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nodSU, Two New *nod* Genes of the Broad Host Range *Rhizobium* Strain NGR234 Encode Host-Specific Nodulation of the Tropical Tree *Leucaena leucocephala*

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Rhizobium **species strain NGR234 nodulates at least 35 diverse genera of legumes as well as the nonlegume *Parasponia andersonii*. Most nodulation genes are located on the 500-kilobase pair symbiotic plasmid, pNGR234*a*. Previously, three plasmid-borne host range determinants (HsnI, HsnII, and HsnIII) were identified by their ability to extend the nodulation capacity of heterologous rhizobia to include *Vigna unguiculata*. In this study, we show that HsnII contains two new *nod*-box linked *hsn* genes, *nodS* and *nodU*. *nodS* controls nodulation of the tropical tree *Leucaena leucocephala*, while the *nodSU* genes regulate nodulation of the pasture legume *Desmodium intortum* and the grain legume *V. unguiculata*. Regulation of the *nod*-box upstream of *nodSU* by the flavonoid naringenin was shown using a fusion with a promoterless *lacZ* gene. Determination of the nucleotide sequence of the *nodS* gene did not reveal homology with any gene in the EMBL library, although *Bradyrhizobium japonicum* USDA110 contains both *nodS* and *nodU* (M. Göttfert, S. Hitz, and H. Hennecke, Molecular Plant-Microbe Interactions 3:308-316, 1990). We suggest that broad host range in NGR234 is controlled in part by a *nodD* gene which interacts with a wide range of flavonoids, and in part by host-specific *nod* genes such as *nodS*.**

Additional keywords: gene regulation, indeterminate nodules.

Legume-*Rhizobium* associations are characterized by varying degrees of specificity. Most *Rhizobium* species nodulate only one or a few genera of legumes. On the other hand, *Bradyrhizobium* species tend to nodulate diverse legumes including a number of the agriculturally most important genera (*Arachis*, *Glycine*, *Vigna*, and so forth). Although both *Rhizobium* and *Bradyrhizobium* species contain plasmids, symbiotic plasmids are almost entirely confined to *Rhizobium*. Since the study of plasmids is simpler than analysis of chromosomes, we sought a *Rhizobium* species with a host range resembling that of *Bradyrhizobium* species. Our data showed that *Rhizobium* species strain NGR234 has a broader host range than any other strain reported (Lewin *et al.* 1987a, 1987b) and is currently known to nodulate 35 different legume genera. NGR234 hosts include the nonlegume *Parasponia*, cover and pasture legumes, grain legumes, and tropical trees (Trinick 1980; Trinick and Galbraith 1980; Broughton *et al.* 1984; Lewin *et al.* 1987a, 1987b; S. G. Pueppke, personal communication).

Genetic analysis of the host range determinants of *Rhizobium* species strain NGR234 showed that at least three different loci on the Sym plasmid pNGR234*a* (HsnI, HsnII, and HsnIII) are involved (Broughton *et al.* 1986; Lewin *et al.* 1987a, 1987b; Nayudu and Rolfe 1987). When transferred to heterologous rhizobia, any of the three loci conferred upon the recipients the ability to nodulate *V. unguiculata* (L.) Walp. In addition, HsnIII, which is linked to the nodulation genes *nodABC*, partially complements mutations in *nodH* of *R. meliloti* for nodulation of *Medicago sativa* L. Also, transconjugants containing HsnI (which is linked to the regulatory *nodD*1 gene) nodulate *G. max* (L.) Merr., *Macroptilium atropurpureum* (DC.) Urb., and *Psophocarpus tetragonolobus* (L.) DC. (Lewin *et al.* 1987b). Since the *nodD*1 gene of NGR234 responds to a large number of plant-produced flavones (Bassam *et al.* 1988; Bender *et al.* 1988; Rolfe 1988), it is at least partly responsible for the extended host range of the HsnI region.

Apart from the ability to extend the host range of heterologous transconjugants to include *V. unguiculata*, HsnII contains a *nod*-box sequence (Lewin *et al.* 1987b). *nod*-boxes are *cis*-acting conserved sequences (approximately 42 base pairs [bp]) in the 5′ region of *nodD*-regulated nodulation (*nod*) genes (Rostas *et al.* 1986; Shearman *et al.* 1986; Schofield and Watson 1986) and are the target sequence of the *nodD* gene product (Hong *et al.* 1987; Fisher *et al.* 1988; Downie and Johnston 1986; Kondorosi and Kondorosi 1986).

Analysis of the region surrounding the *nod*-box in the HsnII locus revealed two new *nod*-box-linked nodulation genes, *nodS* and *nodU*. Expression of *nodSU* is flavonoid-regulated and controls nodulation of the pasture legume *Desmodium intortum* (Mill.) Fawc and Rendle, the grain legume *V. unguiculata*, and the tropical tree *Leucaena*

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Nucleotide and/or amino acid sequence data is to be submitted to GenBank as accession number JO3686.

leucocephala (Lam.) de Wit. Interestingly, homologous genes exist in strain USDA110 of *B. japonicum* (Göttfert *et al.* 1990).

MATERIALS AND METHODS

Microbiological techniques. The bacterial strains, plasmids, and bacteriophages used in this study are listed in Table 1. Strains of *Escherichia coli* were grown either in or on Luria-Bertani medium (Maniatis *et al.* 1982) at 37° C; strains of *Rhizobium* were grown either in or on tryptone-yeast (TY) medium (Beringer 1974) or *Rhizobium* minimal medium (RMM) (Broughton *et al.* 1986) at 28° C. Antibiotic concentrations used were as follows (in micrograms per milliliter): ampicillin, 100; tetracycline (Tc), 10; rifampicin, 50; gentamycin (Gm), 10; spectinomycin (Sp), 50; and kanamycin (Km), 50. Subclones in pRK7813, pMP220, and pPP375 were conjugated into NGR234 or its derivatives either in triparental matings using pRK2013 as a helper plasmid or in diparental matings if the clones had been transformed into S17.1. Matings were performed on filters as described previously (Broughton *et al.* 1986).

