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nodD2 of *Rhizobium* sp. NGR234 is involved in the repression of the *nodABC* operon

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Summary

Transcriptional regulators of the *lysR* family largely control the expression of bacterial symbiotic genes. *Rhizobium* sp. NGR234 contains at least four members of this family: two resemble *nodD*, while two others are more closely related to *syrM*. Part of the extremely broad host range of NGR234 can be attributed to *nodD*1, although the second gene shares a high degree of DNA sequence homology with *nodD*2 of *R. fredii* USDA191. A *nodD*2 mutant of NGR234 was constructed by insertional mutagenesis. This mutant (NGRΩ*nodD*2) was deficient in nitrogen fixation on *Vigna unguiculata* and induced pseudonodules on *Tephrosia vogelii*. Several other host plants were tested, but no correlation could be drawn between the phenotype and nodule morphology. Moreover, *nodD2* has a negative effect on the production of Nod factors: mutation of this gene results in a fivefold increase in Nod factor production. Surprisingly, while the structure of Nod factors from free-living cultures of NGRΩ*nodD*2 remained unchanged, those from *V. unguiculata* nodules induced by the same strain are non-fucosylated and have a lower degree of oligomerization. In other words, developmental regulation of Nod factor production is also abolished in this mutant. Competitive RNA hybridizations, gene fusions and mobility shift assays confirmed that *nodD*2 downregulates expression of the *nodABC* operon.

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Introduction

Signal exchange leads to symbioses between legumes and rhizobia (bacteria of the genera *Azorhizobium*, *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium*). Phenolic compounds, such as flavonoids, in root exudates trigger transcription of bacterial nodulation genes (*nod*, *nol* and *noe*), which play a role in the early stages of the symbiotic interaction (Dénarié *et al*., 1996; Hanin *et al*., 1997a). Some of these genes govern the synthesis of a family of lipochitooligosaccharides (LCOs) called Nod factors, which induce root hair curling and division of cortical cells. LCOs of various rhizobia share a common core, consisting of three to six β-1,4-linked *N*-acetyl-D-glucosamine residues with a fatty acid attached to the nitrogen of the non-reducing sugar moiety (Mergaert *et al*., 1997). This common backbone is reflected in similarities among the *nodABC* genes of the various genera. Most other *nod* genes are not functionally or structurally conserved however, and are involved in strain-specific modifications of the Nod factors. As an example, the *nodSU* genes control the ability of NGR234 to nodulate *Leucaena* through *N*-methylation and 6-*O*-carbamoylation of the non-reducing terminus of the Nod factors (Jabbouri *et al*., 1995).

Although the mechanisms behind regulation of *nod* gene expression are highly variable, they all require the *nodD* gene. NodD acts as both a sensor of the plant signal and a transcriptional regulator of the *nod* genes (Schlaman *et al*., 1992a). NodD proteins belong to the LysR family of transcriptional activators and bind to specific sequences (*nod* boxes) present in the promoter regions of inducible nodulation genes. Activation of *nod* gene expression via *nod* box promoters is not the only regulatory system used in rhizobia, however. *nolX* of *R. fredii* strain USDA257, for example, lacks a *nod* box but is regulated in the conventional flavonoid and NodD-dependent manner (Kovács *et al*., 1995; Bellato *et al*., 1996).

Some species, e.g. *R. leguminosarum* bv. *trifolii*, have only one *nodD* gene, while *B. japonicum*, NGR234, *R. meliloti* and *R. tropici* possess two to five copies of *nodD* (van Rhijn *et al*., 1993). In *R. meliloti*, mutation of all three copies of *nodD* is required to obtain a Nod phenotype (Honma *et al*., 1990). Products of the *nodD* genes of various *Rhizobium* species differ in that they respond, in a species-specific way, to different sets of flavonoids and exudates (Spaink *et al*., 1987a). Furthermore, NodD homologues from the same strain have diverse signal sensitivities

(Györgypal *et al*., 1991). Inducer-independent activator activities of *nod* operons have also been reported: *nodD*3 of *R*. *meliloti* constitutes, together with *syrM*, a self-amplifying positive regulatory circuit which may be important in the regulation of *nod* genes within the developing nodule (Swanson *et al*., 1993). In addition to two copies of NodD, *B*. *japonicum* possesses two genes, *nodVW*, which are members of the family of two-component regulatory systems (Göttfert *et al*., 1990).

nod gene expression is also subject to negative control. In bacteroids, the differentiated nitrogen-fixing form of rhizobia, the expression of *nodD* decreases, and the transcription of inducible nodulation genes is repressed (Schlaman *et al*., 1992b). In *R*. *meliloti*, negative control of *nod* gene expression is provided by the NolR repressor, which contains a putative helix–turn–helix motif that has homology to the N-terminal domain of NodD. NolR appears to function as a repressor of *nod* genes, which provide common nodulation functions (*nodD*1, *nodD*2, *nodD*3, *nodABC* and *nodM*) (Cren *et al*., 1995). In *B*. *japonicum*, repression of *nod* gene expression by NolA is probably an indirect effect, perhaps mediated by other genes (e.g. *nodD*2; Garcia *et al*., 1996). *B*. *japonicum nolA*$^-$ and *R. meliloti nolR*$^-$ mutants retain the ability to nodulate their hosts, albeit at lower efficiency, suggesting that fine tuning of *nod* gene expression is required for optimal nodulation.

NGR234 is able to nodulate more than 110 different plant genera. Part of this broad host range can be explained by the ability of the NodD1 protein to interact with a wide variety of flavonoids and related compounds, such as vanillin (Le Strange *et al*., 1990). NGR234 possesses three other *nodD* homologues: *nodD*2, *syrM*1 and *syrM*2, but a *nodD*1 mutant is unable to initiate nodulation on any plant tested (Relić *et al*., 1993). In this work, we show that the *nodD*2 gene of NGR234 is required for the formation of nitrogen-fixing nodules and is involved in the repression of the *nodABC* operon.

Results

Symbiotic properties of a nodD*2*$^-$ *mutant*

NGRΩ*nodD*2 was constructed by inserting a kanamycin resistance Omega cassette (Fellay *et al*., 1987) into the *Bam*HI site of *nodD*2. Gene replacement was forced using the pJQ200 suicide vector (Quandt and Hynes, 1993). By inoculating various NGR234 hosts with NGRΩ*nodD*2 (Table 1), the phenotype of the mutant was tested. Regardless of the type of nodule formed, three phenotypes (Table 1) were observed: (i) some plants (e.g. *Leucaena leucocephala*) produced a similar number of Fix$^+$ nodules to those inoculated with wild-type NGR234; (ii) others (e.g. *Tephrosia vogelii*) only produced bacteria-free pseudonodules; and (iii) although the nodules of *V*. *unguiculata* and *Cajanus cajan* contained leghaemoglobin, the plants appeared to be starved of nitrogen. Acetylene reduction assays showed that the nitrogenase activity of *V*. *unguiculata* roots infected with NGRΩ*nodD*2 was only 1.5% of that observed in roots inoculated with NGR234 (data not shown). In addition, microscopic analyses (Fig. 1) showed that nodules invoked by the mutant contained fewer bacteria, which were embedded in a fibrillar material resembling the cell wall matrix, where they accumulated large amounts of β-hydroxybutarate. Although a lightly stained, homogeneous, narrow zone surrounded each bacterial cell, the peribacteroidal membrane was not visible. Together, these observations suggest that the processes of bacterial release and differentiation into bacteroids was severely affected.

