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Proteins from Cells of *Rhizobium fredii* Bind to DNA Sequences Preceding *nolX*, a Flavonoid-Inducible *nod* Gene that Is Not Associated with a *nod* Box

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Rhizobium fredii **strains USDA257 and USDA191 both contain a set of nodulation genes termed *nolXWBTUV*. In the USDA257 background, *nolX* prevents infection of soybean cultivars such as McCall, and in both backgrounds, it blocks nodulation of *Erythrina* spp. We report here that expression of *nolX* is differentially responsive to a panel of flavonoids, and that the most potent inducers are also the most active inducers of *nodC*, a conventional, *nod* box-associated gene. Cell-free protein extracts from uninduced and flavonoid-induced cells of strains USDA191 and USDA257 retard the electrophoretic mobility of DNA sequences that lie upstream of *nolX*. Binding is dependent both on *nodD1* and *nodD2*, and it is abolished by the presence of a double-stranded, 23-bp oligonucleotide that lies within a 114-bp *TaqI*/*Sac*II restriction fragment. This oligomer has significant sequence homology to A3, a putative negative regulatory element from *R. leguminosarum* bv. *viciae*. Deletion of the A3-homologous sequences elevates the basal and flavonoid-inducible expression of *nolX* by about 50%.**

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

nolXWBTUV is a sym plasmid locus that imparts cultivar specificity to the interaction of *Rhizobium fredii* strain USDA257 and soybean (Heron et al. 1989; Meinhardt et al. 1993). When the locus is active, nodulation of most agriculturally advanced soybean cultivars, typified by McCall, is checked at the stage of infection (Chatterjee et al. 1990; Balatti and Pueppke 1992). This developmental block is relieved by disruption of any of the six known genes within the locus (Meinhardt et al. 1993). Other soybean cultivars, typified by Peking, are unaffected by *nolXWBTUV* and can be nodulated normally by both the parental strain and mutants (Heron and Pueppke 1984; Heron et al. 1989). The differential symbiotic phenotype of McCall and Peking is controlled by a single dominant gene that is present in Peking (Trese 1995).

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The *nolXWBTUV* locus is not restricted to soybean cultivar-specific strains such as USDA257. Broad host range *Rhizobium* sp. strain NGR234 fails to form nitrogen-fixing nodules of soybean, yet it contains *nolXWBTUV* (Balatti et al. 1995). *R. fredii* strains USDA191 and HH103, which produce normal nitrogen-fixing nodules on both McCall and Peking soybean, also harbor the locus (Bellato, Krishnan, Cubo, Vera, and Pueppke, unpublished). Inactivation of *nolX* allows *R. fredii* strains USDA257, USDA191, and HH103 to nodulate two species of *Erythrina* that are not nodulated by the wild-type parental strains (Bellato, Krishnan, Cubo, Vera, and Pueppke, unpublished), and so the gene is of general symbiotic significance, but in a complex and strain-specific manner.

Expression of *nolX* in strains USDA191 and USDA257 is indistinguishable, and indeed, at least 577 bp of DNA upstream of the *nolX* reading frame are identical in the two strains (Bellato, Krishnan, Cubo, Vera, and Pueppke, unpublished). The activity of *nolX* is dependent on flavonoid signals and under the jurisdiction of the regulatory genes, *nodD1* and *nodD2* (Meinhardt et al. 1993; Kovács et al. 1995; Bellato, Krishnan, Cubo, Vera, and Pueppke, unpublished). NodD proteins, which act as host sensors, bind to a conserved regulatory motif, the *nod* box, which precedes inducible *nod* genes. These proteins are positive transcriptional regulators, but only upon activation by flavonoid signals from the host (Schlaman et al. 1992).

nolX is puzzling because it lacks a *nod* box, yet it is regulated in a conventional flavonoid- and NodD-dependent manner. This property is shared with the *nolBTUV* transcriptional unit of strain USDA257 (Kovács et al. 1995; Krishnan and Pueppke, unpublished) and *nolJ,* a *nod* gene of strain *R. fredii* strain USDA201 (Boundy-Mills et al. 1994). We show here that *nolX* is expressed in a mutant of strain USDA257 lacking *nolXWBTUV* and that the response pattern of *nolX* to flavonoids matches that of *nodABC*. We used gel retardation assays to monitor protein binding to DNA sequences preceding *nolX*. Protein extracts from uninduced and flavonoid-induced cells of strains USDA191 and USDA257 bound to sequences within a 114-bp *TaqI*/*Sac*II fragment that begins 188 bp upstream of the transcriptional initiation site of *nolX* and contains sequence homology to a motif thought to be involved in regulation of *nodD* of *R. leguminosarum* bv. *viciae* (Mao et al. 1994). Protein binding to this region was dependent on both *nodD1* and *nodD2*, but deletion of these sequences enhanced expression of *nolX*.

RESULTS

Flavonoid-inducibility of *nolX*.

The sensitivity of *nolX* to flavonoid signals was assessed by ligating promoter probe pMP220 to a 648-bp *Hin*dIII fragment from strain USDA257. Strain USDA191 contains an identical fragment, which includes the 3′ terminus of *nolW*, the first 71 bp of the *nolX* coding region, and the intervening noncoding sequences (Fig. 1). The resulting plasmid was transferred to strain USDA257 and responsiveness to nine flavonoids measured. Three compounds, the flavone apigenin, the isoflavone daidzein, and the flavone luteolin, elevated expression of the gene by more than 10-fold (Table 1). Three other compounds, the flavone chrysin, the isoflavone genistein, and the flavone naringenin, were less active, increasing expression by five- to sevenfold. The remaining three compounds were essentially inactive.

