Symbiosis-Induced Cascade Regulation of the *Mesorhizobium loti* R7A VirB/D4 Type IV Secretion System

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The *Mesorhizobium loti* R7A symbiosis island contains genes encoding a VirB/D4 type IV secretion system (T4SS) similar to that of *Agrobacterium tumefaciens*. This system has host-dependent effects on symbiosis that probably are due to translocation of two effector proteins, Msi059 and Msi061, into host cells. Here we report that, as in *A. tumefaciens*, the *M. loti* *vir* genes are transcriptionally regulated by a VirA/VirG two-component regulatory system. A *virGN54D* mutant gene of *M. loti* caused constitutive expression of *lacZ* reporter gene fusions to *virB1*, *virD4*, *msi059*, and *msi061*. Expression of these gene fusions also was activated by a NodD gene product from *Rhizobium leguminosarum* in the presence of the inducer naringenin, as was a *virA::lacZ* fusion. This activation was dependent on a *nod* box present 851 bp upstream of *virA*, and a mutant with the *nod* box deleted formed effective nodules on *Leucaena leucocephala*, the same symbiotic phenotype as other *M. loti* *vir* mutants. In contrast, the wild-type strain formed small, empty nodules whereas a *nodD1* mutant was completely Nod[−]. These results indicate that the *M. loti* *vir* genes are induced in a symbiosis-specific manner that involves a two-tiered regulatory cascade, and that the *vir* effectors act after Nod factor during infection thread formation.

Rhizobia are α - and β -proteobacteria that form nitrogen-fixing root nodules on leguminous plants. To establish a successful symbiotic association, molecular signals are exchanged between the two partners (Perret et al. 2000; Spaink 2000; van Rhijn and Vanderleyden 1995). In the early stages of this communication, legumes secrete species-specific flavonoids into the rhizosphere that are recognized in a *Rhizobium* sp.-specific manner by NodD proteins. Members of the LysR family, NodD proteins bind to *nod* boxes in the promoter regions of inducible *nod* genes (Fisher and Long 1993; Long 1996; Peck et al. 2006; Rostas et al. 1986). Rhizobial Nod proteins create *Rhizobium* sp.-specific lipochito-oligosaccharide signaling molecules known as Nod factors (Long 1996; Spaink 2000). Nod factors probably are recognized by plant receptors, and thereby activate signaling pathways required for nodule development and rhizobial invasion (Geurts et al. 2005; Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003).

Depending on the individual *Rhizobium* sp.–legume pairing, rhizobial surface polysaccharides also influence infection thread formation and nodule development (Becker et al. 2005; Fraysse et al. 2003; Gage 2004; Noel and Duelli 2000; Price 1999;

Spaink 2000). In addition, type III secretion systems (T3SS) in *Rhizobium* sp. strain NGR234, *Sinorhizobium fredii*, and *Bradyrhizobium japonicum* have been shown to transport nodulation outer proteins (Nops) to the extracellular milieu. Mutations that perturb the T3SS machinery cause symbiotic phenotypes on some hosts but are phenotypically silent on other hosts (Ausmees et al. 2004; Krause et al. 2002; Krishnan et al. 2003; Lorio et al. 2004; Marie et al. 2003; Viprey et al. 1998). Mutations within genes that code for individual Nops cause different symbiotic phenotypes depending on the host studied (Ausmees et al. 2004). The NopL and NopP effectors of *Rhizobium* NGR234 serve as substrates for plant protein kinases (Bartsev et al. 2003; Skorpil et al. 2005), and expression of NopL in plant cells suppresses a full plant defense response (Bartsev et al. 2004).

The *Mesorhizobium loti* R7A symbiosis island contains genes (*virB1* to *B11* and *virD4*) that encode a type IV secretion system (T4SS) with a high degree of similarity to the *Agrobacterium tumefaciens* *vir* T4SS that transfers T-DNA and several proteins to plants (Christie et al. 2005; Sullivan et al. 2002). Mutations in the *M. loti* *vir* genes have host-dependent effects, causing delayed nodulation on *Lotus corniculatus* and allowing effective nodulation on *Leucaena leucocephala* (Hubber et al. 2004). Additionally, mutants in a gene *msi059* that codes for a putative cysteine protease effector protein transported by the system show symbiotic effects similar to that of the *vir* gene mutants (Hubber et al. 2004). The host-dependent symbiotic phenotypes of the mutants may reflect differences in the hosts' reactions to secreted effector proteins. In *L. leucocephala*, the effector proteins may induce plant defense responses, analogous to the avirulence proteins of plant pathogens, resulting in abortion of infections; whereas, in *Lotus corniculatus*, they may facilitate infection, perhaps by suppressing plant defense reactions (Hubber et al. 2004; Marie et al. 2001).