Comparison of Nod factors produced by NGR234 and the nodD*2*$^-$ *mutant*

Hydrophobic compounds extracted from the supernatants of apigenin-induced cultures grown in the presence of [^{14}C]-acetate were fractionated by reverse-phase thin-layer chromatography (RP–TLC). The TLC profile of NGR234 supernatants showed two major spots corresponding to sulphated (B) and non-sulphated molecules (A) respectively (Hanin *et al*., 1997b). No differences were detected in the TLC profile produced by NGRΩ*nodD*2 (data not shown). FAB/MS analyses of the purified products confirmed that the two strains produced identical Nod factors. Nevertheless, the *nodD*2$^-$ mutant produces substantially more Nod factors. Integration of the two major high-performance liquid chromatography (HPLC) peaks corresponding to the sulphated (B) and acetylated (A) products gave fivefold increases in the amounts of Nod factors produced by the mutant compared with the wild type. This result was confirmed using the tomato cell suspension assay (Staehelin *et al*., 1994).

As it has been reported that Nod factors accumulate in the membranes of *R*. *leguminosarum* bv. *trifolii* cells (Orgambide *et al*., 1995), we also performed TLC analysis of membrane-bound fractions. Spot B (sulphated molecules) was present in the membrane-bound fraction of

Table 1. Phenotype of the NGR234 *nodD*2 mutant.

Plant species	Phenotype
Calopogonium caeruleum	Fix$^+$
Leucaena leucocephala	Fix$^+$
Robinia pseudoacacia	Fix$^+$
Vigna unguiculata	Nod$^+$
Cajanus cajan	Nod$^+$
Lablab purpureus	Pseudonodules
Pachyrhizus tuberosus	Pseudonodules
Psophocarpus palustris	Pseudonodules
Tephrosia vogelii	Pseudonodules
Crotolaria juncea	Pseudonodules
Fleminga congesta	Pseudonodules

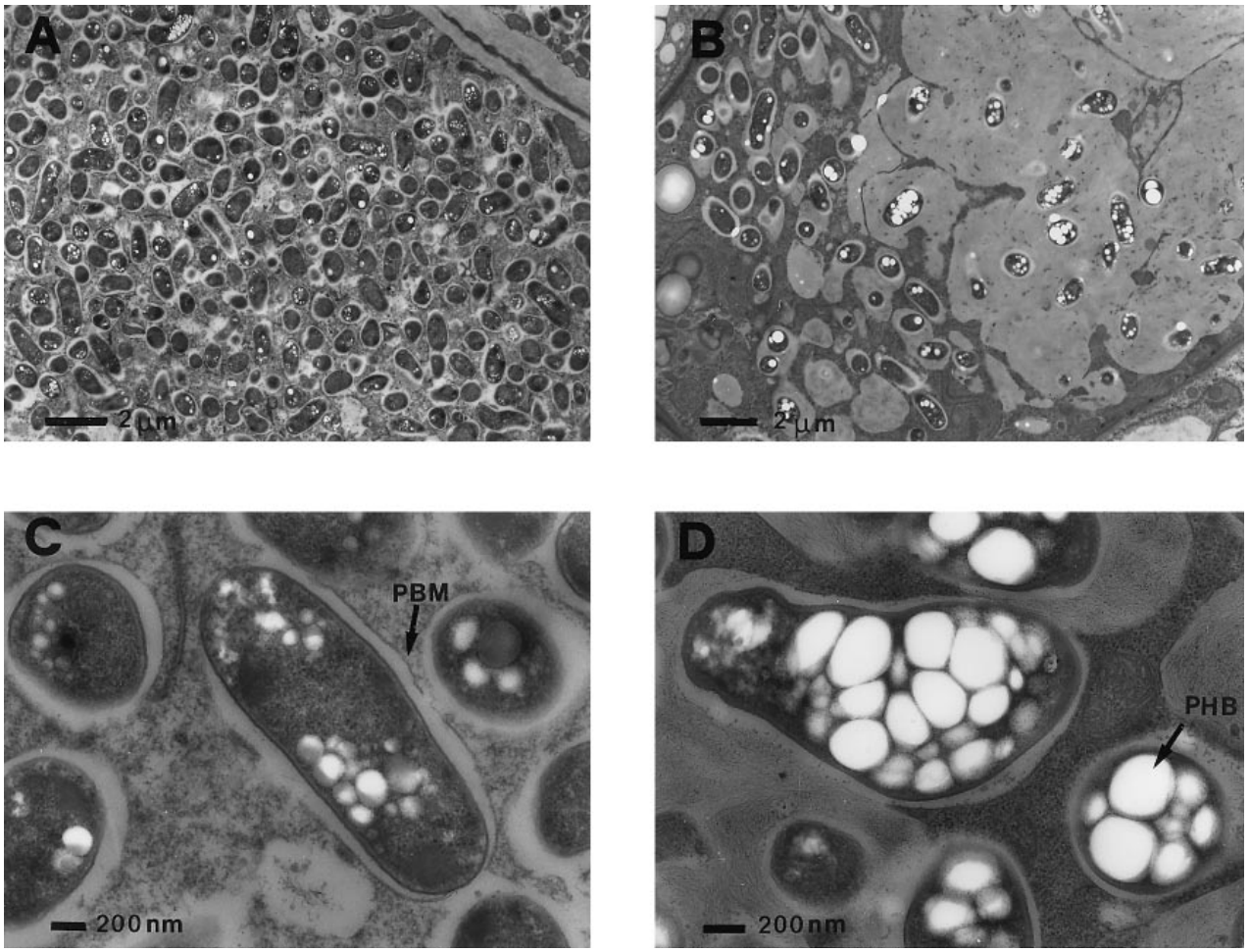
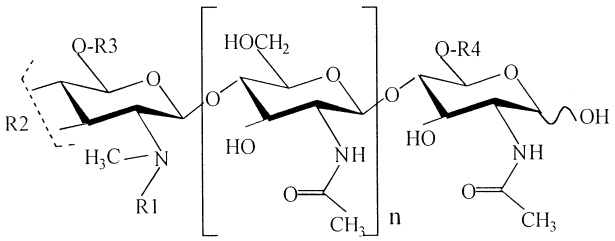


Fig. 1. Electron micrographs showing bacteroids within *Vigna unguiculata* nodules infected by NGR234 (A and C) and NGRΩ*nodD*2 (B and D).Nodules invoked by NGRΩ*nodD*2 were red but contained fewer bacteria, bacteroid release was disturbed and the transformation into bacteroids was incomplete. Bacteria are embedded in a fibrillar material resembling the cell wall matrix. A light, homogenous, narrow zone surrounded each bacterial cell but the peribacteroidal membrane (PBM) was not visible. β-Hydroxybutarate granules (PHB) accumulated in the bacteria, and degeneration of the bacteria was observed.