Responsiveness of the *nolX-lacZ* fusion to apigenin was not influenced by the replacement of *nolXWBTUV* with a *sac* cartridge to create mutant USDA257SD1 (99 ± 25 and 1,270 ± 215 Miller units, respectively, in the absence and the presence of the inducer, a 12.8-fold induction). This indicates that expression of *nolX* is independent of the integrity of the other genes in the *nolXWBTUV* locus. The fusion was not expressed, however, following transfer to *R. meliloti* strain RCR2011 (2 μM luteolin or apigenin as inducer) or to *Agrobacterium tumefaciens* strain C58 or A136 (2 μM acetosyringone as inducer).

Cellular proteins bind to sequences upstream of *nolX*.

We employed gel retardation assays to test the hypothesis that potential regulatory proteins bind to DNA sequences in the promoter region of *nolX*. The 648-bp *Hin*dIII fragment was digested with *Sac*II, and the resulting 206-bp *Hin*dIII/*Sac*II and 442-bp *Sac*II/*Hin*dIII fragments (Fig. 1) mixed separately with proteins from strain USDA257 and assayed. Although we failed to detect retardation of the 442-bp fragment (data not shown), proteins from both uninduced and apigenin-induced cells interacted with the 206-bp fragment so that a retarded band appeared in gels (Fig. 2A). Similar experiments with strain USDA191 confirmed that proteins from this strain can influence mobility of a 339-bp *Hin*dIII/*Bst*EII fragment that overlaps with the 206-bp *Hin*dIII/*Sac*II fragment. Retardation of the labeled fragment was inhibited by addition of unlabeled fragment (Fig. 2B) or by pretreatment of the protein extract with Proteinase K (data not shown).

We assessed the significance of *nodD1* and *nodD2* for protein-DNA complex formation by testing protein extracts from *nodD1-*, *nodD2-*, and double *nodD1/nodD2*-negative mutants of strain USDA191. The 339-bp *Hin*dIII/*Bst*EII fragment (Fig. 1) was used for these experiments, which confirmed that a retarded band does not appear with extracts from any of the three *nodD*-negative mutants (Fig. 3). This indicates that both *nodD1* and *nodD2* are required for complex formation.

We further localized the DNA sequences capable of binding bacterial proteins by examining a series of different restriction fragments by the gel retardation method. As is the case with

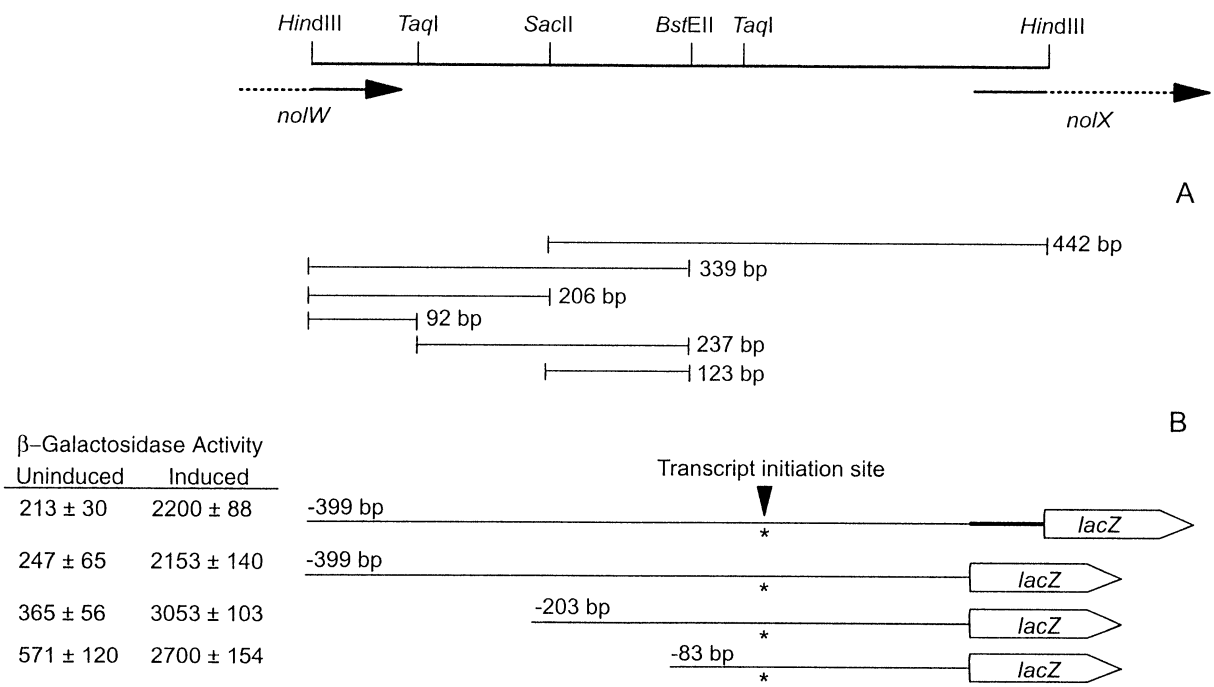

Fig. 1. The *nolX* promoter region.The 3′ terminus of *nolW*, the 5′ terminus of *nolX*, and intervening nontranslated sequences are present on a 648-bp *Hin*dIII fragment. **A,** Restriction fragments used for gel retardation analysis. **B,** DNA fragments (lengths are given with respect to the transcription initiation site) were ligated to pMP220 and used to examine expression of *nolX*. The table on the lower left gives β-galactosidase activities of these constructions (Miller units ± standard deviations), as measured in strain USDA257. Induced cells were cultured in the presence of 2 μM apigenin.

