Functional Analysis of *nodD* Genes of *Rhizobium tropici* CIAT899

Pieternel van Rhijn,[1] Jos Desair,[1] Katrien Vlassak,[1,2] and Jos Vanderleyden[1]

[1]F. A. Janssens Laboratory of Genetics, KU Leuven, W. de Croylaan 42, B-3001 Heverlee, Belgium; [2]CNPAB/EMBRAPA, KM 47, Itaguai 23851, Rio de Janeiro, Brazil

Received 28 February 1994. Accepted 24 May 1994.

***Phaseolus vulgaris* is nodulated by a wide range of rhizobia. A remarkable feature of *Phaseolus* is the release of a large variety of flavonoid molecules (M. Hungria, C. M. Joseph, and D. Phillips, 1991. Plant Physiol. 97:751-758; M. Hungria, C. M. Joseph, and D. Phillips, 1991. Plant Physiol. 97:759-764). Here we describe how three bean symbionts, *R. tropici* CIAT899, *R. etli* CE3, and *Rhizobium* sp. NGR234, differ in their response with pure flavonoid molecules and with HPLC-fractionated root exudates of *Phaseolus* and *Leucaena*. Whereas *R. etli* CE3 and *Rhizobium* sp. NGR234 respond with all flavonoid molecules earlier identified in bean exudates, *R. tropici* CIAT899 shows only induction with naringenin. In contrast, when fractionated exudates of *Phaseolus* and *Leucaena* are used, no remarkable differences in induction profile are observed. Our previous studies (P. J. S. van Rhijn, B. Feys, C. Verreth, and J. Vanderleyden, 1993. J. Bacteriol. 175:438-447) indicated that *R. tropici* CIAT899 contains five *nodD* alleles. The present study indicates that the $nodD_1$ allele contributes most to the NodD activity. Induction experiments show the same flavonoid specificity for the $nodD_1$ as for the wild-type strain CIAT899. Only the $nodD_1$ gene of CIAT899 is able to fully complement the $nodD_1$ mutation of *Rhizobium* sp. NGR234 for the nodulation on *Leucaena, Macroptilium,* and *Phaseolus*. Mutation of $nodD_1$ of CIAT899 completely abolishes the nodulation of *L. leucocephala* and *M. atropurpureum,* and severely reduces the nodulation of *P. vulgaris*.**

Development of symbiotic nodules on leguminous plants by rhizobia is governed by sequential signal exchange between the symbiotic partners. Flavonoids, produced by the host plant, in conjunction with the bacterial transcriptional activator, the NodD protein, are required to induce the expression of the nodulation (*nod*) genes in rhizobia (Firmin *et al.* 1986; Peters *et al.* 1986; Redmond *et al.* 1986). Subsequently, the *nod* gene products are involved in the synthesis of the *Rhizobium* Nod signal molecules which activate plant genes, leading to nodule development (for a review see Dénarié *et al.* 1992).

Flavonoids have been isolated from many legumes, and detailed studies of several legumes, like alfalfa (Maxwell *et al.* 1989; Hartwig *et al.* 1990a; Phillips *et al.* 1992), vetch (Recourt *et al.* 1991; Zaat *et al.* 1989), and common bean (Hungria *et al.* 1991a, 1991b), show that individual species can release numerous *nod* gene inducers. For *R. meliloti,* which contains three copies of the *nodD* allele, the various flavonoids released from their host alfalfa, activate the NodD proteins differently: $NodD_1$ induces the expression of *nod* genes in the presence of luteolin and methoxychalcone (Peters *et al.* 1988; Maxwell *et al.* 1989); $NodD_2$ responds with a methoxychalcone and the nonflavonoids trigonelline and stachydrine (Hartwig *et al.* 1990b; Phillips *et al.* 1992); $NodD_3$ does not need an inducer molecule for *nod* gene activation, but transcription of $nodD_3$ itself is subjected to a complex regulatory circuit involving SyrM (Swanson *et al.* 1993). In contrast, *Phaseolus vulgaris* releases *nod* gene inducers belonging to four different classes of flavonoids, which are all active, but not to the same extent, with the three NodD proteins present in *R. leguminosarum* bv. *phaseoli* strain 4292 (Hungria *et al.* 1991a, 1991b, 1992).

Rhizobia that nodulate *P. vulgaris* comprise at least three species: *R. l.* bv. *phaseoli*, *R. tropici*, and *R. etli* bv. *phaseoli* (Martinez *et al.* 1987; Martinez-Romero *et al.* 1991; Segovia *et al.* 1993). *R. l.* bv. *phaseoli* and *R. e.* bv. *phaseoli*, formerly classified under one species, are chromosomally quite distant but share some plasmid-borne characteristics. They have multiple copies of *nifH* genes (Martinez *et al.* 1985; Quinto *et al.* 1982) and a narrow nodulation host range, and show hybridization with the *psi* (polysaccharide inhibtion) gene (Borthakur *et al.* 1985). *R. tropici* strains have a single copy of the *nifH* gene, have a broad host range spectrum, and do not hybridize with the *psi* gene (Martinez *et al.* 1985; Martinez *et al.* 1988).

In this report we describe how two bean symbionts, namely *R. tropici* CIAT899 and *R. e.* bv. *phaseoli* CE3, and *Rhizobium* sp. NGR234, a broad host range strain, differ by their response with pure flavonoids and with fractionated root exudates of *P. vulgaris* and *Leucaena leucocephala.* A detailed description of strain NGR234 is given by Stanley and Cervantes (1991). Secondly, we describe how the different *nodD* alleles of *R. tropici* CIAT899 are involved in the induction of the *nod* genes and how these alleles participate in nodulation of host plants.

RESULTS

Divergent flavonoid specificity of *Rhizobium* strains nodulating *P. vulgaris*.

In this study we compared *R. tropici* CIAT899, *R. e.* bv. *phaseoli* CE3 and the broad host range strain *Rhizobium* sp. NGR234 for induction of the *nod* genes using authentic

MPMI Vol. 7, No. 5, 1994, pp. 666-677

flavonoids as signal molecules. These include flavonoids that were earlier identified as inducing compounds present in the root (eriodictyol, naringenin, and genistein) and seed (myricetin, quercetin, and kaempferol) exudates of *P. vulgaris* (Hungria *et al.* 1991a, 1991b) and trigonelline, a nonflavonoid *nod* gene inducer present in seed exudates of alfalfa (Phillips *et al.* 1992). The induction capacity of the different *Rhizobium* strains containing pGUS32Km, a *nodABC-uidA* transcription fusion (Materials and Methods), was measured in the presence of authentic flavonoids at a concentration of 100, 400, and 1,600 nM as described in Materials and Methods. The numbers presented in Figure 1 are the mean values of three replicates, and because the variation from each given value is consistently within 10%, exact values are not given. Apparently, *Rhizobium* sp. NGR234 shows high induction with most of the flavonoids tested, except for myricetin and galangin. No β-glucuronidase activity was observed with the betaine trigonelline. *R. e.* bv. *phaseoli* CE3 shows high induction with all the flavones (apigenin, 7 hydroxyflavone, crysin, and luteolin), and the flavonoids present in the root exudates of *P. vulgaris* (eriodictyol, naringenin, and genestein). Low induction was observed with the seed flavonoids of *P. vulgaris* (myricetin, quercetin, and kaempferol), galangin, hesperitin, and the non-flavonoid trigonelline. When the three *Rhizobium* strains are compared for *nod* gene induction with flavonoids known to be present in *P. vulgaris* exudates, it becomes clear that *R. tropici* CIAT899 is quite different from the two other strains. With CIAT899, high induction is only observed with naringenin and to a much lower extent with eriodictyol and genistein. High β-glucuronidase activity was also observed when flavones (apigenin, 7 hydroxyflavone, crysin, and luteolin) were added. It should also be noticed that the background activity (no inducer added) of NGR234, compared to the two other strains tested, is quite high (see Discussion).