NGRΩ*nodD*2 but not in the wild-type strain. HPLC purification and FAB/MS analyses of membrane-bound Nod factors showed that, in addition to acetylated or non-substituted Nod factors, sulphated 2-*O*-methyl-L-fucosylated factors are present in the membrane fraction of the mutant strain.

Analysis of Nod factors from nodule bacteria

As discussed above, *V. unguiculata* plants inoculated with NGRΩ*nodD*2 appeared to be starved of nitrogen, although the nodules contained both bacteria and leghaemoglobin. To test whether deregulation of Nod factor synthesis was responsible for this Fix$^-$ phenotype, nodules obtained by inoculation with NGRΩ*nodD*2 and wild-type NGR234 were collected, ground, filtered and centrifuged. Both the nodule bacteria and the supernatant were extracted separately, as described in the *Experimental procedures*. Reverse-phase HPLC purification followed by FAB/MS analysis of the different fractions revealed the presence of LCOs only in membrane fractions of NGRΩ*nodD*2 nodule bacteria. No significant ions attributable to LCOs could be detected in the supernatant or the membrane fractions of wild-type bacteroids. Retention times of Nod factors isolated from NGRΩ*nodD*2 nodule bacteria correspond to those of non-sulphated products. TLC (data not shown) confirmed the presence of only acetylated molecules. In the low-mass region, FAB/MS analyses gave oxonium ions of *m/z* 526, 483 and 440 corresponding to *N*-methylated *bis*-, mono- or non-carbamoylated D-glucosamine, *N*-acylated by $C_{18:1}$. Molecules acylated by $C_{16:1}$ gave oxonium ions of *m/z* 498, 455 and 412. These fragmentation series are identical to those corresponding to NodNGR factors (Quesada-Vincens *et al*.,



	NGR234 wt	NGR234 NodD$_2^-$ (Bmb)
R1	$C_{18:1}$, $C_{16:1}$, $C_{18:0}$	$C_{18:1}$, $C_{16:1}$
R2	Carb, H	Carb, H
R3	Carb, H	Carb, H
R4	MeFuc, AcMeFuc, SMeFuc	H*
n	3	0, 1, 2, 3

Fig. 2. Structures of the Nod factors isolated from *Vigna unguiculata* nodules infected with NGRΩ*nodD*2. For comparison, the major Nod factors produced by NGR234 *in vitro* are also shown. *n* is the number of *N*-acetyl-D-glucosamine residues. R1 represents acyl chains, the carbon length and double bonds of which are indicated in the table. Carb, carbamate; MeFuc, *2-O*-methylfucose; AcMeFuc, acetylated-*2-O*-methylfucose; SMeFuc, sulphated-*2-O*-methylfucose; wt, wild-type strain; Bmb, bacteroid membrane. *A very small amount of Ac-Me-fucosylated Nod factors was also observed.

1997), proving that the non-reducing terminus of Nod factors isolated from bacteroids induced by NGRΩ*nodD*2 is not modified (Fig. 2). Nevertheless, these fractions yielded pseudomolecular ions $[M^+,H]^+$ that have never been observed in NodNGR factors. These correspond to non-fucosylated Nod factors containing 2, 3, 4 or 5 *N*-acetyl-D-glucosamine residues (Fig. 3). 2-*O*-Methylfucosylated pentamers of *N*-acetyl-D-glucosamine (acetylated or non-acetylated) were also detected, albeit in very small amounts.

nodD*2 is involved in the repression of transcription of the n*odABC *operon*

Most symbiotic determinants of NGR234 are located on a 536 kb plasmid, pNGR234*a*. A competitive hybridization procedure was used to study the organization and timing of flavonoid-inducible transcripts on pNGR234*a* (Fellay *et al*., 1995). A similar approach was used here to identify plasmid-borne genes regulated by NodD2. A set of 23 overlapping cosmids (Fig. 4A) covers the entire pNGR234*a* (Perret *et al*., 1991). Southern blots of endonuclease *Xho*I-digested DNA of these cosmids were hybridized to *in vitro* labelled RNA isolated from NGR234 cells 24 h after induction with 10^{-7} M daidzein (probe RNA). Prehybridization was performed with a 10-fold excess of non-labelled RNA from NGRΩ*nodD*2 cells induced with the same flavonoid (competitor RNA). Only DNA fragments that produced transcripts in excess in NGR234 compared with NGRΩ*nodD*2 should hybridize, revealing loci that are induced by NodD2. Only a 6 kb *Xho*I fragment of pXB64 was clearly visible in the autoradiographs (Fig. 4C). This fragment carries the gene y4wM, the product of which is homologous to putative ABC-type dipeptide binding