the 339-bp *Hin*dIII/*Bst*EII and the 206-bp *Sac*II/*Hin*dIII fragments, a retarded band is apparent with a 237-bp *Taq*I/*Bst*EII subfragment, but it is absent if a 92-bp *Hin*dIII/*Taq*I subfragment or a 123-bp *Sac*I/*Bst*EII subfragment is employed (Fig. 4A and B). Thus, the protein-binding DNA sequences must lie within the 114-bp *Taq*I/*Sac*II subfragment as indicated in Figure 1.

Sequence analysis.

The *Taq*I/*Sac*II subfragment contains a set of sequences with homology to the A3 region of *R. leguminosarum* bv. *viciae*, a small block of dyad symmetry that lies upstream of the single *nodD* copy of this organism (Shearman et al. 1986). The A3 region resembles a portion of the *nodA*-associated *nod* box of *R. leguminosarum* bv. *viciae*, and it is postulated to be a NodD-binding site involved in self-repression of *nodD* (Mao et al. 1994). Homology between the A3 region and sequences upstream of *nolX* is extensive: 14 of 19 bp are conserved, provided that one single base gap is introduced (Fig. 5). The dyad symmetry of A3, however, is not evident in the nolX-associated sequence.

We also aligned the sequences surrounding the A3-homologous region of *nolX* with the *nodA*-associated *nod* boxes of *R. leguminosarum* bv. *viciae* (Mao et al. 1994) and *R. fredii* (Krishnan and Pueppke 1991). Overall, these two *nod* boxes share 72% identity with one another across 47 bp (Fig. 5). This relationship does not extend to the *nolX*-associated sequences, which share only 43 and 30% identity, respectively, to the *nod* boxes of *R. leguminosarum* bv. *viciae* and *R. fredii*.

Significance of the A3-homologous region.

We employed two approaches to assess the significance of the A3-homologous region for expression of *nolX*. First, we synthesized a pair of oligonucleotides: 5′-GATACG-CCCGCCAGGGAATCTGG-3′ and 5′-GGCTATGCGGGCG-GTCCCTTAGA-3′. These were annealed to create a double-stranded 21-mer that corresponds to the entire A3-homologous region (Fig. 5). This fragment was used as a competitor in gel retardation experiments, at stoichiometries similar to those employed by Fisher et al. (1988). These experiments confirmed that 300 ng of this oligonucleotide are sufficient to prevent the appearance of the retarded fragment (Fig. 4C).

Table 1. The *nolX* and *nodABC* promoters of *Rhizobium fredii* USDA257 respond to the same spectrum of flavonoids

Flavonoid	Expression of *nol X* (Miller units ± SE)[a]		Fold-induction	
	Uninduced	Flavonoid (2 µM)	*nolX*	*nodC*[b]
Apigenin	128 ± 22	1,823 ± 65	14.2	5.9
Biochanin A	142 ± 2	256 ± 11	1.8	1.2
Chrysin	126 ± 27	893 ± 55	7.1	5.5
Daidzein	149 ± 27	1,912 ± 119	12.8	5.9
Genistein	152 ± 50	876 ± 20	5.8	6.3
Luteolin	145 ± 21	1,875 ± 90	12.9	6.0
Naringenin	138 ± 41	645 ± 10	4.7	3.5
Quercetin	200 ± 21	298 ± 17	1.5	1.7
Umbelliferone	141 ± 16	125 ± 16	0.9	1.2

[a] Values are the means of seven independent experiments with three replicates each.

[b] Data from Krishnan and Pueppke (1991). Flavonoids were supplied at concentrations of 2 µM.

We also created deletions to assess the impact of upstream sequences on flavonoid-inducible expression of *nolX*. Deletion of the promoter region to position –203 with respect to the transcription initiation site, a procedure that removed the entire A3-homologous region, increased expression of the gene by about 50%, both in the presence and absence of apigenin