Homologues of the *A. tumefaciens* regulatory *virA* and *virG* genes also are present on the *M. loti* R7A symbiosis island (Fig. 1) and strains with mutations in these genes form effective nodules on *Leucaena leucocephala* (Hubber et al. 2004). In *A. tumefaciens*, VirA and VirG constitute a two-component regulatory system that activates the other *vir* genes in response to plant signals (Leroux et al. 1987; Winans et al. 1986). Compounds released from plant wound sites induce the inner membrane-bound VirA dimer to transphosphorylate itself at His-474 (Huang et al. 1990; Jin et al. 1990a and b), and to phosphorylate the cytoplasmic response regulator VirG at Asp-52 (Jin et al. 1990b). VirG then binds at conserved hexameric DNA sequences termed *vir* boxes in *vir* operon promoter regions to induce their transcription (Das et al. 1986; Gao et al. 2006; Jin et al. 1990c; Pazour and Das 1990; Powell et al.

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1989). The dependence of VirG on VirA for activation can be alleviated by a mutation that gives the variant protein VirGN54D; this protein does not require VirA for phosphorylation (Gao et al. 2006) and activates the other *vir* operons constitutively (Han et al. 1992; Jin et al. 1993; Pazour et al. 1992; Scheeren-Groot et al. 1994).

Previously, we reported the presence of two *vir*-box consensus nucleotide repeats within the *M. loti* R7A symbiosis island sequence, one in the promoter region of the *msi061* gene and the other in the intergenic region between the divergently transcribed *virB1* and *virG* genes (Hubber et al. 2004) (Fig. 1). In addition, a putative *nod* box was found 851 bp upstream of *virA* (Fig. 1) and no genes were annotated in the intervening region (Hubber et al. 2004; Sullivan et al. 2002), suggesting a link between *vir* and *nod* gene expression. Here, we report that the *M. loti* *vir* genes are subject to cascade regulation, first through NodD via the *nod* box upstream of *virA* and then through VirA/VirG.

RESULTS

The structural *vir* operons are regulated by VirG.

M. loti VirG and the VirG homologues from several *A. tumefaciens* plasmids share over 72% identity over their entire lengths. The *M. loti* VirG protein contains the asparagine residue at position 54 that, when mutated to an aspartic acid residue, results in VirG variants that constitutively activate *vir* gene expression (Han et al. 1992; Jin et al. 1993; Pazour et al. 1992; Scheeren-Groot et al. 1994). Therefore, an analogous *M. loti* *virGN54D* mutant was constructed by site-directed mutagenesis as described in Materials and Methods, and the gene and its promoter region were cloned into the broad-host-range vector pFAJ1700 to produce pAH4.

The effect of pAH4 on *M. loti* *vir* gene expression was assayed by introducing the plasmid into strains containing *vir::lacZ* reporter gene fusions. These strains were constructed

by homologous recombination-mediated insertion of the suicide vector pFUS2, such that either an insertion duplication mutant (IDM) with a transcriptional fusion between the 5' end of the mutated gene and *lacZ* was created or, in the case of strain AH71 (*msi061*-CMD::*lacZ*), a *cis*-merodiploid (CMD) strain was created that retained an intact copy of *msi061* and its promoter downstream of the integrated plasmid and *msi061::lacZ* fusion. Results of β -galactosidase assays showed that the presence of pAH4 caused increased expression of the *virB1::lacZ*, *virD4::lacZ*, *msi059::lacZ*, *msi061::lacZ*, and *msi061*-CMD::*lacZ* fusions (Table 1). The *msi061*-CMD::*lacZ* fusion also was induced by pAH4 in Δ *virA* and Δ *virG* strains, indicating that induction was independent of the wild-type regulatory genes as expected. In contrast, the *virA::lacZ* and *virG::lacZ* fusions showed similar levels of expression in the presence or absence of pAH4 (Table 1). These results showed that VirG regulates the expression of the structural *vir* operons *virB1*-*virB11* and *msi061*-*virD4*-*msi059*, but not of itself or *virA*.

M. loti *vir* gene induction is under symbiotic control.

The presence of a putative *nod* box in the region upstream of *virA* suggested that *nod* gene inducers might activate *vir* gene expression. Plant molecules capable of activating the *M. loti* NodD protein at physiological concentrations have not been found. However, the NodD protein of *R. leguminosarum* bv. *viciae*, produced from the *nodD* gene cloned in pMP2112, activates *M. loti* *nod* genes in the presence of the flavonoid naringenin (Lopez-Lara et al. 1995; Niwa et al. 2001). The plasmid pMP2112 was introduced into various *M. loti* *lacZ* fusion strains, which then were assayed for β -galactosidase induction. In the presence of both pMP2112 and naringenin, there was significantly greater expression of the positive controls *nodB::lacZ* and *nolO::lacZ*, and also of *virA::lacZ*, *virB1::lacZ*, *virD4::lacZ*, and *msi061::lacZ* fusions (Table 2). This increase was dependent on pMP2112 and inducer, because expression of the fusions was not enhanced by pMP2112 (Table 2) or naringenin

Fig. 1. Genetic organization of the *Mesorhizobium loti* R7A *vir* gene cluster. The locations of various *vir* genes are indicated by arrows. Also shown are the locations of a *nod* box and two putative *vir* boxes (Hubber et al. 2004). The *nod* box consensus was from van Rhijn and Vanderleyden (1995). Y = pyrimidine (C, T); R = purine (G, A); N = any base.