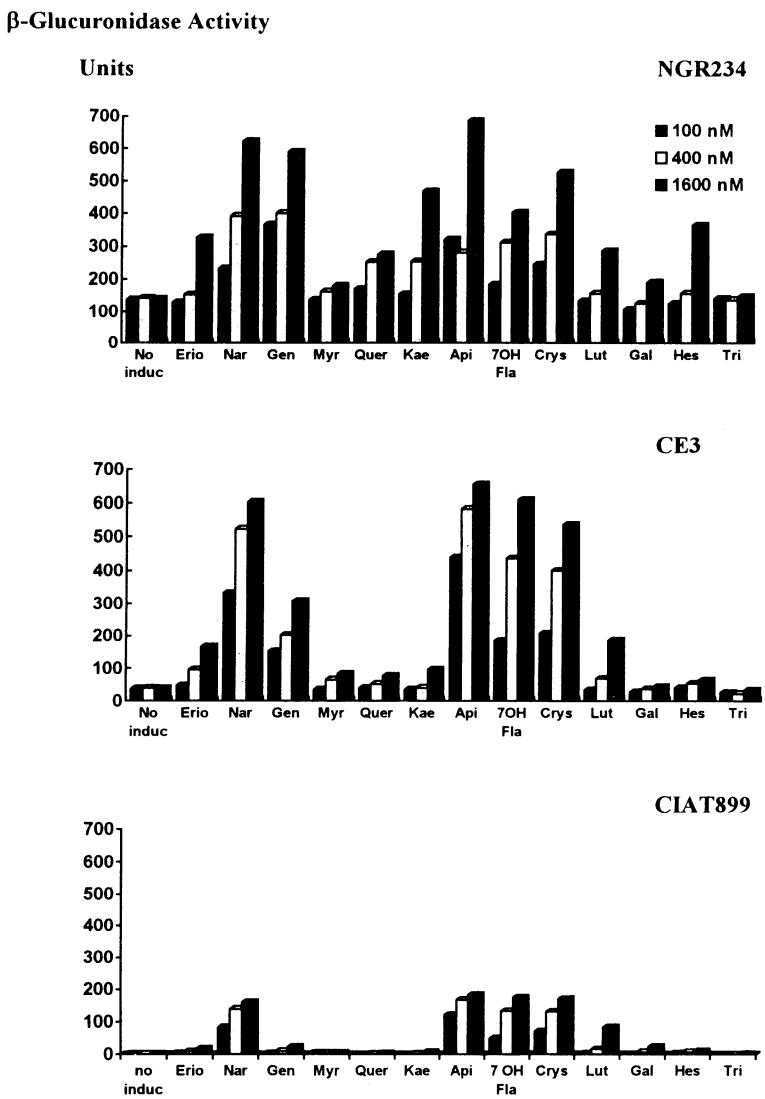

Fig. 1. Induction of the *nodABC-uidA* fusion in the background of strain *Rhizobium* sp. NGR234, *R. etli* bv. *phaseoli* CE3, and *R. tropici* CIAT899 by several flavonoids. Assays were done using flavonoids, eriodictyol (Erio), naringenin (Nar), genistein (Gen), myricetin (Myr), quercetin (Quer), kaempferol (Kaem), apigenin (Api), 7 hydroxyflavone (7OH Fla), crysin (Crys), luteolin (Lut), galangin (Gal), hesperin (Hes), and trigonelline (Tri), at concentrations of 100, 400, and 1,600 nM.

***nod* gene inducers present in root exudates.**

Root exudates of *P. vulgaris* and *L. leucocephala* fractionated on HPLC contain different *nod* gene inducing fractions that are active in different *Rhizobium* strains, as shown in Figures 2 and 3. Apparently, the same fractions show β-glucuronidase activity in both *R. tropici* CIAT899 and *R. e.* bv. *phaseoli* CE3 and this for black bean as well as for *Leucaena* root exudates. In the case of *Rhizobium* sp. NGR234, additional fractions present in *Phaseolus* and *Leucaena* root exudates seem to have induction activity, although the observed activities represent only a slight induction. *nod* gene induction in *R. e.* bv. *phaseoli* is also observed with unfractionated root exudates of *Leucaena.* The following units (as expressed in the caption of Fig. 3) were obtained: NGR234, 400 units;

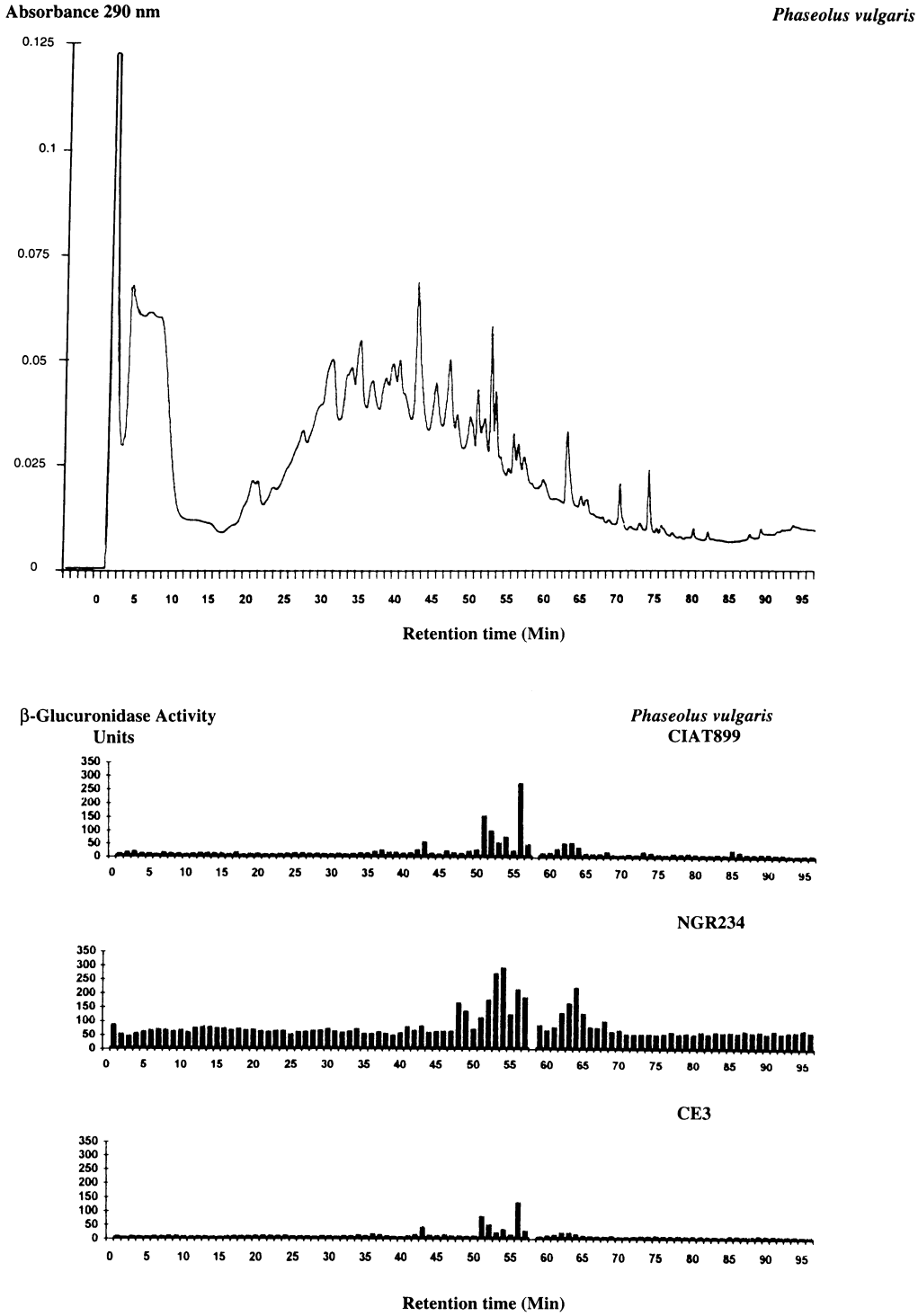

Fig. 2. HPLC characteristics and *nod*-inducing activity of *Phaseolus vulgaris* root exudates fractionated on a reverse-phase C_{18} column. Inducing activity of the root exudate fractions was recorded using *R. tropici* CIAT899, *R. etli* bv. *phaseoli* CE3, and *Rhizobium* sp. NGR234, containing pGUS32Km.