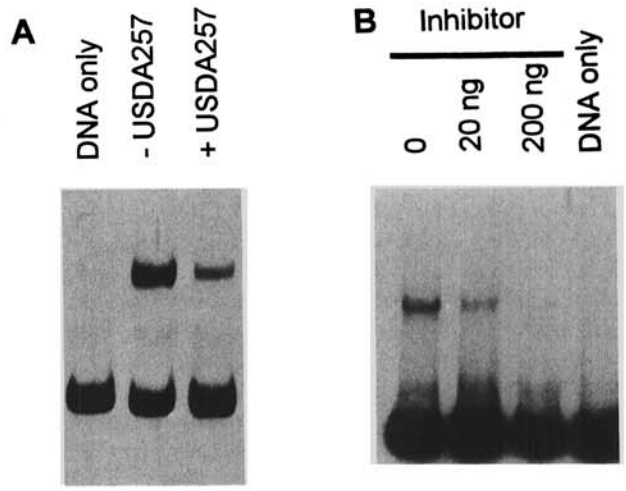

Fig. 2. Proteins from *Rhizobium fredii* cells retard the electrophoretic mobility of DNA sequences that lie upstream of *nolX*. Reaction mixtures contained 30 µg of protein and 1 ng of labeled DNA fragment. **A,** Proteins that retard the mobility of the 206-bp *Hin*dIII/*Sac*II fragment are present in both uninduced and flavonoid-induced cells of strain USDA257. DNA only = control lacking proteins; –USDA257 = proteins from uninduced cells; +USDA257 = proteins from cells that had been grown in the presence of 2 µM apigenin. **B,** Retardation of the 339-bp *Hin*dIII/*Bst*EII fragment is abolished by excess quantities of unlabeled DNA probe.DNA only = control lacking proteins.Lanes labeled "Inhibitor" were loaded with reaction mixtures containing the indicated quantities of unlabeled restriction fragment.

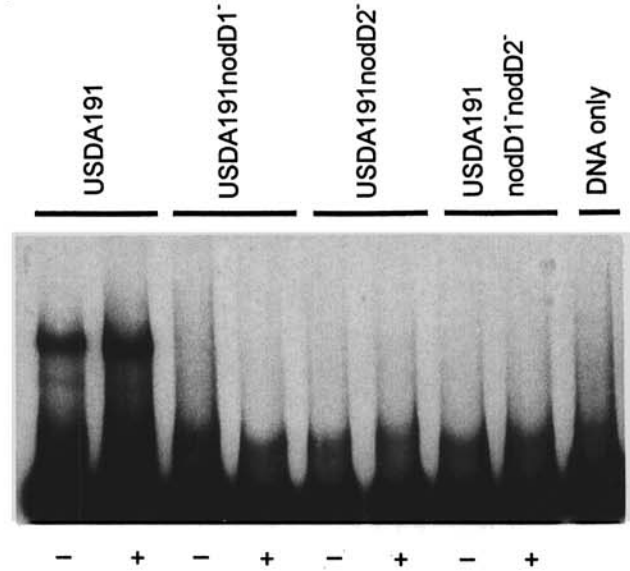

Fig. 3. Formation of the DNA/protein complex depends on both *nodD1* and *nodD2*. A labeled, 339-bp *Hin*dIII/*Bst*EII fragment (approximately 2 ng; see Fig. 1) was mixed with 80 µg of protein from strain USDA191 or one of its *nodD*-mutants and assayed by gel retardation.The "–" and "+" symbols below the lanes indicate that the proteins were from flavonoid-treated (2 µM genistein) or control cells, respectively. The lane marked DNA only was loaded with labeled DNA that had not been exposed to protein.

(Fig. 1). Further deletion to position –83 with respect the transcription initiation site had no additional effect on expression of *nolX*.

DISCUSSION

Rhizobium fredii appears to employ multiple control systems to modulate expression of genes needed to establish symbiosis with its plant hosts. One is a conventional *nodD*-based circuit of the type operative in other nitrogen-fixing rhizobia (Appelbaum et al. 1988; Schlaman et al. 1992; Pueppke 1996) These systems use NodD to sense flavonoid cues from the host and then trigger expression of a set of inducible *nod* genes. *cis*-acting *nod* box promoter sequences are essential players in this process (Rostas et al. 1986; Spaink et al. 1987). They can bind NodD in the absence of flavonoid inducer (Hong et al. 1987; Fisher et al. 1988), but the interaction does not enhance transcription unless the inducer is perceived (Schlaman et al. 1992; Fisher and Long 1993). Although the consensus *nod* box originally was recognized on the basis of sequence conservation (Rostas et al. 1986; Schofield and Watson 1986; Scott 1986), its limits now have been defined by deletion analysis (Spaink et al. 1987), and we know that it is a block of about 47 bp that lies between position –26 and –76 with respect to the transcription initiation site of the regulated gene (Fisher et al. 1987; Mulligan and Long 1989).

The existence of a second *nod*-regulatory system in *R. fredii* can be deduced from analysis of three flavonoid-inducible transcriptional units: *nolJ, nolBTUV,* and *nolX* (Sadowsky et al. 1988; Meinhardt et al. 1993; Boundy-Mills et al. 1994; Kovács et al. 1995; Bellato et al., unpublished). *nodD1* is necessary for inducibility of *nolJ,* and both *nodD1* and *nodD2* are required for *nolX,* yet all three sets of genes lack *nod* boxes, and their promoters share no obvious common structural features. Using *nolX* as a model, we have found that responsiveness of *nolX* to flavonoids depends on both *nodD1* and *nodD2* (Bellato, Krishnan, Cubo, Vera, and Pueppke, unpublished) and that the *nod* box-independent pattern of sensitivity to flavonoids parallels that of a *nod* box-associated gene, *nodC* (Krishnan and Pueppke 1991). Although the maximum levels of induction of *nolX* and *nodC* differ, and the active compounds are grouped more uniformly in the case of *nodC,* Biochanin A, quercetin, and umbelliferone were invariably without activity, and daidzein, the most abundant flavonoid in soybean roots (Cho and Harper 1990; Graham 1990), was a potent inducer of both genes (Table 1).

