding author: Clive Ronson; Telephone: +64 3 479 7701; Fax: +64 3 479 8540; E-mail: Clive.Ronson@otago.ac.nz

*The e-Xtra logo stands for “electronic extra” and indicates that four supplementary tables and one supplementary figure are published online. Also, Figures 1 and 4 appear in color online.

Expression of *Bradyrhizobium japonicum nodZ* in *Rhizobium leguminosarum* bv. *viciae* RBL5560 allowed the strain to nodulate *L. japonicus*, normally a nonhost, but with limited efficiency. Addition of the *M. loti nolL* gene into RBL5560::*nodZ* improved nodulation kinetics to closer to those obtained with *M. loti* R7A (Pacios-Bras et al. 2000). The *nodZ* gene product adds the fucosyl residue to the reducing terminal residue of the NF (Mergaert et al. 1996; Quesada-Vincens et al. 1997) and the *nolL* gene product adds the 4-O-acetyl group to the fucosyl residue (Berck et al. 1999; Corvera et al. 1999). Hence, this study indicated the importance of the modifications to the reducing terminal residue for host-specificity in *L. japonicus*. In contrast, Shibata and associates (2005) reported that a *nolL* mutant of *M. loti* MAFF303099 nodulates *L. japonicus* normally. Several other studies have shown that various NF decorative substitutions can have host-specific effects, presumably reflecting differing specificities of the legume NF receptors. For example, strain RBL5560 carrying the *nodZ* gene also acquired the capacity to induce uninfected nodule-like structures in several other incompatible hosts (López-Lara et al. 1996). A *nolL* mutant of *M. loti* NZP2037 lost the ability to nodulate *L. pedunculatus* but still nodulated *L. corniculatus* (Scott et al. 1996). An *R. etli nolL* mutant showed reduced nodulation of some hosts but not others (Corvera et al. 1999). Transfer of the *Rhizobium* sp. NGR234 *nolL* gene into *Sinorhizobium fredii* USDA257 extended its host range to include *Calopogonium caeruleum*, *Leucaena leucocephala* and *Lotus halophilus* (Berck et al. 1999).

Two legume species, *L. japonicus* and *Medicago truncatula*, have been chosen as models to investigate legume biology due to their small genome size and other favorable characteristics. *L. japonicus* is closely related to the tetraploid pasture species *L. corniculatus* (birdsfoot trefoil) and it is also cross-fertile with the diploid species *L. filicaulis* and *L. burtii*. Genetic maps and recombinant inbred lines derived from crosses of these species with *L. japonicus* Gifu and intraspecific crosses between *L. japonicus* ecotypes Gifu and Miyakojima MG-20 provide powerful tools for efficient genetic analysis in *Lotus* spp. (Hayashi et al. 2001; Kawaguchi et al. 2005; Sandal et al. 2006). Therefore, knowledge of the effects of NF structure on host specificity coupled with approaches such as quantitative trait loci analysis and map-based cloning could contribute to our understanding of the structure and function of NF receptors.

In this study, we have systematically mutagenized the *nod* genes present on ICEMISym^{R7A} and assessed the symbiotic properties of the strain R7A mutants on four *Lotus* spp. Only

three mutants had a clean Nod⁻ phenotype, while the *nodZ* and *nolL* mutants displayed host-specific phenotypes. Our results are consistent with a two-phase model of NF perception, one phase involving root hair deformation and primordium induction and the other infection thread formation and entry.

RESULTS

Mutants in most *Mesorhizobium loti nod* genes do not have an overt symbiotic phenotype.

Mutants with markerless in-frame deletions in 12 of the nodulation genes identified on ICEMISym^{R7A} (all nodulation genes except *nodI*, *nodJ*, *noeJ*, and *noeK*) plus two double mutants (R7AΔ*nodS*Δ*nolO* and R7AΔ*nodD1*Δ*nodD2*) were constructed and their symbiotic properties were assessed on at least two of the four species *L. corniculatus*, *L. japonicus* Gifu, *L. filicaulis*, and *L. burtii* (Table 1). Only strains R7AΔ*nodA*, R7AΔ*nodC*, and R7AΔ*nodD1*Δ*nodD2* failed to induce nodule formation. Strain R7AΔ*nodA* was complemented to Nod⁺ by pJS01 that contains only *nodA*, indicating that the Δ*nodA* mutation did not alter expression of the downstream *nodC* gene. Mutants defective in *nodD2*, *nodM*, *nodB*, *nodS*, and *nolO* formed nitrogen-fixing nodules on all *Lotus* spp. tested, with the same nodulation kinetics as wild-type R7A, as did a Δ*nodS*Δ*nolO* double mutant. Strain R7AΔ*nodD1* induced nitrogen-fixing nodules on *L. japonicus* and *L. corniculatus* with a slight delay (3 days) in the onset of nodulation (data not shown). In contrast, mutants in *nolL* (R7AΔ*nolL*) and in genes of the *nodZ* operon (R7AΔ*nodZ*, R7AΔ*noeL*, and R7AΔ*nolK*) showed a host-specific phenotype, forming effective nodules on *L. burtii* and *L. japonicus* Gifu after a delay but mainly uninfected nodules on at least one other host (see below).

To determine if the efficiency of nodulation was impaired by particular mutations, coinoculation studies of selected mutants with an effective marked strain R7AnifA1::Ωkan were carried out on *L. japonicus* (Table 2). A mixed inoculum of R7A and R7AnifA1::Ωkan showed an average of 40 and 55% of nodules occupied by R7AnifA1::Ωkan and R7A, respectively. This suggests that R7AnifA1::Ωkan was able to invade plant cells and occupy nodules as efficiently as R7A. In contrast, the Δ*nodB*, Δ*nodZ*, and Δ*nolL* strains displayed severely impaired competitive ability because only an average of 3 and 5% of the nodules were occupied by R7AΔ*nodB* and R7AΔ*nodZ*, respectively, compared with an average of more than 80% of nodules occupied by R7AnifA1::Ωkan. None of the nodules were occupied by strain R7AΔ*nolL* alone. The R7AΔ*nodS*, R7AΔ*nolO*,

Table 1. Nodulation phenotypes of *Lotus* spp. inoculated with *Mesorhizobium loti nod* gene mutants observed at week 4 postinoculation