Table 1. Effect of pAH4 (*virG*(N54D)) on expression of *vir::lacZ* fusions in *Mesorhizobium loti*^a

Fusion strain	- pAH4	+ pAH4	Fold induction
AH46 (R7A <i>virA::lacZ</i>)	36.7 ± 2.9 (24)	31.7 ± 2.7 (2)	0.9
AH47 (R7A <i>virG::lacZ</i>)	24.2 ± 3.1 (3)	24.3 ± 3.2 (3)	1.0
AH48 (R7A <i>virB1::lacZ</i>)	14.2 ± 1 (5)	22 ± 0.7 (5)	1.6 *
AH49 (R7A <i>virD4::lacZ</i>)	19.3 ± 1.6 (3)	488.1 ± 40.9 (3)	25.6*
AH50 (R7A <i>msi059::lacZ</i>)	33.6 ± 2.0 (4)	208.8 ± 27.8 (4)	6.8*
AH51 (R7A <i>msi061::lacZ</i>)	77.4 ± 8.5 (5)	1286.1 ± 83.3 (5)	15.5*
AH71 (R7A <i>msi061</i> -CMD:: <i>lacZ</i>)	84.4 ± 12 (3)	851.7 ± 59.0 (4)	10.1*
AH90 (R7A Δ <i>virA</i> <i>msi061</i> -CMD:: <i>lacZ</i>)	10.1 ± 0.2 (5)	55.8 ± 3.2 (5)	5.5*
AH91 (R7A Δ <i>virG</i> <i>msi061</i> -CMD:: <i>lacZ</i>)	19.7 ± 0.1 (3)	259.22 ± 24.3 (5)	14.6*

^a Cultures used for assays were grown in *M. loti* G/RDM broth, pH 6.0, until they reached mid exponential phase (optical density at 600 nm = approximately 0.5). The number of independent assays for each strain is shown in brackets. Asterisks indicate significant induction of the fusion in response to pAH4 (*P* < 0.05 as determined by *t* test).

(data not shown) alone. Addition of pMP2112 and naringenin did not affect the expression of *virG::lacZ* or the negative control *nifA1::lacZ*. These results are consistent with NodD inducing expression of the structural *vir* operons through activating expression of *virA*.

The *nod* box upstream of *virA* is required for *vir* gene activation.

To investigate the role of the *nod* box located 851 bp upstream of *virA* in the transcriptional control of *virA*, a mutant in which the *nod* box was deleted was constructed using a marker replacement strategy. To confirm the role of the *virA* *nod* box in NodD-mediated *vir* gene activation, the gene fusions *virA::lacZ*, *virG::lacZ*, *virB1::lacZ*, and *virD4::lacZ* were each integrated into the Δ *nod*-box strain AH55 to create strains AH63, AH64, AH65, and AH66, respectively. No increases in *vir* gene expression of these strains in the presence of pMP2112 and naringenin occurred compared with uninduced cultures (Table 2).

The Δ *nod*-box:: Ω *kan* strain AH55 also was tested for its nodulation phenotype on *L. leucocephala* plants, together with wild-type and other *vir* mutant strains. The *vir* gene mutants, including AH55, formed large, pink nodules (2 to 5 mm in length) with an oblong and often multilobed structure, whereas strains R7A and N2P2213 formed small, white, round nodules 0.5 to 1 mm in diameter (Fig. 2). Taken together, these results confirm that the *nod* box upstream of the *virA* is required for the symbiotic expression of the *virA*, *virB*, and *msi061* operons.

The *nodD1* gene of *M. loti* most likely mediates *nod* and *vir* gene activation.

To examine the roles of the symbiosis island *nodD1* versus *nodD2* gene products in *vir* gene regulation, mutants were obtained with *EZTn*-GN insertions in either *nodD* gene. The nodu-

lation phenotypes of these mutants were analyzed on *L. leucocephala*. Like R7A, the *nodD2* mutant induced small, white, nodule-like structures, whereas the *nodD1* mutant failed to form any such structures (Fig. 2).

