Research Note

Induction of the *Rhizobium fredii* *nod* Box–Independent Nodulation Gene *nolJ* Requires a Functional *nodD1* Gene

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We previously reported the isolation of a flavonoid-inducible gene from the pSym of *Rhizobium fredii* strain USDA 201 (Sadowsky *et al.* 1988). In this study, we report that a Tn*5* insertion in this gene results in a 6-day delay in the appearance of nodules and a 70% decrease in the number of nodules relative to nodulation by the wild-type *R. fredii* USDA 201. We have named this gene *nolJ*. While the 5′ region of *nolJ* does not contain a highly conserved *nod* box consensus sequence, RNA dot blot hybridization analyses indicated that the flavonoid induction of *nolJ* is dependent on a functional *nodD1* gene. Our data suggest that transcriptional activation of *nolJ* occurs by a mechanism different from that proposed for many other nodulation loci.

Additional keywords: *nod* gene induction, *nod* genes, nodulation efficiency.

Induction of rhizobial nodulation genes requires the interaction of a flavonoid with the NodD protein at the cytoplasmic membrane (Dénarié *et al.* 1992; Fisher and Long 1992; Schlaman *et al.* 1992). The activated NodD protein then binds to a regulatory sequence, the *nod* box, which is found upstream of many nodulation genes (Fisher and Long 1992; Hong *et al.* 1987; Rostas *et al.* 1986; Schlaman *et al.* 1992; Wang and Stacey 1991). Many species of rhizobia have multiple copies of the regulatory *nodD* gene (Honma and Ausubel 1987; Göttfert *et al.* 1986, 1992). *Rhizobium fredii*, the fast-growing microsymbiont of soybeans, has two copies of *nodD*, designated *nodD1* and *nodD2*, which are 69% identical in amino acid sequence (Appelbaum *et al.* 1988). Only *nodD1* is involved in the efficiency of nodulation of soybean and is preceded by a *nod* box (Appelbaum *et al.* 1988; Göttfert *et al.* 1992). Many *R. fredii* nodulation genes, including *nodD1* and *nodD2*, are located on a large Sym plasmid (Appelbaum *et al.* 1988; Olson *et al.* 1985).

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We previously reported the isolation of two flavonoid-inducible genes (ORF1 and ORF2), located in tandem, on a 4.2-kb *Hin*dIII fragment from the pSym of *R. fredii* strain USDA 201 (Sadowsky *et al.* 1988). The genes comprise two separate transcriptional units and are transcribed in the same direction (Fig. 1). Transcription of both genes was induced by daidzein, apigenin, genistein, and several other flavonoids. The 5′ region of each gene shows no homology to a canonical *nod* box sequence (Rostas *et al.* 1986). However, ORF1 does have a possible low-consensus, LysR-type, noncanonical *nod* box (Goethals *et al.* 1992), which ends 42 nucleotides upstream of the transcription start site. While our initial results indicated that ORF1 is involved in interstrain competition for nodulation of soybeans (Sadowsky *et al.* 1988), the symbiotic function of the second inducible gene (ORF2) and the role of *nodD1* and *nodD2* in the flavonoid induction of this gene was unknown.

To determine the function of the second open reading frame, ORF2, we constructed a *R. fredii* USDA 201 strain which had Tn*5* inserted within ORF2. The bacterial strains, phage, and plasmids used and their sources are listed in Table 1. Site-directed mutagenesis of *nolJ* in plasmid pAC2A4 was done with the use of lambda::Tn*5* phage (λ 467) as described by de Bruijn and Lupski (1984). Transposon insertions in the ORF2 coding region were identified and mapped by Southern hybridization (Sambrook *et al.* 1989) to a ^{32}P-labeled Tn*5* gene probe, a 2.1-kb *Xho*I-*Sal*I fragment from pSUP1011 (Simon *et al.* 1983). One plasmid, pRM22, contained Tn*5* inserted 163 base pairs downstream from the start codon of ORF2. To mobilize pRM22 into *R. fredii* USDA 201, we ligated *Eco*RI-digested pRM22 plasmid DNA into the *Eco*RI site of cosmid pLAFR3 (Staskawicz *et al.* 1987). The resulting plasmid, pMSRT22, was conjugated into *R. fredii* USDA 201, and homologous recombination and marker exchange were promoted by using plasmid pPH1JI as described by Ditta (1986). Proper marker exchange was verified by Southern hybridization with ^{32}P-labeled Tn*5* and *nolJ* gene probes (Sadowsky *et al.* 1987). The *nolJ* probe was a 1.7-kb *Xho*I-*Sma*I fragment from pAC2A4. Two ORF2::Tn*5* recombinants, 201-D and 201-G, were selected for further study.