Strain	Nodulation phenotype on <i>Lotus</i> spp. ^z			
	<i>L. japonicus</i>	<i>L. filicaulis</i>	<i>L. corniculatus</i>	<i>L. burtii</i>
R7A	Inf ⁺ Ndv ⁺	Inf ⁺ Ndv ⁺	Inf ⁺ Ndv ⁺	Inf ⁺ Ndv ⁺
R7AΔ <i>nodD1</i>	Inf ⁺ Ndv ^d	Nd	Inf ⁺ Ndv ^d	Nd
R7AΔ <i>nodD2</i>	Inf ⁺ Ndv ⁺	Nd	Inf ⁺ Ndv ⁺	Nd
R7AΔ <i>nodD1</i> Δ <i>nodD2</i>	Inf ⁻ Ndv ⁻	Nd	Inf ⁻ Ndv ⁻	Nd
R7AΔ <i>nodA</i>	Inf ⁻ Ndv ⁻	Nd	Inf ⁻ Ndv ⁻	Nd
R7AΔ <i>nodB</i>	Inf ⁺ Ndv ⁺	Inf ⁺ Ndv ⁺	Inf ⁺ Ndv ⁺	Inf ⁺ Ndv ⁺
R7AΔ <i>nodC</i>	Inf ⁻ Ndv ⁻	Inf ⁻ Ndv ⁻	Inf ⁻ Ndv ⁻	Inf ⁻ Ndv ⁻
R7AΔ <i>nodM</i>	Inf ⁺ Ndv ⁺	Inf ⁺ Ndv ⁺	Inf ⁺ Ndv ⁺	Nd
R7AΔ <i>nodS</i>	Inf ⁺ Ndv ⁺	Nd	Inf ⁺ Ndv ⁺	Inf ⁺ Ndv ⁺
R7AΔ <i>nolO</i>	Inf ⁺ Ndv ⁺	Nd	Inf ⁺ Ndv ⁺	Inf ⁺ Ndv ⁺
R7AΔ <i>nolO</i> Δ <i>nodS</i>	Inf ⁺ Ndv ⁺	Inf ⁺ Ndv ⁺	Inf ⁺ Ndv ⁺	Inf ⁺ Ndv ⁺
R7AΔ <i>nodZ</i>	Inf ⁺ Ndv ^d	Inf ⁻ Ndv ^d	Inf ⁻ Ndv ^d	Inf ⁺ Ndv ⁺
R7AΔ <i>nolL</i>	Inf ⁺ Ndv ^d	Inf ⁻ Ndv ^d	Inf ⁺ Ndv ^d	Inf ⁺ Ndv ⁺
R7AΔ <i>nolK</i>	Inf ⁺ Ndv ^d	Inf ⁻ Ndv ^d	Inf ⁺ Ndv ^d	Inf ⁺ Ndv ⁺
R7AΔ <i>noeL</i>	Inf ⁺ Ndv ^d	Inf ⁻ Ndv ^d	Inf ⁻ Ndv ^d	Inf ⁺ Ndv ⁺

^z Inf⁺ = infected nodules, Inf⁻ = uninfected nodules, Ndv⁺ = normal nodule development, Ndv^d = delayed nodule development, Ndv⁻ = no nodule development, and Nd = not determined.

and R7A Δ nodS Δ nodO strains were as competitive as wild-type R7A because an average of 68% of nodules formed on *L. japonicus* roots were occupied by the former strains whereas less than 30% of the nodules were occupied by R7A Δ nifA1:: Ω kan. Similar results were obtained on *L. filicaulis* (data not shown).

The host-specific nodulation phenotype displayed by the Δ nodL and Δ nodZ mutants.

On *L. filicaulis*, R7A Δ nodL and R7A Δ nodZ both exhibited severely impaired nodulation. Nodule primordia were first observed on day 17 postinoculation. The majority of these structures did not develop further (Fig. 1). By day 38 postinoculation, a few effective nodules were observed on 5 and 15% of *L. filicaulis* plants inoculated with R7A Δ nodZ and R7A Δ nodL, respectively (Fig. 2A). In contrast, nodule primordia were first observed on roots of *L. filicaulis* inoculated with R7A on day 8 postinoculation, and all nodules were effective (Fig. 2A). Strain R7A Δ nodZ had a more severe phenotype on *L. corniculatus* compared with strain R7A Δ nodL (Fig. 2B). Nodule primordia induced by R7A Δ nodZ first appeared on day 14 postinoculation but only a small percentage developed into effective nodules and then only on 10% of the plants. In contrast, nodule primordia induced by R7A Δ nodL were detected on day 9 postinoculation and 75% of the plants developed effective nodules. R7A Δ nodZ and R7A Δ nodL both formed effective nodules on all *L. burtii* and *L. japonicus* plants but nodule formation was delayed compared with plants inoculated with strain R7A. The delay was more pronounced for *L. japonicus* than *L. burtii* (Fig. 2C and D). However, all plants nodulated by day 21 postinoculation and all nodules were pink in color, indicative of infection. The mutant strains were complemented with stable plasmid constructs pPR1 (*nodZ*noe Δ nodK) and pPR2 (*nodL*) and nodulation studies carried out on *L. corniculatus*. The nodulation kinetics of plants inoculated with strain R7A Δ nodZ/pPR1 were similar to those inoculated with the wild-type strain R7A. However, although strain R7A Δ nodL/pPR2 induced a higher number of

nodules than strain R7A Δ nodL, the onset of nodulation was still delayed (Fig. 2B).

The Δ nodL and Δ nodZ mutants are defective for infection thread formation.

The abilities of R7A Δ nodZ and R7A Δ nodL to induce root hair deformation and to form infection threads were assessed on *L. corniculatus* and *L. japonicus*. Root hair branching, root hair tip swelling, and root hair curling were observed on both *Lotus* spp. inoculated with R7A Δ nodZ or R7A Δ nodL (data not shown). To allow infection thread visualization, plasmid pPR3

Table 2. Percentage occupancy of *Lotus japonicus* nodules inoculated with different *Mesorhizobium loti* strains^z

Inoculum	R7AnifA only	R7A or mutant only	Mixed
R7AnifA1:: Ω kan	100	0	0
R7AnifA1:: Ω kan:R7A	40 $\pm$ 6	55 $\pm$ 4	4 $\pm$ 2
R7AnifA1:: Ω kan:R7A Δ nodZ	93 $\pm$ 4	3 $\pm$ 3	4 $\pm$ 2
R7AnifA1:: Ω kan:R7A Δ nodL	87 $\pm$ 7	0	13 $\pm$ 7
R7AnifA1:: Ω kan:R7A Δ nodB	84 $\pm$ 4	5 $\pm$ 5	11 $\pm$ 1
R7AnifA1:: Ω kan:R7A Δ nodS	16 $\pm$ 3	68 $\pm$ 6	16 $\pm$ 3
R7AnifA1:: Ω kan:R7A Δ nodO	29 $\pm$ 2	68 $\pm$ 6	3 $\pm$ 3
R7AnifA1:: Ω kan:R7A Δ nodO Δ nodS	22 $\pm$ 3	68 $\pm$ 12	9 $\pm$ 9

^z Values represent the mean percentage occupancy ($\pm$ standard error of the mean) of at least two experimental replicates.

Fig. 1. A, Effective nodules formed by R7A and B, uninfected nodule primordia formed by R7A Δ nodZ on *Lotus filicaulis* roots at 4 weeks postinoculation.

Fig. 2. Nodulation kinetics on A, *Lotus filicaulis*; B, *L. corniculatus*; C, *L. japonicus* Gifu; and D, *L. burtii* inoculated with *Mesorhizobium loti* strains R7A, R7A Δ nodL, and R7A Δ nodZ. *L. corniculatus* inoculated with complemented strains R7A Δ nodL/pPR2 and R7A Δ nodZ/pPR1 are also shown in B. Twenty plants were used per strain per experiment. Error bars represent $\pm$ standard error of the mean. Only nodules assessed as being infected on the basis of size and color were counted.

that constitutively expresses enhanced green fluorescent protein (eGFP) was introduced into R7A, R7A Δ nodZ, and R7A Δ nodL. The number of infection threads formed by the mutants on *L. japonicus* seedlings was significantly reduced compared with the wild type, with R7A Δ nodL forming even fewer infection threads than R7A Δ nodZ (Fig. 3A). In *L. corniculatus* root hairs, R7A Δ nodL also displayed a delay in infection thread formation but infection thread numbers were not significantly different from the numbers formed by R7A at 10 days postinoculation (Fig. 3B). In contrast, infection thread formation by R7A Δ nodZ was markedly reduced (Fig. 3B).

Transmission electron microscopy performed on sections of 4-week-old *L. japonicus* and *L. corniculatus* nodules formed by R7A Δ nodZ and R7A Δ nodL revealed the presence of infection threads and infected plant cells containing bacteroids. White nodule primordia on *L. corniculatus* roots inoculated with R7A Δ nodZ were devoid of bacteroids (data not shown). Taken together, these results suggest that the delayed nodulation phenotype and the reduced nodulation competitiveness observed with R7A Δ nodZ may be due to the defect in infection thread formation.