CIAT899, 375 units, CE3, 275 units. Therefore, it appears that, although *R. e.* bv. *phaseoli* is able to respond to *Leucaena* root exudates, induction ability does not match nodulation capacity, since *R. e.* bv. *phaseoli* CE3 is unable to nodulate *Leucaena* plants.

Induction of the *nodABC-uidA* fusion by commercial flavonoids and root exudates in the presence of the different *nodD* gene of *R. tropici* CIAT899.

Earlier experiments with crude exudates of the different host plants, *Leucaena,* Siratro, and *Phaselous,* showed that the $nodD_1$ allele of *R. tropici* CIAT899 mostly contributes to the induction of the *nod* genes (van Rhijn *et al.* 1993). Because these induction experiments were carried out in AD822, a pSym-cured derivative of *R. tropici* CIAT899, it was decided to test the different *nodD* alleles also in a Nod⁺Fix⁺ background. Therefore each *nodD* construct, borne on an IncP-1 vector pVK100, was introduced into *Rhizobium* sp. NGR234 $nodD_1$::Ω harboring pGUS32Km, and was tested for induction in the presence of different flavonoids in a similar way as for the wild-type strain *R. tropici* CIAT899. The values presented in Figure 4 are the mean values of three replicates, and variation from each given value is within 10%. Apparently, the $nodD_1$ gene, harboring on pVK24, and to a lower extent the $nodD_3$, on pVK21, of *R. tropici* CIAT899, show some induction with certain flavonoids. The $nodD_1$

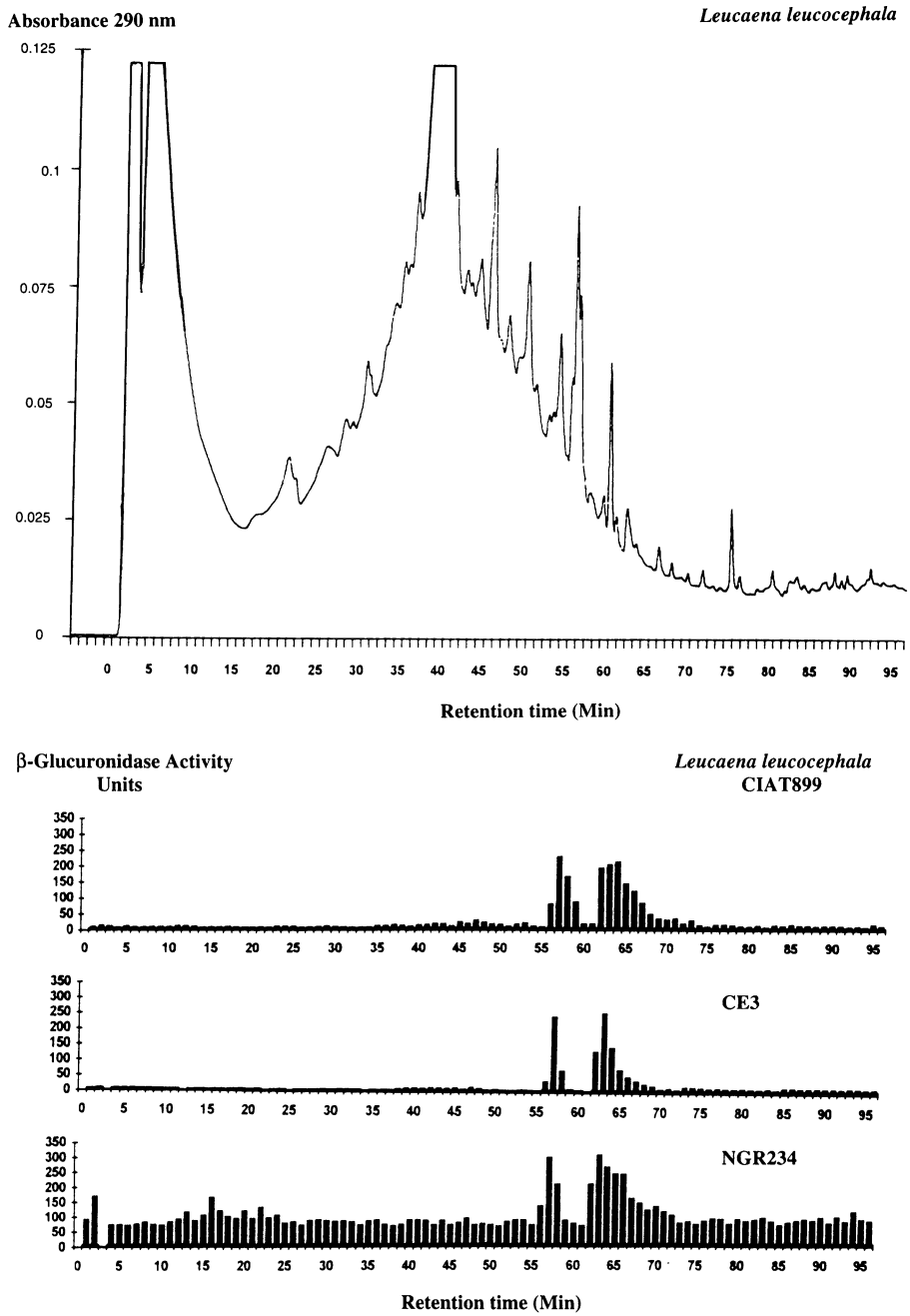

Fig. 3. HPLC characteristics and *nod*-inducing activity of *Leucaena leucocephala* root exudates fractionated on a reverse-phase C_{18} column. Inducing activity of the root exudate fractions was recorded using *R. tropici* CIAT899, *R. etli* bv. *phaseoli* CE3, and *Rhizobium* sp. NGR234, containing pGUS32Km.

gene causes the same induction pattern as the wild-type strain CIAT899, whereas pVK21, containing the $nodD_3$ gene shows a very low induction with naringenin and lacks induction in the presence of luteolin. When NGR234 $nodD_1$::Ω, harboring the plasmids pGUS32 and pVK24, was tested for the fractionated root exudates of *P. vulgaris* and *L. leucocephala* as shown in Figures 5 and 6, the same conclusion can be drawn: The $nodD_1$ gene of *R. tropici* CIAT899 shows largely the same induction pattern as the wild-type strain.

Complementation of NGR234 $nodD_1$::Ω by the different *nodD* genes of *R. tropici* CIAT899 for nodulation of several hosts.

At this point it appears that not all the *nodD* genes of *R. tropici* CIAT899 are involved in the induction of the *nod* genes. This conclusion is based on *ex planta* experiments and needs to be confirmed by nodulation experiments. *Rhizobium* sp. NGR234 is able to nodulate a wide range of plants, including *Phaseolus, Leucaena,* and *Macroptilium* which are also host plants of *R. tropici*. Mutation of the $nodD_1$ gene of *Rhizobium* sp. NGR234 gives a complete Nod⁻ phenotype on the different host plants (Broughton *et al.* 1991). Because NGR234 $nodD_1$::Ω possesses all the host range determinants for nodulating *Phaseolus, Leucaena,* and *Macroptilium,* except a functional *nodD* gene, possible complementation of NGR234 $nodD_1$::Ω by the different *nodD* genes were transferred into *Rhizobium* sp. NGR234 $nodD_1$::Ω, and the transconjugants were used to inoculate *Phaseolus, Leucaeana,* and Siratro plants. The results obtained from eight inoculations are summarized in Table 1. Here it appears that the $nodD_1$ gene

