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Research Note

Deletion Analysis of the 5′ Untranslated Region of the *Rhizobium meliloti nodF* Gene

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Efficient establishment of the symbiosis between rhizobia and their host plants requires precise regulation of bacterial *nod* genes. The *nod* gene transcripts in *Rhizobium meliloti* have approximately 200 nucleotides of untranslated sequence 5′ of the start codon (5′ UTR). We measured the significance of this region by constructing fusions between deletion derivatives of *nodF* and the reporter β-glucuronidase (GUS). Flavonoid-inducible expression of the fusions in *R. meliloti* was evident when extra copies of the positive transcriptional activators NodD1, NodD3, or SyrM were present. The fusions responded normally over a range of inducer concentrations in *Rhizobium leguminosarum* bv. *trifolii*. GUS assays in planta showed no significant difference between the deletion constructs and a wild-type fusion. We conclude that the 5′ UTRs of the *nod* gene transcripts are unlikely to have a significant regulatory role.

Soil bacteria of the genera *Rhizobium, Bradyrhizobium, Sinorhizobium* and *Azorhizobium* can form nitrogen-fixing nodules on legume plants. The symbiosis is selective: individual bacterial strains only nodulate certain hosts. This limitation is partly explained by chemical communication during the establishment of symbiosis (Fisher and Long 1992; Spaink 1994). The plant secretes compounds which induce multiple responses in compatible bacteria, including expression of the *nod* genes required for nodulation (van Rhijn and Vanderleyden 1995; Phillips and Kapulnik 1995). The products of these genes synthesize signaling molecules that can elicit the complex plant responses resulting in nodule formation (Dénarié and Cullimore 1993; Spaink and Lugtenberg 1994).

Precise regulation of the *nod* genes is crucial. Mutants lacking *nod* gene expression fail to induce nodules and mutants that inappropriately express *nod* genes form nodules less efficiently than normal (Knight et al. 1986; Burn et al. 1987; Kondorosi et al. 1989). Basic regulation of these genes is mediated through the interaction between conserved DNA sequences (*nod* boxes) and the positive transcriptional activator NodD proteins (Schlaman et al. 1992a). However, there is considerable additional complexity, including the repressor NolR in some strains of *R. meliloti* (Kondorosi et al. 1991), the regulatory protein SyrM in *R. meliloti* (Mulligan and Long 1989), and possible nodule-specific repression in *R. leguminosarum* bv. *viciae* (Schlaman et al. 1991; Schlaman et al. 1992b). This last form of regulation is especially significant as nodules formed by bacteria that constitutively express *nod* genes are Fix$^-$ (Burn et al. 1987).

These data suggest that there are likely to be both additional genes and *cis* elements involved in *nod* gene regulation. One element was suggested by the fact that several *R. meliloti nod* gene transcripts have 5′ untranslated regions (UTRs) of approximately 200 nucleotides (Fisher et al. 1987; Mulligan and Long 1989). In other prokaryotic systems such unusually long 5′ UTRs have been shown to be involved in regulation at virtually all levels including transcription, translation, and message stability (Landick et al. 1996; McCarthy and Gualerzi 1990; Alifano et al. 1994). The direct influence of the 5′ UTR on translation is generally on initiation and involves regions adjacent (within ~50 nucleotides) to the initiation codon. Other events such as attenuation and polarity that appear translational actually act on downstream genes through effects on transcription itself (Landick et al. 1996; Platt and Bear 1984). Furthermore, nodule-specific suppression of *nod* gene expression in *R. leguminosarum* bv. *viciae* is apparently mediated transcriptionally (Schlaman et al. 1991). Therefore, to test this region for a regulatory role we constructed transcriptional fusions between deletion derivatives of the *nodF* 5′ UTR and GUS such that sequences surrounding the initiation codon are identical in all of the constructs (Fig. 1A, Table 1). We focused on *nodF* because its 5′ UTR has an inverted repeat and two short open reading frames which are important regulatory elements in other systems (Landick et al. 1996; Lovett and Rogers 1996).

We initially tested the effect of leader deletions on expression in *R. meliloti*. We obtained single-copy constructs of each fusion by electroporating MB501 with pGK141-144 and selecting for Nm^r. As these plasmids do not replicate in *R. meliloti*, this results in tandem duplication of the cloned region by recombination at *nodF*, introducing the fusion while leaving an intact copy of *nodF*. We assayed expression of these fusions with plasmids overproducing proteins that either directly or indirectly activate *nod* gene expression (NodD1, NodD3, and SyrM). Induction was undetectable in the absence of additional copies of *nod* gene activator proteins (Table 2, pRmTE3-containing strains). This has been observed with

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some previous *nod* gene fusions (Egelhoff and Long 1985; Schwedock and Long 1989; Györgypal et al. 1991). In the presence of plasmids overproducing NodD1 (pRmE43), NodD3 (pRmE65), or SyrM (pRmS73), the expression of the fusions becomes either highly inducible (pRmE43) or constitutive (pRmE65 and pRmS73) (Table 2). The expression levels between fusions differed by no more than approximately twofold.