Acetylfucosylated NF are required for efficient NF signal transduction.

Previous studies showed that expression of, or balanced signaling through, the *Lotus Nfr1/Nfr5* receptors may influence perception of NF variants (Radutoiu et al. 2007). Therefore, in planta complementation of the non-nodulating *L. japonicus nfr1* and *nfr5* mutants by *Agrobacterium rhizogenes* transformation was employed to determine if the acetylfucose decoration was involved in NF recognition by the NFR1/NFR5 receptors. The *nfr1* and *nfr5* mutant plants were complemented by transformation with vectors containing the *L. japonicus Nfr1* and *Nfr5* genes, respectively, and the transformed hairy

roots were inoculated with R7A or R7A Δ nodZ. At week 3 postinoculation, R7A had formed effective nodules on complemented hairy roots of 71% of *nfr1* mutant plants (25 of 35 plants), whereas R7A Δ nodZ formed nodules on only 7% (3 of 46) of plants with complemented hairy roots. In contrast, R7A formed nodules on approximately 65% (11 of 17 plants) and R7A Δ nodZ on 42% (11 of 26 plants) of the complemented *nfr5* plants. In comparison, strain R7A induced effective nodules on hairy roots of 81% (17 of 21) and R7A Δ nodZ on 70% (21 of 30) of wild-type *L. japonicus* plants transformed with the empty hairy root transformation vector pAR12.

Histochemical staining of *L. japonicus Nin-GUS* roots demonstrated that R7A induced *Nin* expression within 24 h postinoculation and the expression was strongest 10 days postinoculation (Fig. 4). In contrast, induction of *Nin* expression by R7A Δ nodL and R7A Δ nodZ was not observed until 10 days postinoculation and the level of expression was markedly reduced compared with R7A. The zone of expression was essentially limited to the site of nodule primordium development for both mutants, whereas R7A-induced expression was dispersed throughout the root (Fig. 4B). Taken together, these results suggest that the acetylfucose moiety is involved in attaining optimal NF signal transduction initiated by the NFR1/NFR5 receptors.

Determination of NF structures formed by the *M. loti* wild-type and Δ nodZ, Δ nodL, and Δ nodS Δ nodO mutants.

To demonstrate that the observed nodulation behavior of the *M. loti* Δ nodZ, Δ nodL, and Δ nodS Δ nodO mutants was related to the anticipated alterations in their NF structures, the NF from R7A and the mutants were extracted, isolated, and identified using mass spectrometry. The results of analysis of the NF are summarized in Table 3.

R7A produced a range of pentameric NF bearing a fatty acyl group on the nonreducing terminal residue; the acyl groups were saturated or monounsaturated and mainly had 16 or 18 carbon atoms, although two structures were proposed to bear a hydroxylated C22 fatty acyl chain. The majority of the observed structures were assigned as being *N*-methylated, consistent with previous reports of NF structures from *M. loti* strains (López-Lara et al. 1995; Niwa et al. 2001), although it is formally possible that these may be unmethylated species bearing unusual fatty acyl chains with an additional CH₂ moiety and, thus, having an odd number of carbon atoms (these formal possibilities have not been included in the summary table, for

Fig. 3. Average number of infection threads formed in root hairs of **A**, *Lotus japonicus* and **B**, *L. corniculatus* by R7A, R7A Δ nodZ, and R7A Δ nodL containing pPR3 (*nptII_p-gfp*). Numbers of infection threads were monitored at 5, 7, and 10 days postinoculation. Results include the sample size and the mean $\pm$ standard error of the mean. An asterisk represents *P* values < 0.005 and two asterisks represent *P* values < 0.0005. The *P* values were calculated using the alternate Welch *t* test with two-tailed *P* value. Note that the *P* value cannot be calculated for the data with a standard deviation of zero.

Fig. 4. Histochemical staining of *Lotus japonicus Nin-GUS* roots after inoculation with R7A, R7A Δ nodZ, and R7A Δ nodL at **A**, 48 h postinoculation and **B**, 10 days postinoculation.

conciseness). Several species were also *O*-carbamoylated on the nonreducing terminal residue. All the wild-type NF structures observed were substituted on the reducing terminal residue with either an *O*-fucosyl or an *O*-acetylfucosyl residue. The proposed structures are in keeping with the NF structures previously characterized in *M. loti* strains (López-Lara et al. 1995; Olsthoorn et al. 1998; Niwa et al. 2001). The elution order of the NF was consistent with the proposed structural assignments (Supplementary Table 1), with retention increasing with increasing length of fatty acyl chain and increasing fatty acyl saturation. NF bearing hydroxylated fatty acids have been reported previously (Olsthoorn et al. 2000) but not in *M. loti* extracts. The relatively early elution of the proposed hydroxylated NF is consistent with their assignment; it is considerably earlier than would be expected for a nonhydroxylated C22 fatty acyl-bearing homologue. In the previous study, a McLafferty-type rearrangement was observed on CID, resulting in the elimination of a large part of the fatty acid chain (Olsthoorn et al. 2000). This was not observed in the tandem mass spectrometric analyses of the proposed hydroxylated NF in this study

Table 3. Summary of Nod-factor structures produced by R7A and selected mutant strains

Strains	Structures
R7A	V(C16:0, AcFuc) V(C16:0, NMe, Fuc) V(C16:0, NMe, AcFuc) V(C16:0, NMe, Cb, AcFuc) V(C18:0, NMe, AcFuc) V(C18:0, NMe, Cb, AcFuc) V(C18:1, NMe, Fuc) V(C18:1, NMe, AcFuc) V(C18:1, NMe, Cb, AcFuc) V(C22:0-OH, NMe, AcFuc) V(C22:0-OH, NMe, Cb, AcFuc)
R7A Δ <i>nolL</i>	V(C14:2, Cb, Fuc) V(C14:2, NMe, Cb, AcFuc) or V(C14:1-OH, Cb, AcFuc) V(C16:0, NMe, Fuc) or V(C16:1-OH, Fuc) V(C18:0, NMe, Fuc) or V(C18:1-OH, Fuc) V(C18:1, AcFuc) V(C18:1, Cb, Fuc) V(C18:1, Cb, AcFuc) V(C18:1, NMe, Fuc) or V(C18:2-OH, Fuc) V(C18:1-OH, NMe, Fuc) or V(C20:0, Fuc) V(C18:1, NMe, Cb, Fuc) or V(C20:0, Cb, Fuc) V(C18:1, Cb, NMe, Fuc) or V(C18:2-OH, Cb, Fuc) V(C18:0, NMe, Cb, AcFuc) or V(C18:1-OH, Cb, AcFuc)
R7A Δ <i>nodZ</i>	V(C16:0, NMe) V(C16:0, NMe, Cb) V(C18:0, NMe) or V(C19:0) V(C18:0, NMe, Cb) V(C18:1, NMe) V(C18:1, NMe, Cb)
R7A Δ <i>nodS</i> Δ <i>nolO</i>	III(C18:1, Fuc) IV(C16:0, Fuc) IV(C18:1, Fuc) V(C12:1, Cb, Fuc) V(C12:1, Cb, AcFuc) V(C14:0, AcFuc) V(C16:0, AcFuc) V(C16:1, AcFuc) V(C16:0-OH, NMe, AcFuc) V(C18:1) V(C18:1, Fuc) V(C18:0, NMe) or V(C18:1-OH) V(C18:0, AcFuc) V(C18:0-OH, NMe, AcFuc) V(C18:1, AcFuc) V(C18:2-OH, AcFuc) or V(C18:1, NMe, AcFuc) V(C18:1-OH, AcFuc) or V(C18:0, NMe, AcFuc)

(data not shown). However, it should be noted that the product ion spectra in the two studies were recorded under different CID regimes and, thus, are not necessarily directly comparable; in this study, the product ion spectra were recorded using high-energy CID whereas low-energy CID was used in the previous study.