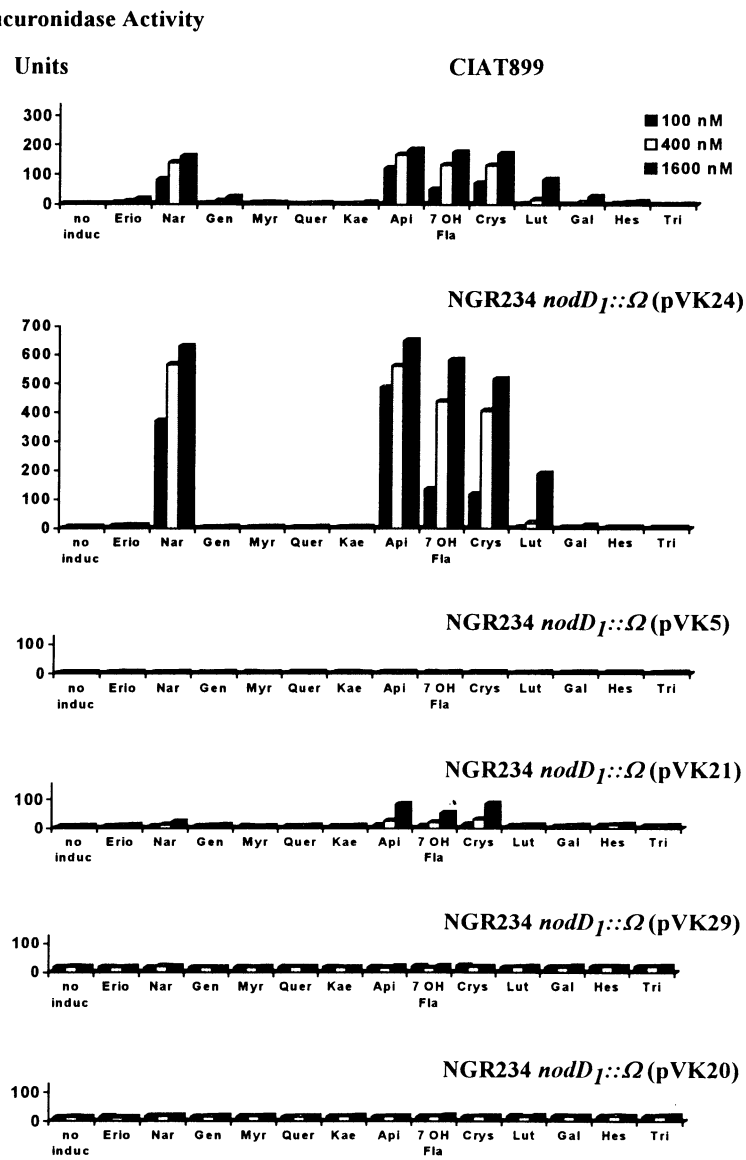

Fig. 4. Induction of the *nodABC-uidA* fusion in the background of strain *R. tropici* CIAT899 and strain *Rhizobium* sp. NGR234 $nodD_1$::Ω containing the different *nodD* alleles of *R. tropici* CIAT899, by several flavonoids. $nodD_1$ is localized on plasmid pVK24, $nodD_2$ of pVK5, $nodD_3$ on pVK21, $nodD_4$ on pVK29, and $nodD_5$ on pVK20. Assays were done using flavonoids, eriodictyol (Erio), naringenin (Nar), genistein (Gen), myricetin (Myr), quercetin (Quer), kaempferol (Kaem), apigenin (Api), 7 hydroxyflavone (7OH Fla), crysin (Crys), luteolin (Lut), galangin (Gal), hesperitin (Hes), and trigonelline (Tri), at a concentration of 100, 400, and 1,600 nM.

of *R. tropici* CIAT899 is able to fully complement the mutation of *Rhizobium* sp. NGR234 on all hosts tested: No delay of nodulation and no decrease of nodule number compared to the wild-type strain NGR234 were observed. On beans, an even higher number of nodules was observed. With the other *nodD* genes, no nodulation or formation of nodule-like structures were observed on *Leucaena* and Siratro plants. Only on bean plants was nodulation observed with pVK20, where 75% of the plants showed nodulation and with pVK21, where only 25% of the bean plants were nodulated, both with a reduced number of nodules per plant.

Mutagenesis of $nodD_1$ of *R. tropici* CIAT899 and its effect on nodulation.

Finally we constructed a $nodD_1$ mutant of CIAT899 to confirm our hypothesis that only the $nodD_1$ gene is involved in

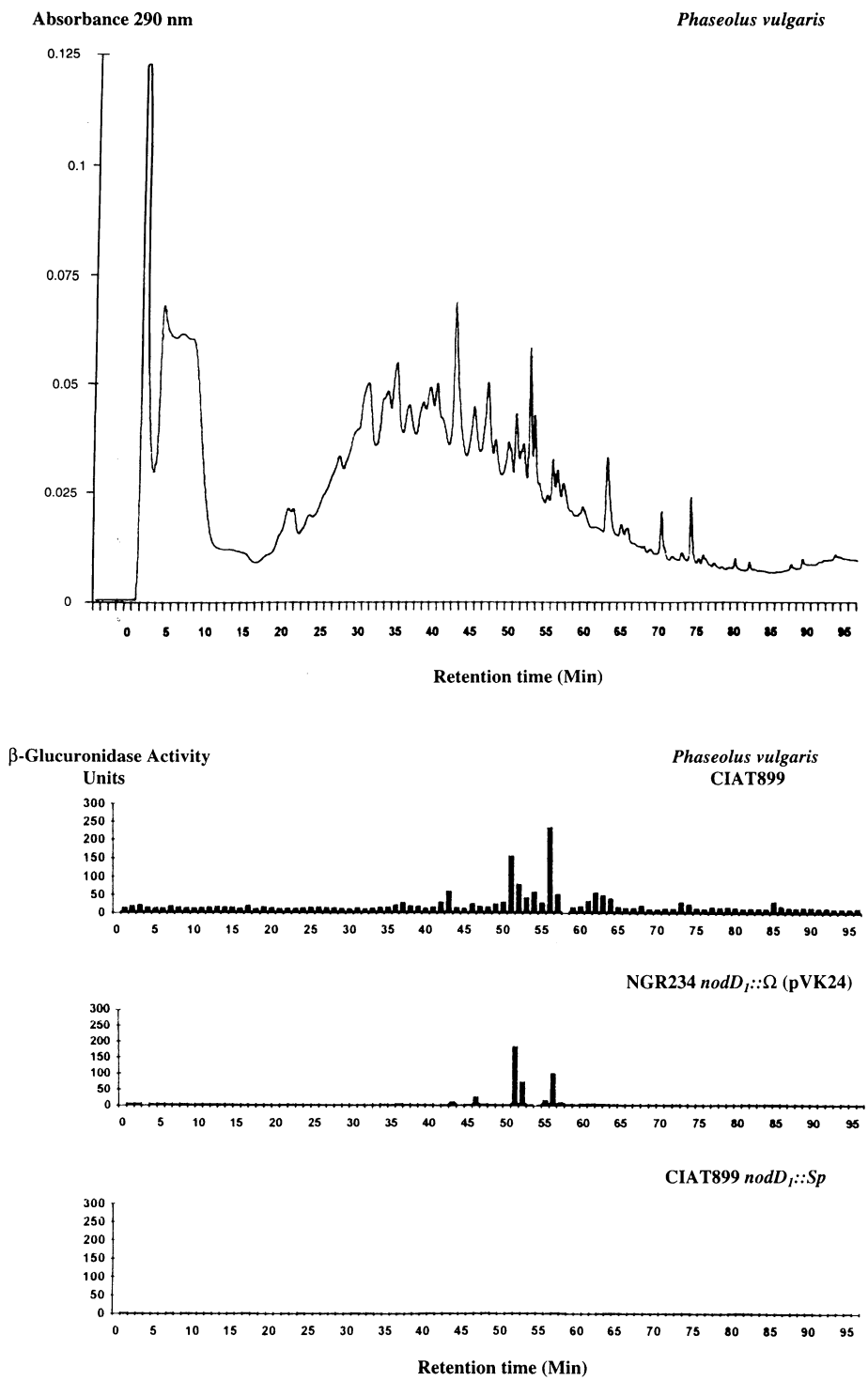

Fig. 5. HPLC characteristics and *nod*-inducing activity of *Phaseolus vulgaris* root exudates fractionated on a reverse-phase C_{18} column. Inducing activity was measured for *R. tropici* CIAT899, *Rhizobium* sp. NGR234 $nodD_1$::Ω (pVK24), and *R. tropici* CIAT899*nodD1::Sp*.