DNA manipulation. Isolation of DNA, DNA analysis, and cloning procedures have been described previously (Broughton *et al.* 1985; Broughton *et al.* 1986; Maniatis *et al.* 1982). DNA probes were ^{32}P-labeled either by nick translation (Maniatis *et al.* 1982) or by oligo-labeling (Feinberg and Vogelstein 1984). Nonradioactive DNA-DNA hybridizations were performed with a DNA Labeling and Detection Kit (nonradioactive) from Boehringer Mannheim (Mannheim, Federal Republic of Germany) according to the manufacturer's recommendations. End labeling of oligonucleotide probes was as described in Lewin *et al.* (1987b).

Construction of NGR234 mutants. To construct NGR234Δ1, the two *Bam*HI fragments of pJFA31 surrounding the *nod*-box (Fig. 1) were replaced by the Ω-Sp fragment to generate clone pA8. For the construction of NGR234Ω25, a Ω-Km fragment was inserted into the *Bam*HI site of pA18 (to create pA25). Clone pA26 resulted from the insertion of an Ω-Sp fragment into the *Hpa*I site of pA18. Then, pA8, pA25, and pA26 were introduced into NGR234 by conjugation resulting in strains NGR234(pA8) (Tc-resistant [Tc^r], Sp^r), NGR234(pA25) (Tc^r, Km^r), and NGR234(pA26) (Tc^r, Sp^r). The IncP1-IncP2 plasmid R751-pMG2 (Gm^r) was transferred into these strains by conjugation. This plasmid is incompatible with pRK7813 (Tc^r, IncP1), so that selection for Gm^r and Sp^r (for NGR234Δ1 and NGR234Ω26) or Gm^r and Km^r (for NGR234Ω25), coupled with screening for Tc sensitivity, permitted selection of transconjugants which had lost the vector pRK7813. The inability to retain

Table 1. Bacterial strains, plasmids, and bacteriophages used in this study

Designation	Characteristics[a]	Source or reference
Rhizobium species		
NGR234 (Rif^r)	Rif^r derivative of strain NGR234	Trinick 1980; Stanley *et al.* 1988
NGR234Δ1	NGR234 mutant with a 9-kb deletion in HsnII, Rif^r, Sp^r	This study
NGR234Ω25	*nodS* mutant of NGR234, Rif^r, Km^r	This study
NGR234Ω26	*nodU* mutant of NGR234, Rif^r, Sp^r	This study
Escherichia coli		
FM15	F$^-$ Δ(*lac pro*) Δ*thi* Δ*lacZ recA*	Dowling *et al.* 1987
S17.1	RP4 Tc::Tn*7* integrated in the chromosome	Simon 1984
TG1	Δ(*lac pro*) *supE thi hsd5* F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ* ΔM15	Debellé and Sharma 1986
JM101	Δ(*lac pro*) *supE thi*, F′*traD36 proAB lacI*^{q} ZΔM15	Messing 1983
JM103	Δ(*lac pro*) *supE thi strA sbcB15 endA hspR4* F′*traD36 proAB lacI*^{q} ZΔM15	Messing 1983
Plasmids		
pRK7813	Broad host range IncP1 costramid, Tc^r	Jones and Gutterson 1987
pMP220	Broad host range IncP promoter cloning vector with a promoterless *lacZ* gene	Spaink *et al.* 1987
pHP45	Vector containing an Ω Sp interposon, Sp^r, Ap^r	Prentki and Krisch 1984
pHP45ΩKm	Vector containing an Ω Km interposon, Km^r, Ap^r	Fellay *et al.* 1987
pRK2013	Tra$^+$ helper plasmid for mobilization	Figurski and Helinski 1979
R751-pMG2	IncP1-IncP2 fusion plasmid, Sm^r, Gm^r	Jacoby *et al.* 1976
pWA58	pNGR234*a* subclone in cosmid pJB8 containing the HsnII region, Ap^r	Broughton *et al.* 1986
pJFA31	*Sau*3A subclone of pWA58 in pRK7813, 18.5-kb insert, Tc^r	This study
pJFA53	*Sau*3A subclone of pWA58 in pRK7813, 7.5-kb insert, Tc^r	This study
pA8	Derivative of pJFA31 with replacement of two *Bam*HI fragments (9 kb) by the Ω Sp interposon	This study
pA27	Subclone of the 1.8-kb *Eco*RI fragment of pA16 in pMP220, *nod*-box-*lacZ* fusion, Tc^r	This study
pA13, 16, 18, 19, 20, 21, 22, 25, 26; pJS5	Subclones of different sizes of the HsnII region, with or without deletions or Ω insertions used for complementation analysis; vector pRK7813, Tc^r (see Fig. 1)	This study
pRJ453	Subclone of the *nodSU* genes from *Bradyrhizobium japonicum* USDA110 cloned in pPP375, Tc^r	Göttfert *et al.* 1990
Phages		
M13mp10	M13 cloning vector used for sequencing	Messing 1983
M13mp11	M13 cloning vector used for sequencing	Messing 1983

[a] Rif, rifampicin; Sp, spectinomycin; Km, kanamycin; Tc, tetracycline; Ap, ampicillin; Sm, streptomycin; Gm, gentamycin; ^{r}, resistant; and kb, kilobase.

pRK7813 was due to homologous recombination and insertion of the Ω fragment into the target sequences of the NGR234 genome. Possible homogenotes were verified for integration of the Ω fragment into the correct position by Southern blot analysis.

Measurement of β-galactosidase activity. Five-milliliter precultures were grown to stationary phase in TY medium containing Tc (to select for pMP220 and subclones therein). Fifty microliters of this preculture was inoculated into 5 ml of RMM medium (containing 10 mM glucose instead of mannitol) with Tc and, if needed, 10^{-7} M naringenin as inducer. To measure β-galactosidase activity, 0.5 ml of a 2- to 3-day-old culture was used according to the method of Miller (1972).

Sequence analysis. DNA sequences were analyzed by the dideoxy chain termination method (Sanger *et al.* 1977) as described in the Amersham Handbook (Amersham Corporation, Braunschweig, Federal Republic of Germany) or in the Sequenase protocol (U.S. Biochemical Corporation, Cleveland, OH). Sequences were determined using restriction fragments cloned in M13mp10 and M13mp11 and by creating deletions using DNase I (Barnes *et al.* 1983).

