MPMI Vol. 8, No. 5, 1995, pp. 693–699.

Rhizobium sp. NGR234 Contains a Functional Copy of the Soybean Cultivar Specificity Locus, *nolXWBTUV*

Pedro A. Balatti, László G. Kovács, Hari B. Krishnan, and Steven G. Pueppke

Department of Plant Pathology, University of Missouri, Columbia 65211 U.S.A.
Received 9 March 1995. Accepted 30 May 1995.

***Rhizobium* sp. NGR234, a broad host range nitrogen-fixing symbiont of many legumes, is closely allied to *Rhizobium fredii*, but there are conflicting reports of its ability to nodulate soybean, the normal host of *R. fredii*. We evaluated the symbiotic interaction of NGR234 with 89 soybean cultivars and detected only disorganized structures, which had central vascular connections and lacked bacteroids. NGR234 contains *nolXWBTUV*, a locus responsible for cultivar-specific nodulation of soybean by *R. fredii* USDA257. We cloned the NGR234 locus as an 8.0-kb *Eco*RI fragment and found that its restriction map differs from that of the corresponding fragment from USDA257 at only two sites. RNA dot blot analysis indicated that the NGR234 locus is expressed in response to flavonoid signals. This observation was confirmed by probing Western blots with polyclonal antibodies directed against NolX of USDA257 and by monitoring levels of expression of a fusion between the *nolX* allele of NGR234 and *lacZ*. Histochemical observations with the gene fusion documented that *nolX* is expressed by rhizobia in the rhizosphere of soybean roots and in infection threads and mature nodules of cowpea.**

Additional keywords: cultivar specificity, *Glycine max,* nodulation genes, symbiosis.

The soybean, *Glycine max* (L.) Merr., can form nitrogen-fixing nodules with three well-known species of symbiotic bacteria, *Bradyrhizobium japonicum, B. elkanii,* and *Rhizobium fredii* (Jordan 1982; Scholla and Elkan 1984; Kuykendall et al. 1992). It also nodulates with the newly described species, *R. tianshanense* (Chen et al. 1995). The first three microorganisms, and likely the fourth, differ greatly in their physiology and genetics, and each can nodulate legume genera other than *Glycine.* Although bradyrhizobia and *R. fredii* strains such as USDA191 and QB1130 are broadly adapted in their abilities to form Fix$^+$ nodules on soybean cultivars (Jordan 1982; Israel et al. 1986; Lin et al. 1987; Kuykendall et al. 1992), most *R. fredii* strains nodulate soybean in a cultivar-specific manner (Keyser et al. 1982; Balatti and Pueppke 1992). Many primitive and a few agronomically improved cultivars yield nitrogen-fixing nodules with these strains, but nodules on most improved cultivars are either absent or structurally and functionally abnormal, depending on growth conditions (Heron and Pueppke 1984; Balatti and Pueppke 1992). The *nolXWBTUV* locus is a negative regulator of such cultivar specificity in *R. fredii* strain USDA257 (Heron et al. 1989). Inactivation of any of the genes in this locus conditions fully Fix$^+$ nodulation of all soybean cultivars that have been tested (Heron and Pueppke 1984; Heron et al. 1989; Meinhardt et al. 1993; Krishnan and Pueppke 1994).

Yet another organism, *Rhizobium* sp. NGR234, has also been reported to nodulate soybean (Trinick 1980; Lewin et al. 1987, 1990). It has a very broad host range (Pueppke and Broughton 1995) and is closely related to *R. fredii* (Jarvis et al. 1992). The interaction has been characterized as abnormal and ineffective (Morrison et al. 1986; Nayudu and Rolfe 1987; Relic et al. 1994), and we failed to detect any responses in plastic growth pouches (Balatti and Pueppke 1990). One possible explanation for these inconsistencies is that NGR234, like many *R. fredii* strains, nodulates soybean in a cultivar-dependent manner. Alternatively, nodulation of soybean by NGR234 may be highly sensitive to the environment or to some other unknown variable in nodulation tests.

We have become intrigued by the fact that NGR234 is the only organism other than *R. fredii* that is known to harbor the cultivar-specificity genes *nolXWBTUV* (Perret et al. 1991; Meinhardt et al. 1993; Krishnan and Pueppke 1994). This observation raises a series of fundamental questions about the possible function of the locus in NGR234 and its relationship to cultivar-specific interactions with soybean. We consequently established the nodulation phenotypes of NGR234 on a large number of soybean cultivars, and we examined the structure and function of the cultivar-specificity locus in this strain.

RESULTS

NGR234 produces aberrant root proliferations on soybean.

A total of 89 soybean cultivars belonging to seven different maturity groups were inoculated with NGR234, and responses were assessed under defined growth conditions. The roots of all soybean cultivars inoculated with *Rhizobium* sp. NGR234 contained globose structures that generally appeared as small, irregular swellings, but they lacked the parallel elongated ridges characteristic of Fix$^+$ nodules produced by USDA191. We were unable to recover bacteria from represen-

Corresponding author: Steven G. Pueppke;
E-mail: plantsgp@mizzou1.missouri.edu

Present address of Pedro A. Balatti: Instituto de Fisiologia Vegetal, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, CC31 La Plata 1900, Argentina

tative structures; leghemoglobin was inevitably absent, and acetylene-reduction activity was undetectable.