the nodulation of *Leucaena* and Siratro. A spectinomycin cassette was introduced into the $nodD_1$ allele of *R. tropici* CIAT899 as described in Materials and Methods. The induction of the *nodABC-uidA* fusion in this mutant (CIAT899 $nodD_1$::*Sp*) was measured in the presence of fractionated root exudates of *P. vulgaris* (Fig. 5) and unfractionated root exudates of *Leucaena.* From Figure 5 it can be seen that inactivation of the $nodD_1$ allele abolishes completely *nod* gene induction with fractionated bean exudates. With unfractionated *Leucaena* root exudates, CIAT899 gave induction up to 280 units while the $nodD_1$ CIAT899 mutant did not show any activity. Next, the mutated strain was tested for nodulation on *Leucaena, Macroptilium,* and *Phaseolus.* As expected, no nodules were formed with the $nodD_1$ mutant of CIAT899 on *Leucaena* and *Macroptilium* plants. On *P. vulgaris*, all plants inoculated with CIAT899, $nodD_1$::*Sp* were nodulated, although with a reduced nodule number compared to the wild-type strain. This indicates that $nodD_1$ is responsible for the nodulation of *Leucaena* and *Macroptilium* by CIAT899, but that for the nodulation of *P. vulgaris* the other *nodD* alleles can also function.

DISCUSSION

P. vulgaris is nodulated by a wide range of rhizobia, among which are *R. l.* bv. *phaseoli*, *R. e.* bv. *phaseoli*, and *R. tropici* strains. A remarkable feature of *Phaseolus* is the release of a large variety of flavonoid molecules, which are identified as natural *nod* gene inducers for *R. l.* bv. *phaseoli* strain 4292 and *R. etli* CE3 (Hungria *et al.* 1991a, 1991b). When testing

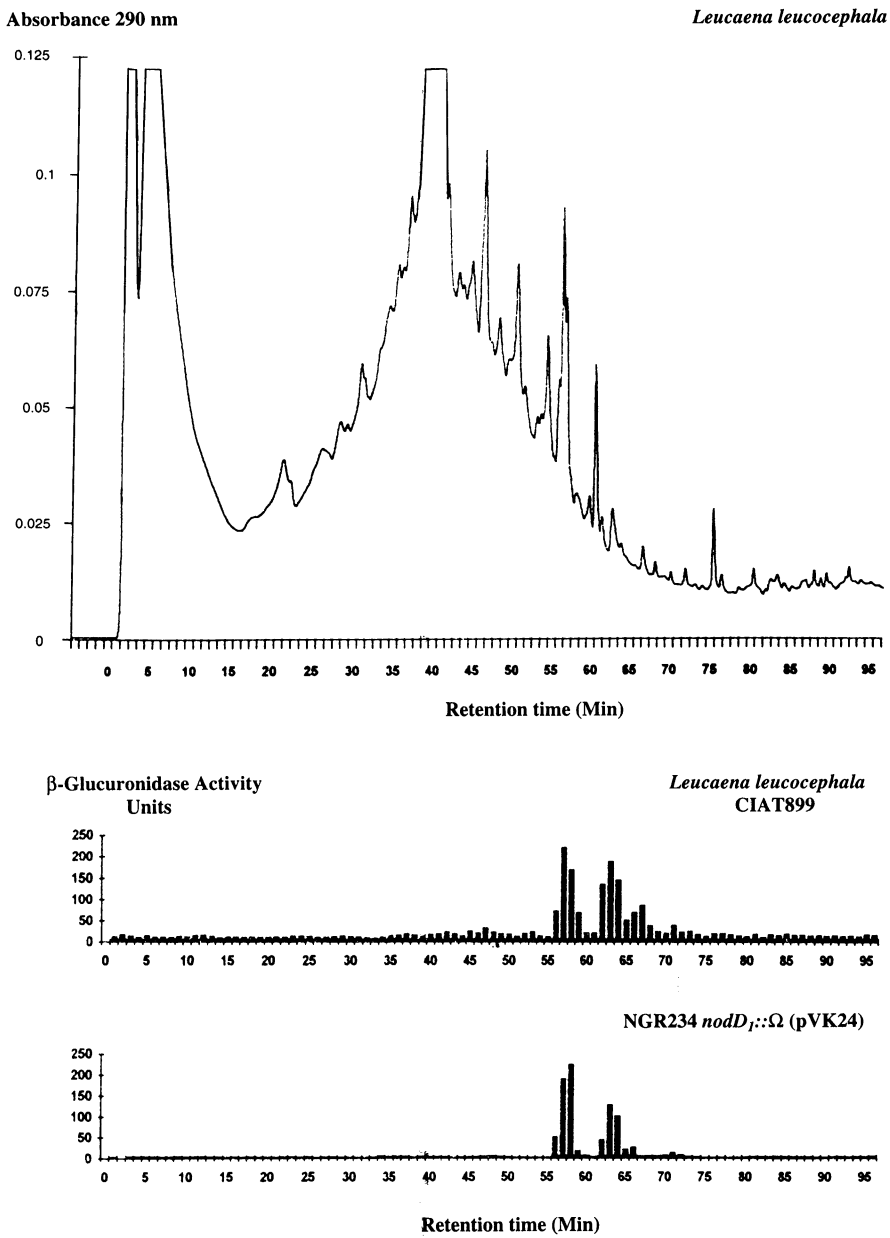

Fig. 6. HPLC characteristics and *nod*-inducing activity of *Leucaena leucocephala* root exudates fractionated on a reverse-phase C_{18} column. Inducing activity was measured for *R. tropici* CIAT899 and *Rhizobium* sp. NGR234 $nodD_1$::Ω (pVK24).

these defined flavonoid compounds on *R. tropici* strains CIAT899 and CFN299 (data not shown), only naringenin showed a high induction of the *nodABC-uidA* operon. The contribution of the flavonols (myricetin, quercetin, and kaempferol), the flavone eriodictyol, and the isoflavone genistein, to the induction of the *nodABC*-operon in the *R. tropici* strains is remarkably low (Fig. 1). On the other hand, when fractionated root exudates of *P. vulgaris* and *L. leucocephala* were tested, the inducing profiles of *R. tropici* CIAT899 and *R. etli* CE3 were quite similar. Even for the broad host range strain NGR234, responding to a very wide spectrum of inducing molecules, only a few additional fractions, with a slight induction, could be observed (Figs. 2 and 3). The apparent contradiction between our results and those obtained by Hungria *et al.* (1991a, 1991b), possibly reflects a difference in bean cultivar used. Alternatively, it could be that for CIAT899, the glycoside derivatives of flavonoids, rather than the pure flavonoids, as used in our assay, are the main inducing compounds. A quantitative difference in inducing activity for the naturally occurring glycoside derivatives and hydrolysates has indeed been observed by Hungria *et al.* (1991b).