To assay expression without additional *nod* gene regulatory proteins, plasmid-borne fusions (Fig. 1A) were tested in *R.*

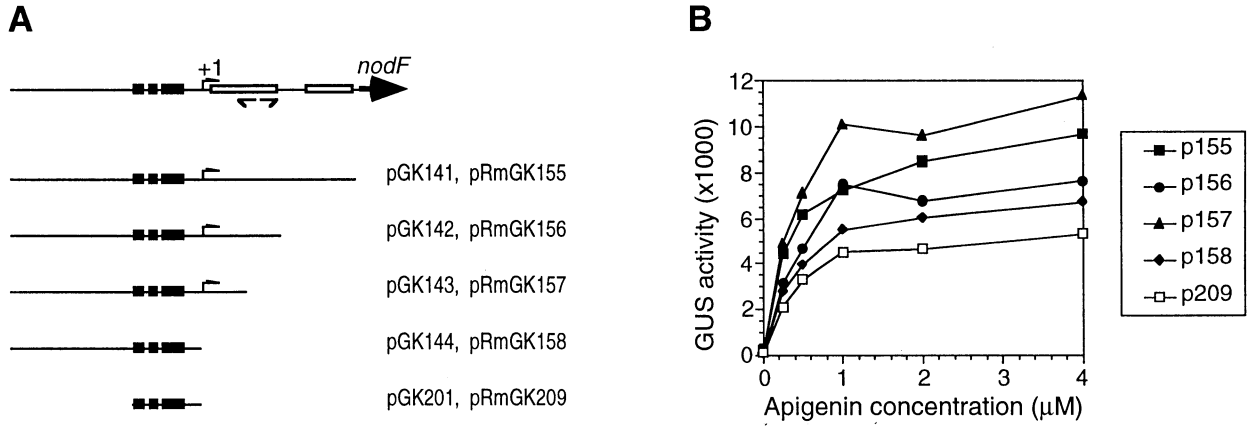

Fig. 1. Construction of fusions and assay in *Rhizobium leguminosarum* bv. *trifolii*. **A,** The *nodf* regulatory region is shown with the *nod* box represented by black boxes and the transcription initiation site marked +1. Two upstream open reading frames are shown as white boxes and the inverted repeat indicated by arrows below the main line. Deletions of the *nodf 5′* UTR were constructed by PCR using two upstream primers (5′-GAGAGGGATCCAGCCTG-3′, *5′*-CACAGGGATCCATTTCAC-3′) and four downstream primers (5′CGTGGGAAGCTI'GTGCTAC-3′, 5′-GCAGAAGCTTCCGATGACG-3′, *5′*GGCTAAGCTTAATGCCCGC-3′, 5′-GT-rCCC-17AAGCTTGTGTTCG-3′). The resulting fragments were cloned into pGK22 as *BamHI-HinDIII* fragments (pGKI41-4, pGK201) and subcloned with GUS into pSW213 to obtain pRmGKI55-8 and pRmGK209. pGKI41-144 were also used directly in the generation of *R. meliloti* strains GK3-6. **B,** GUS activity was assayed after 3 h inductions with apigenin at the concentrations shown. Each data point is the average of three independent experiments performed in duplicate.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics or genotypes[a]	Source or reference
Rhizobium meliloti		
Rml02l	Wild type; Sm^r-derivative of RCR2011	Meade et al. 1982
MB501	Tn*5*-Tp^r; derivative of WM249, uncharacterized Rm1021::Tn *5*-233 with increased electroporation competence	M. Barnett, this laboratory
JAS154	Rm102l; *nodE*-GUS (Nm^r)	J. Swanson, this laboratory
GK3	Chromosomal insertion of pGK141 in Rml02l	This study
GK4	Chromosomal insertion of pGK142 in Rml02l	This study
GK5	Chromosomal insertion of pGK143 in Rml02l	This study
GK6	Chromosomal insertion of pGK144 in Rml02l	This study
Rhizobium leguminosarum bv. *trifolii*		
ANU843	Wild type	Rolfe et al. 1980
Plasmids		
pBluescript SK+	Ap^r; high-copy-number ColEl cloning vector	Stratagene
pRK600	Cm^r; mobilizing plasmid	Finan et al. 1986
pSW213	Tc^r; broad-host-range cloning vector	Chen and Winans 1991
pJOGus2	Ap^r; pUC1813 with cloned GUS-Nm^r (Km^r) cassette	Ogawa 1994
pRmJT5	Tc^r; large *nod* gene region cloned into pLAFRl	Swanson et al. 1987
pRmTE3	Tc^r; pLAFRl with *trp* promoter and polylinker	Egelhoff and Long 1985
pRmE43	*nodD1* expressed from *trp* promoter in pRmTE3	Fisher et al. 1988
pRmE65	*nodD3* expressed from *trp* promoter in pRmTE3	Fisher et al. 1988
pRmS73	*syrM* expressed from *trp* promoter in pRmTE3	Swanson et al. 1993
pGK19	pJOGus2 with internal *Hin*dIII site removed	This study
pGK20	pBluescript SK+ with *Bgl*II linker inserted at *Hin*cII	This study
pGK22	GUS-Nm^r fragment of pGK19 cloned into pGK20	This study
pGK141	399-bp *nodF* PCR product cloned into pGK22	This study
pGK142	318-bp *nodF* PCR product cloned into pGK22	This study
pGK143	274-bp *nodF* PCR product cloned into pGK22	This study
pGKI44	230-bp *nodF* PCR product cloned into pGK22	This study
pGK201	72-bp *nodF* PCR product cloned into pGK22	This study
pRmGK155	pGK141 fusion cloned into pSW213	This study
pRmGK156	pGK142 fusion cloned into pSW213	This study
pRmGK157	pGK143 fusion cloned into pSW213	This study
pRmGK158	pGK144 fusion cloned into pSW213	This study
pRmGK209	pGK201 fusion cloned into pSW213	This study