The lines yielding abnormal responses to NGR234 are as follows: Maturity group 00 = McCall; Maturity Group II = Corsoy; Maturity Group III = Asgrow A3733; Buchanan; Dekalb CX366; Dunfield; Eisenhower II; Express; Fontanelle 5999; Garfield; Golden Harvest H1358; Hamilton; Hubner H375; JMS3609; Kaiser Estech 358; Kaiser Estech 371; Lewis 367; Lynks 5387; Mosoy 8838; NECO 1200; NECO 1051N; Omega; Pilgrim; Pioneer 9381; Recyster; Reeds 330; Reeds 333; Roosevelt; Sherman; Super Crost Diam. D301; Truman III; Maturity Group IV = Asgrow 4595; Atlanta II; Crawford; Dallas; Dekalb CX415; Dekalb CX458; Dekalb CX469C; Delsoy 4500; ExoSC400; Mosoy 8842; Mosoy 9043; NECO 1300; NECO Exp. 0401; Northrup King S42-40; Northrup King S42-50; Northrup King S44-77; Northrup King S48-84; Peking; Pioneer 9442; Ripley; Ray Carroll 941; Sooty; Supercrest; Triumph 426; Maturity Group V = Asgrow 5979; Coker 6955; Dyer; Essex; Forrest; Hyperformer 89-167B; Hyperformer 89-576; Hyperformer HSC 591; Hyperformer HSC 501. Midwest 5220; NC + 5H61; Northrup King X59-60; Northrup King 69-55; Pioneer 9531; Pioneer 9581; Pioneer 9591; Rhodes; S85-1084; Stone; Terra VIG 515; Terra VIG 5693; United Agri UAPX-42; Maturity Group VI = Asgrow 6297; Davis; Hartz 6200; Northrup King S61-89; Northrup King X9161; Pioneer 9641; United Agri UAPX-27; United Agri UAPX-34; United Agri UAPX-38; United Agri UAPX-46; Maturity Group VII = Bragg; Maturity Group IX = Viçoja.

Strain NGR234 has been reported to form nonfixing nodules on the soybean cultivar Bragg (Morrison et al. 1986), and so we inoculated Bragg seedlings with NGR234, as well as *R. fredii* USDA257 and USDA191 as negative and positive controls, respectively (Fig. 1). USDA191 produced typical, nitrogen-fixing nodules with characteristic peripheral vascular bundles, a layer of cortical cells with thickened walls, and

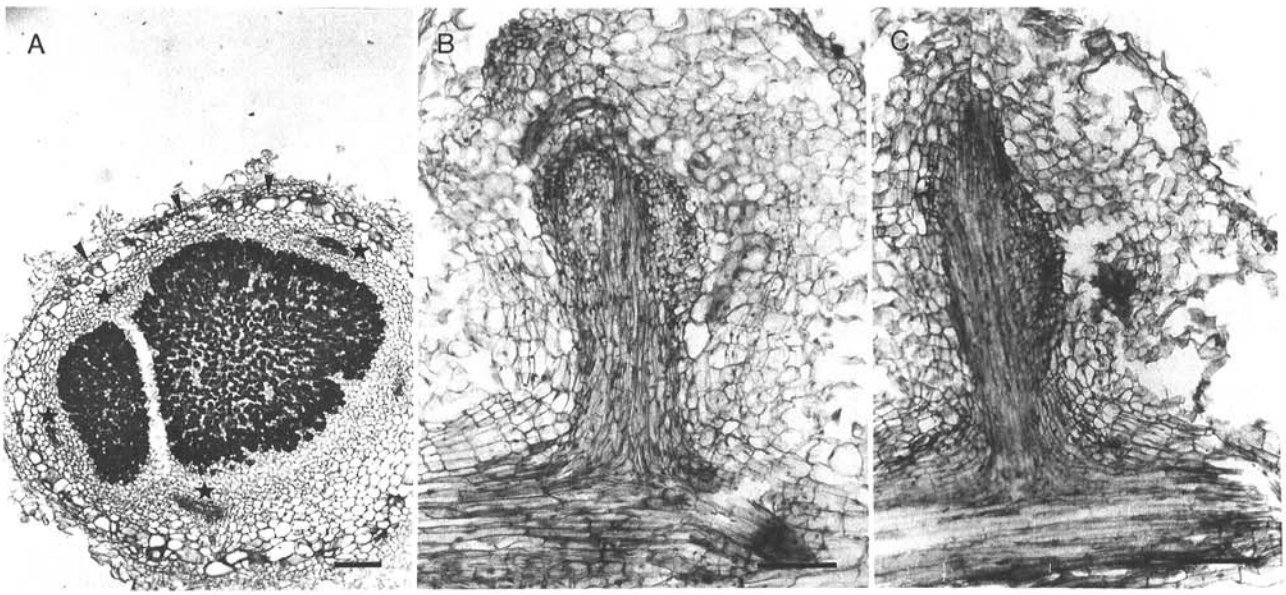

Fig. 1. Responses of Bragg soybean roots to rhizobia. **A,** *Rhizbium fredii* USDA191. This nodule has typical morphology: a central bacteroid zone, a layer of cortical cells with thickened walls (arrowheads), and peripherally arranged vascular bundles (stars). **B,** *Rhizobium* sp. NGR234 and, **C,** *R. fredii* USDA257. These abnormal proliferations lack bacteroids, and their vascular regions are centrally located and perpendicular to those of the root. Bars = 500 μm.