Earlier analysis showed the presence of five different *nodD* alleles in *R. tropici* CIAT899 (van Rhijn *et al.* 1993). The present study indicates that the $nodD_1$ allele of *R. tropici* CIAT899 contributes most to the NodD activity. 1) Induction experiments, using defined compounds or fractionated root exudates of *Phaseolus* and *Leucaena,* show the same flavonoid specificity and induction profile for the $nodD_1$ gene as for the wild-type strain CIAT899 (Figs. 4–6). 2) Only the $nodD_1$ gene of CIAT899 is able to fully complement the $nodD_1$ mutation of *Rhizobium* sp. NGR234 for nodulation of *Leucaena, Macroptilium,* and *Phaseolus* (Table 1). 3) The mutation of the $nodD_1$ of CIAT899 completely abolishes the nodulation on *L. leucocephala* and *M. atropurpureum,* and severely reduces the nodulation of *P. vulgaris.* For the nodulation of *P. vulgaris* a supplementary, but minor role of $nodD_3$ or $nodD_5$ of CIAT899 can be expected. This is apparently in contrast to the results of the induction experiments, since little or no activity is observed with the $nodD_3$ or $nodD_5$ alleles. It should be noted that our induction experiments were done with non-glycosylated molecules, whereas Hungria *et al.* (1991a) have shown that bean root exudates mostly glycosylated derivatives of the active *nod*-inducing molecules, the glycosides

Table 1. Complementation of NGR234 $nodD_1$::Ω with the *nodD* genes of *Rhizobium tropici* CIAT899 for nodulation[a] of different hosts

Strain	Plasmid	*Leucaena leucocephala*	Siratro	*Phaseolus vulgaris*
CIAT899	–	+ (5)	+ (9)	+ (85)
NGR234	–	+ (7)	+ (10)	+ (13)
NGR234 $nodD_1$::Ω	–	–	–	–
NGR234 $nodD_1$::Ω	pVK24 ($nodD_1$)	+ (8)	+ (9)	+ (39)
	pVK5 ($nodD_2$)	–	–	–
	pVK21 ($nodD_3$)	–	–	± (9)
	pVK29 ($nodD_4$)	–	–	–
	pVK20 ($nodD_5$)	–	–	± (20)

[a] Average nodule number of eight independent plants are indicated between brackets; +: fully developed and nitrogen-fixing nodules; –: only 25% of the plants were nodulated in the case of NGR234 $nodD_1$::Ω (pVK21) and 75% in case of NGR234 $nodD_1$::Ω (pVK20), both with a reduced number of nodules.

Table. 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Rhizobium strains		
CIAT899	Wild-type isolate from *P. vulgaris*	EMBRAPA, Brazil
CIAT899 $nodD_1$::*Sp*	$nodD_1$ mutant of CIAT899, Sp^r	This study
BR816	Wild-type isolate from *L. leucocephala*	EMBRAPA, Brazil
CFN299	Wild-type isolate from *P. vulgaris*	Martinez *et al.* 1987
NGR234 (Rif^r)	Rif^r derivative of *Rhizobium* sp. NGR234	Lewin *et al.* 1990
NGR234 $nodD_1$::Ω	$nodD_1$ mutant of NGR234, Rif^r, Sp^r	Broughton *et al.* 1991
CE3	St^r derivative of wild-type strain CFN42	Noel *et al.* 1984
Plasmids		
pVK24	pVK100 recombinant cosmid containing the $nodD_1$ of CIAT899, Tc^r	van Rhijn *et al.* 1993
pVK5	pVK100 recombinant cosmid containing the $nodD_2$ of CIAT899, Tc^r	van Rhijn *et al.* 1993
pVK21	pVK100 recombinant cosmid containing the $nodD_3$ of CIAT899, Tc^r	van Rhijn *et al.* 1993
pVK29	pVK100 recombinant cosmid containing the $nodD_4$ of CIAT899, Tc^r	van Rhijn *et al.* 1993
pVK20	pVK100 recombinant cosmid containing the $nodD_5$ of CIAT899, Tc^r	van Rhijn *et al.* 1993
pGUS32	pRG960SD containing the *nodABC* promoter of *Rhizobium* sp. BR816 toward *uidA*	van Rhijn *et al.* 1993
pGUS32Km	Derivative of pGUS32 containing a Km^r gene	This study
pRK2013	ColE1, helper plasmid for tripartite mating, Km^r	Figurski and Helsinki 1979
pCD24	pUC19 derivative containing the $nodD_1$ gene of CIAT899	van Rhijn *et al.* 1993
pSUP401	ColE1, cloning vector, Km^r	Simon *et al.* 1983
pHP45	Vector containing an Ω Sp interposon, Ap^r, Sp^r	Prentki and Kirsch 1984
pJQ200SK	Suicide vector, based on P15A *ori*, and contains *sacB* gene of *Bacillus subtilis*, Gen^r	Quandt and Hynes 1993
pJQ24Sp	pJQ200SK containing a 4-kb fragment with $nodD_1$::*Sp* of *R. tropici* CIAT899	This study

being more active than the aglycones.

The capacity of a flavonoid to act as a coinducer with a NodD protein is strongly affected by its molecular structure. *Rhizobium* strain NGR234 and *R. etli* CE3 show a relatively low specificity of response with signal molecules: from the compounds tested only trigonelline showed no induction. A detailed study by Györgpal *et al.* (1991) showed that NodD protein of MPIK3030, which is closely related to NGR234, only requires the hydroxylation of the C7 on the flavonoid ring for substantial *nod* gene induction. Some structural characteristics for flavonoids to act as coinducer for the $NodD_1$ protein of CIAT899 can be deduced. 1) The flavone-ring skeleton together with a hydroxylation at C7 are sufficient to induce *nod* gene activity at high level. Additional hydroxylation at positions C5 (crysin) or at positions C5 and C′3 (apigenin) has little effect, whereas hydroxylation on C5, C′3, and C′4 (luteolin) remarkably reduces the capacity to act as coinducer (Fig. 3). 2) For ring structures other than the flavone-type, only naringenin appears to be active. Isoflavone (genistein), flavonols (myricetin, quercetin, kaempferol), and the flavanone (eriodictyol), which only differs from naringenin by a hydroxylation on C′3, have no inducing activity.

The broad host range spectrum of *Rhizobium* sp. NGR234 is clearly reflected in the induction studies (Fig. 1; Györgpal *et al.* 1991). Le Strange *et al.* (1990) showed even that the $NodD_1$ protein responds to activation by simple phenolic compounds like vanillin nad isovanillin. This feature of NGR234 can be used to screen for a broad spectrum of molecules that can act as coinducers, present in exudates of a wide variety of plants. Compared to *R. tropici* CIAT899 and *R. etli* CE3, NGR234, shows a high background activity (no inducer added) of the *nodABC-uidA* fusion. This is probably due to the reporter system pGUS32Km used in our system, and not necessarily an intrinsic property of NGR234, since the rather high background activity has not been observed consistently throughout different experimental systems. 1) A *nodSU*-fusion from NGR234, tested in NGR234 and *R. fredii,* did not show a difference in background activity (Krishnan *et al.* 1993). 2) When induction of *nodC-lacZ* of *R. meliloti* was tested in NGR234 or *R. meliloti*, hardly any background activity was observed (Horvath *et al.* 1987). 3) The *nodC-lacZ* of *R. meliloti*, in a *R. meliloti* background, gave almost the same basal activity when the *nodD* gene of *R. meliloti* was replaced by the $nodD_1$ of NGR234 (Györgpal *et al.* 1991).

Several nodulation studies indicate that *P. vulgaris* is a promiscuous host for microsymbionts (Martinez *et al.* 1987). Studies on structure analysis of coinducers released from bean and Nod metabolites synthesized by the various rhizobia nodulating bean, allow us to understand some aspects of this promiscuity at molecular level. 1) *Phaseolus* releases a large variety of flavonoid structures which may act as natural *nod* gene inducers on the different host (Hungria *et al.* 1991a, 1991b; Fig. 2). 2) *P. vulgaris* is nodulated by a wide range of *Rhizobium* species, among which *R. etli*, *R. l.* bv. *phaseoli*, *R. tropici*, *Rhizobium* sp. NGR234 (this study), and *Azorhizobium caulinodans* (data not shown). The Nod metabolites from most of the reference strain of these species have been identified: NGR234 (Price *et al.* 1992), *A. caulinodans* (Mergaert *et al.* 1993), *R. tropici* CFN299 (Puopot *et al.* 1993). When comparing these structures, we can deduce that they are all very different and that they only have a vaccenic acid and a methyl group at the nonreducing end of the polysaccharide in common. But taking into account some preliminary data from some other bean symbionts, *R. tropici* CIAT899 (Spaink *et al.* 1993) and *R. etli* CE3 (E. Martinez, personal communication), we can conclude that the Nod metabolites able to trigger nodule organogenesis on bean have only a methyl group in common.

MATERIALS AND METHODS

Bacterial strans and plasmids.