[a] The following abbreviations indicate: Tc^r, tetracycline resistant; Sm^r, streptomycin resistant; Tp^r, trimethoprim resistant; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant.

leguminosarum bv. *trifolii*, where we have previously reported Long 1993). As in *R. meliloti*, all constructs were highly inducible and expression levels varied approximately twofold (Fig. 1B). We also asked whether there was variation in sensitivity to inducer concentration. All fusions behaved essentially identically over a range of inducer concentrations from 0 to 4 μM (Fig. 1B). The induction of expression reached a plateau that expression of *nod* gene fusions is more robust (Fisher and

Table 2. GUS activity[a] of fusions in *Rhizobium meliloti* overproducing *nod* gene regulators in the absence (–) and presence of (+) 3 μM luteolin

	pRmTE3 P_{trp}		pRmE43 P_{trp}-*nodDI*		pRmE65 P_{trp}-*nodD3*		pRmS73 P_{trp}-*syrM*	
Strain	–	+	–	+	–	+	–	+
GK3	13 ± 2 b	18 ± 2	16 ± 1	92 ± 30	433 ± 53	402 ± 67	159 ± 17	176 ± 24
GK4	12 ± 8	6 ± 2	12 ± 4	63 ± 8	310 ± 19	283 ± 23	60 ± 13	74 ± 19
GK5	8 ± 1	9 ± 1	11 ± 0	136 ± 45	609 ± 105	548 ± 179	242 ± 68	181 ± 20
GK6	11 ± 7	10 ± 3	7 ± 1	56 ± 9	616 ± 61	500 ± 74	108 ± 40	89 ± 13

[a] GUS units are: $[OD_{415} \times 10^5]/[\text{time (min)} \times \text{cell volume (ml)} \times OD_{600}]$.
[b] Results are reported as the average of three duplicate experiments ± standard error.