Table 1. Strains and plasmids used in this study

Strain/plasmid	Description	Source/reference
Rhizobium		
NGR234	Wild-type; Fix$^+$ on *Leucaena leucocephala*	Trinick 1980
NGR234Ω*nodD1*	*nodD1*-mutant of NGR234	W. J. Broughton
USDA191	Wild-type; Fix$^+$ on McCall and Peking soybean	Keyser et al. 1984
USDA257	Wild-type; Fix$^+$ on Peking soybean	Keyser et al. 1984
Plasmids		
pRK415	Wide host range *incP1*; Tc^R	Keen et al. 1988
pRK2013	*incP1* helper plasmid; Km^R	Ditta et al. 1980
pGEM7Zf$^+$	Multicopy sequencing vector, Ap^R	Promega Biotech
pMP220	*incP1-lacZ* reporter plasmid; Tc^R	Spaink et al. 1987
pRfDH412	8.0-kb *Eco*RI fragment containing *nolXWBTUV* of USDA257 in pGEM7Zf$^+$	Meinhardt et al. 1993
pXB901	Lorist2 cosmid containing *nolXWBTUV* of NGR234	Perret et al. 1991
pRNDH411	*nolXWBTUV*-containing insert of pXB901 in pRK415	This work
pRNDH412	*nolXWBTUV*-containing insert of pXB901 in pGEM7Zf+	This work
pMP650XN	650-bp *Hin*dIII fragment containing the promoter of *nolX* from NGR234 in pMP220	This work

bacteroid-filled cells in their interiors (Fig. 1A). The structures induced by NGR234 were much different, resembling modified lateral roots. Bacteroids were absent, and only a few scattered cells had thickened walls. The vascular bundles were centrally located, appearing as a column extending perpendicularly from those of the root (Fig. 1B). USDA257 also elicited abnormal proliferations on soybean cultivar Bragg (Fig. 1C). Plant responses to this strain were indistinguishable from those to NGR234 and included central vascular bundles, a few unorganized cells with thickened cell walls, and no bacteroids.

Characterization of the *nolXWBTUV* locus of NGR234.

Cosmid clone pXB901, which contains an 8-kb *Eco*RI fragment that hybridizes with the 8.0-kb, *nolXWBTUV*-containing fragment from USDA257, was obtained from a Lorist2 cosmid library by colony hybridization (Perret et al. 1991). The insert of pXB901 subsequently was subcloned into pGEM7Zf$^+$ and pRK415 (Table 1). We performed restriction enzyme analysis and side-by-side hybridization comparisons of restricted subfragments from USDA257 and NGR234 (data not shown) and found that there are only two differences in the restriction maps (Fig. 2). The 3′ portion of the coding sequence of *nolU,* the gene that was originally inactivated by Tn*5* mutagenesis in USDA257 and that was first associated with the extended host range phenotype on McCall (Meinhardt et al. 1993), lacks an *Apa*I site in NGR234. There is also a region of variability well downstream from *nolX*, in an area that has not yet been characterized. NGR234 contains an *Xho*I site not present in USDA257, and NGR234 has a *Pst* I site that is missing in NGR234 (Fig. 2).

Expression of the *nolXWBTUV* locus.

Expression of the *nolXWBTUV* locus in NGR234 was assessed by three different experimental approaches. In the first, we isolated RNA from control and apigenin-induced cells of NGR234 and blotted it to membrane filters. After confirming that equivalent amounts of RNA were loaded in each well, we evaluated transcription by probing with DNA fragments internal to *nolV* and spanning the entire 8-kb *nolXWBTUV*-containing fragment of USDA257. Transcripts complementary to both probes were barely detectable in RNA from non-induced cells, but they were readily apparent in RNA from cells that had been induced with the flavonoid (Fig. 3). We failed to find any message homologous to the 8.0-kb fragment in total RNA from NGR234*nodD1*::Ω (data not shown), a mutant that lacks an intact copy of the regulatory gene, *nodD1* (Broughton et al. 1991).

We selected the *nolX* promoter for use in a second set of experiments to evaluate transcription of the cultivar-specificity locus in NGR234. This promoter, which is flavonoid-inducible to high levels of expression in USDA257 (Meinhardt et al. 1993), was cloned into the *lacZ* reporter pMP220 (Spaink et al. 1987) and mobilized into wild-type NGR234. The level of expression of *nolX* of NGR234 was elevated 14-fold upon induction with 2 μM apigenin for 16 hr in culture medium, from 113 ± 10 Miller units to 1,542 ± 130 Miller units. Corresponding levels in controls containing the vector only were 142 ± 15 Miller units and 112 ± 20 Miller units.

NGR234(pMP650XN) was inoculated onto soybean and cowpea seedlings grown in plastic growth pouches and Magenta-type jars. *nolX* was expressed by bacteria on the surface of McCall soybean roots as assessed histochemically, but no infection structures were detected (data not shown). In cowpea, a fully compatible, Fix$^+$ interaction, the *nolX–lacZ* fusion was expressed at all stages of symbiosis, ranging from infection threads (Fig. 4) to mature nodules (data not shown).