The bacterial strains and plasmids used in this study are listed in Table 2. *E. coli* strains were maintained on LB agar (Miller 1972) and grown in LB broth supplemented with the appropriate antibiotics. The concentrations of antibiotics used for *E. coli* were (per milliliter) 10 μg of tetracycline, 50 μg of spectinomycin, and 100 μg of ampicillin. *Rhizobium* strains were maintained on yeast extract-mannitol (YM) medium (Hooykaas *et al.* 1977) or on tryptone-yeast (TY) medium (Beringer 1974). The concentrations of antibiotics used for *Rhizobium* species were (per milliliter) 10 μg of tetracycline, 150 μg of spectinomycin, 50 μg of kanamycin, and 30 μg of nalidixic acid.

Construction of reporter gene fusion.

A *nodABC-uidA* transcription fusion was constructed in pRG960SD (Van den Eede *et al.* 1992). Starting with pGUS32 (van Rhijn *et al.* 1993), the *Bam*HI site of the cloning site, linked to the *uidA* reporter gene, was eliminated by a small deletion, starting at the *Sma*I site located at then end of the *nodABC* operon towards the *Sma*I site of the polycloning site. Subsequently, a Km resistance cassette was introduced in the remaining *Bam*HI site, in such a way that the transcription of the *npt*II gene is opposite to transcription of the *uidA* gene, resulting in a very low background β-glucuronidase activity in *Rhizobium* sp. BR816.

Construction of CIAT899 $nodD_1$ mutant.

The 4-kb *Pst*I fragment of pCD24, containing the $nodD_1$ gene of CIAT899, was cloned in pSUP401 (Simon *et al.* 1983). The 200-bp *Eco*RI fragment within $nodD_1$ was replaced by the Ω-Sp fragment of pHP45 (Prentki and Kirsch 1984). Subsequently, the *Xho*I-*Pst*I fragment, containing the mutated $nodD_1$ gene, was subcloned into the suicide vector pJQ200SK (Quandt and Hynes 1993), digested with *Sal*I and *Pst*I, resulting in pSQ24Sp. pJQ200SK contains the *SacR/B* gene, encoding levansucrase, which is lethal to Gram-negative bacteria when plated on sucrose containing media. pJQ24Sp was introduced into CIAT899 by a triparental mating using pRK2013 as helper. Homologous recombination resulting from a double cross-over could be selected by resistance to spectinomycin and to 5% sucrose. The mutations were subsequently checked by Southern hybridization with both Sp cassette and the $nodD_1$ gene of CIAT899 as probe DNA.

Preparation and fractionation of root exudates.

Seeds of *P. vulgaris* 'Negro-Argel' and *L. leucocephala* were sterilized as described previously by van Rhijn *et al.* (1993). The seeds were germinated for 3 days on water agar in a humid atmosphere at 30° C. Root exudates were collected by growing germinated sprouts in 5 ml of Jensen medium

(Vincent 1970) for 3 wk in the case of *P. vulgaris* and 6 wk for *L. leucocephala* plants. The collected root exudates of 50 plants were freeze dried and extracted with 1 ml of methanol 100%. The compounds were separated for *nod*-inducing assays by diluting the exudates first with a same volume of water. A 300-µl aliquot was injected in an HPLC system (Waters 600E) fitted with a reverse-phase C-18 column (Phenomenex Bondclone 10C18 300x3,9) and eluted with 0.5 ml/min from 0 to 4 min with 89:10:1 (v/v) water/methanol/acetic acid, and from 4 to 84 min with a linear gradient from 89:10:1 (v/v) water/methanol/acetic acid to 99:1 (v/v) methanol/acetic acid, followed by 8 min of isocratic chromatography with 99:1 (v/v) methanol/acetic acid. Compounds were monitored with a UV detector that measured absorbance at 290 nm every second. Five minutes after the injection, eluent fractions were collected at 1-min intervals, vacuum dried, and dissolved in 100 µl of water. A 20-µl sample was used for *nod* gene induction assay.

Assay for *nod* gene induction.

Different *Rhizobium* strains containing the transcription fusion, pGUS32Km, were tested for *nod* gene induction using methods described earlier (van Rhijn *et al.* 1993). The standard flavonoids were present at a final concentration at 100, 400, and 1,600 nM. The sources of flavonoid standards were: Aldrich (7-hydroxyflavone, hespertin, luteolin, galangin), Sigma (naringenin, quercetin, kaempferol, crysin, trigonelline), ICN (genestein, apigenin), D. A. Phillips, University of California, Davis (eriodictyol, myricetin). For the fractionated root exudates, the assay was done in a similar way using 20-µl sample in a total induction volume at 75 µl.

Plant tests.

Nodulation capacity of *P. vulgaris* 'Negro-Argel,' *L. leucocephala,* and *M. atropurpureum* (Siratro) was assayed in glass tubes (3 × 20 cm) filled with a 1:1 mixture of vermiculite and perlite saturated with Jensen medium (Vincent 1970). Surface-sterilized and germinated seeds (van Rhijn *et al.* 1993) were inoculated with 250 µl of an overnight bacterial culture, and maintained in a growth cabinet with 12-hr day-length at 28° C/24° C daylight temperature. Nodulation data were recorded 4 wk (*Phaseolus*) or 5 wk (*Leucaena* and *Macroptilium*) after inoculation and bacteria were reisolated from the nodules according to Vincent (1970).

ACKNOWLEDGMENTS

We gratefully acknowledge W. Broughton and A. Franco for providing strains, and D. Phillips and M. Holsters for the flavonoids. This work was supported in part by the STDII program (TS2-0100-C[GDF]) of the Commission of the European Communities. PvR acknowledges the receipt of a predoctoral fellowship from the Belgian Instituut ter Aanmoediging van het Wetenschappelijk Onderzoek in de Nijverheid en in de Landbouw.

LITERATURE CITED

Beringer, J. E. 1974. R-factor transfer in *Rhizobium leguminosarum.* J. Gen. Microbiol. 120:421-429.

Borthakur, D., Downie, J. A., Johnston, A. W. B., and Lamb, J. W. 1985. *psi,* a plasmid-linked *Rhizobium phaseoli* gene that inhibits exopolysaccharide production and which is required for symbiotic nitrogen fixation. Mol. Gen. Genet. 200:278-282.

Broughton, W. J., Krause, A., Lewin, A., Perret, X., and Price, N. P. J. 1991. Signal exchange mediates host-specific nodulation of tropical legumes by the broad host-range *Rhizobium* species NGR234. Pages 162-167 in: Advances in Molecular Genetics of Plant-Microbe Interactions. Vol. 1. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Dénarié, J., Debellé, F., and Rosenberg, C. 1992. Signaling and host range variation in nodulation. Annu. Rev. Microbiol. 46:497-531.

Figurski, D. H., and Helsinki, D. R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans.* Proc. Natl. Acad. Sci. USA 76:1648-1652.

Firmin, J. L., Wilson, K. E., Rossen, L., and Johnston, A. W. B. 1986. Flavonoid activation of nodulation genes in *Rhizobium* reversed by other compounds present in plants. Nature 342:90-92.

Györgpal, Z., Kondorosi, E., and Kondorosi, A. 1991. Diverse signal sensitivity of NodD protein homologs from narrow and broad host range rhizobia. Mol. Plant-Microbe Interact. 4:356-364.

Hartwig, U. A., Maxwell, C. A., Joseph, C. M., and Phillips, D. A. 1990a. Chryseoriol and luteolin released from alfalfa seeds induce *nod* genes in *Rhizobium meliloti.* Plant Physiol. 92:116-122.

Hartwig, U. A., Maxwell, C. A., Joseph, C. M., and Phillips, D. A. 1990b. Effects of alfalfa *nod* gene-inducing flavonoids on *nodABC* transcription in *Rhizobium meliloti* strains containing different *nodD* genes. J. Bacteriol. 172:2769-2773.

Hooykaas, P. J. J., Klapwijk, P. M., Nuti, M. P., Schilperoort, R. A., and Rörsch, A. 1977. Transfer of the *Agrobacterium* Ti plasmid to avirulent agrobacteria and to rhizobia ex planta. J. Gen. Microbiol. 98:477-484.