Using gel retardation assays of the sort applied earlier to NodD, we readily demonstrated that cellular proteins can bind to sequences upstream from *nolX*. The target site for binding maps to a 114-bp *TaqI*/*Sac*II fragment that lies between positions –402 and –188 with respect to the transcriptional initiation site for the gene. This fragment contains a run of 19 bases with 74% identity to the A3 region of *R. leguminosarum* bv. *viciae* (Mao et al. 1994), and inhibition studies confirmed that the A3-homologous region is likely to function as a protein-binding site (Fisher et al. 1988). The A3 element of *R. leguminosarum* bv. *viciae* lies upstream of *nodD* and is thought to mediate self-repression of this gene (Mao et al. 1994). Deletion analysis confirmed that expression of *nolX* is enhanced if the A3-homologous region is removed, and so the *R. fredii* binding protein(s) is likely to act as a repressor. Since the A3 element is characterized by homology to a portion of the *nod* box consensus sequence (Shearman et al. 1986; Mao et al. 1994), we have examined the structure of the homologous region from *R. fredii* in detail. Overall, homology to the consensus *nod* box is very low (Spaink et al. 1987). Only 12 of 32 conserved *nod* box positions are retained and the distance between the element and *nolX* is much greater than that expected for a *nod* box. Highly divergent, yet functionally active *nod* boxes are known in *Azorhizobium caulinodans* and *Bradyrhizobium japonicum,* two nitrogen-fixing species that are only distantly related to the genus *Rhizobium* (Goethals et al. 1989; Wang and Stacey 1991; Goethals et al. 1992). The promoter from *A. caulinodans* is characterized by an ATC-N_9-GAT motif (Goethals et al. 1992), and that from *B. japonicum* by a 9-bp modular structure (Wang and Stacey 1991). Both of

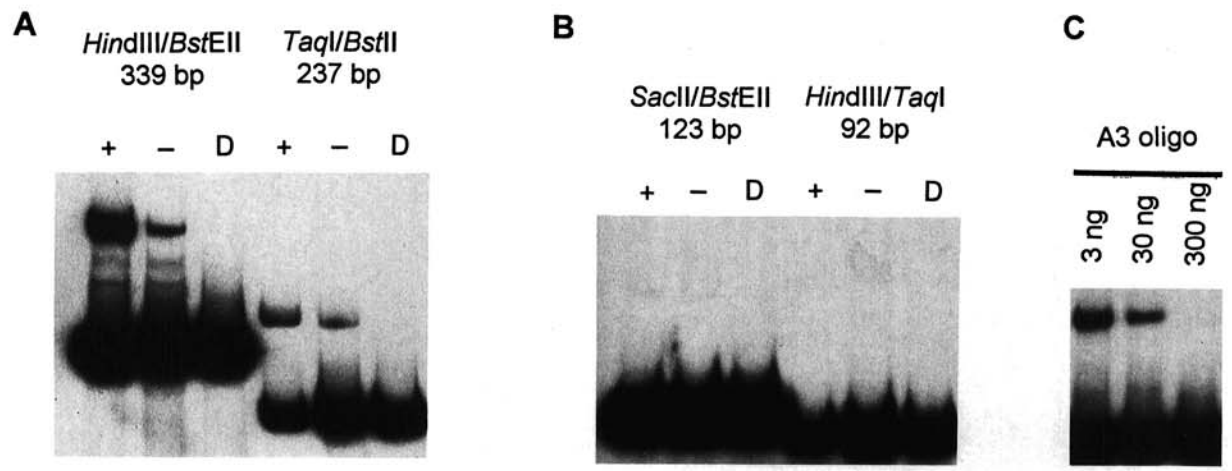

Fig. 4. Localization of sequences upstream from *nolX* that are capable of binding proteins from *R. fredii* cells. **A** and **B,** The indicated restriction fragments (ca. 2 ng each; see Fig. 1) were labeled and mixed with 80 μg of protein from genistein-induced strain USDA191. The "+" and "–" minus symbols below the lanes indicate that the proteins were from flavonoid-treated (2 μM genistein) or control cells, respectively. The letter D indicates that the lane was loaded with DNA that had not been exposed to protein. **C,** An unlabeled, double-stranded oligonucleotide with homology to the A3 region of *Rhizobium leguminosarum* bv. *viciae* inhibits complex formation between the 339-bp *Hin*dIII/*Bst*EII fragment (about 2 ng; see Fig. 1) and 80 μg of protein from genistein-induced strain USDA191.

these structural features are believed to be important for promoter activity, but neither is associated with *nolX*. We also sought, but failed to find, sequences related to the binding site of NolR, a repressor protein from *R. meliloti*, a species closely related to *R. fredii* (Kondorosi et al. 1989, 1991; Cren et al. 1995). The A3-homologous region thus lacks a series of features diagnostic of both the *nod* box and target sites for the NolR repressor protein, and so it is likely that protein binding is conditioned by some other feature of the sequence.