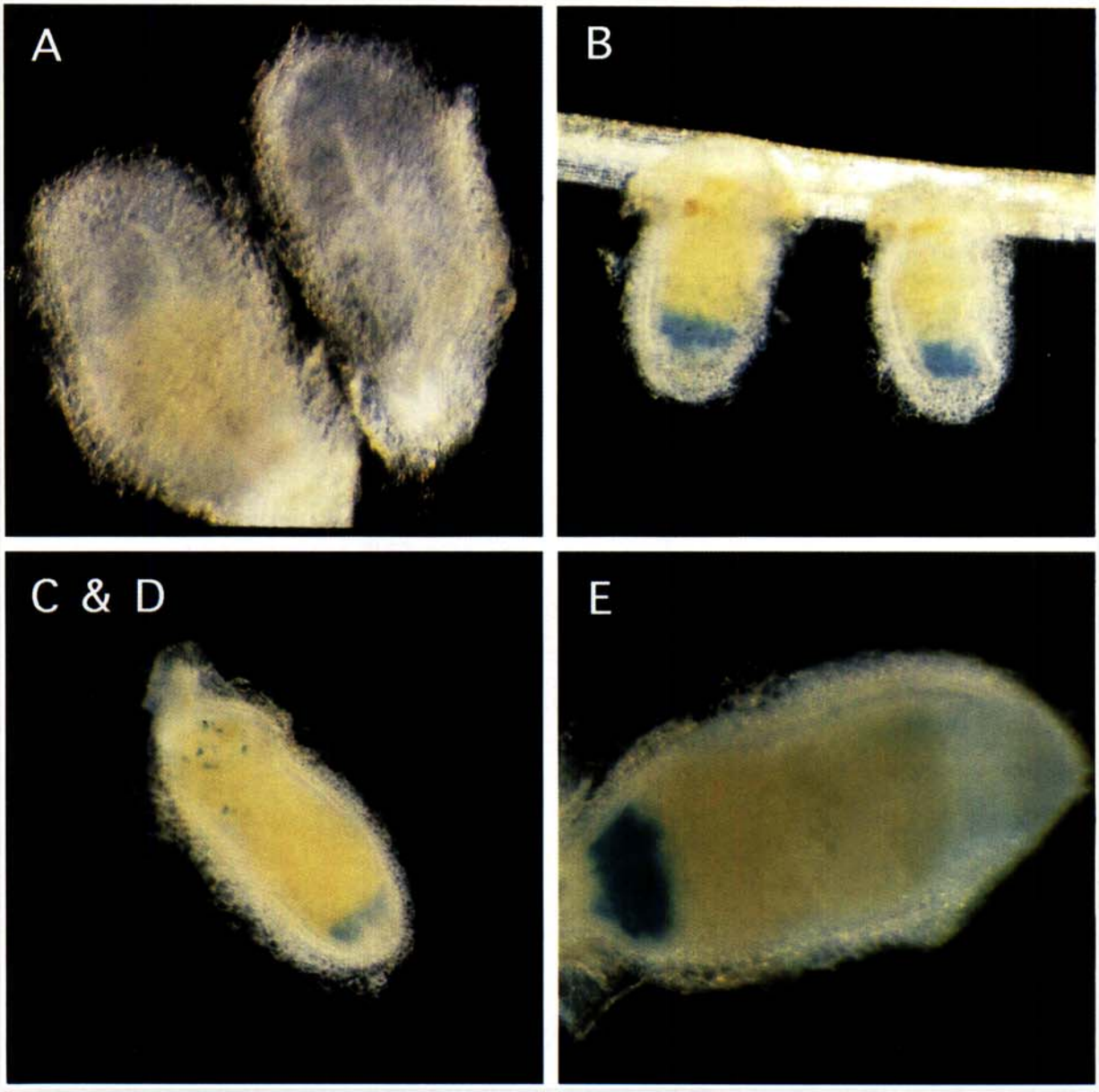

Fig. 2. In situ staining of P-glucuronidase fusions in *Rhizobium meliloti* on alfalfa. The five patterns of staining observed as well as the approximate frequency observed with all constructs were: A, no staining (–10%); **B,** tip staining (–70%); **C,** tip staining with punctate staining at the base of the nodule (–10%); **D,** only punctate staining at the base of the nodule (~5%); **E,** more extensive localized staining at the base of the nodule (–5%).

at 1 to 2 μM which is consistent with previous observations of flavonoid responses (Peters and Long 1988; Hartwig et al. 1991; Hungria et al. 1992).

Previous work suggested that *nod* gene expression in *R. leguminosarum* bv. *viciae* is subject to nodule-specific regulation (Schlaman et al. 1992b). We tested the possibility that similar regulation in *R. meliloti* would affect expression of the deletion fusions by nodulating alfalfa with the single-crossover derivatives. Thirty nodules for each bacterial strain were harvested and stained for GUS expression at 21 days postinoculation as described previously (Swanson et al. 1993). The staining patterns observed are shown in Figure 2. Most nodules exhibited a tip-staining pattern identical to that previously described for expression of *nod* genes in alfalfa nodules (Sharma and Signer 1990). A few nodules exhibited staining at the base which was generally in addition to the typical tip staining. We observed the same range and variability of staining from control nodules generated using a *nodE*-GUS fusion (strain JAS154/pRmJT5).

We found that constructs lacking the entire 5′ UTR showed the same overall pattern of expression under several assay conditions, strongly suggesting that the 5′ UTRs of the *nod* gene transcripts play no significant regulatory role. This result was unexpected, but is consistent with the lack of sequence conservation between the different 5′ UTRs and the fact that some other *R. meliloti* transcripts have long 5′ UTRs (Barnett et al. 1996). Alternatively, because precise control of *nod* gene regulation is important for efficient nodulation (Kondorosi et al. 1989), the twofold differences in expression observed in this study might be significant. This possibility could be addressed by fusing the deletions directly to the *nodF* gene and comparing nodulation behavior. Lastly, it is possible that a greater difference would be revealed by assaying expression under conditions that more closely mimic the natural environment. In summary, the data reported here suggest that in our strain of *R. meliloti*, the sequences of the 5′ UTR are not required for qualitative *nod* gene regulation in either free-living culture or nodules.

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