As expected, the *nodZ* mutant produced unfucosylated NF (Table 3). In keeping with the results of the wild-type strain analysis, all the NF structures consisted of a pentameric GlcNAc backbone bearing a fatty acyl group on the nonreducing terminal residue; the length of the acyl chains (C16 and C18) and the presence of *N*-methylation and *O*-carbamoylation were all analogous to the results of the wild-type extract analysis. Again, the elution order of the NF was consistent with the proposed structural assignments (Supplementary Table 2).

The *nolL* mutant produced pentameric NF structures with C14-C18 fatty acyl groups on the nonreducing terminal residue. However, in addition to the expected fucosylated NF, acetyl-fucosylated structures were also unexpectedly detected. Although there were fewer different such acetylfucosyl structures produced by the mutant than the wild type, these were not minor components. The major NF species bore C16 and C18 saturated or monosaturated acyl groups. In addition, two minor, previously unreported C14 fatty acyl-bearing species were also observed (Supplementary Table 3).

The NF structures of the R7A Δ *nolO* Δ *nodS* double-mutant strain mainly consisted of a backbone structure of five GlcNAc residues, together with a single three-GlcNAc-residue NF and two GlcNAc₄ structures. The NF from the R7A double-mutant strain mostly bore *O*-acetyl-fucose or *O*-fucose on the reducing terminal residue, and the nonreducing terminal residue was *N*-acylated with predominantly C16:0, C16:1, C18:0, or C18:1 chains. Due to the deletion of *nolO* which is responsible for carbamoylation and *nodS* for *N*-methylation of the NF, this strain was not expected to produce carbamoyl and *N*-methyl decorations. However, the mass spectrometric and CID tandem mass spectrometric data demonstrated the presence of two very minor carbamoylated species (Supplementary Table 4). However, these species were not detected in NF preparations from the *nolO* single mutant (data not shown).

DISCUSSION

Mutants in most of the nodulation genes on ICEMISym^{R7A} did not have an overt symbiotic defect. Of the common *nod* genes, *nodA* and *nodC* mutants showed a Nod⁻ phenotype as expected but, surprisingly, the *nodB* mutant retained the ability to induce nodules on all *Lotus* spp., although it was severely compromised in nodulation competition studies. NodB is a deacetylase that removes the *N*-acetyl moiety from the NF nonreducing terminal residue to allow the addition of an acyl chain to the nitrogen of the resulting primary amine by NodA (John et al. 1993). In most rhizobia, *nodABC* form an operon and the *nodBC* genes are phylogenetically congruent whereas, in *M. loti*, *nodB* is in a separate operon to *nodAC* (Scott et al. 1996; Sullivan et al. 2002) and *nodB* and *nodC* are phylogenetically incongruent (Wernegreen and Riley 1999). It seems possible that *nodB* mutants examined previously were Nod⁻ because of polar effects on the expression of *nodC*. Our results suggest that other deacetylases may substitute for the role of NodB, allowing sufficient NF to be made to induce nodulation. Mutants in the regulatory *nodD1* and *nodD2* genes were Nod⁺ but nodulation was abolished in the *nodD1**nodD2* double mutant, indicating that NodD1 and NodD2 can functionally complement one another. However, the *nodD1* mutant showed delayed nodulation on *Lotus* spp. (this work) and is unable to nodulate *Leucaena leucocephala* (Hubber et al. 2007), indicat-

ing that *nodD1* is the more important of the two *nodD* genes. No role for *nodD2* was apparent.

Chemical substituents on the nonreducing terminal residues of NF are important for the initiation and extension of infection threads in *S. meliloti* and *R. leguminosarum* (Downie and Surin 1990; Ardourel et al. 1994; Walker and Downie 2000). However *M. loti* Δ *nodS*, Δ *noI*O, and Δ *nodS* Δ *noI*O mutants all nodulated normally and did not display competitive defects in coinoculation studies. NF structures from the Δ *nodS* Δ *noI*O mutant revealed that only NF species with an atypical fatty acid (C12) carried a carbomoyl group, which may reflect the activity at a very low level of another transferase. Carbomoyl groups were not detected in NF with C16 or C18 fatty acids or in NF from the single Δ *noI*O mutant, suggesting that the carbomoyl group is not required for the host-specificity or efficiency of nodulation of *Lotus* spp. The importance of the methyl group in nodulation is still unclear because these NF structural analyses did not distinguish between, for example, *N*-methylated C18:0 or the (less likely) unmethylated C19:0 NF.

Pacios-Bras and associates (2000) showed that the addition of *nodZ* and *noI*L to *R. leguminosarum* resulted in a strain, strain DZL, able to nodulate *Lotus japonicus*, again suggesting a low-stringency requirement for the nonreducing terminal decorations. However, strain DZL is unable to nodulate *L. filicaulis*, a host-specific nodulation phenotype that has been delimited to differences in the *Nfr5* receptor genes of *L. japonicus* and *L. filicaulis* (Radutoiu et al. 2007). The NF produced by strain DZL differ from those produced by *M. loti* R7A not only in the decorations on the nonreducing terminal residue but also in the nature of the attached fatty acid. Given that the nonreducing-terminal carbamoylation decoration is not required for NF perception by *L. filicaulis*, our results suggest that the fatty acid may be the more important host-specific determinant.

As expected, strain R7A Δ *nodZ* produced NF species lacking the acetylucose on the reducing terminal residue. Thus, the symbiotic defects induced by R7A Δ *nodZ* indicate the importance of the acetylucose in early symbiotic interactions. Surprisingly, NF from R7A Δ *noI*L were a mixture of fucosylated NF and acetylucosylated NF species, indicating that other acetyltransferases can functionally complement the *noI*L enzyme. Acetylated structures were also seen in NF species produced by another *M. loti* *noI*L mutant (Shibata et al. 2005). However, the fucosylated NF species were more common in R7A Δ *noI*L than in R7A. The symbiotic phenotypes of R7A Δ *noI*L may be the result of a reduced level of acetylucosylated NF species, or possibly the acetyl group may influence the stability and, hence, concentration of the NF signal. It has previously been suggested that the yield of NF obtained from the *R. leguminosarum* DZL strain was higher than that obtained for *R. leguminosarum* DZ that lacks *noI*L (Pacios-Bras et al. 2000).

The host-specific nodulation phenotype induced by R7A Δ *nodZ* and R7A Δ *noI*L on four *Lotus* spp. may reflect the diversity of plant receptor structures or differences in the sensitivity of the receptors to NF. The ability of both the R7A Δ *nodZ* and R7A Δ *noI*L mutants to induce root hair curling on *L. corniculatus* and *L. japonicus* plants indicates that the acetylucose was not required for this early response. The delayed formation of nodule primordia on roots of *L. filicaulis* suggests that the acetylucose facilitated, but was not an absolute requirement for, the induction of cortical cell division. Strains R7A Δ *nodZ* and R7A Δ *noI*L exhibited a significantly reduced capacity to elicit infection thread formation on both *L. japonicus* and *L. corniculatus*. Aberrant infection threads were not observed, suggesting that there was no defect in infection thread extension and maintenance. In contrast, aberrant infection thread structures were observed with *S. meliloti* *nodF**nodL*

mutants that produce NF with modified nonreducing terminal residues (Ardourel et al. 1994; Limpens et al. 2003) and *S. meliloti* *exo* mutants that produce altered succinoglycans (Cheng and Walker 1998; Pellock et al. 2000). The delayed and reduced infection thread initiation observed with R7A Δ *nodZ* and R7A Δ *noI*L is consistent with the delayed and reduced nodulation phenotypes and impaired competitiveness of the mutants. Taken together, these results indicate that the acetylucose moiety on the reducing terminal residue of the *M. loti* NF plays a significant role at the stage of infection thread initiation and rhizobial entry into the root hairs. In the case of *L. japonicus*, sufficient signaling eventually occurs to allow infection and effective nodule formation whereas, in *L. corniculatus* and *L. filicaulis*, signaling does not reach the threshold required to allow bacterial entry.