Plant tests. Nodulation capacity was assayed in tubes, in Magenta jars, or in growth pouches. In tubes, plants

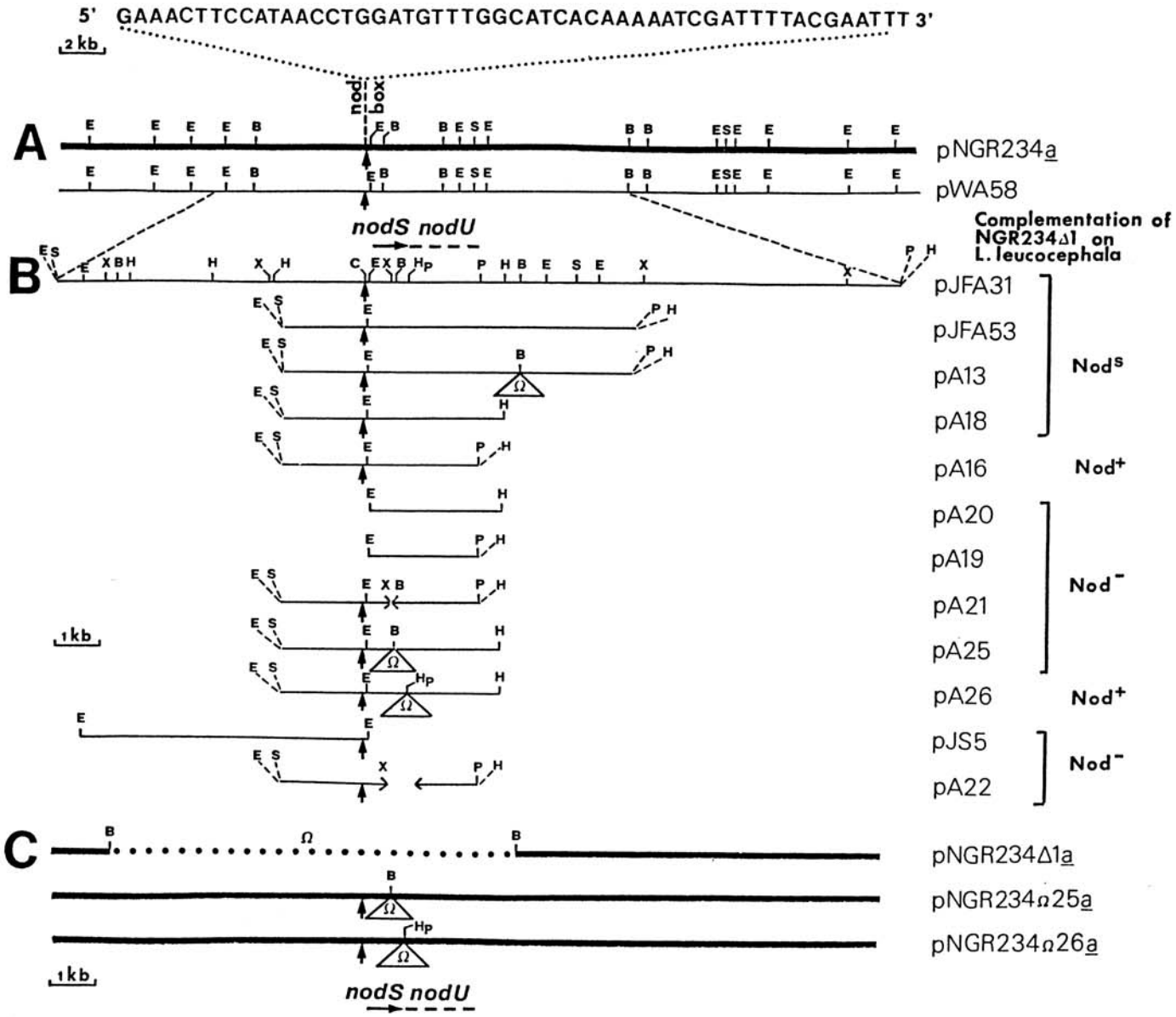


Fig. 1. **A**, Physical map of the pNGR234*a* HsnII locus contained on pWA58 showing restriction sites for *Bam*HI = B, *Eco*RI = E, and *Sma*I = S as well as the location and sequence of the *nod*-box. **B**, Subclones used for complementation of the mutant NGR234Δ1 together with the phenotype of the transconjugants on *Leucaena leucocephala* and the position of the genes *nodS* and *nodU*. On clone pJFA31, restriction sites for *Bam*HI, *Eco*RI, *Hin*dIII = H, *Xho*I = X, and *Sma*I as well as some relevant sites for *Cla*I = C, *Hpa*I = Hp, and *Pst*I = P are indicated. For the other clones, only restricton sites used in their construction are shown. **C**, Mutants NGR234Δ1, NGR234Ω25, and NGR234Ω26 with *Bam*HI and *Hpa*I sites relevant to their construction. Restriction sites derived from the polylinker of vector pRK7813 are represented by dashed lines. Arrows pointed upward indicate the position of the *nod*-box.

were grown on B+D agar (Broughton and John 1979). Magenta jars were constructed from two Magenta growth vessels (Magenta Corporation, Chicago), which were filled with B+D solution in the lower part and with washed vermiculite in the upper part. A wick made from cotton wool allowed the B+D solution to rise into the vermiculite in the upper chamber. Growth pouch (Northrup King Co., Minneapolis, MN) experiments were performed according to the manufacturer's recommendations using B+D nutrient solution. Almost complete sealing of the pouches allowed semisterile growth of the root systems. The following legume hosts were tested for their ability to nodulate: *D. intortum* cv. Greenleaf, *D. uncinatum* (Jacq) DC. cv. Silver-Leaf, *G. max* cv. Peking, *Lablab purpureus* (L.) Sweet, *L. leucocephala* cv. Cunningham, *M. atropurpureum* cv. Siratro, *Moghania congesta* Roxb., *P. tetragonolobus*, and *V. unguiculata* cv. Red Caloona.

Seeds were sterilized by immersion in H_2SO_4 for 10 min, washed twice in H_2O, and rinsed in 5% H_2O_2 for 10 min, followed by six washes in sterile water. Afterward, they were allowed to germinate on B+D agar at 28° C for 2 to 6 days depending on the host plants. Germinated seeds were transferred into the appropriate growth system and inoculated 1 to 3 days after transfer. Bacteria for inoculation were grown to stationary phase in TY medium containing antibiotics, centrifuged, and resuspended in B+D medium to a concentration of about 10^9 cells per milliliter.