We utilized polyclonal antibodies against NolX of strain USDA257 for a third set of experiments. These antibodies react specifically with polypeptide of about 66-kDa from protein extracts of USDA257; expression of this polypeptide is dependent on flavonoid induction and on the presence of an intact copy of *nolX*, indicating that it is the product of the *nolX* gene (Kovacs et al. 1995). We prepared total protein extracts from apigenin-induced and uninduced cells of NGR234 and USDA257 and probed them with antibodies specific to NolX of USDA257. As expected, a ca. 66 kD polypeptide band was present in extracts of induced cells of USDA257 (Fig. 5). A polypeptide of identical mobility but less intensity was present in extracts of flavonoid-induced cells of NGR234.

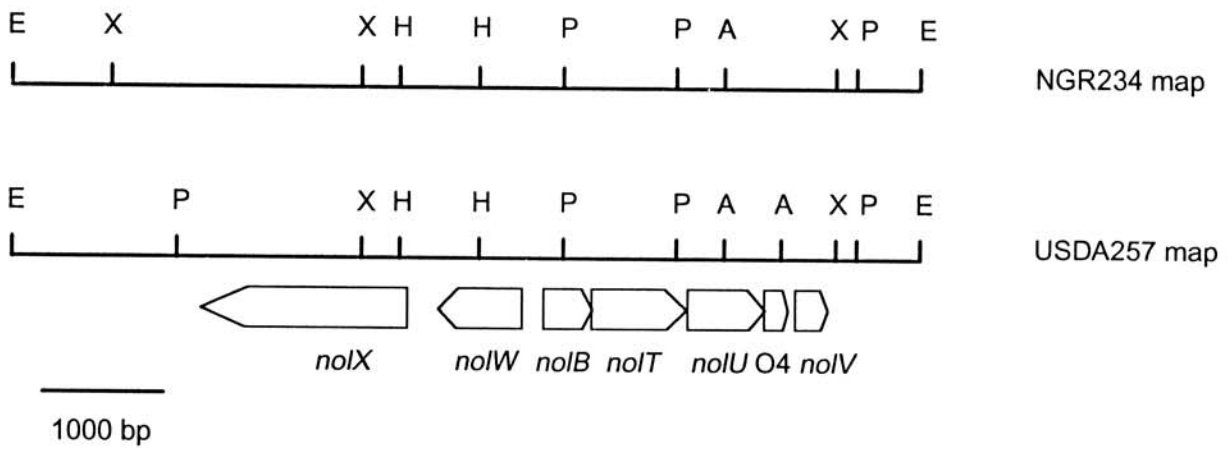

Fig. 2. Relationship between the 8.0-kb cultivar specificity locus of *Rhizobium fredii* USDA257 and a homologous 8.0-kb *Eco*RI fragment from *Rhizobium* sp. NGR234. The positions of *nolXWBTUV* are indicated on the USDA257 map. O4 refers to open reading frame 4, a locus of no known function (Meinhardt et al. 1993).

DISCUSSION

Rhizobium sp. NGR234 was first isolated from *Lablab purpureus* in Papua New Guinea (Trinick 1980), and it has attracted great interest because of its broad host range (Fellay et al. 1995). Trinick (1980), the first to systematically examine the host range of NGR234, indicated that this strain forms "effective" nodules on an unspecified soybean cultivar. After microscopic analysis of root sections, we concluded that soybean cultivar McCall is neither infected nor nodulated by NGR234 in plastic growth pouches (Balatti and Pueppke 1990). Yet NGR234 has been reported to nodulate the soybean cultivars Bragg, Williams, Peking, Preston, and Calland (Broughton et al. 1984; Morrison et al. 1986; Nayudu and Rolfe 1987; Relic et al. 1994), and there are reports of cultivar specificity (Lewin et al. 1987, 1990).

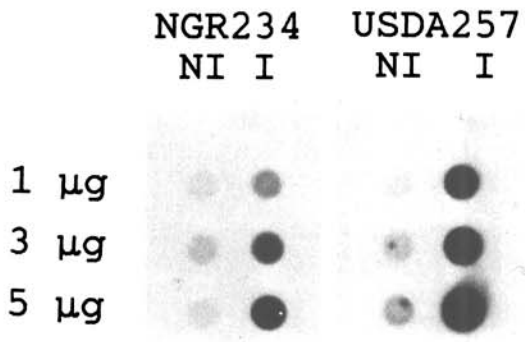

Fig. 3. RNA dot blot analysis of the expression of *nolXWBTUV* in *Rhizobium* sp. NGR234 and *R. fredii* USDA257. Sequences internal to *nolV* were used as probe. N = RNA from noninduced, control cultures. I = RNA from cultures that had been induced for 12 h with 2 µM apigenin. The calculated concentrations of RNA added to the wells are indicated on the left.