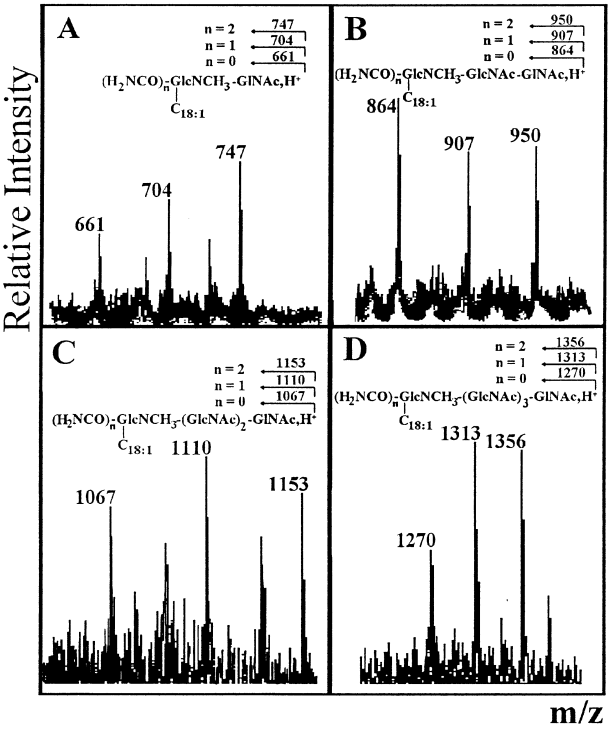
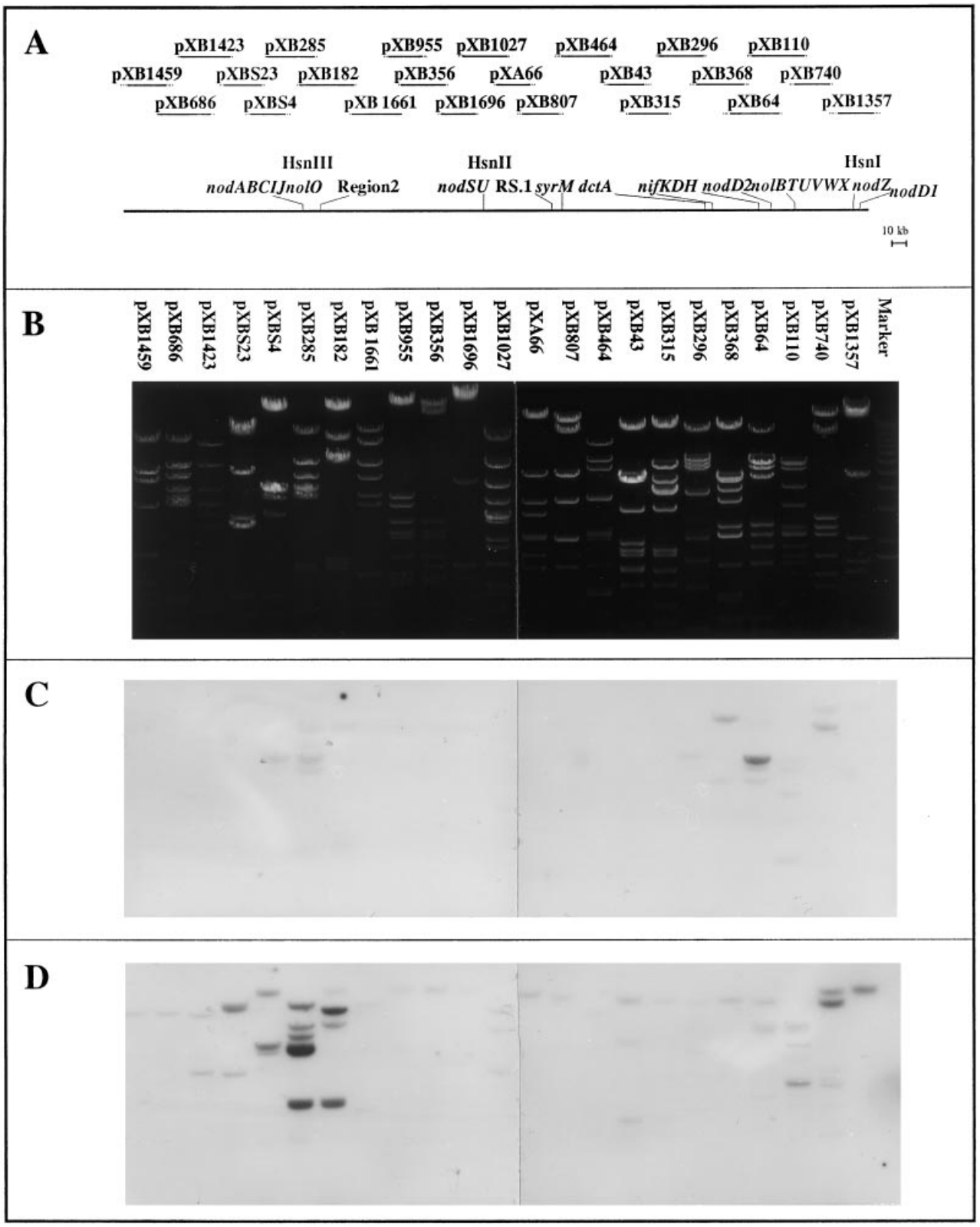


Fig. 3. Pseudomolecular ions from the FAB mass spectra region of Nod factors extracted from the bacteria extracted from *Vigna unguiculata* nodules infected by NGRΩ*nodD*2. A–D. Scans of the $[M+H]^+$ ions corresponding, respectively, to the *N*-methyl, *N*-vaccenoyl, bi-, tri-, tetra- or pentamers, which may be mono- or *bis*-carbamoylated at the non- reducing terminus.

Fig. 4. Competitive hybridizations to Southern blots of cosmid DNA covering pNGR234*a*.
A. Physical map of pNGR234*a*. The ordered cosmid library is shown on top of the genetic map.
B. Ethidium bromide-stained electrophoresis gel showing the restriction patterns of *Xho*I-digested cosmid DNA.
C. Loci whose transcription is NodD2 dependent: hybridizations were performed as described in *Experimental procedures* using ^{32}P-radiolabelled RNA probes from *Rhizobium* sp. NGR234 cultures induced by daidzein for 24 h and competitor RNA from NGRΩ*nodD*2 cultures induced under the same conditions.
D. NodD2-dependent repression: autoradiograms were obtained using RNA probes from NGRΩ*nodD*2 and competitor RNA from NGR234 cultures induced with daidzein for 24 h.


A
pXB1423 pXB285 pXB955 pXB1027 pXB464 pXB296 pXB110
pXB1459 pXBS23 pXB182 pXB356 pXA66 pXB43 pXB368 pXB740
pXB686 pXBS4 pXB 1661 pXB1696 pXB807 pXB315 pXB64 pXB1357
HsnIII
HsnII
HsnI
nodABCIJnolO Region2
nodSU RS.1 syrM dctA
nifKDH nodD2nolBTUVWX nodZ nodD1
10 kb
B
pXB1459 pXB686 pXB1423 pXBS23 pXBS4 pXB285 pXB182 pXB 1661 pXB955 pXB356 pXB1696 pXB1027 pXA66 pXB807 pXB464 pXB43 pXB315 pXB296 pXB368 pXB64 pXB110 pXB740 pXB1357 Marker
C
D

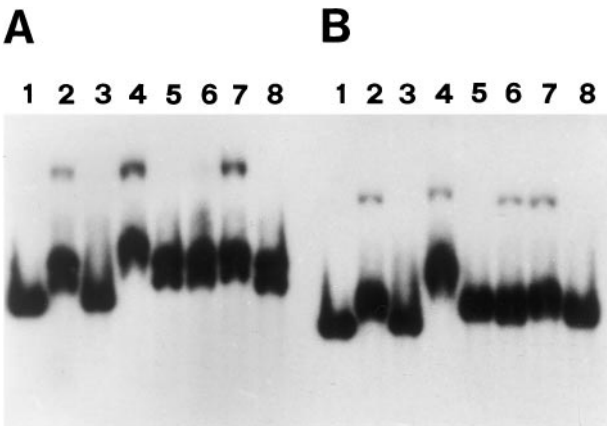


Fig. 5. Complex formation between the *nodABC* and *noeE nod* boxes and protein extracts of NGR234 strains.
A. Retardation of the 275 bp DNA fragment carrying the *nodABC nod* box.
B. Retardation of the 240 bp DNA fragment carrying the *noeE nod* box. Source of extracts: none (lane 1); NGR234 induced with daidzein for 4 h (lanes 2 and 3) or 24 h (lane 4); NGRΩ*nodD*1 (lane 5); NGRΩ*nodD*2 (lane 6); NGRΩ*syrM*1 (lane 7); and NGRΩ*nodD*1/*D*2 (lane 8) induced for 4 h. Lane 3 shows competition using a 50-fold excess of unlabelled *nod* box DNA.

proteins and is preceded by a *nod* box-like sequence (Freiberg *et al*., 1997). Faint labelling of two *Xho*I fragments (3.8 kb and 4.2 kb) shared by cosmids pXB285 and pXBS4 was also detected.