For tests in Magenta jars, 1 ml of inoculum per plant was used, while in tubes and growth pouches, 0.25 ml of inoculum per plant was added. Plants in tubes were kept in a growth room with a daytime temperature of 25° C, a nighttime temperature of 20° C, and a light phase of 16 hr (30 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ photosynthetically active radiation [PAR]). Magenta jars were kept in a plant growth chamber with a daytime temperature of 30° C, a nighttime temperature of 20° C, and a light phase of 16 hr (including a 1-hr stepped "sunrise" and a 1-hr stepped "sunset", maximum intensity 350 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ PAR). Growth pouches were kept in a growth room with a daytime temperature of 28° C, a nighttime temperature of 20° C, and a light phase of 16 hr (150 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ PAR). In tubes and growth pouches, nodule numbers were counted every day during the first 20 days after inoculation, then every 2 days. To distinguish between Nod^+ and Nod^- phenotypes in the Magenta jars, plants were harvested 6 wk after inoculation. Acetylene reduction of whole rooting systems was measured using the methods of Williams and Broughton (1979).

Light and electron microscopy. Microscopic techniques were performed as described previously (Wong *et al.* 1983).

RESULTS

HsnII carries determinants for host-specific nodulation of *L. leucocephala* and influences the kinetics of nodulation of *V. unguiculata* and *D. intortum*. Since our previous data had shown that the HsnII locus contains a *nod*-box (see Introduction), we began to analyze the function of the *nod*-box by deleting the two surrounding *Bam*HI fragments (total size, 9 kilobases [kb], Fig. 1). These were replaced by the Ω fragment, an interposon carrying a Sp resistance gene. Putative homogenates were examined by Southern blot analysis (Fig. 2), then tested (wild-type NGR234 as well as the mutant NGR234Δ1) on nine NGR234 host plants. Eight of these plants were Nod^+ with NGR234Δ1 (*D. intortum*, *D. uncinatum*, *G. max*, *L. purpureus*, *M. atropurpureum*, *Moghania congesta*, *P. tetragonolobus*, and *V. unguiculata*) ($Nod^+ = >70\%$ of inoculated plants nodulated). With *L. leucocephala* it was Nod^- ($Nod^- =$ no nodules appearing when plant tests were performed in growth pouches, while in Magenta jar experiments <20% of the inoculated plants had a few nodules [see Tables 2 and 3]). Bumps surrounded by thick and short secondary roots were also observed (Fig. 3B). In comparison, NGR234 formed fully effective nodules (no bumps) on 100% of inoculated *L. leucocephala* plants (Fig. 3A).

Kinetics of nodulation were analyzed using *D. intortum* and *V. unguiculata*, since these plants grow well in transparent nodulation systems. In both cases the mutant strain was delayed in nodulation compared to the wild type; the percentage of nodulated plants was lower; and nodule numbers were reduced (Figs. 4A, 4B, and 5). Since NGR234Δ1 is Nod^- on *L. leucocephala* but Nod^+ Fix^+ on *M. atropurpureum* and *V. unguiculata* (for example Fig. 3D), the deletion in the region surrounding the *nod*-box restricts host range.

Confirmation that plasmid R751-pMG2 (which had been introduced into the mutant strain for homogenotization [see Materials and Methods]) had no effect on nodulation was obtained by the Nod^+ phenotype of NGR234(R751-pMG2) when tested on *L. leucocephala* (data not shown). Then to prove that these changes in nodulation behavior

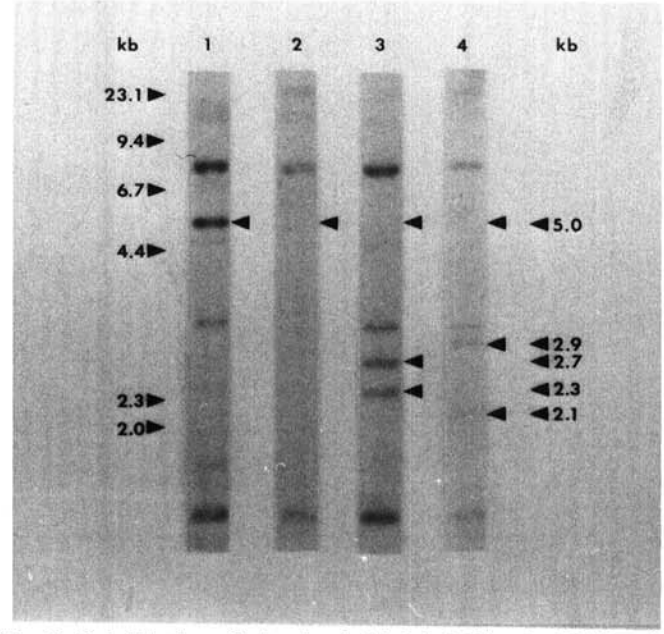


Fig. 2. Hybridization of digoxigenin-labeled DNA of pJFA31 with *Hin*dIII-digested DNA from NGR234, lane 1; NGR234Δ1, lane 2; NGR234Ω25, lane 3; and NGR234Ω26, lane 4. The 5-kilobase (kb) *Hin*dIII fragment containing the *nod*-box (Fig. 1) is missing in all three mutants. The Ω fragment (which is flanked by *Hin*dIII sites) breaks the 5-kb fragment into 2.3- and 2.7-kb fragments in strain NGR234Ω25 and 2.1- and 2.9-kb fragments in strain NGR234Ω26.

were due to the deletion of the 9-kb fragment, we introduced plasmid pJFA31 (Fig. 1) into NGR234Δ1 by conjugation, and the transconjugants were tested on *D. intortum* and *L. leucocephala*. These transconjugants were Nod^+ on *L. leucocephala* and showed no delay in nodulation on *D. intortum* (Fig. 4B).