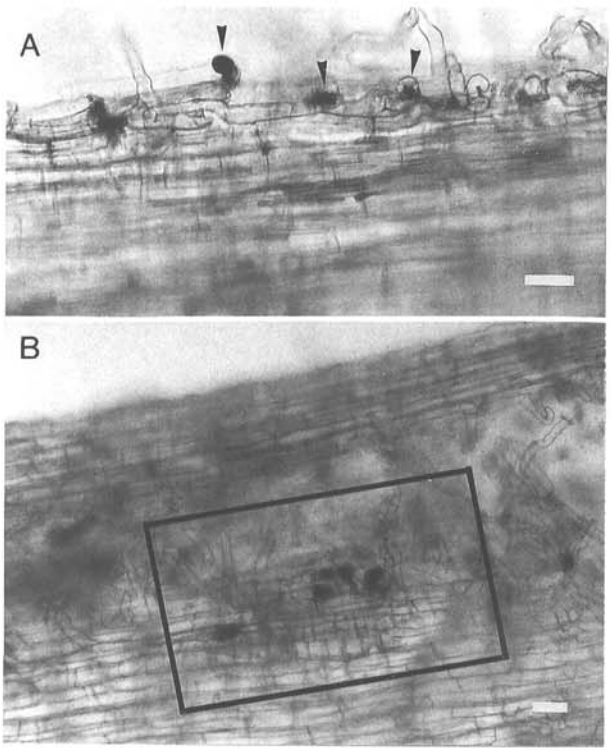

Fig. 4. Expression of a *nolX-lacZ* gene fusion in cells of *Rhizobium* sp. NGR234(pMP220XN) that have been inoculated onto cowpea roots. Roots were stained histochemically with X-Gal 10 days after inoculation with rhizobia as described in Materials and Methods. **A,** Side-on view of infected root hairs, which are stained black (arrowheads). **B,** Surface view of a zone of cortical cell divisions (boxed) with associated infected, stained root hairs. Bars = 50 µm.

Although the precise nature of the interactions of NGR234 and soybean remain unresolved, R-primes carrying regions of the symbiosis plasmid of NGR234 have been constructed (Nayudu and Rolfe 1987). When mobilized into the sym plasmid-cured derivative ANU265, one of these, pMN49, carried the entire host range of parent strain NGR234, with the exception of genes required to nodulate an unspecified soybean cultivar. This led to the suggestion that nodulation of soybean by NGR234 may be relatively complex, requiring more loci than needed for symbiosis with other hosts.

We evaluated the responses of 89 soybean cultivars to inoculation with NGR234 under standardized conditions, both to assess cultivar specificity and to generate background data that could be used to explain the presence of *nolXWBTUV*, a soybean cultivar-specificity locus that is known to function in *R. fredii* USDA257 (Meinhardt et al. 1993). Under uniform growth conditions in vermiculite, *Rhizobium* sp. NGR234 did not form normal Fix^+ nodules on any of a wide variety of soybean cultivars. In contrast to our previous experiments with growth pouches (Balatti and Pueppke 1990), though, NGR234 did elicit morphological responses by all of the cultivars tested, including McCall. These reactions took the form of abnormal outgrowths that in some cases superficially resembled nodules. Several lines of evidence suggest that they

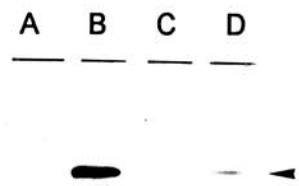

Fig. 5. NolX protein is detectable in flavonoid-induced cultures of *Rhizbium fredii* USDA257 and *Rhizobium* sp. NGR234. Cells were induced with 2 µM apigenin, and total proteins were isolated, electrophoresed, blotted to nitrocellulose, and probed with antibodies against NolX protein of USDA257 as described in Materials and Methods. Lane A = uninduced USDA257; Lane B = induced USDA257; Lane C = uninduced NGR234; Lane D = induced NGR234. The arrowhead marks the position of the approximately 66-kDa NolX protein.

are not normal nodules: They lacked leghemoglobin, they did not reduce acetylene, and we were unable to isolate bacteria from them. Moreover, these outgrowths had central vascular strands, unlike conventional Fix$^+$ nodules, in which xylem and phloem are always peripheral (Truchet et al. 1989). Although we cannot rule out the possibility that NGR234 may occasionally form Fix$^+$ nodules on soybean under some conditions, our data support the view that the responses of soybean to NGR234 are not greatly influenced by cultivar and that they are aberrant.

Our observations of NGR234 document an interesting relationship with the known behavior of *R. fredii*. Strains such as USDA257 form Fix$^+$ nodules with some cultivars, while strains such as USDA191 form Fix$^+$ nodules with all cultivars (Keyser et al. 1982; Heron and Pueppke 1984; Balatti and Pueppke 1992). The outgrowths induced by NGR234 and USDA257 on Bragg soybean in fact are indistinguishable, and thus the symbiotic interactions of USDA191, USDA257, and NGR234 with soybean cultivars seem to form a progression from full compatibility with all cultivars to compatibility with some cultivars to lack of compatibility with any cultivar.

nolXWBTUV regulates cultivar specificity of *R. fredii* on soybean, and inactivation of any of the genes in the locus extends the host range of USDA257 to improved soybean cultivars (Heron et al. 1989; Balatti and Pueppke 1990; Meinhardt et al. 1993). But why does NGR234, a strain that uniformly elicits only abnormal root outgrowths on soybean, carry a soybean cultivar specificity locus (Perret et al. 1991; Meinhardt et al. 1993; Fellay et al., 1995)? We have addressed two possibilities. One, that the DNA homology between USDA257 and NGR234 is incidental, has been ruled out. The *nolXWBTUV*-hybridizing *Eco*RI fragment of NGR234 retains most restriction sites found in the fragment from USDA257, and thus the regions appear to be structurally homologous.