After initial induction, transcription of the common *nod* genes decreased to undetectable levels 24 h after incubation with purified flavonoids. This repression is probably linked to NodD2 (Fellay *et al*., 1995). To test this, we screened the symbiotic plasmid for genes that are down-regulated by NodD2. Inverting the probe and the competitor RNAs in competitive RNA hybridization experiments allowed us to test this hypothesis. Probe RNA was extracted from NGRΩ*nodD*2 cells treated for 24 h with 10^{-7} M daidzein, while the competitor RNA was taken from induced NGR234 cells. Restriction fragments of cosmids pXB285 and pXB182 containing the *nodABCIJnolOnoeI* operon were strongly labelled (Fig. 4D), demonstrating the requirement for a functional *nodD*2 gene in the repression of this operon.

Protein extracts retard nod *boxes in gels*

Specific DNA binding of NodD2 to the *nodABC* promoter region was studied by means of gel retardation assays using total cellular protein extracts isolated from daidzein-induced NGR234 and NGRΩ*nodD*2 cells. Polymerase chain reaction (PCR)-amplified fragments from the *nodABC* and *noeE nod* boxes (275 bp and 240 bp respectively) were used as probes. When protein extracts from NGR234 were mixed with radioactively labelled *nodABC* promoter probes, three retarded fragments were seen (Fig. 5A). Specificity in the protein–DNA interactions was demonstrated by titrating the signal away using unlabelled *nodABC nod* box DNA. The upper retarded band was not present when crude protein extracts isolated from NGRΩ*nodD*2 were used in the binding reaction (Fig. 5A, line 6). Mutation of *nodD2* had no effect on the gel retardation pattern of the *noeE nod* box fragment however (Fig. 5B), indicating that NodD2 primarily affects the *nodABC nod* box.

Expression of a transcriptional nodABC–β*-galactosidase fusion*

A PCR fragment containing the *nod* box of the *nodABC* operon was cloned upstream of the β-galactosidase gene into the broad-host-range vector pMP220. Triparental matings were used to mobilize the resulting construct, pNBA, into NGR234 and NGRΩ*nodD*2. β-Galactosidase activities of the transconjugants grown in liquid cultures were monitored after induction with apigenin. Different dilutions of the cultures were used to minimize the influence of growth phase on gene expression. In the wild-type bacterium, after an initial induction, the β-galactosidase activity decreased, whereas mutation of *nodD*2 results in continuous expression of the *nodABC* operon (Fig. 6A). Moreover, the introduction of *nodD*2 on a multicopy plasmid (pRAF130) into NGR234 (pNBA) as well as into NGRΩ*nodD*2 (pNBA) completely abolished *nodABC* expression. As this data accords with that from competitive RNA hybridizations, it seems likely that the repressor activity is linked to NodD2.

Sequence analysis and site-directed mutagenesis of nodD*2*

*nodD*2 is one of the 416 open reading frames (ORFs) of pNGR234*a* that have been sequenced entirely (Freiberg *et al*., 1997). Homologies with *nodD*3 of *R. meliloti* and *nodD*1 of NGR234 range from 73% to 76% at the DNA level. In addition, *nodD*2 of NGR234 shares 98% identity with that of *R. fredii* USDA191. Only five basepairs are different within the coding region: two of these are silent, two lead to conservative amino acid changes, while one results in an amino acid change at the C-terminus of the protein (Fig. 7B). Homology also extends to the 5′ untranslated region. Nevertheless, owing to a single base change, the ORF of NodD2 of NGR234 is extended by an 55 additional amino acids at the N-terminus.

To determine whether NodD2 of NGR234 is actually longer than its USDA191 homologue, a stop codon was introduced by site-directed mutagenesis immediately upstream of the third possible start codon, which corresponds to the translational start of *nodD*2 of USDA191. The 2.1 kb *Pst*I–*Not*I fragment containing *nodD*2 and its promoter region was cloned into pBluescriptKS+ giving plasmid pRAF120.

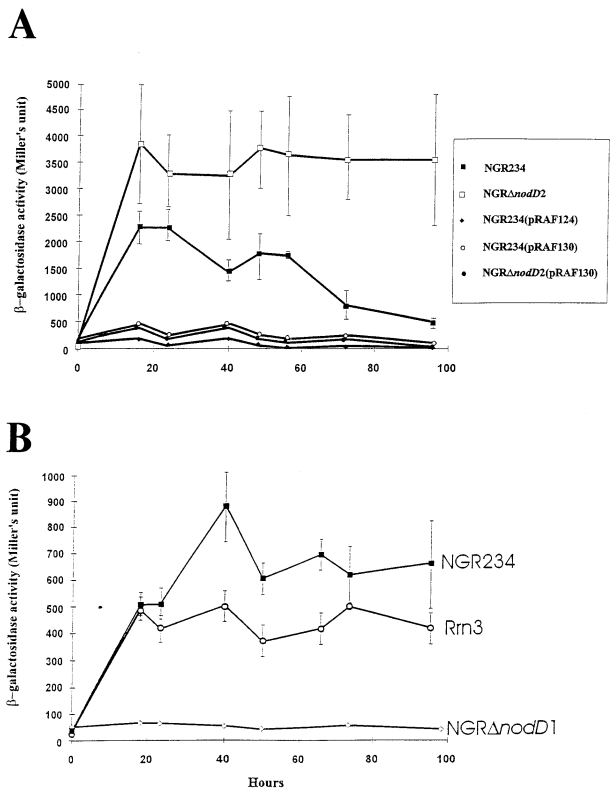


Fig. 6. β-Galactosidase activity of the *nodABC*$^-$ and *nodD*2$^-$ promoter–*lacZ* fusions.
A. Plasmid pNBA containing the *nodABC nod* box fused to *lacZ* was introduced in NGR234 (black rectangles) and NGRΩ*nodD*2 (open rectangles). Black diamonds represent the activity of NGR234(pNBA) complemented with the crippled copy of *nodD*2 on plasmid pRAF124. Activities resulting from the introduction of the wild-type copy of *nodD*2 carried on pRAF130 into NGR234 (pNBA) (open circles) and into NGRΩ*nodD*2 (pNBA) (dark circles) are also included.
B. Plasmid pRAF115 containing the *nodD*2 promoter fused to *lacZ* was introduced in NGR234 (black rectangles), its *rpoN* mutant derivative Rrn3 (open circles) and *nodD*I mutant NGRΩ*nodD*1 (open diamonds).

Site-directed mutagenesis of pRAF120 was performed by recombinant polymerase chain reaction (PCR; Jones and Howard, 1991), as described in *Experimental procedures*. The insert was then subcloned into the broad-host-range vector pBBR1MCS-5 yielding pRAF124, which was fully sequenced to verify the mutation. The introduction of pRAF124 into NGR234(pNBA) completely abolished the expression of the β-galactosidase activity driven by the *nodABC* promoter (Fig. 6A). A similar result was obtained upon the introduction of pRAF124 into NGRΩ*nodD*2 (pNBA) (data not shown).

Transcription of nodD2 *is flavonoid dependent*

Transcription of *nodD*2 was monitored by cloning its promoter region upstream of the promoterless *lacZ* gene of the pMP220 vector (Spaink *et al*., 1987b). β-Galactosidase assays of transconjugants harbouring the resulting plasmid, pRAF115, showed that transcription of *nodD*2 is inducible by flavonoids (Fig. 6B). Induction is NodD1 dependent and is affected by a mutation in the *rpoN* gene, which encodes σ^{54}. Rather than having a *nod* box-like sequence, *nodD*2 possesses regions upstream of the coding sequences that strongly resemble promoters used by the σ^{54} and σ^{28} holoenzymes (Fig. 7A).