DISCUSSION

We have shown that the expression of the *M. loti* T4SS machinery is tightly controlled and subject to cascade regulation, requiring first the perception of plant-derived signals through NodD1 and subsequently activation by the VirA/VirG two-component regulatory system. Like the *A. tumefaciens* T4SS, regulation of the *M. loti* *vir* system by VirA/VirG probably occurs via VirG binding to *vir* boxes present upstream of the *virB1* and *msi061* genes. Also inherent in the regulation of both systems is the perception of environmental signals. However, unlike *A. tumefaciens* *virA*, the *M. loti* *virA* gene is under transcriptional regulation, its expression being mediated through the *nod* box located 851 bp upstream of the start codon of the gene. This key feature of the *M. loti* *vir* gene regulatory cascade links the timing of *vir* gene expression to the early stages of nodule invasion.

The involvement of the *nod* box in *vir* gene expression was demonstrated by the findings that the Δ *nod* box mutant strain AH55 showed the same distinctive phenotype as *vir* mutants on *L. leucocephala*, and that NodD-mediated induction of *vir* gene expression was abolished in the mutant. The *virA* *nod* box is one of only two *nod* boxes on the symbiosis island of *M. loti* R7A not associated with genes involved in Nod factor synthesis (Sullivan et al. 2002). The distances from the start codons of the *nod* operons to the *nod* box consensus varies greatly, from 67 to 658 bp; however, at 851 bp upstream of the start codon, the *virA* *nod* box is unusually far upstream of the gene it controls. The transcriptional start sites have not been determined for the *M.*

Table 2. Expression of *nod::lacZ* and *vir::lacZ* fusions in *Mesorhizobium loti* R7A containing *nodD* from *Rhizobium leguminosarum* (pMP2112)^a

Fusion strain	+ pMP2112 – Naringenin	+ pMP2112 + Naringenin	Fold induction
AH46 (R7A <i>virA::lacZ</i>)	27.4 ± 1.4 (5)	55.6 ± 4.3 (5)	2.0*
AH47 (R7A <i>virG::lacZ</i>)	39.39 ± 3.6 (3)	44.27 ± 7.7 (3)	1.1
AH48 (R7A <i>virB1::lacZ</i>)	10.3 ± 1.8 (5)	64.3 ± 10.1 (7)	6.2*
AH49 (R7A <i>virD4::lacZ</i>)	21.5 ± 1.5 (4)	76 ± 9.1 (4)	3.5*
AH50 (R7A <i>msi059::lacZ</i>)	22.6 ± 2.2 (6)	87.9 ± 21.3 (3)	3.9*
AH51 (R7A <i>msi061::lacZ</i>)	44.4 ± 2.7 (4)	90.3 ± 12.4 (4)	2.0*
AH52 (R7A <i>nifA1::lacZ</i>)	2.54 ± 0.3 (3)	1.95 ± 0.6 (3)	0.8
AH61 (R7A <i>nolO::lacZ</i>)	9.99 ± 0.3 (3)	109.33 ± 17.6 (3)	10.9*
AH62 (R7A <i>nodB::lacZ</i>)	23.05 ± 3.4 (3)	256.40 ± 56.8 (3)	11.1*
AH63 (R7A Δ <i>nod</i> -box:: Ω <i>kan</i> , <i>virA::lacZ</i>)	10.94 ± 1.5 (3)	9.68 ± 0.7 (3)	0.9
AH64 (R7A Δ <i>nod</i> -box:: Ω <i>kan</i> , <i>virG::lacZ</i>)	30.6 ± 0.3 (3)	30.4 ± 0.4 (3)	1.0
AH65 (R7A Δ <i>nod</i> -box:: Ω <i>kan</i> , <i>virB1::lacZ</i>)	8.47 ± 1.5 (3)	8.77 ± 1.5 (3)	1.0
AH66 (R7A Δ <i>nod</i> -box:: Ω <i>kan</i> , <i>virD4::lacZ</i>)	12.11 ± 0.8 (3)	14.9 ± 1.6 (3)	1.2

^a Cultures used for assays were grown in *M. loti* G/RDM broth, pH 6.0, until they reached stationary phase (optical density at 600 nm > 1.0). In each assay, samples were assessed in duplicate. The number of independent assays for each strain is shown in brackets. Asterisks indicate significant induction of the fusion in response to naringenin ($P < 0.05$ as determined by *t* test).

Fig. 2. Photographs of 12-week-old *Leucaena leucocephala* nodules from plants inoculated with strains of *Mesorhizobium loti*. Plants inoculated with N2P2213, R7A, and AH93 (*nodD2::EZTn*-GN) had small (0.5 to 1 mm), round nodules, whereas plants inoculated with N2P2037, AH49 (*virD4::lacZ*), and AH55 (Δ *nod*-box:: Ω *kan*) had large (2 to 5 mm), pink, elongated nodules, often with a multilobate structure. Plants inoculated with AH92 (*nodD1::EZTn5*) were devoid of nodule-like structures.

loti nod genes or *virA*; however, for several characterized *nod* genes, the transcriptional start sites are 23 to 35 bp downstream of the conserved 26-bp section of the *nod*-box consensus (Schlaman et al. 1998). If this is the case for *virA*, then its 5' transcribed but untranslated region is approximately 800 bp. The strong similarity between the *A. tumefaciens* and *M. loti vir* systems indicate that they share a relatively recent common ancestor; therefore, the *M. loti nod* box upstream of *virA* probably was acquired by a recent recombinational event. Hence, the spacing may be suboptimal and it could be continuing to evolve. Alternatively, the region may be involved in fine-tuning the level of *virA* expression; it also is possible that this region contains a pseudogene. Several *nod* genes in other rhizobial species have long untranslated leader sequences but the regions do not show sequence conservation and no function has been assigned to them (Schlaman et al. 1998).