Horvath, B., Bachem, C. W., Schell, J., and Kondorosi, A. 1987. Host-specific regulation of nodulation genes in *Rhizobium* is mediated by a plant signal interacting with the *nodD* gene product. EMBO J. 6:841-848.

Hungria, M., Johnston, A. W. B., and Phillips, D. 1992. Effect of flavonoids released naturally from bean (*Phaseolus vulgaris*) on *nodD* regulated gene transcription in *Rhizobium leguminosarum* bv. *phaseoli.* Mol. Plant-Microbe Interact. 5:199-203.

Hungria, M., Joseph, C. M., and Phillips, D. 1991a. Anthocyanidins and flavonols, major *nod* gene inducers from seeds of black-seeded common beans (*Phaseolus vulgaris* L.) Plant Physiol. 97:751-758.

Hungria, M., Joseph, C. M., and Phillips, D. 1991b. *Rhizobium nod* gene inducers exuded naturally from roots of common beans (*Phaseolus vulgaris* L.). Plant Physiol. 97:759-764.

Krishnan, H. B., Lewin, A., Feally, R., Broughton, W. J., and Pueppke, S. G. 1992. Differential expression of *nodS* accounts for the varied abilities of *Rhizobium fredii* USDA257 and *Rhizobium* sp. strain NGR234 to nodulate *Leucaena* spp. Mol. Microbiol. 6:3321-3330.

Le Strange, K. K., Bender, G. L., Djordjevic, M. A., Rolfe, B. G., and Redmond, J. W. 1990. The *Rhizobium* strain NGR234 *nodD1* gene product responds to activation by simple phenolic compounds vanillin and isovanillin present in wheat seedling extracts. Mol. Plant-Microbe Interact. 3:214-220.

Lewin, A., Cervantes, E., Wong, C. H., and Broughton, W. 1990. *nodSU,* two new *nod* genes of the broad host range *Rhizobium* strain NGR234 encode host-specific nodulation on the tropical tree *Leucaena leucocephala.* Mol. Plant-Microbe Interact. 3:317-326.

Martinez, E., Pardo, M. A., Palacios, R., and Cevallos, M. A. 1985. Reiteration of nitrogen fixation gene sequences and specificity of *Rhizobium* in nodulation and nitrogen fixation in *Phaseolus vulgaris.* J. Gen. Microbiol. 131:1779-1786.

Martinez, E., Flores, M., Brom, S., Romero, D., Davila, G., and Palacios, R. 1988. *Rhizobium phaseoli:* A molecular genetics view. Plant Soil. 108:179-184.

Martinez, E., Palacios, R., and Sanchez, F. 1987. Nitrogen-fixing nodules induced by *Agrobacterium tumefaciens* harboring *Rhizobium phaseoli* plasmids. J. Bacteriol. 169:2828-2834.

Martinez, E., Puopot, R., Promé, J. C., Pardo, M. A., Segovia, L., Truchet, G., and Dénarié, J. 1993. Chemical signaling of *Rhizobium* nodulating bean. Pages 171-176 in: New Horizons in Nitrogen Fixation. R. Palacios, J. Mora, and W. E. Newton, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Marrtinez-Romero, E., Segovia, L., Mercante, F. M., Franco, A. A., Graham, P., and Pardo, M. A. 1991. *Rhizobium tropici,* a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int. J. Syst. Bacteriol. 41:417-426.

Maxwell, C. A., Hartwig, U. A., Joseph, C. M., and Phillips, D. A. 1989.

A chalcone and two related flavonoids released from alfalfa roots induce *nod* genes of *Rhizobium meliloti*. Plant Physiol. 91:842-847.

Mergaeret, P., Van Montagu, M., Promé, J.-C., and Holsters, M. 1993. Three unusual modifications, a D-arabinosyl, a *N*-methyl, and a carbamoyl group, are present on the Nod factors of *Azorhizobium caulinodans* strain ORS571. Proc. Natl. Sci. USA 90:1551-1555.

Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Noel, K. D., Sánchez, A., Fernández, L., Leemans, J., and Cevallos, M. A. 1984. *Rhizobium phaseoli* symbiotic mutants with Tn*5* insertions. J. Bacteriol 158:148-155.

Peters, N. K., and Long, S. R. 1988. Alfalfa root exudates and compounds which promote or inhibit induction of *Rhizobium meliloti* nodulation genes. Plant Physiol. 88:396-400.

Peters, N. K., Frost, J. W., and Long, S. R. 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* *nodulation genes. Science 233:977-980.

Phillips, D. A., Joseph, C. M., and Maxwell, C. A. 1992. Trigonelline and stachydrine from alfalfa seed activate NodD2 protein in *Rhizobium meliloti*. Plant Physiol. 99:1526-1531.

Poupot, R., Martinez-Romero, E., and Prome, J.-C. 1993. Nodulation factors from *Rhizobium tropici* are sulfated or nonsulfated chitopentasaccharides containing an *N*-methyl-*N*-acylglusaminyl terminus. Biochemistry 32:10430-10435.

Prentki, P., and Kirsch, H. M. 1984. *In vitro* insertional mutagenesis with a selectable DNA fragment. Gene 29:303-313.

Price, N. P., Relic, B., Talmont, F., Lewin, A., Promé, D., Pueppke, S. G., Maillet, F., Dénarié, J., Promé, J.-C., and Broughton W. J. 1992. Broad-host-range *Rhizobium* species strain NGR234 secretes a family of carbamoylated, and fucosylated, nodulation signals that are *O*-acetylated or sulphated. Mol. Microbiol. 6:3575-3584.

Quandt, J., and Hynes, M. F. 1993. Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. Gene 127:15-21.

Quinto, C., de la Vega, H., Flores, M., Fernández, L., Ballado, T., Soberón, G., and Palacios, R. 1982. Reiteration of nitrogen fixation gene sequences in *Rhizobium phaseoli*. Nature (London) 299:724-726.

Recourt, K., Schripsema, J., Kijne, J. W., van Brussel, A. A. N., and Lugtenberg, B. J. J. 1991. Inoculation of *Vicia sativa* subsp. *nigra* roots with *Rhizobium leguminosarum* biovar *viciae* results in release of *nod* gene activating flavonones and chalcones. Plant Mol. Biol. 16:841-852.

Redmond, J. W., Batley, M., Djordjevic, M. A., Innes, R. W., Kuempel, P. L., and Rolfe, B. G. 1986. Flavones induce expression of nodulation genes in *Rhizobium*. Nature (London) 323:632-635.

Segovia, L., Young, P. W., and Martinez-Romero, E. 1993. Reclassification of American *Rhizobium leguminosarum* biovar *phaseoli* type I strains as *Rhizobium etli* sp. *nov*. Int. J. Syst. Bacteriol. 43:374-377.

Simon, R., Priefer, U., and Pühler, A. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteia. BioTechnology 1:784-791.

Stanley, J., and Cervantes, E. 1991. Biology and genetics of the broad host range *Rhizobium* sp. NGR234. J. Appl. Bacteriol. 70:9-19.

Swanson, J. A., Mulligan, J. T., and Long, S. R. 1993. Regulation of *syrM* and *NodD3* in *Rhizobium meliloti*. Genetics 134:435-444.

Van den Eede, G., Deblaere, R., Goethals, K., Van Montagu, M., 1992. Broad host range and promoter selection vectors for bacteria that interact with plants. Mol. Plant-Microbe Interact. 5:228-234.

van Rhijn, P. J. S., Feys, B., Verreth, C., and Vanderleyden, J. 1993. Multiple copies of *nodD* in *Rhizobium tropici* CIAT899 and BR816. J. Bacteriol. 175:438-447.

Vincent, J. M. 1970. A Manual for the Practical Study of Root-Nodule Bacteria. International Biological Programme Handbook. Blackwell Scientific Publications Ltd., Oxford. pp. 73-97.

Zaat, A. J., Schripsema, J., Wijfelman, C. A., van Brussel, A. A. N., and Lugtenberg, B. J. J. 1989. Analysis of the major inducers of the *Rhizobium nodA* promoter from *Vicia sativa* root exudate and their activity with different *nodD* genes. Plant Mol. Biol. 13:175-188.