Discussion

Precise regulation of *nod* genes is required for effective symbiosis. Constitutive expression of the *R. leguminosarum nodABC* genes cloned on a multicopy plasmid and reintroduced into the wild-type strain inhibited nodulation (Knight *et al*., 1986). In *R. meliloti*, genes determining synthesis of the core Nod factor structure are subject to negative regulation by NolR (Cren *et al*., 1995). This control is needed for optimal nodulation, as NolR$^-$ *R. meliloti* strains nodulate *Medicago sativa* less efficiently than NolR$^+$ strains (Kondorosi *et al*., 1989). After an initial *nodD*1-dependent activation, transcription of all three Hsn loci of pNGR234*a* is repressed (Fellay *et al*., 1995).

In this work, many lines of evidence point to a role for *nodD*2 in the repression of the *nodABCIJnolOnoeI* operon. First, the NodD2 mutant produces five times more Nod factors than the wild-type strain. Nod factor production is also continuous, as their accumulation in nodules of *V. unguiculata* infected with NGRΩ*nodD*2 was observed. Secondly, competitive RNA hybridizations as well as reporter gene fusions confirmed the increase in *nodABC* transcripts in the mutant and showed that their repression is dependent on *nodD*2. Thirdly, mobility shift experiments indicate a specific interaction between NodD2 and the *nodABC* promoter.

Flavonoid signal molecules induce the expression of *nodD*2. Induction is *nodD*1 dependent even though *nodD*2 lacks a *nod* box-promoter. The presence of a σ^{54} consensus promoter sequence upstream of the ORF correlates with the reduction in *nodD*2 expression in the NGR234 *rpoN* mutant; the remaining activity can be explained by a second promoter located on the DNA fragment used for the β-galactosidase fusion. Although *nodD*2 of NGR234 and USDA191 (Appelbaum *et al*., 1988) have nearly identical DNA sequences, a single nucleotide difference extends the ORF of NGR234 *nodD*2 by 55 amino acids. A site-directed mutant of *nodD*2 removing this extension results in strong repression of activity of the *nodABC nod* box. The presence of *nodD*2 on a multicopy number plasmid leads to enhanced gene expression, resulting in more severe *nodABC* repression than that found in the wild-type background. Interestingly, by transferring *nodD*2 (pRAF130) into NGR234, the same repression was observed as in NGR234 (pNBA) and NGRΩ*nodD*2 (pNBA). It

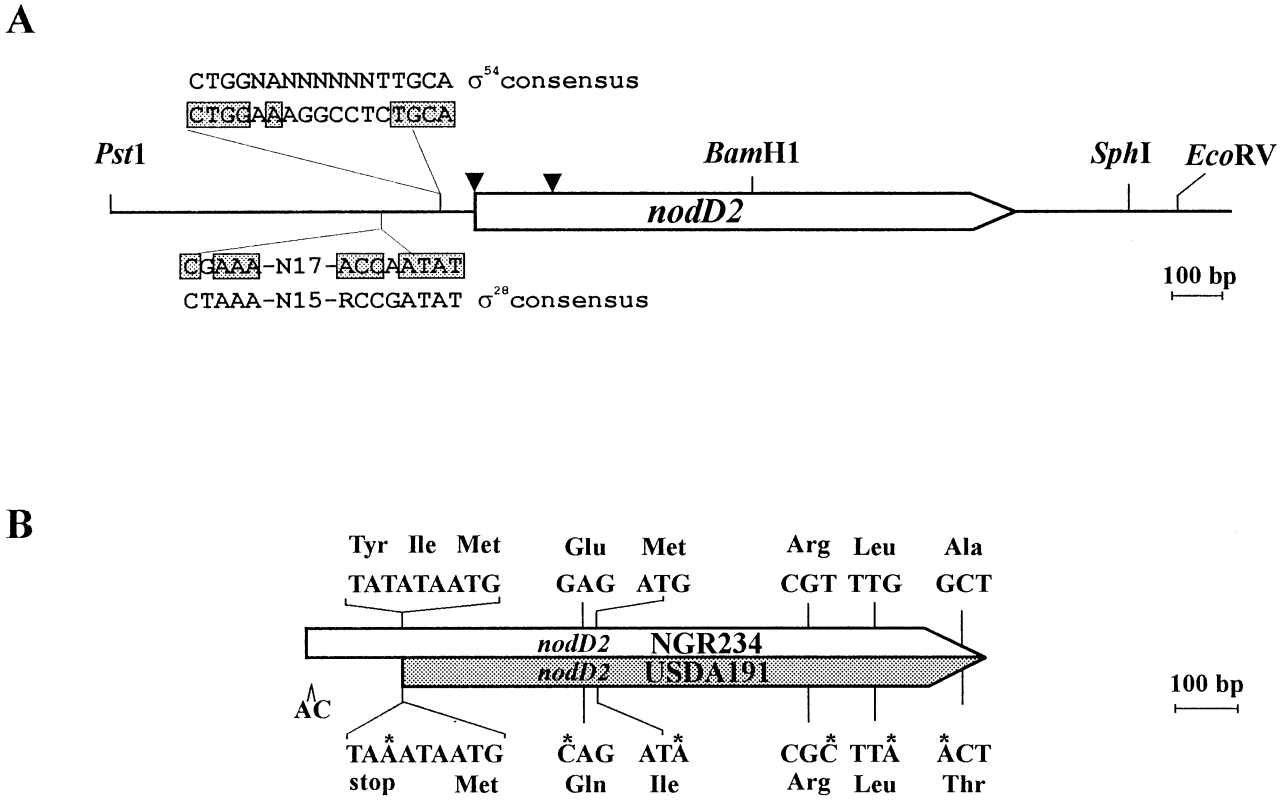


Fig. 7. A. Physical map of the NGR234 *nodD*2 locus. The white arrow indicates the open reading frame. Possible start codons are indicated by black triangles. The sequence of the putative σ^{54} (Shingler, 1996) and σ^{28} (Arnosti and Chamberlin, 1989) promoters are also shown. B. Comparison of the nucleotide sequences of NGR234 and USDA191 *nodD*2 genes. The open reading frames of NGR234 (white) and USDA191 (shaded) are aligned, and the nucleotide differences are marked by an asterisk. Codons containing these changes are shown together with their corresponding amino acids.

cannot be excluded, however, that two NodD2 proteins are produced from different initiation codons and that the shorter of the two is responsible for repressor activity. In this scenario, pRAF130 would encode two NodD2 forms, the longer of which might play a role(s) other than regulation of *nodABC* (e.g. activation of y4wM).