Previously, we have shown that T3SS mutants of *M. loti* MAFF303099 exhibit a similar phenotype to the strain R7A VirB/D4 T4SS mutants on *L. leucocephala*, suggesting that T3SS and T4SS systems play interchangeable roles in different strains of *M. loti* (Hubber et al. 2004). The NodD-mediated activation of the *M. loti* T4SS observed in this study is similar to that reported for T3SS from *S. fredii*, *B. japonicum*, and *Rhizobium* sp. strain NGR234. In these systems, transcription of a regulatory gene *ttsI* is activated by NodD in a flavonoid-dependent manner, and TtsI, in turn, activates operons required for T3SS synthesis and function (Krause et al. 2002; Marie et al. 2004; Viprey et al. 1998). Although TtsI is a member of the two-component response regulator family, it is thought to be a constitutive activator because a cognate sensor histidine kinase has not been identified and it contains a glutamate residue instead of the conserved aspartate residue shown to be the primary phosphoacceptor in OmpR from *Escherichia coli* and NtrC of *Salmonella typhimurium*. The analogous aspartate to glutamate substitution in OmpR or NtrC leads to their constitutive activation (Delgado et al. 1993; Klose et al. 1993; Lan and Igo 1998; Marie et al. 2004). It is proposed that the cascade regulation of the T3SS system in these rhizobia allows for temporal expression such that the T3SS is formed after production of Nod factors, reflecting the likely function of the T3SS in suppressing plant defense responses during infection thread formation and development (Kobayashi et al. 2004; Marie et al. 2004).

The *A. tumefaciens* VirA protein activates VirG by phosphorylation in response to a combination of three plant-released chemical signals, namely phenolic compounds such as acetosyringone, particular monosaccharides involved in plant cell wall synthesis, and acidic pH (Brencic and Winans 2005; Zhu et al. 2000). These signals reflect the plant wound response and new cell-wall synthesis at the site of the wound. The NodD-dependent activation of the various *vir::lacZ* fusions was highly reproducible (Table 2), indicating that *M. loti* VirA must be at least partially active as a VirG protein kinase under our in vitro assay conditions. Nevertheless, it seems likely that the *M. loti* VirA also senses plant-derived signals when activating VirG, a suggestion supported by our observation that VirGN54D gave much higher levels of β -galactosidase activity from *msi059::lacZ*, *virD4::lacZ*, and *msi061::lacZ* fusions than did the NodD protein from *R. leguminosarum* in the presence of naringenin (Tables 1 and 2). However, the *virB1::lacZ* fusion showed the opposite pattern of expression (Tables 1 and 2), for reasons that are currently not clear. It may be that high expression of the *virB::lacZ* mutant was deleterious to the cell because a truncated VirB1 peptide would be produced. VirB1 is a lytic transglycosylase that normally is translocated across the inner membrane (Christie et al. 2005) and, because the truncated peptide contains a signal sequence, it may exhibit toxicity.

Infection thread initiation is thought to require localized degradation of the root hair wall at the site of infection, followed by new cell-wall synthesis (Gage 2004). It is tempting to speculate that, analogous to *A. tumefaciens*, the *M. loti* VirA is detecting the plant cell wall degradation and synthesis associated with infection thread initiation during the nodulation process and then activating VirG. The effector proteins transported by the T4SS then would act to suppress plant defense responses in compatible legumes such as *Lotus corniculatus*; whereas, in incompatible legumes such as *Leucaena leucocephala*, they are intercepted by the plant as avirulence proteins, leading to rapid induction of the defense responses. We are currently testing these hypotheses.