The in planta complementation experiment showed that introduction of *Nfr1* and *Nfr5* to nonnodulating *L. japonicus* *nfr1* and *nfr5* plant mutants, respectively, restored the nodulating ability of the composite plants when inoculated with R7A, as previously reported (Radutoiu et al. 2007). However, nodulation of the *nfr1* plants transformed with *Nfr1* and inoculated with R7A Δ *nodZ* was severely impaired. These results are in accordance with results obtained using the *R. leguminosarum* DZL strain (Radutoiu et al. 2007) and suggest that perception of modified NF is particularly sensitive to *Nfr1* expression levels or NFR1-mediated signaling. The receptor function obtained was sufficient to allow a normal response to complete NF but not to NF lacking the acetylucose, providing further evidence that the acetylucose on the reducing terminal residue of NF is required for efficient signal transduction mediated by NF perception at the extracellular domain of the NFR1 receptor.

The acetylucose NF decoration also strongly influenced the induction of *L. japonicus* *Nin* expression, as shown by histochemical staining. *Nin* mutants are devoid of infection thread and nodule primordium formation and, consistent with this, reduced *Nin* expression was observed in *L. japonicus* *Nin*-*GUS* nodule primordia induced by the mutants. Indeed, the expression pattern induced by R7A Δ *nodZ* was localized to the site of nodule primordium development whereas *Nin* expression induced by R7A was dispersed along the plant roots. The reason for this differential expression pattern of *L. japonicus* *Nin* induced by different NF structures may again be linked to the efficiency or magnitude of NF signal transmission between cells. The expression of *Nin* is dependent on the Ca^{2+} -calmodulin dependent kinase that is presumed to be activated by Ca^{2+} oscillation (Oldroyd and Downie 2004; Marsh et al. 2007). Higher concentrations of NF with altered structures compared with NF with wild-type structures were required to provoke Ca^{2+} spiking in *Medicago truncatula*. For example, *S. meliloti* NF lacking a sulfate group on the reducing terminal residue had a 30,000-fold-reduced capacity to induce Ca^{2+} spiking, and NF containing a modified acyl chain showed a 100-fold reduction in activity (Oldroyd et al. 2001; Wais et al. 2002). It is possible that the NF lacking the acetylucose group produced by strain R7A Δ *nodZ* are required at a higher concentration to trigger signal transduction to the same level as that triggered by wild-type NF. The localized expression of *Nin* in the nodule primordia at 10 days postinoculation may be explained by a weaker signal transduction or reduced infection that is limiting *Nin* expression in the epidermal cell layer at earlier time points.

Two models have been proposed to explain the perception of NF that leads to the induction of the NF signal transduction cascade. Ardourel and associates (1994) suggested a two-receptor model, with a signaling receptor responsible for the induction of plant responses leading to the formation of nodule primordia and an entry receptor responsible for the regulation

of bacterial entry (infection thread formation). The signaling receptor is proposed to have a minimal requirement for NF structure whereas the entry receptor requires specific NF decorations. A one-receptor model was proposed by Oldroyd and associates (2001), with differential activation of the receptor or receptor complex having different effects on the downstream signaling cascade, resulting in the different stringencies that are observed between early plant responses and bacterial infection. Our study shows that R7A Δ nodZ was able to induce root hair curling and cortical cell division with only a delay but was severely defective in infection thread formation. These results are consistent with a two-phase recognition model, with early plant responses resulting from low-stringency recognition and rhizobial invasion requiring more stringent recognition. Our data also suggest that the NFR1/NFR5 receptors respond most efficiently to NF containing the acetylucose residue, consistent with a single-receptor complex with two output phases.

MATERIALS AND METHODS

Bacterial strains and *Lotus* spp. seed.

Mesorhizobium loti R7A (Sullivan et al. 1995) was used as the wild-type strain and for generation of all mutants. Strain R7AnifAI:: Ω kan was described by Sullivan and associates (2001). Seed of *L. filicaulis*, *L. burtii*, and *L. japonicus* Gifu was provided by N. Sandal, University of Aarhus, Denmark. *L. corniculatus* seed was supplied by AgResearch, New Zealand.

Media and growth conditions.

M. loti strains were grown at 28°C in or on Rhizobium defined medium (RDM) with either 4% (wt/vol) glucose (G/RDM) or 5% (wt/vol) sucrose (S/RDM) (Sullivan et al. 2001) or in B medium (van Brussel et al. 1977). Media were supplemented with antibiotics as required at the following concentrations: neomycin at 200 μ g/ml, gentamicin at 40 μ g/ml, and spectinomycin at 250 μ g/ml.

Construction of *M. loti* mutants.

All *nod* gene mutants were constructed as in-frame markerless deletions. Oligonucleotide primer pairs were designed to amplify two 1- to 1.2-kb regions, one of which contained a short region of the 5' end of the gene and approximately 1 kb of preceding sequence and the other a short region of the 3' end and 1 kb of downstream sequence. Restriction endonuclease sites were incorporated into the primers to facilitate directional cloning. The two polymerase chain reaction (PCR) products were cloned into pJQ200SK (Quandt and Hynes 1993) such that the PCR products were fused in appropriate orientation to produce an insert containing an in-frame deletion derivative of the gene and flanking regions. The resulting constructs were confirmed by sequencing and then transferred from *Escherichia coli* S17-1/ λ pir (Herrero et al. 1990) into *M. loti* R7A by biparental spot matings (Hubber et al. 2004). Exconjugants were plated onto G/RDM containing gentamicin and single crossover events were confirmed by PCR. Suitable gentamicin-resistant isolates were then plated on S/RDM and resulting sucrose-resistant, gentamicin-sensitive colonies were screened by PCR and DNA sequencing to confirm the mutants.

Plasmid constructs.

To construct plasmids pPR1 and pPR2, oligonucleotide primer pairs were designed to amplify the *nodZ* operon that contains the *nodZ*, *nolK*, and *noeL* genes or the *nolL* gene and their respective promoter regions. Restriction endonuclease sites were incorporated into each end of the PCR products. The *nodZ* operon PCR product and the *nolL* PCR product were ligated into pBluescript II KS and pLitmus28 (New England BioLabs, Bev-

erly, MA, U.S.A.), respectively. The inserted fragments were then excised and ligated into pFAJ1700 (Dombrecht et al. 2001) to give pPR1 and pPR2. To produce pJS01, the *nodA* gene and preceding promoter region was amplified and cloned into pFAJ1700. Plasmid pPR3 was constructed by inserting a 336-bp PCR product amplified from the *nptII* promoter within plasmid pFAJ1708 (Dombrecht et al. 2001) into pPROBE-KT (Miller et al. 2000) as a *HindIII-EcoRI* fragment upstream of the *gfp* gene. All plasmid inserts were confirmed by sequencing.

Nodulation assays.