Table 2. Complementation analysis of the *Rhizobium* deletion mutant NGR234Δ1

Strain[a]	Nodule numbers[b]
NGR234	24 ± 4
NGR234Δ1[c]	5 ± 2
NGR234Δ1(pA18)	55 ± 6
NGR234Δ1(pA16)	16 ± 3
NGR234Δ1(pA26)	15 ± 4
NGR234Δ1(pA25)	5 ± 2
NGR234Δ1(pA21)	6 ± 2
NGR234Δ1(pJS5)	0
NGR234Δ1(pA22)	0

[a] *Leucaena leucocephala* plants were inoculated with NGR234, NGR234Δ1, or NGR234Δ1 carrying various plasmids.
[b] Data are from a typical Magenta jar experiment (six to eight plants per strain tested) and show average nodule numbers per plant 40 days after inoculation (± standard error = SE).
[c] In growth pouches, NGR234Δ1 did not form any nodules.

A 3-kb region of HsnII is essential for nodulation of *L. leucocephala* by strain NGR234. To further localize the host range determinants within the 9-kb fragment, we assayed complementation of the Nod^- phenotype on *L. leucocephala*, first with subclones of different sizes (pJFA31, pJFA53, pA18, pA16, pA19, pA20, and pJS5), then with subclones carrying deletions (pA21 and pA22) or Ω insertions (pA13, pA25, and pA26) at different positions (Fig. 1). Each of these subclones was introduced

Table 3. Ω Insertion analysis of the HsnII locus of *Rhizobium* sp. strain NGR234

	Nodule numbers[b]			
Plant[a]	NGR234	NGR234Δ1	NGR234Ω25	NGR234Ω26
L. leucocephala	21 ± 2	2 ± 1[c]	2 ± 1[c]	27 ± 5
V. unguiculata	138 ± 19	39 ± 3	35 ± 5	101 ± 12

[a] *Leucaena leucocephala* and *Vigna unguiculata* were inoculated with NGR234, NGR234Δ1, and two Ω insertion mutants.
[b] Data are from a typical Magenta jar experiment (8 to 10 plants per strain tested) and show average nodule numbers per plant (± SE) 46 days after inoculation for *L. leucocephala* and 34 days after inoculation for *V. unguiculata*.
[c] In growth pouches, NGR234Δ1 and NGR234Ω25 did not form any nodules on *L. leucocephala*.

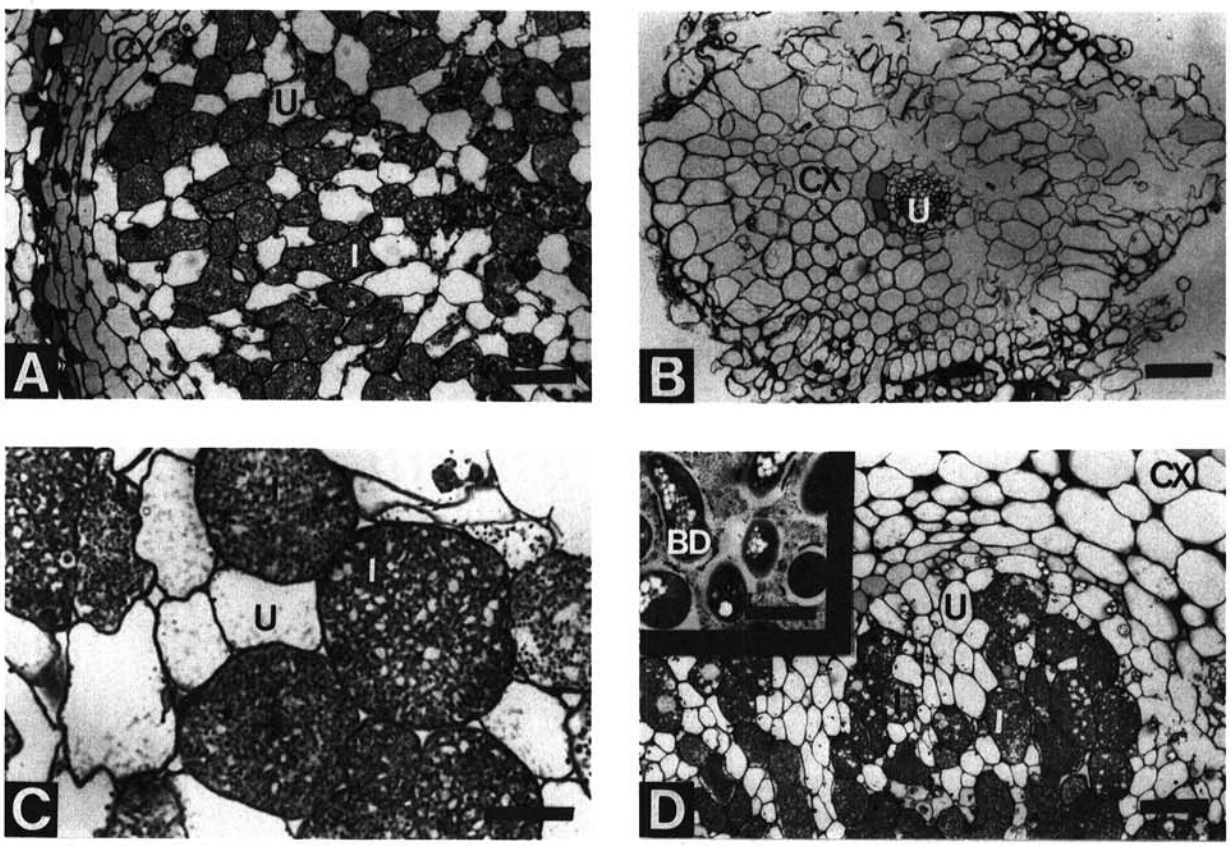


Fig. 3. **A**, Light micrograph of a *Leucaena leucocephala* nodule containing NGR234 showing central infected cells interspersed with uninfected cells and a narrow cortical region; bar = 100 μm. **B**, Light micrograph of a bump formed on *L. leucocephala* inoculated with NGR234Δ1 showing a wide cortex with a small central uninfected region resembling the vascular region of a root; bar = 200 μm. **C**, Light micrograph of a *L. leucocephala* nodule containing NGR234Δ1(pA18) showing large infected cells with dense bacteroid populations and uninfected cells; bar = 30 μm. **D**, Light micrograph of a *Macroptilium atropurpureum* nodule containing NGR234Δ1 showing groups of central infected cells with dense bacteroid populations interspersed with uninfected cells and large cortical cells; bar = 100 μm. The insert shows healthy bacteroids with numerous β-polyhydroxybutyrate granules; bar 1 μm. BD = bacteroid, CX = cortex, I = infected cell, and U = uninfected cell.

into the deletion mutant NGR234Δ1 by conjugation and tested for complementation. Based on nodulation of *L. leucocephala*, the transconjugants could be divided into three classes: Nod^S = supernodulating (nodule numbers more than twice the wild-type level), Nod^+ (wild-type level), and Nod^- (no complementation) (Fig. 1 and Table 2). The Nod^S phenotype of clones pJFA31, pJFA53, pA18, and pA13 can be explained by a copy number effect. The nodules were Fix^+ and showed a high density of bacteroids (Fig. 3C). The plants had a higher shoot dry weight than those inoculated with the NGR234 wild type (data not shown).