M. loti R7A contains two copies of the regulatory *nodD* gene on its symbiosis island, *nodD1* that is downstream of the nodulation gene *nolL* and *nodD2* that is upstream of *nolL* and divergently transcribed from it (Sullivan et al. 2002). We isolated mutants in each of these genes and assayed their symbiotic phenotype on *L. leucocephala*. Both the wild-type and the *nodD2* mutant formed small white nodules. These nodule structures were more defined than those previously observed (Hubber et al. 2004) and are similar to those formed by *M. loti* NZP2213 on *L. leucocephala* as reported by Pankhurst and associates (1987). These nodules do not contain infected plant cells; however, a few aborted infection threads containing rhizobia are present. The *L. leucocephala* seed used in this study was from a different source and variety than the seed used by Hubber and associates (2004), which likely explains the different wild-type nodulation responses. In contrast, the *nodD1* mutant was completely Nod⁻ on *L. leucocephala*. Thus, *nodD1* is the important regulatory *nod* gene required for Nod factor synthesis in the interaction of *M. loti* with *L. leucocephala*. It, rather than *nodD2*, must also induce *virA* expression, because the *nodD2* mutant showed a wild-type rather than *vir* phenotype on *L. leucocephala*. The fact that the wild-type and *nodD2* mutant induced empty nodules strongly suggests that, although regulated by NodD1, the *vir* gene products act after Nod factor in the nodule developmental program.

In summary, this work establishes the involvement of NodD and VirA/VirG in regulation of the *M. loti vir* system. In the future, the involvement of secondary signals in optimizing *vir* gene expression in *M. loti* will be further investigated.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The bacterial strains and plasmids used in this study are listed in Table 3.

Media and growth conditions.

E. coli strains were cultured at 37°C on Luria-Bertani agar or in 2YT broth (Sambrook et al. 1989). Strains of *M. loti* were grown at 28°C in TY (Beringer 1974) or *Rhizobium*-defined media with 4% (wt/vol) glucose (Hubber et al. 2004). Media were supplemented with antibiotics as required at concentrations as described by Hubber and associates (2004).

DNA manipulations, bacterial crosses, and electroporations.

DNA manipulations, bacterial crosses, and electroporations were performed as described previously (Hubber et al. 2004).

Construction of the *M. loti* R7A *virGN54D* mutant gene by site-directed mutagenesis.

The mutation (a nucleotide substitution from A to G at position 160) that produces the VirGN54D protein was introduced into a cloned copy of *M. loti virG* using the Gene Editor in vitro Site-Directed Mutagenesis System (Promega). To create the

template vector, a 2.7-kb *Pst*I fragment containing the entire *virG* gene and the preceding 1,045 bp (nucleotides 62,892 to 64,854 of the symbiosis island) was cloned in the same orientation as the *Amp*^r gene into pIC20R. Mutagenesis was carried out using the oligonucleotide *virG*SDMR (Table 4). The mutation was confirmed by sequencing, and a confirmed clone was cleaved with *Xho*I and *Xba*I to obtain the mutated *virG* fragment. This fragment was ligated into pFAJ1700 to create pAH4.

Creation of *nodD* mutants and transcriptional *lacZ* fusions.

A pIJ3200 cosmid clone designated pSC7 containing a partial *Sau*3a restriction fragment spanning approximately 25 kb of the

symbiosis island (approximate coordinates 130 to 155,000) was subjected to transposon mutagenesis using an in vitro transposon designated *EZTn*-GN. To construct *EZTn*-GN, a cassette containing the *nptII* gene and a promoterless *gusA* gene was subcloned from pCRS487 (Reeve et al. 1999) into the *EZ::TN* pMOD3<R6K γ ori/MCS> transposon construction vector (Epicentre) forming p*EZTn*-GN. The transposon, *EZTn*-GN, was amplified from p*EZTn*-GN by polymerase chain reaction (PCR) using the pMOD<MCS> forward and reverse primers (Epicentre), and then used to perform an in vitro transposition reaction on pSC7 using conditions recommended by the manufacturer. The mutagenized DNA was transformed into *E. coli* HB101 by