Lotus spp. seed were scarified by immersion in sulfuric acid for 5 min followed by washing in sterile water. The seed were then surface-sterilized by immersion in 10% (vol/vol) commercial bleach and 0.02% (vol/vol) Tween 80 for 20 min and washed five times in sterile water. Seed were germinated in the dark for 2 days on 0.8% (wt/vol) water agar. For nodulation tests, seedlings were grown individually in test tubes containing 8-ml slopes of Jensen's agar (Vincent 1970) capped with foam plugs that allowed unimpeded gas exchange, and left to grow for 2 days prior to inoculation. The plants were observed for nodule formation every 2 days for at least 28 days. In all, 15 to 20 plants were assessed per experiment. The experiments were repeated two to three times. For infection thread assays, seedlings were grown between nylon pillows as previously described (Szczyglowski et al. 1998) with some modifications. The pillows (5 by 5 by 19 cm) were soaked in Hoaglund's nitrogen-free medium (Hotter and Scott 1991) for 30 min before planting. Seedlings were left to grow for 2 days before inoculation. Roots were stained with propidium iodide at 10 μ g/ml and observed using an Olympus BX51 microscope with fluorescence illuminator (BX-RFA) at days 5, 7, and 10 post-inoculation. GFP-labeled bacterial cells were visualized using a fluorescence mirror unit (U-MWIB3). For root hair deformation assays, plants were grown on a Fahraeus slide (Vincent 1970) with some modifications. Hoaglund's medium was used and the coverslip was removed from the slide after the agar was set on the Fahraeus slides. Inoculum was spotted at the tip of the root. Root hair deformation was observed under a light microscope at 3 days postinoculation. Ten plant roots were assessed at each time point per assay for each treatment. The assays were repeated twice.

Coinoculation studies.

These were performed as previously described (Hubber et al. 2004) with some modifications. Plants were inoculated with 200 μ l of 1:1 mixed suspensions of R7AnifAI:: Ω kan and R7A or R7A Δ nodZ or R7A Δ nolL, each at 10³ CFU/ml. Bacteria were isolated from all nodules formed on *Lotus* plants and were cultured on G/RDM plates. To determine the ratio of different *M. loti* strains, 10 bacterial colonies from each plate were patched onto nonselective G/RDM and G/RDM containing neomycin at 200 μ g/ml.

Hairy root transformation and nodulation tests of composite plants.

L. japonicus *nfr1* and *nfr5* mutant complementation studies were performed as previously described (Radutoiu et al. 2007), except that composite plants were analyzed for nodulation at 3 weeks postinoculation.

NF purification.

Plasmid pMP2112 (Spaink et al. 1989) that contains the *nodD* gene of *R. leguminosarum* was transferred to the relevant strain so that *nod* gene expression could be induced with naringenin (López-Lara et al. 1995). NF were extracted using *n*-butanol as described (Niwa et al. 2001). The crude NF extracts were pre-

fractionated using solid-phase extraction (SPE) on a C18 column (Strata C18-E 70Å), essentially as described (Soria-Díaz et al. 2003). The NF were eluted from the SPE columns using sequentially two different aqueous acetonitrile [ACN (aq)] solutions (45 and 60%, vol/vol) to produce two fractions, which were then dried under vacuum. The SPE fractions were reconstituted overnight in 700 µl of 60% ACN (aq). This was diluted with H₂O to a final volume of 2 ml prior to reversed-phase high-performance liquid chromatography (HPLC) fractionation. The HPLC fractionation was carried out on a C18 column (5 µm OSD2, 4.6 by 250 mm) with two subsequent 1-ml injections, and eluted using a gradient elution of 20% ACN (aq) for 20 min and 20 to 60% ACN (aq) for 20 min flowing at 1 ml min⁻¹. The column was washed and reequilibrated with 90% ACN (aq) for 5 min and 20% ACN (aq) for 5 min. During the gradient, 1-min fractions were collected. Fractions corresponding to peaks in the UV absorbance were pooled, dried under vacuum, and reconstituted in 100 µl of ACN:H₂O (1:1 vol/vol) before mass spectrometric analysis.

Mass spectrometric analysis.

Matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS) and tandem mass spectrometry (MS/MS) spectra were recorded using an Applied Biosystems 4700 proteomics analyzer with time-of-flight/time-of-flight optics. The HPLC fraction (0.5 µl) was mixed with an equal volume of the matrix solution, α -cyano-4-hydroxy-cinnamic acid at 7 mg/ml, before being spotted on a MALDI target plate. Mass spectra were recorded over the range m/z 800 to 4,000, with a "focus mass" of m/z 1,500 using the 4700 Explorer software. The laser was set at an intensity of 5,000 units; each spectrum was acquired using 1,000 laser shots in total, made up of 20 sub-spectra, each of which consisted of 50 laser shots. MS/MS spectra were acquired with an average of 2,500 laser shots per spectrum. The collision energy was set at 1 keV and the ions were collided in the CID cell with nitrogen as the collision gas.

Additional data were obtained on some fractions and structures using the same matrix and spot preparation method, recording the MALDI MS and MS/MS data using a Bruker ultraflex III mass spectrometer with LIFT-MS/MS facility (Centre of Excellence in Mass Spectrometry, University of York). Mass spectra were obtained in the range m/z 500 to 3,000, using the FlexControl software; each spectrum was acquired manually, and involved the summing of laser shots (up to 4,000) in order to generate spectra of an acceptable signal-to-noise ratio. MS/MS spectra were obtained on precursor ions accelerated to 8 kV and selected in the timed ion gate. Fragment ions produced on laser-induced dissociation were accelerated using a further 18 to 20 kV in the LIFT cell, and separated in the reflectron; metastable ion suppression was used during acquisition of the fragment ions.

ACKNOWLEDGMENTS

This work was supported by grants from the Marsden Fund administered by the Royal Society of New Zealand and the Danish National Research Foundation. P. Rodpithong thanks the European Molecular Biology Organization for a short-term fellowship and the Department of Microbiology, University of Otago for a Ph.D. scholarship. We thank N. Sandal for his generous supply of *Lotus* seed and R. Easingwood and the Electron Microscopy Unit, University of Otago, for assistance with the transmission electron microscopy work. D. Sumpton's studentship was supported by the Engineering and Physical Sciences Research Council U.K. (EPSRC) and the European Union (ICA4-CT-2001-10056). K. Songsrirote is grateful for Ph.D. support via a Royal Thai Scholarship, and J. Thomas-Oates gratefully acknowledges funding from the Analytical Chemistry Trust Fund, the RSC Analytical Division, and EPSRC. The Centre of Excellence in Mass Spectrometry at York is supported by Science City York and Yorkshire Forward using funds from the Northern Way Initiative.