The nodulation phenotype of mutants 201-D and 201-G on *Glycine max* cv. Peking was determined in Leonard Assemblies as described by Sadowsky and Cregan (1992). Seeds were surface-sterilized (Vincent 1970) before planting and were inoculated with 10^4, 10^6, or 10^8 cells of *R. fredii* strain 201-D or 201-G or wild-type USDA 201. After inoculation, plants were grown in a plant growth chamber as described by Sadowsky and Cregan (1992). Nodule numbers were determined 30 days after planting. In both mutants, nodule numbers were significantly reduced, to about 30% of those produced by wild-type USDA 201 (data not shown). Impaired nodulation ability in the mutant strains was independent of the inoculum dosage. Plants inoculated with the mutants were smaller and chlorotic and did not turn green until 6 wk after inoculation. In addition, the nodules were located farther down the root system than those produced by the wild-type USDA 201. This suggested that nodule initiation by the mutants is slower than that by the wild-type strain.

The effect of the ORF2::Tn*5* mutation on the kinetics of nodulation of *G. max* cv. Peking in growth pouches is shown in Figure 2. Mutant strain 201-D showed a 6-day delay in nodulation, relative to USDA 201. By day 17, the mutant had nodulated only 75% of the plants, whereas the wild-type parent had formed nodules on 100% of the plants by 10 days after inoculation. Moreover, the mutant produced an average of 1.3 nodules per plant, while the wild-type strain produced 6.4 nodules per plant. These results show that ORF2 is involved in the efficiency of soybean nodulation. We have called this gene *nolJ*.

Mutations in other nodulation genes, including the *B. japonicum nod*D1 and *nod*VW genes and the *R. meliloti nod*F, *nod*E, and *nod*G genes, have also been reported to cause a delay in nodulation and reduced nodule number (Göttfert *et al.* 1986, 1990; Horvath *et al.* 1986; Kondorosi *et al.* 1985). Most of these genes, however, are involved in the determination of the host range for nodulation. The *nolJ* gene does not appear to be a host specificity determinant, since mutant 201-D was not impaired in its ability to form nodules on several hosts nodulated by *R. fredii,* including *Albizia lebbeck, Cajanus cajan, Desmodium canadense, D. illinoense, Flemingia congesta, Glycine soja, Hardenbergia comptoniana, Indigofera tinctoria, Kummerowia stipulacea, Lotus corniculatus,*

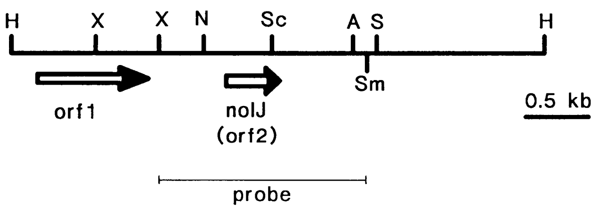

Fig. 1. Restriction map and physical relationship of the two root exudate–inducible genes, ORF1 and *nolJ*, cloned in pAC2A4. ORF1, 950 nucleotides long, codes for a gene which has been shown to be involved in interstrain competition for nodulation. The *nolJ* gene, 450 nucleotides long, is involved in the efficiency of soybean nodulation. Restriction enzyme sites: H, *Hin*dIII; X, *Xho*I; N, *Nae*I; Sc, *Sac*II; A, *Ava*I; Sm, *Sma*I; S, *Sal*I. The fragment used as a probe for RNA dot blot hybridization analyses is indicated. The arrows indicate the direction of transcription. Adapted from Sadowsky *et al.* (1988).