The second possibility is that the two *nolXWBTUV* regions, although structurally conserved, are regulated differentially. We detected message corresponding to *nolXWBTUV* in apigenin-treated cells of NGR234 (Fig. 3), just as is the case in USDA257 (Kovacs et al. 1995), and using a *lacZ* fusion to the promoter region of *nolX*, we have shown that the gene is flavonoid-inducible in NGR234. In USDA257, *nolX* is expressed from preinfection to the stage of mature nodules and dependent on the regulatory gene *nodD1* (Meinhardt et al. 1993). Similar expression of the gene in NGR234 could not be evaluated with soybean, where infection threads did not form. But in a compatible interaction between NGR234 and cowpea, the gene was expressed and dependent on *nodD1*. Moreover, we also detected NolX protein in extracts of apigenin-induced NGR234 cells. These observations all are consistent with a common mode of regulation for *nolXWBTUV* in both USDA257 and NGR234.

It thus seems likely that NGR234 lacks one or more genes necessary to form Fix$^+$ nodules on the *Glycine max*, and that these genes are distinct from *nolXWBTUV*. Although expressed in NGR234, *nolXWBTUV* would not be of significance until basic compatibility has been established. If this interpretation is correct, it should be possible both to establish such basic compatibility by transferring appropriate genes from *R. fredii* to NGR234 and to detect and manipulate cultivar specificity in such genetic backgrounds (Krishnan et al. 1992; Perret et al. 1994).

MATERIALS AND METHODS

Plant tests.

Seeds of the soybean (*Glycine max* (L.) Merr.) cultivars McCall and Peking were from Eric Pueppke, Erie, ND, and the University of Missouri Bradford Research Farm, respectively. Seeds of other soybean cultivars were donated by H. Minor, Department of Agronomy, University of Missouri. Cowpea seeds *(Vigna unguiculata* (L.) Walp. cv. Pink Eye Purple Hull) were obtained from Hastings Seeds, Atlanta, GA. Seeds were surface-disinfested (Pueppke 1983) and sown in Magenta-type Leonard jars containing sterile vermiculite (Krishnan and Pueppke 1991) and Jensen's N-free nutrient solution (Vincent 1970). Rhizobia are listed in Table 1 and were stored in 7.5% glycerol at –70°C. Cultures were maintained for short time periods as slants on yeast extract-mannitol (YEM) medium (Vincent 1970) at 8°C. Rhizobia were grown in liquid YEM medium at 28 to 30°C and rotated at 150 rpm.

The general procedure for testing nodulation has been described elsewhere (Balatti and Pueppke 1992). The plants were grown at 28°C under a 12-h photoperiod (about 500 μE m^{-2} s^{-1}, photosynthetically active radiation). Nodulation was assessed 25 days after inoculation, when plants were scored according to the following scheme: Fix$^+$ = normal spherical nodule shape with internal red pigmentation due to leghemoglobin on the inside. These nodules were often characterized by series of raised parallel ridges on their surfaces. Abnormal nodules = irregularly shaped growths with no leghemoglobin or bacteria inside. Nod$^-$ = plants with roots lacking any visible responses to inoculation. Ten plants of each cultivar were tested, and if the original phenotype was not clearcut, the experiment was repeated. Acetylene-reduction assays with the cultivars Davis, McCall, and Peking were conducted as described previously (Schwinghamer 1970; Balatti and Pueppke 1992).

Ultrastructural analysis.

Sets of McCall seedlings that had been pregerminated for 48 h were transferred to autoclaved plastic pouches. Seedlings were dip-inoculated with USDA257 or NGR234 as required, and the location of the root tip of each seedling was marked on the surface of the pouch. *R. fredii* USDA191, which forms Fix$^+$ nodules on McCall, was included as a positive control. The plants were grown for 5 days at 28°C under a 12-h photoperiod. Lengths of primary root extending from 1 mm above to 9 mm below the RT mark then were harvested, dehydrated, fixed, and embedded as described (Chatterjee et al. 1990). Serial 15-μm longitudinal sections were stained with safranin-fast green and examined under bright-field optics at magnifications ranging from 40 to 400×. In planta expression of *nolX* was measured histochemically as described by Krishnan and Pueppke (1992). Since soybean did not form bacteroid-containing nodules upon inoculation with NGR234, cowpea was selected for these experiments. Seedlings were in-oculated as described above with NGR234 or NGR234(pMP650XN) and examined 10 days later.

Molecular methods.