Inactivation of either *nodD1* or *nodD2* prevents formation of the retarded protein-DNA complex in *R. fredii*, and thus both NodD proteins have a role in the process. The functions of individual copies of *nodD* in regulating flavonoid-inducible genes have been studied systematically in *R. meliloti* (Györgypal et al. 1988; Mulligan and Long 1989; Honma et al. 1990), *R. leguminosarum* bv. *phaseoli* (Davis and Johnston 1990), *R. tropici* (van Rhijn et al. 1993, 1994), and *Rhizobium* sp. strain BR816 (van Rhijn et al. 1993, 1994). Within a given organism, different copies of *nodD* often vary widely in their abilities to enhance expression of such *nod* genes, but in all cases single *nodD* copies are sufficient for activity (Schlaman et al. 1992; Pueppke 1996). Such independence is also characteristic of the binding of NodD1 and NodD3 of *R. meliloti* to the *nod* box of *R. meliloti* (Fisher and Long 1989). One possible explanation for our observations is that NodD1 and NodD2 both bind to the target DNA sequence, perhaps as a heterodimer. Alternatively, NodD1 and NodD2 may both be necessary for some other process that is indirectly required for binding. We currently are preparing antibodies against NodD proteins from *R. fredii*, so that these possibilities can be sorted out.

MATERIALS AND METHODS

Strains and plasmids.

Strains and plasmids used in this study are listed in Table 2. Bacteria were stored at –70°C in 7.5% glycerol. Rhizobia were maintained for short periods on slants at 8°C and cultured in yeast extract-mannitol medium (Vincent 1970); *Escherichia coli* was grown in Luria-Bertani broth (Sambrook et al. 1989). When appropriate, antibiotics were supplied at the following concentrations in μg/ml: ampicillin, 50; kanamycin, 50; tetracycline, 10.

DNA manipulations and nucleic acid biochemistry.

Cloning, digestion with restriction enzymes, ligations, plasmid isolation, end-labeling, and other commonly employed protocols have been described by Sambrook et al. (1989). Deletions within the *nolX* promoter region were prepared by polymerase chain reaction with plasmid pRfDH421 as double-stranded template. In each case, one primer was 5′-GCCGACATTGTGCATCCTCA-3′, a sequence that corresponds to positions +8 to –12 with respect to the translational start site of the gene. The other primers were located at positions –262 to –242, –382 to –368, and –578 to –568 with respect to the transcriptional start site and primed the synthesis of 270-, 390-, and 586-bp DNA fragments, respectively. Each fragment was cloned into the polylinker site of pGEM7Z(f)+, and then restriction sites from the polylinker were used to prepare restriction fragments suitable for cloning into the promoter probe, pMP220 (Table 2). Oligonucleotides were synthesized by the DNA Core Facility, University of Missouri.

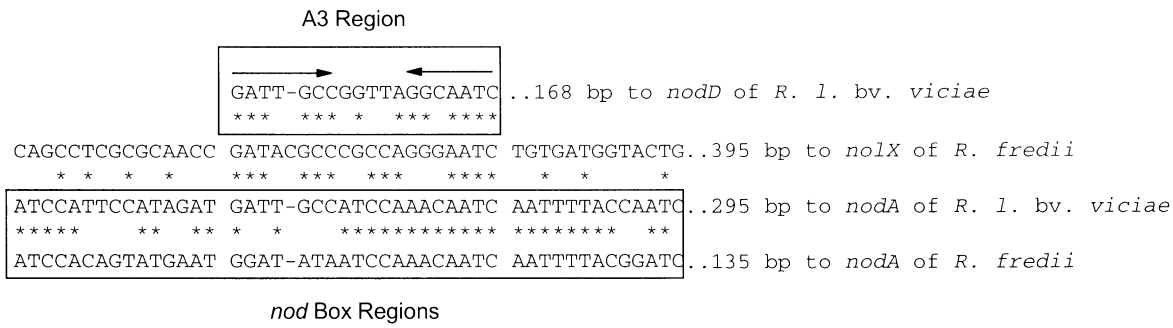

Fig. 5. Alignment of the A3 region of *Rhizobium leguminosarum* bv. *viciae* with *R. fredii* sequences that lie upstream of *nolX* and that inhibit formation of the retarded DNA/protein complex. The arrows mark the inverted repeat that characterizes the A3 sequence.

Table 2. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source
E. coli DH5α	Plasmid host	Promega Biotech.
Rhizobium fredii		
USDA191	Wild-type; Fix$^+$ nodules on Peking and McCall soybean	Keyser et al. 1982
USDA191nodD1$^-$	USDA191 containing a Km-resistance cassette in *nodD1*	Appelbaum et al. 1988
USDA191nodD2$^-$	USDA191 containing a Km-resistance cassette in *nodD2*	Appelbaum et al. 1988
USDA191nodD1$^-$nodD2$^-$	USDA191 containing an Ω insertion in *nodD1* and a Km-resistance cassette in nodD2	Boundy-Mills et al. 1994
USDA257	Wild-type; Fix$^+$ nodules on Peking, Inf$^-$ on McCall	Keyser et al. 1982
257SD1	USDA257 with 4.2 kb, *nolXWBTUV*-containing fragment replaced with a *sac* cartridge; Fix$^+$ nodules on Peking, Fix$^+$ on McCall	L. Kovács
Plasmids		
pGEM7Zf(+)	Multicopy sequencing vector; Ap^R	Promega Biotech.
pMP220	*lacZ*-containing, broad host range promoter probe; Tc^R	Spaink et al. 1987
pRfDH421	pGEM7Zf(+) carrying *nolXWBTUV* of USDA257 on a 4.2-kb *Bam*HI insert, Ap^R	Meinhardt et al. 1993
kpMP650.7	pMP220 carrying the *nolX* promoter region as a 648-bp *Hin*dIII fragment; Tc^R	This study
pMP400.7	pMP220 carrying 578 bp 5′ to the *nolX* coding region; Tc^R	This study
pMP200.7	pMP220 carrying 382 bp 5′ to the *nolX* coding region; Tc^R	This study
pMP083.7	pMP220 carrying 262 bp 5′ to the *nolX* coding region; Tc^R	This study

Assessment of flavonoid-inducibility.