These analyses showed that 1) NGR234Δ1(pA18) had a Nod^S phenotype; 2) NGR234Δ1(pA25) was Nod^-; 3) NGR234Δ1(pA26) was Nod^+ (Table 2); and 4) the only difference among clones pA18, pA25, and pA26 was the presence or absence and location of the Ω fragment. This suggested the existence of two genes, one at the insertion site of the Ω fragment in pA25 and the other at the site of insertion in pA26. The differences in nodule number between strains NGR234Δ1(pA25) and NGR234Δ1(pA26) can be explained either by a more important function of the first locus or by a polar effect of the Ω fragment. Transconjugants containing pJS5 were Nod^-, indicating that the whole region to the left of the *nod*-box is not important for nodulation of *L. leucocephala* (Fig. 1).

Clones pA19 and pA20 are derivatives of pA16 and pA18 lacking the *nod*-box plus 1.8 kb of the region to the left of it (Fig. 1). Analysis of their capacity to complement the phenotype of the deletion mutant for nodulation of *L. leucocephala* showed that only background levels of nodules were observed. This, together with data from a *lacZ* fusion in the *nod*-box region (see below), points to a regulatory function of the *nod*-box. Thus we conclude that the 3-kb region extending from the *nod*-box to the *Hin*dIII site of clone pA18 is essential for nodulation of *L. leucocephala*.

HsnII contains two genes, *nodS* and *nodU*, that are involved in nodulation of *L. leucocephala*, *V. unguiculata*, and *D. intortum*. Since the complementation analysis described above suggested the existence of two host-specific *nod* genes, we sought confirmation by generating insertion mutants within each putative locus. This was done by homogenotization of the Ω insertions of clones pA25 (to create strain NGR234Ω25) and pA26 (to create strain NGR234Ω26) (Fig. 1). NGR234Ω25 and NGR234Ω26 were tested on *L. leucocephala*, *V. unguiculata*, and *D. intortum*. On *L. leucocephala*, NGR234 formed typical cigar-shaped nodules, while NGR234Ω26 formed smaller, round nodules (Fix^+). On the other hand, inoculation with NGR234Ω25 produced bumps or very few nodules (Fix^-) that were less developed than those produced by NGR234Ω26 and similar to those formed by NGR234Δ1 (Table 3). These data confirm the complementation analysis. The insertion in NGR234Ω26 had almost no effect on final nodule numbers of *V. unguiculata*, while the insertion in NGR234Ω25 reduced nodule numbers to the level of NGR234Δ1 (Table 3). Similarly, NGR234Ω25 and NGR234Ω26 were both delayed in nodulation of *V. unguiculata* and *D. intortum* (the delay was more pronounced with NGR234Ω25 than with NGR234Ω26 [compare Figs. 4A and 5]).

The effect of Ω insertions in these two loci on nodulation of at least three NGR234 host plants suggests that the target sequences represent two functional genes. Further support for the existence of these genes, *nodS* (represented by the position of the Ω insertion in clone pA25) and *nodU* (represented by the position of the Ω insertion in clone pA26), was obtained by hybridization and sequence comparisons (see below) with the homologous genes identified in *B. japonicum* USDA110 (Göttfert *et al.* 1990).

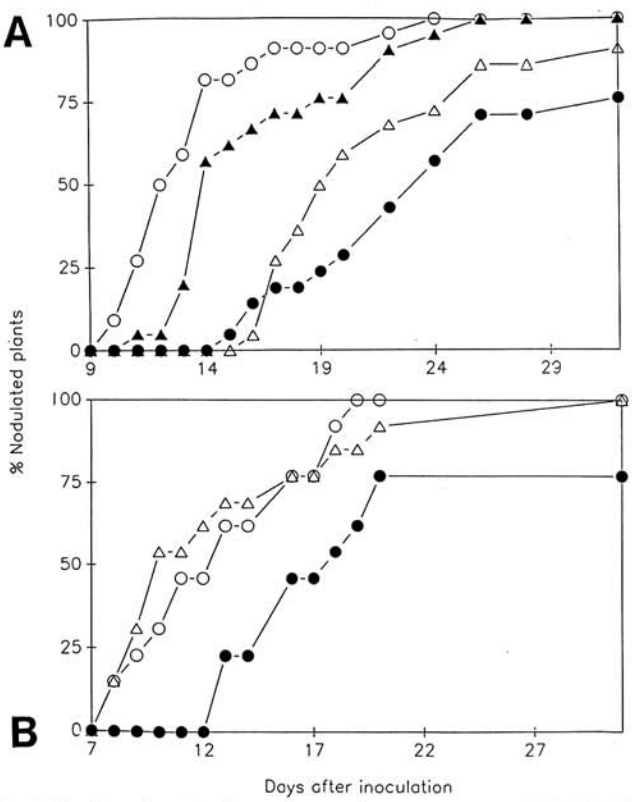


Fig. 4. Kinetics of nodulation on *Desmodium intortum*. **A**, NGR234 (○), NGR234Δ1 (●), NGR234Ω25 (△), and NGR234Ω26 (▲); experiment in tubes, 21 to 22 plants per strain tested. **B**, NGR234 (○), NGR234Δ1 (●), and NGR234Δ1(pJFA31) (△); experiment in tubes, 13 to 14 plants per strain tested.