nodZ, which is responsible for the fucosylation of NodNGR factors (Quesada *et al*., 1997) is initially activated then repressed in a NodD2-independent manner. Repression of *nodZ* in the NodD2$^-$ background could explain the presence of non-fucosylated Nod factors in the bacteroids of *V. unguiculata* nodules. This was confirmed by the observation that β-galactosidase activity of the *nodZnod* box–*lacZ* fusion is *nodD*2 independent (Fellay *et al*., 1995). Another repressor, whose activity is independent of *nodD*2 and which plays a role in the repression of *nodZ*, must exist. Examination of promoter regions for DNA motifs possibly involved in the repression of *nod* genes was performed by aligning the *nod* boxes of *nodABC* (Relić *et al*., 1994), *nodSU* (Lewin *et al*., 1990) and *nodZ* (R. Fellay, unpublished). A *nolR* binding site motif matching the *R. meliloti* consensus sequence was found downstream of the *nod* box of *nodABC* and *nodZ* but not of *nodSU* (data not shown). A single, chromosomal copy of *nolR* was confirmed using PCR, sequencing and hybridization techniques (data not shown). If NolR is responsible for the inactivation of *nodZ* and *nodABC*, the requirement of *nodD*2 for the repression of *nodABC* remains to be explained. Possibly, co-operation between NolR and NodD2 (or a NodD2-linked) repressor is needed for interaction with the *nodABC* promoter.

It seems likely that mutation of *nodD*2 should lead to a pleiotropic phenotype. Plant tests have shown that NGRΩ*nodD*2 does not affect the nodulation of *Leucaena leucocephala* but results in the formation of Fix$^-$ and empty nodules on *V. unguiculata* and *Tephrosia vogelii* respectively. Thus, *nodD*2 of NGR234 is involved in host specificity, perhaps at an intermediary stage in the symbiosis, at which it inhibits Nod factor production, thus permitting bacteroid establishment.

Experimental procedures

Media, growth conditions and bacterial matings

Bacteria were grown at 28°C in *Rhizobium* minimal medium (RMM) (Broughton *et al*., 1986) or RMM3 (Price *et al*., 1992)

Table 2. Bacterial strains and plasmids.

Strain/plasmid	Characteristics	Source
Strains		
NGR234(Rif^R)	Rif^R derivative of wild-type strain NGR234 from *Lablab purpureus*	Lewin *et al*. (1990)
NGRΩ*nodD*1	NGR234 derivative containing an Ω insertion in the *Bam*HI site of *nodD*1, Sp^R	Relić *et al*. (1993)
NGRΩ*nodD*2	NGR234 derivative containing an Ω insertion in the *Bam*HI site of *nodD*2, Km^R	Fellay *et al*. (1995)
NGRΩ*nodD*1/*D*2	NGRΩ*nodD*1 carrying an Ω insertion in *nodD*2, Km^R, Sp^R	This work
NGRΔ*syrM*1	*syrM*1- deletion mutant of NGR234, Sp^R	Hanin *et al*. (1998)
NGR234R*rn*3	NGR234, *rpoN Bgl*IIf::GmI-3, Gm^R	van Slooten *et al*. (1990)
Plasmids		
pBluescript-KS+	Phage f1, *lac*αZ$^+$, Ap^R	Stratagene
Lorist 2	A 5.6 kb cosmid vector, Km^R	Gibson *et al*. (1987)
pBS+	pMB1, phage f1, *lac*αZ$^+$, Ap^R	Stratagene
pMP220	IncP expression vector containing a promoterless *lacZ* gene, Tc^R	Spaink *et al*. (1987b)
pBBR1MCS-5	Broad-host-range cloning vector, Gm^R	Kovach *et al*. (1994)
pXB64	A Lorist 2 clone from pNGR234*a*, Km^R	Perret *et al*. (1991)
pGM2	A 6 kb *Eco*RI fragment from pXB64 containing *nodD2* cloned in pBS+	This work
pRAF130	A 2.1 kb *Pst*I–*Bgl*II fragment from pGM2 cloned in pBBR1MCS-5	This work
pRAF124	A 2.1 kb *Pst*I–*Not*I fragment containing site-directed mutation in *nodD*2. Cloned in pBBR1MCS-5	This work
pRAF115	A 1300 bp *Pst*I–*Bam*HI fragment containing the promoter of *nodD*2 cloned in pMP220	This work
pRK2013	ColE1 replicon containing the *tra* genes of RK2, Nm^R Km^R	Figurski and Helinski (1979)
pRK600	pRK2013 Nm^s::Tn*9*, Km^R Cm^R	Finan *et al*. (1986)

containing succinate as the carbon source. Apigenin and daidzein were added to concentrations of 1×10^{-6} M and 2×10^{-7} M, respectively, to induce *nod* gene expression. Rifampicin and tetracycline were added at 100 and 25 μg ml^{-1} respectively. Broad-host-range plasmids were mobilized from *E. coli* DH5α to *Rhizobium* by triparental matings using pRK2013 or pRK600 as helper plasmids (Ditta *et al*., 1980).

Plant assays

Nodulation tests were performed in Magenta jars (Magenta Corp.) (Lewin *et al*., 1990). All plants were grown at a day temperature of 26°C, a night temperature of 20°C and a light phase of 16 h. To distinguish between Nod$^+$ and Nod$^-$ phenotypes, plants were harvested 6 weeks after inoculation. Nodules were prepared for electron microscopy as described by Golinowski *et al*. (1987).

Purification of extracellular Nod factors

Each strain was grown at 27°C in 2 l Erlenmeyer flasks containing 1 l of RMM3 medium with or without 1×10^{-6} M apigenin (Price *et al*., 1992). Cells were grown to an A_{600} of 1. After centrifugation, extracellular Nod factors were extracted from the supernatant as described previously (Price *et al*., 1992; Jabbouri *et al*., 1995). Nod factors were labelled by raising the bacteria in RMM3 supplemented with 10 mCi of [^{14}C]-acetate.

Purification of membrane-bound Nod factors

Bacterial cells taken from Nod factor preparations were washed twice with 0.1 M NaCl to remove the interstitial Nod factors. Membrane-bound Nod factors were extracted with chloroform–propanol–methanol–water followed by C_{18} reverse-phase chromatography, as described by Orgambide *et al*. (1995).

Purification of Nod factors from the membranes of nodule bacteria

Nodules of *V. unguiculata* were collected 21 days after inoculation with NGR234 or NGRΩ*nodD*2. Nodules were homogenized in 5 mM Tris-HCl and 1 mM $MgSO_4$ (pH 7.2) (Ching and Hedtke, 1977). Then, the homogenate was filtered through four layers of Miracloth and centrifuged at 200 × *g* for 2 min at 4°C. The supernatant was clarified by centrifugation for 30 min at 4000 × *g* at 4°C and extracted twice with butanol. Membrane lipids were extracted from the pellet as described by Orgambide *et al*. (1995). Hydrophobic membrane or extracellular fractions were purified by reverse-phase HPLC on semi-preparative columns (Price *et al*., 1992; Jabbouri *et al*., 1995). Analysis of each fraction by FAB/MS in the positive

ionization mode was performed at an acceleration voltage of 8 kV (Jabbouri *et al*., 1995).