LITERATURE CITED

- Amor, B. B., Shaw, S. L., Oldroyd, G. E., Maillet, F., Penmetsa, R. V., Cook, D., Long, S. R., Dénarié, J., and Gough, C. 2003. The NFP locus of *Medicago truncatula* controls an early step of Nod factor signal transduction upstream of a rapid calcium flux and root hair deformation. *Plant J.* 34:495-506.
- Ardourel, M., Demont, N., Debellé, F., Maillet, F., de Billy, F., Promé, J., Dénarié, J., and Truchet, G. 1994. *Rhizobium meliloti* lipooligosaccharide nodulation factors: Different structural requirements for bacterial entry into target root hair cells and induction of plant symbiotic development responses. *Plant Cell* 6:1357-1374.
- Berck, S., Perret, X., Quesada-Vincens, D., Promé, J. C., Broughton, W. J., and Jabbouri, S. 1999. NoIL of *Rhizobium* sp. strain NGR234 is required for O-acetyltransferase activity. *J. Bacteriol.* 181:957-964.
- Cheng, H. P., and Walker, G. C. 1998. Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. *J. Bacteriol.* 180:5183-91.
- Corvera, A., Promé, D., Promé, J. C., Martínez-Romero, E., and Romero, D. 1999. The *nolL* gene from *Rhizobium etli* determines nodulation efficiency by mediating the acetylation of the fucosyl residue in the nodulation factor. *Mol. Plant-Microbe Interact.* 13:236-246.
- Dombrecht, B., Vanderleyden, J., and Michiels, J. 2001. Stable RK2-derived cloning vectors for the analysis of gene expression and gene function in gram-negative bacteria. *Mol. Plant-Microbe Interact.* 14:426-430.
- Downie, J. A., and Surin, B. P. 1990. Either of two *nod* gene loci can complement the nodulation defect of a *nod* deletion mutant of *Rhizobium leguminosarum* biovar *viciae*. *Mol. Gen. Genet.* 222:81-86.
- Fisher, R. F., and Long, S. R. 1993. Interaction of NodD at the *nod* box: NodD binds to two distinct sites on the same face of the helix and induces a bend in the DNA. *J. Mol. Biol.* 233:336-348.
- Geurts, R., Fedorova, E., and Bisseling, T. 2005. Nod factor signaling genes and their function in the early stages of *Rhizobium* infection. *Curr. Opin. Plant Biol.* 8:346-352.
- Hayashi, M., Miyahara, A., Sato, S., Kato, T., Yoshikawa, M., Taketa, M., Hayashi, M., Pedrosa, A., Onda, R., Imaizumi-Anraku, H., Bachmair, A., Sandal, N., Stougaard, J., Murooka, Y., Tabata, S., Kawasaki, S., Kawaguchi, M., and Harada, K. 2001. Construction of a genetic linkage map of the model legume *Lotus japonicus* using an intraspecific F2 population. *DNA Res.* 8:301-310.
- Herrero, M., de Lorenzo, V., and Timmis, K. N. 1990. Transposon vectors containing stable non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.* 172:6557-6567.
- Hotter, G. S., and Scott, D. B. 1991. Exopolysaccharide mutants of *Rhizobium loti* are fully effective on a determinate nodulating host but are ineffective on an indeterminate nodulating host. *J. Bacteriol.* 173:851-859.
- Hubber, A., Vergunst, A. C., Sullivan, J. T., Hooykaas, P. J. J., and Ronson, C. W. 2004. Symbiotic phenotypes and translocated effector proteins of the *Mesorhizobium loti* strain R7A VirB/D4 type IV secretion system. *Mol. Microbiol.* 54:561-574.
- Hubber, A., Sullivan, J. T., and Ronson, C. W. 2007. Symbiosis-induced cascade regulation of the *Mesorhizobium loti* R7A VirB/D4 type IV secretion system. *Mol. Plant-Microbe Interact.* 20:255-261.
- John, M., Rohrig, H., Schmidt, J., Wieneke, U., and Schell, J. 1993. *Rhizobium* nodB protein involved in nodulation signal synthesis is a chitooligosaccharide deacetylase. *Proc. Natl. Acad. Sci. U.S.A.* 90:625-629.
- Kawaguchi, M., Pedrosa, A., Yano, K., Hayashi, M., Murooka, Y., Saito, K., Nagata, T., Namai, K., Nishida, H., Shibata, D., Sato, S., Tabata, S., Hayashi, M., Harada, K., Sandal, N., Stougaard, J., Bachmair, A., and Grant, W. F. 2005. *Lotus burtii* takes a position of the third corner in the *Lotus* molecular genetics triangle. *DNA Res.* 12:69-77.
- Limpens, E., Franken, C., Smit, P., Willemse, J., Bisseling, T., and Geurts, R. 2003. LysM domain receptor kinases regulating rhizobial Nod factor-induced infection. *Science* 302:630-633.
- López-Lara, I. M., van den Berg, J. D. J., Thomas-Oates, J. E., Glushka, J., Lugtenberg, B. J., and Spaik, H. P. 1995. Structural identification of the lipo-chitin oligosaccharide nodulation signals of *Rhizobium loti*. *Mol. Microbiol.* 15:627-638.
- López-Lara, I. M., Blok-Tip, L., Quinto, C., Garcia, M. L., Stacey, G., Bloemberg, G. V., Lamers, G. E. M., Lugtenberg, B. J. J., Thomas-Oates, J. E., and Spaik, H. P. 1996. NodZ of *Bradyrhizobium* extends the nodulation host range of *Rhizobium* by adding a fucosyl residue to nodulation signals. *Mol. Microbiol.* 21:397-408.
- Madsen, E. B., Madsen, L. H., Radutoiu, S., Olbryt, M., Rakwalska, M., Szczygłowski, K., Sato, S., Kaneko, T., Tabata, S., Sandal, N., and Stougaard, J. 2003. A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. *Nature* 425:637-640.