Table 1. Bacteria, plasmids, and phage used and their sources

	Relevant characteristics	Reference or source
Rhizobium fredii		
USDA 201	Wild type	USDA[a]
201-D and 201-G	*nolJ*::Tn*5*, nod delayed, eff$^-$	This study
USDA 191	Wild type	USDA
191 *nod*D1::Kan	*nod*D1$^-$, fix$^-$, eff$^-$	Appelbaum *et al.* 1988
191 *nod*D2::Kan	*nod*D2$^-$, nod delayed	Appelbaum *et al.* 1988
191 *nod*D1::Ω plus *nod*D2::Kan	*nod*D1$^-$, *nod*D2$^-$	This study
EA213C3	Sym$^-$, nod$^-$	Appelbaum *et al.* 1988
λ 467	λ b221 rex::Tn*5* c1857, Oam29, Pam80	Nancy Kleckner
PLAFR3	Lac$^+$, IncP-1	Staskawicz *et al.* 1987
pSUP1011	pSUP101::Tn*5*	Simon *et al.* 1983
pPH1JI	IncP-1, gentamicin^r	Ditta 1986
pRK2073	Helper plasmid	Ditta 1986
pEA4-19	*nod*D1 in pUC19	Appelbaum *et al.* 1988
pHP45Ω	Source of Ω interposon	Prentki and Krisch 1984
pSUP202	Suicide vector	Simon *et al.* 1983
pRK290	Cloning vector	Ditta *et al.* 1980
pAC2A4	ORF1 and *nolJ* from USDA 201 in pACYC184	Sadowsky *et al.* 1988
pKB3	*nod*D1::Ω in pSUP202	This study
pRM22	*nolJ*::Tn*5* in pAC2A4	This study
pMSRT22	pRM22 cloned into *Eco*RI site of pLAFR3	This study

[a] USDA = U.S. Department of Agriculture, Agricultural Research Service, Beltsville, Maryland.

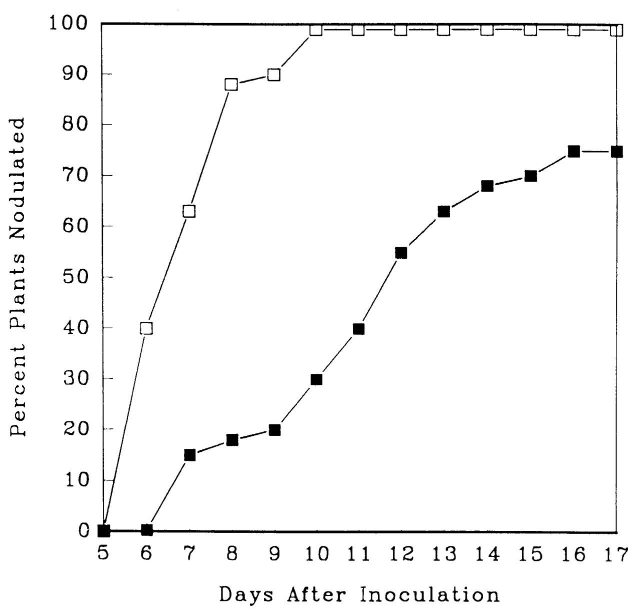

Fig. 2. Nodulation kinetics of *Rhizobium fredii* strains USDA 201 (□) and 201-D (*nolJ*::Tn*5*) (■) on *Glycine max* cv. Peking. Forty sterile seedlings were each inoculated with 1.0 ml (5 × 10^6 cells) of TY-grown (Beringer *et al.* 1978) cultures of strain USDA 201 or the ORF2::Tn*5* mutant 201-D. Uninoculated seedlings (20) served as negative controls. The plants were incubated in a growth chamber at 25° C with an 18-hr photoperiod and were watered daily. Nodules were counted daily until 17 days postinoculation. The number of days taken to form visible nodules was evaluated. The plotted values are the percentages of plants nodulated at each time point.

Macroptilium atropurpureum, Macrotyloma axillare, Phaseolus angularis, P. vulgaris, Psophocarpus tetragonolobus, Tephrosia vogelii, Vigna caracalla, V. radiata, and *V. vexillata* (data not shown). Moreover, *nolJ* shares no sequence homology with any of the previously reported common host-specific or genotype-specific nodulation genes. Thus, *nolJ* appears to be unique among the rhizobia.

Plant-mediated expression of many *nod* genes requires *nodD* (Fisher and Long 1992; Schlaman *et al.* 1992) and a highly conserved *nod* box consensus sequence (Rostas *et al.* 1986) located upstream from the transcription start site. Since flavonoid induction of *nolJ* apparently does not require a *nod* box consensus sequence, it was of interest to determine if NodD is involved in the induction of *nolJ*. To study this, we constructed a *R. fredii* USDA 191 derivative which lacks both copies of *nodD*. *R. fredii* strain USDA 191 was chosen for this study because the sequences of both *nodD* genes are known, and because it is genetically closely related to USDA 201. The *nodD1* gene was subcloned as an *EcoRI* fragment from pEA4-19 (Appelbaum *et al.* 1988) into the *EcoRI* site of pRK290 (Ditta *et al.* 1980). The gene was disrupted by insertion of the Ω interposon as a *BamHI* fragment from pHP45Ω (Prentki and Krisch 1984) into the *BamHI* site in the middle of the *nodD1* open reading frame (Appelbaum *et al.* 1988). The *nodD1*::Ω construct was subcloned into the *EcoRI* site of pSUP202 (Simon *et al.* 1983), to produce plasmid pKB3. pKB3 was transformed into *Escherichia coli* strain S17-1 and conjugated into *R. fredii* USDA 191 *nodD2*::Kan (Appelbaum *et al.* 1988). The presence of the *nodD1*::Ω allele was confirmed by Southern hybridization analysis.