Digestion with restriction endonucleases, bacterial transformations, cloning, plasmid isolations, and preparation of

filters for Southern blot analysis followed the guidelines of Sambrook et al. (1990). DNA was labeled with [^{32}P]-dCTP by the random priming method (United States Biochemical Corp.). RNA was isolated by the hot phenol method essentially as described by Wang and Stacey (1991). The RNA was exposed to RNAse-free DNAse for 30 min at 37°C and then treated with phenol-chloroform and reprecipitated. The RNA was dissolved in RNAse-free water and stored at –70°C. The RNA was blotted to nitrocellulose membranes and amounts verified by hybridization with a radioactively labeled DNA probe homologous to rRNA sequences (Scott-Craig et al. 1991). Expression of the *nolXWBTUV* locus was assessed with two probes. One was the insert of pRfDH412, an 8-kb *Eco*RI fragment containing the entire locus and flanking regions. The other was a 300-bp *Nar*I fragment located within the 3′ end of *nolV.*

The flavonoid-inducibility of the *nolX* promoter region from NGR234 was tested with cells of NGR234 containing pMP650XN (Krishnan and Pueppke 1991). The inducer was 2 μM apigenin. The preparation and purification of NolX-specific antibodies has been described (Kovacs et al. 1995). Cells were centrifuged from 5-ml cultures containing about 1.5×10^9 cells per ml and resuspended in 400 μl of 1× treatment buffer (Harlow and Lane 1988). The resulting lysates were boiled for 5 min to produce total protein extracts. Equal amounts of protein samples, about 150 μg, were electrophoresed in 12% SDS polyacrylamide gels and transferred to nitrocellulose by electroblotting. The resulting blots were reacted with antibodies, and the antibody-protein complexes were detected with ^{125}I-labeled protein A (New England Nuclear; Sp. Act. 8.81 μCi/μg.) as described by Harlow and Lane (1988).

ACKNOWLEDGMENTS

This work was supported by competitive research grants from the United States Department of Agriculture, National Research Initiative and is Journal Series 12322 of the Missouri Agricultural Experiment Station.

LITERATURE CITED

Balatti, P. A., and Pueppke, S. G. 1990. Nodulation of soybean by a transposon-mutant of *Rhizobium fredii* USDA257 is subject to competitive nodulation blocking by other rhizobia. Plant Physiol. 94:1276-1281.

Balatti, P. A., and Pueppke, S. G. 1992. Identification of North American soybean lines that form nitrogen fixing nodules with *Rhizobium fredii* USDA257. Can. J. Plant Sci. 72: 49-55.

Broughton, W. J., Heycke, N., Meyer, H., and Pankhurst, C. E. 1984. Plasmid linked *nif* and *"nod"* genes in fast-growing rhizobia that nodulate *Glycine max, Psophocarpus tetragonolobus,* and *Vigna unguiculata*. Proc. Natl. Acad. Sci. USA 81:3093-3097.

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. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, Netherlands.

Chatterjee, A., Balatti, P. A., Gibbons, W., and Pueppke, S. G. 1990. Interaction of *Rhizobium fredii* USDA257 and nodulation mutants derived from it with the agronomically improved soybean cultivar McCall. Planta 180:303-311.

Chen, W., Wang, E., Wang, S., Li, Y., Chen, X., and Li, Y. 1995. Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, People's Republic of China. Int. J. Syst. Bacteriol. 45:153-159.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. 1980. Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti.* Proc. Natl. Acad. Sci USA 77:7347-7351.

Fellay, R., Rochepeau, P., Relic, B., and Broughton, W. J. 1995. Signals to and emanating from *Rhizobium* largely control symbiotic specificity. In: Pathogenicity and Host Specificity in Plant Diseases. K. Kohmoto, R. P. Singh and U. S. Singh, eds. Elsevier, Oxford, in press.

Harlow, E., and Lane, D. 1988. Antibodies, A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Heron, D. S., Érsek, T., Krishnan, H. B., and Pueppke, S. G. 1989. Nodulation mutants of *Rhizobium fredii* USDA257. Mol. Plant-Microbe Interact. 2:4-10.

Heron, D. S., and Pueppke, S. G. 1984. Mode of infection, nodulation specificity, and indigenous plasmids of 11 fast-growing *Rhizobium japonicum* strains. J. Bacteriol. 160:1061-1066.

Israel, D. W., Mathis, J. N., Barbour, W. M., and Elkan, G. H. 1986. Symbiotic effectiveness and host-strain interactions of *Rhizobium fredii* USDA 191 on different soybean cultivars. Appl. Environ. Microbiol. 51:898-903.

Jarvis, B. D. W., Donner, H. L., and Young, J. P. W. 1992. Phylogeny of fast-growing soybean nodulating rhizobia supports synonymy of *Sinorhizobium* and *Rhizobium* and assigment to *Rhizobium fredii.* Int. J. Syst. Bacteriol. 42:93-96.

Jordan, D. C. 1982. Transfer of *Rhizobium japonicum* Bachanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants. Int. J. Syst. Bacteriol. 32:136-139.

Keen, N. T., Tamaki, S., Kobayashi, D., and Trollinger, D. 1988. Improved broad-host range plasmids for DNA cloning in Gram-negative bacteria. Gene 70:191-197.

Keyser, H. H., Bohlool, B. B., Hu, T. S., and Weber, D. F. 1982. Fast growing rhizobia isolated from root nodules of soybean. Science 215:1631-1632.

Kovacs, L. G., Balatti, P. A., Krishnan, H. B., and Pueppke, S. G. 1995. Transcriptional organization and expression of *nolXWBTUV,* a locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA257. Mol. Microbiol., in press.

Krishnan, H. B., Lewin, A., Fellay, 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. NGR234 to nodulate *Leucaena* spp. Mol. Microbiol. 6:3321-3330.