- Marsh, J. F., Rakocevic, A., Mitra, R. M., Brocard, L., Sun, J., Eschstruth, A., Long, S. R., Schultze, M., Ratet, P., and Oldroyd, G. E. 2007. *Medicago truncatula* NIN is essential for rhizobial-independent nodule organogenesis induced by autoactive calcium/calmodulin-dependent protein kinase. *Plant Physiol.* 144:324-335.
- Mergaert, P., D'Haese, W., Fernández-López, M., Geelen, D., Goethals, K., Promé, J. C., Van Montagu, M., and Holsters, M. 1996. Fucosylation and arabinosylation of Nod factors in *Azorhizobium caulinodans*: Involvement of *nolK*, *nodZ* as well as *noeC* and/or downstream genes. *Mol. Microbiol.* 21:409-419.
- Miller, W. G., Leveau, J. H., and Lindow, S. E. 2000. Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. *Mol. Plant-Microbe Interact.* 13:1243-1250.
- Niwa, S., Kawaguchi, M., Imazumi-Anraku, H., Chechetka, S. A., Ishizaka, K., Ikuta, A., and Kouchi, H. 2001. Responses of a model legume *Lotus japonicus* to lipochitin oligosaccharide nodulation factors purified from *Mesorhizobium loti* JRL501. *Mol. Plant-Microbe Interact.* 14:848-856.
- Oldroyd, G. E., and Downie, J. A. 2004. Calcium, kinases and nodulation signaling in legumes. *Nature Rev. Mol. Cell Biol.* 5:566-576.
- Oldroyd, G. E., and Downie, J. A. 2008. Coordinating nodule morphogenesis with rhizobial infection in legumes. *Annu. Rev. Plant Biol.* 59:519-546.
- Oldroyd, G. E., Mitra, R. M., Wais, R., and Long, S. R. 2001. Evidence for structurally specific negative feedback in the Nod factor signal transduction pathway. *Plant J.* 28:191-199.
- Olsthoorn, M. M., López-Lara, I. M., Petersen, B. O., Bock, K., Haverkamp, J., Spaik, H. P., and Thomas-Oates, J. E. 1998. Novel branched Nod factor structure results from alpha-(1→3) fucosyl transferase activity: The major lipo-chitin oligosaccharides from *Mesorhizobium loti* strain NZP2213 bear an alpha-(1→3) fucosyl substituent on a nonterminal backbone residue. *Biochemistry* 37:9024-9032.
- Olsthoorn, M. M., Stokvis, E., Haverkamp, J., Spaik, H. P., and Thomas-Oates, J. E. 2000. Growth temperature regulation of host-specific modifications of rhizobial lipo-chitin oligosaccharides: The function of *nodX* is temperature regulated. *Mol. Plant-Microbe Interact.* 13:808-820.
- Pacios-Bras, C., Jordá, M. A., Wijfjes, A. H. M., Harteveld, M., Stuurman, M., Thomas-Oates, J. E., and Spaik, H. P. 2000. A *Lotus japonicus* nodulation system based on heterologous expression of the fucosyl transferase NodZ and the acetyl transferase NodL in *Rhizobium leguminosarum*. *Mol. Plant-Microbe Interact.* 13:475-479.
- Pellock, B. J., Cheng, H. P., and Walker, G. C. 2000. Alfalfa root nodule invasion efficiency is dependent on *Sinorhizobium meliloti* polysaccharides. *J. Bacteriol.* 182:4310-4318.
- Quandt, J., and Hynes, F. M. 1993. Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. *Gene* 127:15-21.
- Quesada-Vincens, D., Fellay, R., Nasim, T., Viprey, V., Burger, U., Promé, J. C., Broughton, W. J., and Jabbouri, S. 1997. *Rhizobium* sp. strain NGR234 NodZ protein is a fucosyltransferase. *J. Bacteriol.* 179:5087-5093.
- Radutoiu, S., Madsen, L. H., Madsen, E. B., Felle, H. H., Umehara, Y., Gronlund, M., Sato, S., Nakamura, Y., Tabata, S., Sandal, N., and Stougaard, J. 2003. Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* 425:585-592.
- Radutoiu, S., Madsen, L. H., Madsen, E. B., Jurkiewicz, A., Fukai, E., Quistgaard, M. M., Albrechtsen, A. S., James, E. K., Thirup, S., and Stougaard, J. 2007. LysM domains mediate lipochitin-oligosaccharide recognition and Nfr genes extend the symbiotic host range. *EMBO (Eur. Mol. Biol. Organ.) J.* 26:3923-3935.
- Ramsay, J. P., Sullivan, J. T., Stuart, G. S., Lamont, I. L., and Ronson, C. W. 2006. Excision and transfer of the *Mesorhizobium loti* R7A symbiosis island requires an integrase IntS, a novel recombination directionality factor RdfS, and a putative relaxase RlxS. *Mol. Microbiol.* 62:723-734.
- Riely, B. K., Mun, J. H., and Ané, J. M. 2006. Unraveling the molecular basis for symbiotic signal transduction in legumes. *Mol. Plant Pathol.* 7:197-207.
- Rostas, K., Kondorosi, E., Horvath, B., Simoncsits, A., and Kondorosi, A. 1986. Conservation of extended promoter regions of nodulation genes in *Rhizobium*. *Proc. Natl. Acad. Sci. U.S.A.* 83.
- Sandal, N., Petersen, T. R., Murray, J., Umehara, Y., Karas, B., Yano, K., Kumagai, H., Yoshikawa, M., Saito, K., Hayashi, M., Murakami, Y., Wang, X., Hakoyama, T., Imazumi-Anraku, H., Sato, S., Kato, T., Chen, W., Hossain, M. S., Shibata, S., Wang, T. L., Yokota, K., Larsen, K., Kanamori, N., Madsen, E. B., Radutoiu, S., Madsen, L. H., Radu, T. G., Krusell, L., Ooki, Y., Banba, M., Betti, M., Rispaill, N., Skøt, L., Tuck, E., Perry, J., Yoshida, S., Vickers, K., Pike, J., Mulder, L., Charpentier, M., Müller, J., Ohtomo, R., Kojima, T., Ando, S., Marquez, A. J., Gresshoff, P. M., Harada, K., Webb, J., Hata, S., Suganuma, N., Kouchi, H., Kawasaki, S., Tabata, S., Hayashi, M., Parniske, M., Szczygłowski, K., Kawaguchi, M., and Stougaard, J. 2006. Genetics of symbiosis in *Lotus japonicus*: Recombinant inbred lines, comparative genetic maps, and map position of 35 symbiotic loci. *Mol. Plant-Microbe Interact.* 19:80-91.
- Scott, D. B., Young, C. A., Collins-Emerson, J. M., Terzaghi, E. A., Rockman, E. S., Lewis, P. E., and Pankhurst, C. E. 1996. Novel and complex chromosomal arrangement of *Rhizobium loti* nodulation genes. *Mol. Plant-Microbe Interact.* 9:187-197.
- Shibata, S., Mitsui, H., and Kouchi, H. 2005. Acetylation of fucosyl residue at the reducing end of *Mesorhizobium loti* Nod factors is not essential for nodulation of *Lotus japonicus*. *Plant Cell Physiol.* 46:1017-1020.
- Smit, P., Limpens, E., Geurts, R., Fedorova, E., Dolgikh, E., Gough, C., and Bisseling, T. 2007. Medicago LYK3, an entry receptor in rhizobial nodulation factor signaling. *Plant Physiol.* 145:183-191.
- Soria-Díaz, M. E., Tejero-Mateo, P., Espartero, J. L., Rodríguez-Carvajal, M. A., Moron, B., Sousa, C., Megias, M., Amarger, N., Thomas-Oates, J. E., and Gil-Serrano, A. M. 2003. Structural determination of the lipochitin oligosaccharide nodulation signals produced by *Rhizobium giardinii* bv. giardinii H152. *Carbohydr. Res.* 338:237-250.
- Spaik, H. P. 2000. Root nodulation and infection factors produced by rhizobial bacteria. *Annu. Rev. Microbiol.* 54:257-288.
- Spaik, H. P., Okker, R. J., Wijffelman, C. A., Tak, T., Goosen-de Roo, L., Pees, E., van Brussel, A. A., and Lugtenberg, B. J. 1989. Symbiotic properties of rhizobia containing a flavonoid-independent hybrid *nodD* product. *J. Bacteriol.* 171:4045-4053.
- Sullivan, J. T., and Ronson, C. W. 1998. Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. *Proc. Natl. Acad. Sci. U.S.A.* 95:5145-5149.
- Sullivan, J. T., Patrick, H. N., Lowther, W. L., Scott, D. B., and Ronson, C. W. 1995. Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. *Proc. Natl. Acad. Sci. U.S.A.* 92:8985-8989.
- Sullivan, J. T., Brown, S. D., Yocum, R. R., and Ronson, C. W. 2001. The *bio* operon on the acquired symbiosis island of *Mesorhizobium* sp. strain R7A includes a novel gene involved in pimeloyl-CoA synthesis. *Microbiology* 147:1315-1322.
- Sullivan, J. T., Trzebiatowski, J. R., Cruickshank, R. W., Gouzy, J., Brown, S. D., Elliott, R. M., Fleetwood, D. J., McCallum, N. G., Rossbach, U., Stuart, G. S., Weaver, J. E., Webby, R. J., de Bruijn, F. J., and Ronson, C. W. 2002. Comparative sequence analysis of the symbiosis island of *Mesorhizobium loti* strain R7A. *J. Bacteriol.* 184:3086-3095.
- Szczygłowski, K., Shaw, R. S., Wopereis, J., Copeland, S., Hamburger, D., Kasiborski, B., Dazzo, F. B., and de Bruijn, F. J. 1998. Nodule organogenesis and symbiotic mutants of the model legume *Lotus japonicus*. *Mol. Plant-Microbe Interact.* 11:684-697.
- van Brussel, A. A., Planqué, K., and Quispel, A. 1977. The wall of *Rhizobium leguminosarum* in bacteroid and free-living forms. *J. Gen. Microbiol.* 101:51-56.
- Vincent, J. M. 1970. A Manual for the Practical Study of Root Nodule Bacteria. Blackwell Scientific Publications, Oxford.
- Wais, R. J., Keating, D. H., and Long, S. R. 2002. Structure-function analysis of Nod factor-induced root hair calcium spiking in rhizobium-legume symbiosis. *Plant Physiol.* 129:211-224.
- Walker, S. A., and Downie, J. A. 2000. Entry of *Rhizobium leguminosarum* bv. *viciae* into root hairs requires minimal Nod factor specificity, but subsequent infection thread growth requires *nodO* or *nodE*. *Mol. Plant-Microbe Interact.* 13:754-762.
- Wernegreen, J. J., and Riley, M. A. 1999. Comparison of the evolutionary dynamics of symbiotic and housekeeping loci: A case for the genetic coherence of rhizobial lineages. *Mol. Biol. Evol.* 16:98-113.