Table 3. Bacterial strains and plasmids used in this study

Strain	Relevant characteristics	Reference, source
<i>Mesorhizobium loti</i>		
R7A	Nod ⁺ Fix ⁻ on <i>Leucaena leucocephala</i>	Sullivan et al. 2002
NZP2213	Type strain; Nod ⁺ Fix ⁻ on <i>L. leucocephala</i>	Jarvis et al. 1982
NZP2037	Nod ⁺ Fix ⁺ on <i>L. leucocephala</i>	Jarvis et al. 1982
AH46	R7A <i>virA::lacZ</i> , pFUS2 IDM, Gm ^r	Hubber et al. 2004
AH47	R7A <i>virG::lacZ</i> , pFUS2 IDM, Gm ^r	Hubber et al. 2004
AH48	R7A <i>virB1::lacZ</i> , pFUS2 IDM, Gm ^r	Hubber et al. 2004
AH49	R7A <i>virD4::lacZ</i> , pFUS2 IDM, Gm ^r	Hubber et al. 2004
AH50	R7A <i>msi059::lacZ</i> , pFUS2 IDM, Gm ^r	Hubber et al. 2004
AH51	R7A <i>msi061::lacZ</i> , pFUS2 IDM, Gm ^r	Hubber et al. 2004
AH52	R7A <i>nifA1::lacZ</i> , pFUS2 IDM, Gm ^r	Hubber et al. 2004
AH55	R7A Δ <i>nod-box</i> (5' <i>virA</i> gene):: Ω <i>kan</i> , Nm ^r	This work
AH61	R7A <i>nolO::lacZ</i> , pFUS2 IDM, Gm ^r	J. Sullivan, unpublished
AH62	R7A <i>nodB::lacZ</i> , pFUS2 IDM, Gm ^r	J. Sullivan, unpublished
AH63	AH55 containing <i>virA::lacZ</i> , Nm ^r , Gm ^r	This work
AH64	AH55 containing <i>virG::lacZ</i> , Nm ^r , Gm ^r	This work
AH65	AH55 containing <i>virB1::lacZ</i> , Nm ^r , Gm ^r	This work
AH66	AH55 containing <i>virD4::lacZ</i> , Nm ^r , Gm ^r	This work
AH71	R7A <i>msi061-CMD::lacZ</i> , pFUS2 CMD, Gm ^r	This work
AH90	R7A Δ <i>virA</i> containing <i>msi061-CMD::lacZ</i> , Gm ^r	This work
AH91	R7A Δ <i>virG</i> containing <i>msi061-CMD::lacZ</i> , Gm ^r	This work
AH92	R7A <i>nodD1::EZTn</i> -GN, Nm ^r	This work
AH93	R7A <i>nodD2::EZTn</i> -GN, Nm ^r	This work
Plasmids		
pAH4	pFAJ1700 containing the <i>M. loti</i> <i>vir</i> GN54D mutant gene and its promoter cloned as an <i>Eco</i> RI fragment, Tc ^r	This work
pFAJ1700	Broad-host-range vector, Tc ^r	Dombrecht et al. 2001
pFUS2	<i>oriV</i> ^{ColE1} <i>oriT</i> ^{RK2} <i>lacZ</i> transcriptional reporter; suicide vector, Gm ^r	Antoine et al. 2000
pHP45 Ω -Km	Contains Ω fragment; Km ^r	Fellay et al. 1987
pIC20R	<i>oriV</i> ^{ColE1} , <i>lacZ</i> α , Ap ^r	Marsh et al. 1984
pIJ3200	Broad-host-range cosmid, Tc ^r	Liu et al. 1990
pJQ200SK	pACYC derivative, <i>oriV</i> ^{p15A} <i>oriT</i> ^{RK2} <i>sacB</i> ; Gm ^r	Quandt and Hynes 1993
pMP2112	pMP2733 containing the <i>Rhizobium leguminosarum</i> bv. <i>trifolii</i> <i>nodD</i> , Sp ^r	Lopez-Lara et al. 1995
pPH1J1	IncP plasmid, Gm ^r	Hirsch and Beringer 1984

^a Gm^r, Nm^r, Tc^r, Km^r, Ap^r, and Sp^r = gentamicin, neomycin, tetracycline, kanamycin, ampicillin, and spectinomycin resistant.

Table 4. Primers used in this study

Name	Sequence 5' to 3' ^a	Used for ^b	Position ^c
Gus-out	TTCGCGATCCAGACTGAATG	S	
<i>msi061</i> CMDf	TTATTA <u>GGATCC</u> ATCCATTGCTCGGCCCTCC	CMD	73,593–73,610
<i>msi061</i> CMDr	ATAATTAAGCTTAGGTAGTCTCAGCAAGGT	CMD	73,911–73,928
Δ <i>nod-box</i> df	AAATTTA <u>AGATCT</u> CTTCTTGAAAGCTAACCCCTTG	ME	49,727–49,747
Δ <i>nod-box</i> dr	TAATTTTCTAGATGCGTAGTTGATCTGAATCGT	ME	50,777–50,797
Δ <i>nod-box</i> uf	TTTAAAACTAGTATTAGGCAGGCATTGGAGAAG	ME	48,671–48,691
Δ <i>nod-box</i> ur	ATAAAATAGATCTCGGTTTTCGTTAGATGCGAGC	ME	49,640–49,660
<i>virG</i> SDMR	CCCGGCCTAGGTGAGGTCACAAC	SDM	64,084–64,108
Δ <i>virG</i> UF	AAATTTACTAGTCTGCATACTCAGGCATTGGTT	MD	63,085–63,105
Δ <i>virG</i> UR	ATAATTAGATCTTTGTCTAAAGCTGTTGCCGAG	MD	63,890–63,910
Δ <i>virG</i> DF	AAATTTAGATCTACGCTTAGCTACGAGGCA	MD	64,629–64,648
Δ <i>virG</i> DR	GAATTCCTCGAGCAGCAGCTGCAGCAGGCC	MD	66,230–66,259
Δ <i>virA</i> UF	TTTAAAACTAGTGCATCTCGACCATCTCAAGAAGC	MD	49,049–49,072
Δ <i>virA</i> UR	AATAATGGATCCGCTCAAGCCATCACGCAC	MD	50,146–50,163
Δ <i>virA</i> DF	TTTAAAGGATCCCGAGACGTTGTTCTAGC	MD	53,191–53,207
Δ <i>virA</i> DR	TTTAAACTCGAGTATAACGATCTGGAGGACATC	MD	54,073–54,093

^a Restriction sites incorporated into primers are underlined.