Krishnan, H. B., and Pueppke, S. G. 1991. Sequence and analysis of the *nodABC* region of *Rhizobium fredii* USDA257, a nitrogen-fixing symbiont of soybean and other legumes. Mol. Plant-Microbe Interact. 4:512-520.

Krishnan, H. B., and Pueppke, S. G. 1992. A *nolC–lacZ* gene fusion in *Rhizobium fredii* facilitates direct assessment of competition for nodulation of soybean. Can. J. Microbiol 38:515-519.

Krishnan, H. B., and Pueppke, S. G. 1994. Host range, RFLP, and antigenic relationships between *Rhizobium fredii* strains and *Rhizobium* sp. NGR234. Plant Soil 161:21-29.

Kuykendall, L. D., Saxena, B., Devine, T. E., and Udell, S. E. 1992. Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii.* sp. nov. Can. J. Microbiol. 38:501-505.

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

Lewin, A., Rosenberg, H., Meyer, H., Wong, C. H., Nelson, L., Manen, J., Stanley, J., Dowling, D. N., Dénarié, J., and Broughton, W. J. 1987. Multiple host-specificity loci of the broad host-range *Rhizobium* sp. NGR234 selected using the widely compatible legume *Vigna unguiculata.* Plant. Mol. Biol. 8:447-459.

Lin, J., Walsh, K. B., Johnson, D. A., Canvin, D. T., Shujin, W., and Layzell, D. B. 1987. Characterization of *R. fredii* QB1130, a strain effective on commercial soybean cultivars. Plant Soil 99:441-446.

Meinhardt, L. W., Krishnan, H. B., Balatti, P. A., and Pueppke, S. G. 1993. Molecular cloning and characterization of a sym plasmid locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA257. Mol. Microbiol. 9:17-29.

Morrison, N. A., Trinick, M. J., and Rolfe, B. G. 1986. Comparison of the host range of fast-growing *Rhizobium japonicum* strains with a fast-growing isolate from *Lablab*. Plant Soil 92:313-321.

Nayudu, M., and Rolfe, B. G. 1987. Analysis of R-primes demonstrates that genes for broad host range nodulation of *Rhizobium* strain NGR234 are dispersed on the sym plasmid. Mol. Gen. Genet. 206:326-337.

Perret, X., Broughton, W. J., and Brenner, S. 1991. Canonical ordered cosmid library of the symbiotic plasmid of *Rhizobium* species NGR234. Proc. Natl. Acad. Sci. USA 88:1923-1927.

Perret, X., Fellay, R., Bjourson, A. J., Cooper, J. E., Brenner, S., and Broughton, W. J. 1994. Subtraction hybridisation and shot-gun sequencing: A new approach to identify symbiotic loci. Nucleic Acids Res. 22:1335-1341.

Pueppke, S. G. 1983. *Rhizobium* infection threads in root hairs of *Glycine max* (L.) Merr., *Glycine soja* Sieb. & Zucc., and *Vigna unguiculata* (L.) Walp. Can. J. Microbiol. 29:69-76.

Pueppke, S. G., and Broughton, W. J. 1995. Symbiotic specificity in the legume-*Rhizobium* symbiosis: *Rhizobium* sp. NGR234 and *R. fredii* USDA257 have exceptionally broad, interrelated host ranges. Plant Soil, in press.

Relic, B., Perret, X., Estrada-Garcia, M. T., Kopcinska, J., Golinowski, W., Krishnan, H. B., Pueppke, S. G., and Broughton, W. J. 1994. Nod factors of *Rhizobium* are the key to the legume door. Mol. Microbiol. 13:171-178.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scholla, M. H., and Elkan, G. H. 1984. *Rhizobium fredii* sp. nov., a fast-growing species that effectively nodulates soybean. Int. J. Syst. Bacteriol. 34:484-486.

Schwinghamer, E. A., Evans, H. J., and Dawson, M. D. 1970. Evaluation of the effectiveness in mutant strains of *Rhizobium* by acetylene reduction relative to other criteria for N_2 fixation. Plant Soil 33:192-212.

Scott-Craig, J. S., Guerinot, M. L., and Chelm, B. K. 1991. Isolation of *Bradyrhizobium japonicum* DNA sequences that are transcribed at high levels in bacteroids. Mol. Gen. Genet. 228:356-360.

Spaink, H. P., Okker, R. J. H., Wijffelman, C. A., Pees, E., and Lugtenberg, B. J. J. 1987. Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. Plant Mol. Biol. 9:27-39.

Trinick, M. J. 1980. Relationships amongst the fast growing rhizobia of *Lablab purpureus, Leucaena leucocephala, Mimosa* sp., *Acacia farnesiana* and *Sesbania grandiflora* and their affinities with other rhizobial groups. J. Appl. Bacteriol. 49:39-53.

Truchet, G., Camut, S., de Billy, F., Odorico, R., and Vasse, J. 1989. The *Rhizobium*-legume symbiosis. Two methods to discriminate between nodules and other root-derived structures. Protoplasma 149:82-88.

Vincent, J. M. 1970. A Manual for the Practical Study of Root-Nodule Bacteria. Blackwell Scientific Publications, Oxford.

Wang, S., and Stacey, G. 1991. Studies of the *Bradyrhizobium japonicum nodD1* promoter: A repeated structure for the *nod*-box. J. Bacteriol. 173:3356-3365.