The effect of isoflavonoid and flavonoid molecules on expression of *nolX* was assessed with the promoter probe plasmid, pMP220 (Spaink et al. 1987) in strain USDA257. Cells were grown in a prescribed manner (Krishnan and Pueppke 1991), and β-galactosidase activity was measured by the method of Miller (1972). Inducers were purchased from Sigma Chemical Co., St. Louis, MO.

Protein-DNA binding assays.

Rhizobium cells were grown in 100-ml liquid cultures at 30°C with agitation at 150 rpm for 24 h, at which time genistein was added to a final concentration of 1 μM. After incubation for an additional 24 h, cells were harvested by centrifugation for 10 min at 7,700 × *g*, washed in 0.9% NaCl, recentrifuged, and suspended in 3 ml of 50 mM Tris-HCl containing 1.5 mM $MgCl_2$, 15 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, and 115 μM phenylmethylsulfonylfluoride, final pH 8.0 (Dusha and Kondorosi 1993). Cells were disrupted by sonication for 30 s, and the extract was clarified by centrifugation at 40,000 × *g* for 1 h at 4°C. Glycerol was added to the supernatant solution to achieve a concentration of 10%, and the extract was stored at –70°C. Protein was determined by the method of Bradford (1976) with bovine serum albumin as standard.

The gel retardation assay followed the general protocol of Kondorosi et al. (1989). Briefly, DNA fragments were end-labeled with [^{32}P]dCTP and Klenow polymerase. Following removal of unincorporated label, approximately 1 ng of labeled DNA was mixed with 20 to 100 μg of protein in 10 mM Tris-HCl containing 100 mM NaCl, 3 mM dithiothreitol, 0.5 mM EDTA, 2 μg of sonicated salmon sperm DNA, and 5% glycerol, final pH 7.5. After incubation for 30 min at 23°C, the reaction was stopped by adding 5 μl of a dye solution containing 0.25% bromophenol blue and 0.25% xylene cyanol in 30% aqueous glycerol. Protein-DNA complexes were separated from unbound DNA by electrophoresis on 5 or 8% polyacrylamide gels in Tris-borate-EDTA buffer (Kondorosi et al. 1989). Gels were dried under vacuum and then subjected to autoradiography. In some experiments, extracts were pretreated with proteinase K as described (Hong et al. 1987). Experiments with a double-strained oligonucleotide as a competitive inhibitor of binding were patterned after those of Fisher et al. (1988) and used stoichiometric ratios of restriction fragment and oligonucleotide that were found previously to be appropriate.

ACKNOWLEDGMENTS

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LITERATURE CITED

Appelbaum, E. R., Thompson, D. V., Idler, K., and Chartrain, N. 1988. *Rhizobium japonicum* USDA191 has two *nodD* genes that differ in primary structure and function. J. Bacteriol. 170:12-20.

Balatti, P. A., Kovács, L. G., Krishnan, H. B., and Pueppke, S. G. 1995. *Rhizobium* sp. NGR234 contains a functional copy of the soybean cultivar specificity locus, *nolXWBTUV.* Mol. Plant-Microbe Interact. 8:693-699.

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.

Boundy-Mills, K.L., Kosslak, R.M., Tully, R.E., Pueppke, S.G., Lohrke, S., and Sadowsky, M. J. 1994. Induction of the *Rhizobium fredii nod* box-independent nodulation gene *nolJ* requires a functional *nodD1* gene. Mol. Plant-Microbe Interact. 7:305-308.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

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.

Cho, M.-J., and Harper, J. E. 1990. Effect of inoculation and nitrogen on isoflavonoid concentration in wild-type and nodulation-mutant soybean roots. Plant Physiol. 95:435-442.

Cren, M., Kondorosi, A., and Kondorosi, E. 1995. NolR controls expression of the *Rhizobium meliloti* nodulation genes involved in the core Nod factor synthesis. Mol. Microbiol. 15:733-747.

Davis, E. O., and Johnston, A. W. B. 1990. Regulatory functions of the three *nodD* genes of *Rhizobium leguminosarum* biovar *phaseoli.* Mol. Microbiol. 4: 933-941.

Dusha, I., and Kondorosi, A. 1993. Genes at different regulatory levels are required for the ammonia control of nodulation in *Rhizobium meliloti.* Mol. Gen. Genet. 240:435-444.

Fisher, R. F., Brierley, H. L., Mulligan, J. T., and Long, S. R. 1987. Transcription of *Rhizobium meliloti* nodulation genes: Identification of a *nodD* transcription initiation site, *in vitro* and *in vivo.* J. Biol. Chem. 262:6849-6855.