^b S = sequencing, CMD = *cis*-merodiploid pFUS2 fusion, ME = marker exchange, SDM = site-directed mutagenesis, and MD = markerless deletion.

^c Symbiosis island coordinates, numbered according to annotated R7A symbiosis island sequence (Sullivan et al. 2002).

electroporation and kanamycin- and tetracycline-resistant transformants were selected. The positions of *EZTn*-GN insertions were mapped by restriction endonuclease analysis and by cloning *Eco*RI fragments containing the insertions into pBluescript SKII and sequencing these clones using the primer Gus-out (Table 4). pSC7 clones containing transposon insertions which mapped to the *nodD1* and *nodD2* genes were transferred to R7A by electroporation, and marker exchange of the transposon insertions was forced by plasmid incompatibility using pPH1JI (Ruvkun and Ausubel 1981). pPH1JI was cured from strains by mating in pLAFR1 and selecting for gentamicin- and tetracycline-resistant colonies. The resulting mutants, designated AH92 (*nodD1::EZTn*-GN) and AH93 (*nodD2::EZTn*-GN), were confirmed by Southern hybridization.

Replacement of the *nod*-box region upstream of *virA* with the *Ωkan* interposon (Fellay et al. 1987) was achieved using marker exchange (Ruvkun and Ausubel 1981). Regions flanking the *nod* box were obtained by PCR using oligonucleotide primer pairs Δnod -boxuf/ Δnod -boxur and Δnod -boxdf/ Δnod -boxdr (Table 4) containing restriction endonuclease sites (*Spe*I and *Bgl*II for the upstream flanking region and *Bgl*II and *Xba*I for the downstream flanking region). The gene replacement cassette containing the *Ωkan* cassette inserted between the two PCR products was constructed in pIJ3200 and the resulting plasmid was used to achieve marker exchange as described above to produce mutant strain AH55, which was confirmed by PCR analysis.

To construct the CMD fusion strain AH71, oligonucleotide primer pairs *msi061*CMDF and *msi061*CMDR (Table 4) were designed to amplify a 331-bp region containing the putative promoter and 5' region of *msi061*. The PCR product was cloned into the suicide vector pFUS2 (Antoine et al. 2000) adjacent to its promoterless *lacZ* gene and the clone confirmed by DNA sequencing. Double *nodD*/IDMs and double *nod*-box::*Ωkan*/IDM mutants were constructed using the pFUS2 clones that were used to construct the single *vir* mutants (Hubber et al. 2004). The pFUS2 clones were transferred from *E. coli* S17-1 into the *M. loti* strains by conjugation. Transconjugants were passaged four times on selective media, and the strains were confirmed by Southern hybridization analysis.

Markerless deletions of the *virA* and *virG* genes were constructed in *M. loti* R7A using the suicide vector pJQ200SK (Quandt and Hynes 1993). Oligonucleotide primer pairs which contained restriction endonuclease sites to facilitate cloning (Table 4) were designed to amplify 1- to 2-kb regions that flanked the regulatory *vir* genes. The primer pairs used were Δvir GUF/ Δvir GUR and Δvir GDF/ Δvir GDR for *virG* and Δvir AUF/ Δvir AUR and Δvir ADF/ Δvir ADR for *virA*. The PCR products were digested with appropriate enzymes and ligated into pJQ200SK digested with *Spe*I and *Xho*I. Clones containing both PCR products were confirmed by sequencing and then transferred to R7A, followed by selection for Gent^r isolates. Integration at the correct site was confirmed by Southern hybridization. A second homologous recombination event that excised the vector was detected by loss of sucrose sensitivity, due to the loss of the *sacB* gene present on pJQ200SK. Strains with the desired deletion were confirmed by PCR analysis and Southern hybridization.

Analysis of *vir* gene expression.

β -Galactosidase assays were performed on *M. loti* G/RDM broth (pH 6.0) cultures in midexponential or stationary phase as described previously (Miller 1972). Naringenin (1 μ M) was added to broths where required.

Nodulation tests.

L. leucocephala var. Cunningham was grown under controlled environmental conditions of 70% humidity, 22 to 25°C during

the day, and 14°C at night on a cycle of 16 and 8 h of day and night, respectively. Seed were placed in a small amount of concentrated sulfuric acid for 5 to 10 min. After removing the acid, the seed were washed, incubated overnight in fresh sterile distilled water, washed twice, and transferred to watery agar (0.8%) for 2 days in the dark to germinate. Seedlings were grown, inoculated, and analyzed as described previously (Hubber et al. 2004).

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