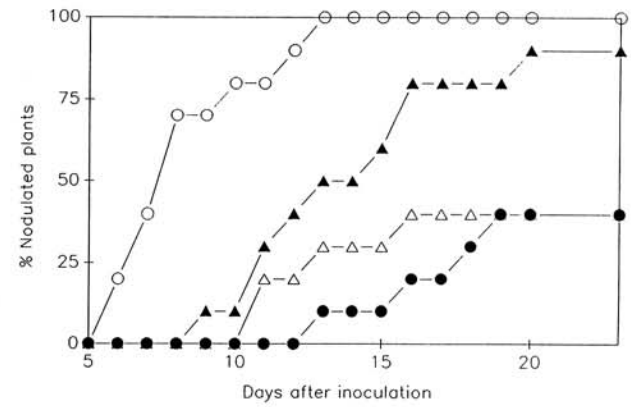
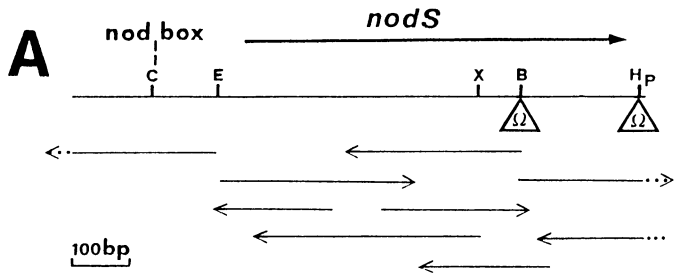


Fig. 5. Kinetics of nodulation of NGR234 (○), NGR234Δ1 (●), NGR234Ω25 (△), and NGR234Ω26 (▲) on *Vigna unguiculata*; growth pouch experiment, 10 plants per strain tested.

Since the *nodSU* genes from NGR234 and *B. japonicum* USDA110 showed strong homology at the nucleotide level, we assayed complementation of the NGR234 *nodS* mutant NGR234Ω25 with clone pRJ453, which contains the *nodSU* genes from *B. japonicum* USDA110 (see Göttfert *et al.* 1990). Complementation for nodulation of the NGR234-Ω25(pRJ453) transconjugants was tested on *L. leucocephala* held in growth pouches, and they were Nod$^-$. Thus, a mutation in the *nodS* gene of NGR234 cannot be complemented by the corresponding gene of *B. japonicum* USDA110.

Sequence analysis of *nodS*. Since genetic analysis showed that the locus directly to the right of the *nod*-box (*nodS*) (Fig. 1) is essential for nodulation of *L. leucocephala*, we determined the sequence of the regulatory and coding regions. A typical *nod*-box was identified surrounding a *Cla*I site (Figs. 1 and 6). There are three possible start codons downstream of the *nod*-box that code for proteins



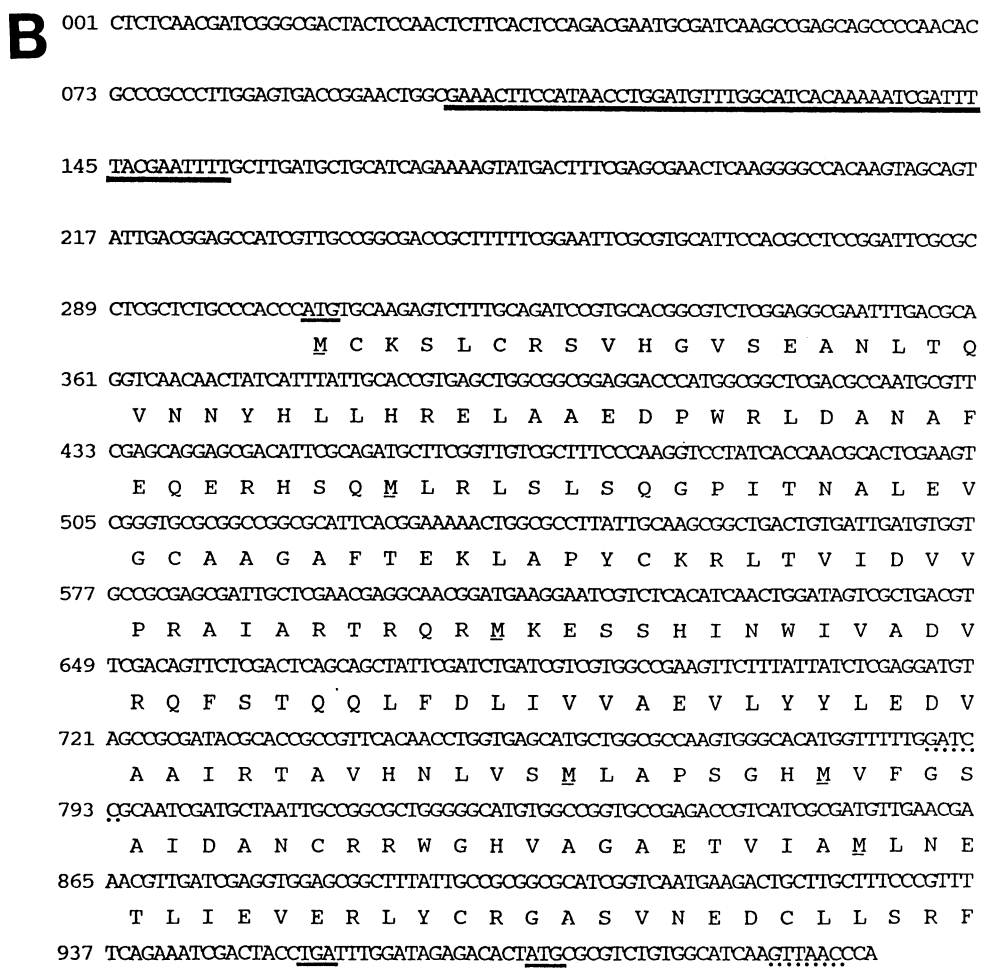


Fig. 6. **A**, Region sequenced showing location of *nodS* as well as restriction sites for *Bam*HI = B, *Cla*I = C, *Eco*RI = E, *Hpa*I = Hp, and *Xho*I = X. Triangles indicate the position of the Ω fragments in the mutants NGR234Ω25 and NGR234Ω26. **B**, Nucleotide sequence of a 1,000-base pair region containing the *nodS* gene (and its deduced amino acid sequence) and the 5′ upstream *nod*-box region as well as the beginning of *nodU*. The *nod*-box sequence, the start and stop codons of *nodS*, and the start of *nodU* are underlined. The position of the Ω insertions in mutants NGR234Ω25 and NGR234Ω26 are marked by points.