Fisher, R. F., Egelhoff, T. T., Mulligan, J. T., and Long, S. R. 1988. Specific binding of proteins from *Rhizobium meliloti* cell-free extracts containing NodD to DNA sequences upstream of inducible nodulation genes. Genes Devel. 2:282-293.

Fisher, R. F., and Long, S. R. 1989. DNA footprint analysis of the transcriptional activator proteins NodD1 and NodD3 on inducible *nod* gene promoters. J. Bacteriol. 171:5492-5502.

Fisher, R. F., and Long, S. R. 1993. Interactions of NodD at the *nod* box: NodD binds to two distinct sites on the same face of the helix and induces a bend in the DNA. J. Mol. Biol. 233:336-348.

Goethals, K., Gao, M., Tomekpe, K., Van Montagu, M., and Holsters, M. 1989. Common *nodABC* genes in *Nod* locus 1 of *Azorhizobium caulinodans:* Nucleotide sequence and plant-inducible expression. Mol. Gen. Genet. 219:289-298.

Goethals, K., Van Montagu, M., and Holsters, M. 1992. Conserved motifs in a divergent *nod* box of *Azorhizobium caulinodans* ORS571 reveal a common structure in promoters regulated by LysR-type proteins. Proc. Natl. Acad. Sci. USA 89:1646-1650.

Graham, T. L. 1991. Flavonoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates. Plant Physiol. 95:594-603.

Györgypal, Z., Iyer, N., and Kondorosi, A. 1988. Three regulatory *nodD* alleles of diverged flavonoid-specificity are involved in host-dependent nodulation by *Rhizobium meliloti.* Mol. Gen. Genet. 212:85-92.

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.

Hong, G.-F., Burn, J. E., and Johnston, A. W. B. 1987. Evidence that DNA involved in the expression of nodulation (*nod*) genes in *Rhizobium* binds to the product of the regulatory gene *nodD.* Nucleic Acids Res. 15:9677-9690.

Honma, M. A., Asomaning, M., and Ausubel, F. M. 1990. *Rhizobium meliloti nodD* genes mediate host-specific activation of *nodABC.* J. Bacteriol. 172:901-911.

Kondorosi, E., Gyuris, J., Schmidt, J., John, M., Duda, E., Hoffmann, B., Schell, J., and Kondorosi, A. 1989. Positive and negative control of *nod* gene expression in *Rhizobium meliloti* is required for optimal nodulation. EMBO J. 8:1331-1340.

Kondorosi, E., Pierre, M., Cren, M., Haumann, U., Buiré, M., Hoffmann, B., Schell, J., and Kondorosi, A. 1991. Identification of NolR, a negative transacting factor controlling the *nod* regulon in *Rhizobium meliloti*. J. Mol. Biol. 222:885-896.

Kovács, 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. 17:923-933.

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.

Mao, C., Downie, J. A., and Hong, G. 1994. Two inverted repeats in the *nodD* promoter region are involved in *nodD* regulation in *Rhizobium leguminosarum.* Gene 144:87-90.

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.

Mulligan, J. T., and Long, S. R. 1989. A family of activator genes regulates expression of *Rhizobium meliloti* nodulation genes. Genetics 122:7-18.

Pueppke, S. G. 1995. The genetic and biochemical basis for nodulation of legumes by rhizobia. Crit. Rev. Biotechnol. 16:1-51.

Rostas, K., Kondorosi, E., Horvath, B., Simoncsits, A., and Kondorosi, A. 1986. Conservation of extended promoter regions of nodulation genes in *Rhizobium.* Proc. Natl. Acad. Sci. USA 83:1757-1761.

Sadowsky, M. J., Olson, E. R., Foster, V. E., Kosslak, R. M., and Verma, D. P. S. 1988. Two host-inducible genes of *Rhizobium fredii* and characterization of the inducing compound. J. Bacteriol. 170:171-178.

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

Schlaman, H. R. M., Okker, R. J. H., and Lugtenberg, B. J. J. 1992. Regulation of nodulation gene expression by NodD in rhizobia. J. Bacteriol. 174:5177-5184.

Schofield, P. R., and Watson, J. M. 1986. DNA sequence of *Rhizobium trifolii* nodulation genes reveals a reiterated and potentially regulatory sequence preceding *nodABC* and *nodFE.* Nucleic Acids Res. 14: 2891-2903.

Scott, K. F. 1986. Conserved nodulation genes from the non-legume symbiont *Bradyrhizobium* sp. (*Parasponia*). Nucleic Acids Res. 14: 2905-2919.

Shearman, C. A., Rossen, L., Johnston, A. W. B., and Downie, J. A. 1986. The *Rhizobium leguminosarum* nodulation gene *nodF* encodes a polypeptide similar to acyl-carrier protein and is regulated by *nodD* plus a factor from pea root exudate. EMBO J. 5:647-652.

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.

Trese, A. T. 1995. A single dominant gene in McCall soybean prevents effective nodulation with *Rhizobium fredii* USDA257. Euphytica 81:279-282.

van Rhijn, P., Desair, J., Vlassak, K., and Vanderleyden, J. 1994. The NodD proteins of *Rhizobium* sp. strain BR816 differ in their interactions with coinducers and in their activities for nodulation of different host plants. Appl. Environ. Microbiol. 60:3615-3623.

van Rhijn, P., 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. 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.