RNA extraction and labelling

Cultures were harvested at a cell density of $A_{600} = 0.4–0.6$ and resuspended in 10 ml of a solution consisting of equal volumes of phenol saturated with sodium acetate (pH 4.5) and 20 mM Tris-HCl containing 600 mM NaCl, 1 mM EDTA and 1% (w/v) SDS. The solution was prewarmed to 90°C before use. RNA was extracted with phenol–chloroform, precipitated with ethanol and purified by centrifugation through a CsCl cushion at 115 000 × *g* for 1 h, using a Beckman TLA-100.3 rotor (Ausubel *et al*., 1987). To prepare probes, 10–15 μg of RNA was partially hydrolysed in 125 mM NaOH for 25 min on ice and labelled using T4 polynucleotide kinase and [γ-^{32}P]-ATP for 90 min at 37°C (Apte and Haselkorn, 1990). The upper part of an UltraFree-MC filter unit (Millipore) was filled with 700 μl of Sephadex G50 (Pharmacia Biotech) and washed with TE8 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Probes (in a final volume of 100 μl) were loaded onto Sephadex G50 held in Eppendorf tubes and centrifuged for 5 s. The flow-through was added directly to the prehybridization solution.

Competitive hybridizations

Endonuclease-digested cosmid DNA was electrophoretically separated on 0.8% agarose gels and transferred to GeneScreen Plus (DuPont NEN) membranes. Prehybridization was performed overnight at 65°C in 15 ml of 50 mM Tris-HCl (pH 7.4) containing 0.2% (w/v) BSA, 0.2% (w/v) Ficoll (Pharmacia Biotech), 0.1% (w/v) sodium pyrophosphate, 1% (w/v) SDS, 1 M NaCl and 100–150 μg of total competitor RNA. Hybridizations were performed by adding $5–10 \times 10^7$ cpm of the probe to the prehybridization solution and incubating for 20 h at 65°C in a hybridization oven. Three washings (30 min each) were performed at 65°C in 1% (w/v) SDS dissolved in 1× SSC (Sambrook *et al*., 1989), followed by a further washing for 15 min in 0.2× SSC at room temperature.

Mobility shift DNA binding assays

PCR with the following primers was used to label *nod* boxes of the *nodABC* operon and the *noeE* gene: NBA1 (CTGCAGCCTGTTCTTTGATCCAACTTCC); NBA2 (GAATTCCCGACCTTCCCGTATCACTCG); NOE1 (GGGGCAGAATGCAGTCTGCACC) and NOE2 (CGCCACCGGTTGGTTACAGTGG).

PCR reactions in a final volume of 20 μl were prepared in 0.2 μM deoxyribonucleoside triphosphates, 1× $Vent_R$ (New England Biolabs) reaction buffer, 1.5 mM $MgSO_4$, 1 μM each of the primers, 25 μCi of ^{32}P-labelled dCTP and 1 unit of $Vent_R$ DNA polymerase. The reactions were subjected to 35 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 20 s. After completion of cycling, the reactions were incubated at 72°C for 5 min, and the DNA fragments were purified on Bio-Spin chromatography columns (Bio-Rad). Crude extracts were obtained by resuspending the bacterial pellets in 50 mM phosphate buffer (pH 7.2) containing 1 mM dithiothreitol (DTT) and 100 mM phenylmethylsulphonyl fluoride (PMSF) and passing the cells twice through a French press. After centrifugation (20 min at 10 000 × *g*, 4°C) to remove cell debris, binding reactions were performed in a 15 μl total volume (room temperature for 15 min) of the following: 10^4 dpm of probe DNA, 1 μl of crude extract in 10 mM KCl, 100 mM NaCl, 0.5 mM DTT, 1.4 mM EDTA, 50 mM Tris-HCl (pH 8), 5% (v/v) glycerol and 50 μg ml^{-1} BSA. Then, the samples were submitted to electrophoresis on 5% polyacrylamide gels for 150 min at 100 V in 90 mM Tris borate buffer containing 2 mM EDTA (pH 8.0).

Construction of gene fusions and monitoring of β-galactosidase activity

The promoter region of the *nodABC* operon was amplified by PCR from cosmid pXB285 using NBA1 and NBA2 as primers and $Vent_R$ DNA polymerase. This 275 bp fragment was then cloned into the *Eco*RV site of pBluescriptKS+ (Stratagene) and later, as an *Kpn*I–*Xba*I insert, into pMP220 (resulting in plasmid pNBA). A 1.3 kbp *Pst*I–*Bam*HI fragment containing the first 550 bp of the *nodD*2 ORF and an additional 750 bp of upstream sequences was cloned into pBluescriptKS+ giving pRAF110. This *Pst*I–*Bam*HI fragment was excised from pRAF110 with *Xba*I and *Eco*RI and recloned into pMP220, which was itself linearized with the same two enzymes (yielding pRAF115). *Rhizobium* strains containing pNBA or pRAF115 were grown in RMM media supplemented with 25 μg ml^{-1} tetracycline to an A_{600} of 0.1. Apigenin or daidzein was then added to the cultures to a concentration of 1×10^{-6} M and 2×10^{-7} M respectively. At different times, β-galactosidase activity was determined according to Miller (1972).

Cloning of nodD2

To clone *nodD2*, a *Pst*I–*Bgl*II fragment (2.1 kbp) containing *nodD*2 and its promoter was extracted from pGM2 plasmid and cloned into the *Bam*HI–*Pst*I sites of pBBR1MCS-5. The resulting plasmid was called pRAF130.

Mutagenesis of nodD2

The 2.1 kb *Pst*I–*Not*I fragment containing *nodD*2 and its promoter region was cloned into pBluescriptKS+ resulting in pRAF120. Site-directed mutagenesis was performed by recombinant PCR (Jones and Howard, 1991). The entire pRAF120 was amplified using the divergent and overlapping primers, D2mut1 (GCAGAAACGCTTTGGTTGAATTAAATAATGCGTTTTAAGG) and D2mut2 (TTAATTCAACCAAAGCGTTTCTGCCCTCATAGAACAGG), which generated DNA segments possessing ends that were homologous to each other. PCR reactions in a 50 μl final volume were prepared containing 0.2 μM deoxyribonucleoside triphosphates, 1× $Vent_R$ reaction buffer, 1.5 mM $MgSO_4$, 1 μM each primer and 1 unit of $Vent_R$ DNA polymerase. Each reaction mixture was subjected to 35 amplification cycles (94°C for 30 s, 62°C for 30 s and 72°C for 5 min). After the completion of cycling, the reactions were incubated at 72°C for 5 min and subjected to gel electrophoresis on 0.8% agarose. The amplification products were extracted from the gel using the Nucleotrap system (Macherey & Nagel) and used to transform competent *E. coli* DH5α cells. Point mutations were verified by manual dideoxy sequencing.

To generate a site-specific mutation in NGR234, the insert was subcloned as a *Sal*I–*Pst*I fragment into the broad-host-range vector pBBR1MCS-5 giving pRAF124. Before conjugation into NGR234, the insert was fully sequenced to ensure that only the desired mutation was obtained. DNA sequence analysis was performed with the Prism Ready Reaction Dye Deoxy Terminator sequencing kit and the Applied Biosystem model AB373 DNA sequencer (Applied Biosystems/Perkin-Elmer/Cetus).

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