composed of 216, 166, and 155 amino acids, respectively. Evidence suggesting that the open reading frame starts with the first ATG comes from comparisons between the data presented in Figure 6 and the sequence of the *nodS* gene of *B. japonicum* USDA110 (Göttfert *et al.* 1990). The NodS protein, which has a molecular weight of 24,169, shows no homology with any protein in the European Molecular Biology Organization and Swiss-Prot data banks. The *nodS* sequence data confirm the data of the genetic analysis on the location of the *nodS* gene (Figs. 1 and 6). To confirm the location of the *nodU* gene at the position of the Ω insertion in clone pA26 (Fig. 1), parts of the *nodU* gene were sequenced, and the sequence compared with the sequence of the *B. japonicum nodU* gene. Sequence homology confirmed the location of *nodU* adjacent to *nodS*. In addition, it was shown that the Ω insertion in strain NGR234Ω26 (Fig. 1) is indeed located in *nodU* (Fig. 6).

Transcriptional *lacZ* fusion of the *nod*-box region. To analyze the regulation of *nodS* and *nodU*, we constructed a transcriptional fusion to the *nod*-box region by cloning the 1.8-kb *Eco*RI fragment from pA16 into the promoter fusion vector pMP220 (clone pA27). pA27 and pMP220 were transferred into NGR234 by conjugation to yield transconjugants NGR234(pA27) and NGR234(pMP220).

Since naringenin has been shown to be one of the inducer substances secreted by plant roots and is able to induce *nod* genes of NGR234 (Bassam *et al.* 1988; Rolfe 1988), we tested the influence of naringenin on expression of the β-galactosidase fusion in NGR234(pA27). Addition of naringenin (10^{-7} M) to a culture of NGR234(pA27) increased β-galactosidase activity ninefold (Table 4). This indicates that the *nod*-box-linked genes in the HsnII region are induced by plant-secreted flavonoids.

DISCUSSION

Initially, the three Hsn loci of NGR234 were identified by their ability to extend the nodulation capacity of heterologous transconjugants to *V. unguiculata* (Broughton *et al.* 1986; Lewin *et al.* 1987a, 1987b). Ironically, extension of this principle to fine structure analysis of the loci was unsuccessful. Apparently the RP4-based Tn*5*-Mob system of Simon (1984) that we used initially produces cointegrated plasmids which are sufficiently stable over a period of weeks to allow nodulation and reisolation of the invasive bacteria. All cosmid or plasmid vectors derived from RP4 (and RSF1010) that we have tested since then have not been stable enough to allow nodulation. We were only able to solve the problem by deleting a large region containing the *nod*-box and seeking an NGR234 host on which the deletion mutant was Nod$^-$. When we then reintroduced various cosmid clones covering the deleted region into the deletion mutant and assayed nodulation capacity under previously Nod$^-$ conditions, selection pressure was for maintenance of the plasmids and allowed efficient nodulation.

Table 4. Induction of the HsnII *nod*-box promoter by naringenin

Strain	β-Galactosidase activity[a] (Miller units)	
	Without naringenin	With naringenin
NGR234(pA27)	146 ± 1	1337 ± 230
NGR234(pMP220)	276 ± 15	285 ± 8

[a] β-Galactosidase activity of a *nod*-box-*lacZ* fusion was measured in cultures with or without naringenin (10^{-7} M). Data show the average of three determinations (± SE).

Like *Medicago* and *Mimosa*, *Leucaena* possesses indeterminate nodules. Furthermore, *L. leucocephala* is a fast-growing, deep-rooted, tropical tree of great importance in tropical agriculture. Thus the *nodS* gene is unique in two ways: it is the first demonstrated to control nodulation of a tree in a host-specific manner, and the first among the three *hsn* loci of pNGR234*a* that clearly regulates an indeterminate symbiosis.

Genes homologous to *nodSU* have also been identified in *B. japonicum* USDA110 (Göttfert *et al.* 1990). A *nodS* mutant of NGR234 could, however, not be complemented by the *nodSU* genes of *B. japonicum*. This lack of complementation may be explained by the fact that *L. leucocephala* is not a host plant of *B. japonicum* USDA110 and underlines the host-specific character of the function of the *nodSU* gene products. Lack of interspecies complementation of host-specific *nod* genes has been reported by several researchers (for example Debellé *et al.* 1986; Djordjevic *et al.* 1985; Horvath *et al.* 1986; Kondorosi *et al.* 1984).

Faucher *et al.* (1988, 1989) proposed a model for the regulation of host specificity in legume-*Rhizobium* associations that can be generalized as follows: first, the plant excretes flavonoids that interact with the DNA-binding *nodD* gene product; second, *nodD* activates transcription of the *nodABC* operon producing a "common" factor capable of deforming root hairs (= Nod factor); third, host specificity genes (for example *nodH* and *nodQ* of *R. meliloti*) tailor this "common" Nod factor to specific plants; and fourth, the now completely host-specific Nod factor is released from the *Rhizobium* where it causes specific root hair deformation and the onset of nodulation. Recently, the structure of this specific Nod factor of *R. meliloti*, called NodRm-1, has been shown to be a sulfated β-1,4-tetrasaccharide of D-glucosamine in which three amino groups are acetylated and the fourth is acetylated with a C_{16} bis-unsaturated fatty acid (Lerouge *et al.* 1990).

Control of broad host range of strains like NGR234 differs from that exercised by specific strains like *R. leguminosarum*, *R. meliloti*, and so forth, in two important ways. Rather than having a *nodD*1 that responds to one or at most a few flavonoids, NGR234 possesses a broad host range *nodD*1; that is a *nodD* whose gene product is able to interact with a large number of flavones, flavanones, isoflavones, and coumarins (Bassam *et al.* 1988; Bender *et al.* 1988; Rolfe 1988). In addition, NGR234 possesses multiple host range determinants (Broughton *et al.* 1986; Lewin *et al.* 1987a, 1987b; Nayudu and Rolfe 1987). If the operon structure of HsnIII is similar to *nodSU* (that is a *hsn* operon preceded by a plant-regulated *nod*-box), then broad host range could be controlled in part by a *nodD*1 gene that interacts with a variety of plant excretion products, and in part by a series of *hsn* genes that tailor the Nod product to specific plants. We are currently testing this hypothesis.

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