We analyzed RNA from *R. fredii* USDA 191 wild-type, *nodD1*::Kan, *nodD2*::Kan, and *nodD1*::Ω plus *nodD2*::Kan strains grown in the presence or absence of the flavonoid apigenin, which induces the expression of *nolJ*. RNA dot blot hybridization analyses (Fig. 3) showed that transcription of *nolJ* is induced about fourfold in the wild-type strain and in a *nodD2*::Kan mutant strain, but is not induced in *nodD1*::Kan and *nodD1*::Ω plus *nodD2*::Kan strains. Similar results were obtained when strains were grown in the presence of the isoflavone genistein (data not shown). These results show that the induction of *nolJ* does not require *nodD2*, but does require a functional *nodD1* gene. The *nolJ* transcript was not detected in flavonoid-induced or uninduced strain EA213C3, which lacks the Sym plasmid (Appelbaum *et al.* 1988).

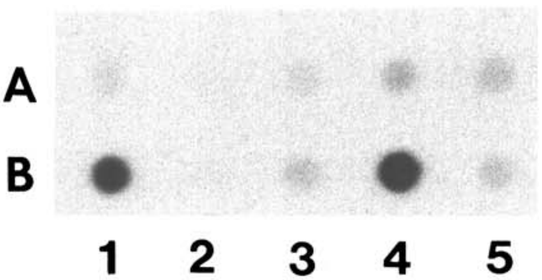

Fig. 3. RNA dot blot hybridization analysis of isoflavonoid-induced RNA from wild-type and *nodD* insertion mutants of *Rhizobium fredii* strain USDA 191. Total RNA from flavonoid induced and uninduced *R. fredii* cultures was isolated using a modification of the hot-phenol extraction procedure described by Meyer and Schottel (1992). Cultures were induced for 18 hr with 1 μM apigenin as described by Sadowsky *et al.* (1988). RNA (5 μg per well) was dot-blotted onto nitrocellulose filters (Bethesda Research Laboratories) with the use of a Hybri-Dot Manifold (Bethesda Research Laboratories), and filters were hybridized to a ^{32}P-labeled 1.7-kb *XhoI-SmaI* fragment from pAC2A4 containing *nolJ* as described by Sambrook *et al.* (1989). RNA was isolated from cultures grown in the absence of apigenin (row A) or in its presence (row B). Lane 1, USDA 191 wild type; lane 2, EA213C3, Sym-plasmid cured derivative of USDA 191; lane 3, USDA 191 *nodD1*::Kan; lane 4, USDA 191 *nodD2*::Kan; lane 5, USDA 191 *nodD1*::Ω plus *nodD2*::Kan.

Since there are currently no physical or genetic maps of the *R. fredii* Sym plasmid, the physical location of *nolJ* and *nodD1* relative to each other is presently unknown. To determine the location of both genes, we used field inversion gel electrophoresis (Sobral *et al.* 1990) of genomic DNA from USDA 201 digested with *HpaI*, which rarely cuts *R. fredii* genomic DNA, and hybridized Southern blots to *nodD1* and *nolJ* probes. Our results indicate that *nolJ* and *nodD1* are located within 100 kb of each other on the 190-MDa Sym plasmid (data not shown).

Taken together, our results indicate that the induction of transcription of *nolJ* does not require a canonical upstream *nod* box consensus sequence, but does require a functional *nodD1* gene. Thus, transcriptional activation of *nolJ* most likely requires a different type of promoter than that seen for many other nodulation loci. While other *nod* box–independent nodulation genes have been reported in *R. fredii* (Meinhardt *et al.* 1993) and other rhizobia (Barbour *et al.* 1992; Dénarié *et al.* 1992; Göttfert *et al.* 1990), many of these genes encode host range functions. Our data, however, indicate that *nolJ* does not appear to be a host specificity determinant. Lastly, since induction of *nolJ* does not require a canonical *nod* box consensus sequence, our data suggest that transcriptional activation of *nolJ* occurs by a mechanism different from that proposed for other nodulation loci. We are currently investigating whether NodD acts directly or indirectly to control the induction of *nolJ*.

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